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Use of Reduced Sulfur Compounds by *Beggiatoa* sp.

DOUGLAS C. NELSON AND RICHARD W. CASTENHOLZ*
Department of Biology, University of Oregon, Eugene, Oregon 97403

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A strain of *Beggiatoa* cf. *leptomitiformis* (OH-75-B, clone 2a) was isolated which is unique among reported strains in its ability to deposit internal sulfur granules from thiosulfate. It also deposited these characteristic granules (as all *Beggiatoa* species do) from sulfide. In cultures where growth was limited by exhaustion of organic substrates, these granules generally comprised about 20% of the total cell weight. With medium containing acetate and thiosulfate, no measurable utilization of thiosulfate or deposition of elemental sulfur (S^0) took place until after the exponential growth phase. Neither sulfide nor thiosulfate added an increment to heterotrophic growth yield except for the weight of the deposited S^0. The deposition of S^0 from thiosulfate was probably a disproportionation in which S^0 and sulfate were produced in a 1:1 ratio. Some of the S^0 was further oxidized to sulfate. No autotrophic or mixotrophic growth was demonstrated for this strain. When inoculated in small, well-dispersed quantities into yeast extract medium, this strain grew only after long lags. Addition of the enzyme catalase eliminated initial lags and increased growth rates slightly. In contrast, catalase had no influence on growth rate when added to mineral medium containing acetate. In yeast extract medium, the inhibition of growth rate was presumably because of peroxides. Addition of thiosulfate was almost as effective as catalase in eliminating this inhibition. The S^0 granules which, in this case, were deposited during the exponential growth phase, appeared to be partly responsible for this relief. This strain of *Beggiatoa* sp. remained active for at least 5 days under strictly anaerobic conditions, and under those conditions, it increased its dry weight by about 2.5-fold. Anaerobic "growth" and maintenance required the presence of an energy source, such as acetate. When cells containing much internal S^0 were transferred to an organic anaerobic medium, a substantial portion of the internal S^0 was eventually converted to sulfide.

Probably the most striking feature of the genus *Beggiatoa* is its ability to deposit granules of elemental sulfur (S^0). This deposition of sulfur granules in the presence of a source of H_2S is the only taxonomic feature which separates the genus *Beggiatoa* from *Vitreoscilla* (18). These granules are deposited between the cell wall and the cell membrane within invaginations of that membrane (33). They will be referred to as internal S^0 granules in this paper, to distinguish them from external granules of S^0, which are sometimes found completely free, especially in old cultures. What benefit *Beggiatoa* species obtain from the deposition, and possible subsequent oxidation, of internal S^0 granules has been the subject of much inquiry and controversy.

Winogradsky (36, 37) believed that oxidation of sulfide to sulfate, via this S^0 intermediate, was the source of both energy and reductant in *Beggiatoa* species. In fact, the general concept of chemoautotrophy, which has since then been verified for other bacterial types (22), stems from this pioneering work with *Beggiatoa* species. However, Winogradsky's experimental cultures of *Beggiatoa* species were enrichment cultures, and the medium that he used was based on natural spring water which contained traces of organic material. His studies, though suggestive of autotrophic metabolism for *Beggiatoa* species, by no means confirmed the matter. A significant number of more recent studies have searched in vain for autotrophy among a wide variety of strains of *Beggiatoa* species (5, 8, 26–28, 33).

Keil (14) reported autotrophic growth of *Beggiatoa* sp. in pure cultures under a restricted range of environmental conditions. His cultures grew in inorganic liquid medium under reduced partial pressures of oxygen and narrow ranges of CO_2 and H_2S and only with ammonium (not nitrate) as a source of nitrogen.

More recently, Pringsheim deemed it necessary to repeat the work of his student, Keil. Kowallik and Pringsheim (16) designed and used

a liquid culture vessel which allowed generation of opposed vertical concentration gradients of oxygen and sulfide. Utilizing inorganic medium containing this unknown range of sulfide/oxygen ratios, they submitted the only report of autotrophy for *Beggiatoa* sp. within the last 50 years. There are several problems with their claim. Their definition of autotrophic growth consisted of a nonquantitative determination of growth through two transfers in inorganic, sulfide-containing medium. The inoculum was from a heterotrophic source culture, and there were apparently no controls lacking sulfide. Pringsheim later retracted the claim of autotrophy for *Beggiatoa* sp. (26, 27).

In heterotrophically grown cultures exposed to H_2S, Burton and Morita (5) observed S^0 deposition within 15 to 20 min, and Winogradsky (37) saw deposition within even a shorter time. Because of the rapidity of the appearance of S^0 granules, it has been argued that *Beggiatoa* sp. does not derive useful energy from this initial oxidation of sulfide (5). This would represent a loss of about one-fourth of the energy available from the oxidation of sulfide to sulfate.

Whether the internal S^0 granules of *Beggiatoa* sp. are further oxidized to sulfate was not clearly proven in previous studies. There are reports of S^0 disappearing slowly from filaments when the source of sulfide was removed (36, 37). However, if these filaments were continuing to grow on organic substances, the "disappearance" of S^0 could simply reflect dilution of the density of existing granules by growth rather than their oxidation. Pringsheim (26) claimed to have shown sulfate production from *Beggiatoa* cultures grown in sulfate-free medium when they were supplied with sulfide. However, because of the culture conditions employed, his test for sulfate production was equivocal.

The oxidation of sulfide to S^0 and eventually to sulfate has also been proposed as the basis of a mixotrophic metabolism in *Beggiatoa* sp. (26). The postulated effect of the inorganic oxidation in this case is to supply the cells with energy, and organic carbon compounds are presumed to be necessary as precursors of at least some cell components. The evidence in support of mixotrophy is varied and nonquantitative (8, 27, 28). An example can be found in the work of Strohl and Larkin (33). They reported that 30 of their 32 strains grew poorly on mineral medium (presumably containing agar) when acetate ranged in concentration from 0.1 to 500 mg/liter. The same medium supplemented with 300 mg of sodium sulfide per liter supported good growth of all strains. It is difficult to determine whether this type of observation supports the concept of mixotrophic utilization of reduced sulfur compounds or the postulated function discussed in the next paragraph.

In addition to the possible functions of H_2S or S^0 granules or both in mixotrophic or autotrophic metabolism, a third function of the reduced sulfur compounds has been suggested by Burton and Morita (5). They worked with a strain of *Beggiatoa* sp. which proved to be catalase negative and found that its growth in complex organic media was greatly enhanced when filter-sterilized catalase was added. A 24-fold increase in wet weight was noted when compared with a control flask containing denatured catalase. They theorized that in the absence of catalase, sulfide oxidation at localized sites of peroxide production might result in peroxide destruction and growth stimulation. Since the initial findings of Burton and Morita, all strains of *Beggiatoa* species tested have been found to be catalase negative (33). Thus, the potential of reduced sulfur compounds to substitute for catalase function becomes significant.

A number of subclones of *Beggiatoa* (*B.* cf. *leptomitiformis*) strain OH-75-B have been isolated which are capable of depositing S^0 from either thiosulfate or sulfide (20). This previously undiscovered metabolic flexibility makes it easier to explore the utilization of sulfur in *Beggiatoa* species. Compared with sulfide, thiosulfate is easier to supply in known quantities. It is not nearly so poisonous, and it does not have the undesirable property, which H_2S has, of combining rapidly and spontaneously with oxygen (12). This strain of *Beggiatoa* sp. was used to examine the ability of sulfide, thiosulfate, and the resultant S^0 granules to support mixotrophic or autotrophic growth or both. The ability of these reduced sulfur compounds to replace the beneficial effects of catalase was explored, and preliminary experiments on the possibility of anaerobic S^0 use were also undertaken.

MATERIALS AND METHODS

Throughout this study, unless otherwise noted, the strain of *Beggiatoa* sp. used was OH-75-B subclone 2a (abbreviated, clone 75-2a). The isolation and maintenance of this strain were on a thiosulfate-containing medium with only NTA (nitrilotriacetic acid, a chelator), agar (Difco Laboratories), and organic impurities in agar as possible sources of organic carbon (20). A year after the isolation of OH-75-B, a number of other clones of *Beggiatoa* sp. (OH-763-B, OH-765-B, and OH-766-B) were isolated from the same environment and in the same fashion. The most striking difference between the subclones of OH-75-B and the more recently isolated clones is the fact that only the subclones of OH-75-B deposit characteristic granules of S^0 when presented with thiosulfate as a reduced sulfur

source. Periodically, however, it was desirable to employ a control strain for experiments on thiosulfate utilization, and clone 763 was chosen.

Many of the experiments used flasks on shakers for determining growth yields and rates as previously described (20). Unless stated otherwise, this replicate flask method was employed for growing inoculation cultures as well as for performing experiments. However, when special gas mixtures or large culture volumes were desired, bubbler vessels were employed (Fig. 1). The vessel contained 400 ml of appropriately supplemented medium which was mixed by the flow of bubbles from the bottom of the vessel. Gas mixtures from one or two sources were first passed through sterile cotton filters and then admitted into a humidifying flask. If two mixtures were employed simultaneously, the relative proportions were quantified approximately by counting the bubbling rates at both in-flow tubes in the humidifier flask. Sampling was performed by use of a sterile syringe which was attached to a Luer Lock fitting at the upper end of a sampling tube. The sampling tube extended from near the bottom of the bubbler vessel through the plastic cap and terminated in the fitting. The Nalgene cap fitted loosely over the vessel, allowing gasses to escape around the edges, and the slight positive pressure was usually sufficient to prevent contamination through this gap. The whole apparatus could be autoclaved.

Some experiments required a medium lacking sulfate ions and other forms of sulfur. To that end, the following medium (medium NC) was devised by modification of "medium-C" of Sheridan and Castenholz (29): 1.00 liter of distilled water; 0.10 g of nitrilotriacetic acid; 0.50 ml of trace element solution [1.00 liter of distilled water-3.0 ml of concentrated HCl-2.0 g of $MnCl_2 \cdot 4H_2O$-0.50 g of $Zn(NO_3)_2 \cdot 6H_2O$-0.50 g of H_3BO_3-0.025 g of $CuCl_2 \cdot 2H_2O$-0.025 g of $Na_2MoO_4 \cdot 2H_2O$-0.025 g of $CoCl_2 \cdot 6H_2O$-0.025 g of $VOSO_4$]; 1.0 ml of $FeCl_3$ solution (0.29 g/liter); 0.082 g of $Ca(NO_3)_2 \cdot 4H_2O$; 0.0080 g of NaCl; 0.103 g of KNO_3; 0.69 g of $NaNO_3$; 0.111 g of Na_2HPO_4; 0.103 g of $Mg(NO_3)_2 \cdot 6H_2O$; 0.100 g of $CaCl_2 \cdot 2H_2O$; and 0.120 g of NH_4NO_3. The pH was adjusted to 7.0 with NaOH before autoclaving. This medium is essentially our medium A (20) modified by replacing sulfate salts with equivalent nitrate salts.

S^0 was extracted from cells by a modification of the procedure of Doemel and Brock (7). The method is based on the fact that S^0 can be quantitatively extracted from dried material by carbon disulfide. The details of the procedure are given below.

After the dry weight of the *Beggiatoa* material had been determined (20), filters containing known amounts of *Beggiatoa* sp. were placed in screw-capped test tubes containing 10 ml of spectral-grade CS_2. The tubes were sealed tightly with Teflon-lined caps, and the filters were extracted at room temperature for at least 12 h with vigorous shaking. The CS_2 was then removed to clean test tubes, and the filters were extracted again for 12 h with additional 5-ml volumes of CS_2. The extracts were added to the first ones, and the CS_2 was evaporated by passing a stream of filtered air over the liquid. This left a dried residue containing the extracted S^0. To assay the S^0 in a single tube, the residue was dissolved in 3 ml of ligroine (practical grade; boiling point, 90 to 120°C) with vigorous shaking for at least 12 h. The colorimetric procedure of Bartlett and Skoog (3) was then employed.

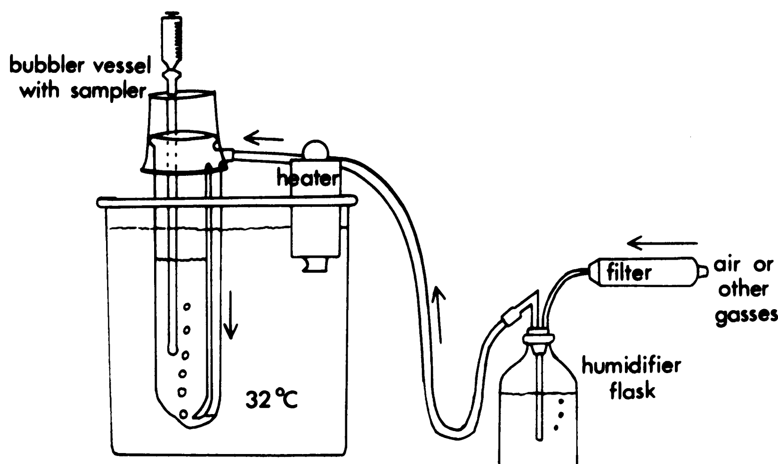


FIG. 1. *Bubbler vessel with sampling device. Culture vessel holds about 400 ml of medium. Medium was bubbled with air or other gas mixtures (arrows indicate gas flow direction). When two gasses (or gas mixtures) were employed simultaneously, a second tube into the humidifier was used. Vessel was kept at a constant temperature (usually 32°C) by suspending it in an aquarium of heated water. Apparatus was modified when rigorously anaerobic conditions were desired. A tightly fitting stopper (not shown) replaced the loosely fitting Nalgene cap. An exhaust tube was added through the stopper, and the exhaust gasses were bubbled through a sterile tube filled with a 100 mM zinc acetate-10 mM acetic acid solution. This served as a trap when testing for sulfide evolution under anaerobic conditions. The efficacy of the trap was verified by inserting a second trap in series with the first. Nitrogen gas used to establish anaerobic conditions in bubblers was first passed through a tube containing copper mesh heated to 150°C to remove traces of oxygen.*

Replicate reagent blanks were constructed by extracting empty filters and treating the extracts in the above fashion. The filters used (Nuclepore) did not contribute significantly to the value of the blank, but the CS_2 did. Different brands of spectral-grade CS_2 made significant but acceptable contributions to the value of the blank, whereas the use of reagent grade CS_2 gave excessive background values. To construct a standard curve, known amounts (in the range of 5 mg) of precipitated sulfur (Mallinckrodt, USP grade) were dissolved in 100-ml quantities of ligroine. Various dilutions of these standards were then treated by the above-described methods.

Analysis for sulfate was performed by a slight modification of the turbidometric procedure found in *Standard Methods* (2). Our procedure used 2.5 ml of a 30% (wt/vol) $BaCl_2$ solution instead of crystals. Turbidity was monitored at 420 nm in a spectrophotometer (Gilford-modified Beckman DU). The standard curve was nonlinear and could not be extended above 40 mg/liter (0.4 mM). Thiosulfate appears to interfere with sulfate analyses of both the turbidometric and gravimetric types. In addition to interfering with the above method, it was also found to interfere with the methods of van Gemerden (34) and Gleen and Quastel (9). No simple correction factor or extrapolation method could be devised to make the sulfate analysis employed trustworthy in the presence of thiosulfate. For 0.2 mM sulfate, concentrations of thiosulfate less than 0.02 mM did not interfere significantly.

Determinations of thiosulfate concentrations were made by the method of Sörbo (32). Soluble sulfide was measured by the method of Pachmayr as reported by Castenholz (6).

Incorporation of [^{14}C]bicarbonate by *Beggiatoa* sp. was determined with cells grown in medium A supplemented with 3.7 mM acetate or various combinations of acetate (0.74 to 7.4 mM) and thiosulfate (0.40 to 4.0 mM). Cells were concentrated by centrifugation and resuspended in 50 ml of medium A. This process was repeated twice more, with the final resuspension begun in sufficient medium to provide 8 ml of *Beggiatoa* mixture per incubation vial. Dry weights of the contents of replicate vials were determined, and the range for the various experiments was 5 to 50 μg/ml. Uptake experiments were performed in 11-ml screw-capped vials with the concentrations of the bicarbonate, acetate, thiosulfate, sulfide, and ascorbate solutions adjusted so that the additions to the *Beggiatoa* sp. of the various combinations tested yielded a final volume of 10 ml. The resuspended *Beggiatoa* mixture was aerated for about 10 min before apportioning it into the incubation vials. The incorporation experiments were initiated by the addition of [^{14}C]$NaHCO_3$ (20 μCi/ml, 0.100 μCi/μg; New England Nuclear Corp.) to a final specific activity of 0.20 μCi/ml. The vials were incubated at 32°C for 0.5 to 3 h, and the experiments were terminated by the addition of 0.4 ml of Formalin to each vial.

The contents of each vial were collected on a separate filter (Schleicher and Schuell, Selectron; 0.45-μm pore size), with an acid wash after the filtration step. The filters were glued to steel planchets, and when dry, the activity of each sample was counted on a thin-window, low-background, automatic planchet counter (Nuclear Chicago, model 4342 with scaler/timer). The efficiency of the counter was determined by a barium carbonate precipitation method (35) and was found to have a value of 6.70%. A standard ^{14}C polymer disk (1.3 μCi/g; Amersham Corp.) was counted with the samples from every experiment. This made it possible to correct for slight changes in counting efficiency from day to day.

The method of Vollenweider (35) was employed to convert counts per minute (corrected for efficiency of counting) to units of milligrams of carbon fixed per hour per milligram of cell (dry weight). The total CO_2 in replicate incubation mixtures (with distilled water substituted for additions of acetate) was determined by a potentiometric method (10).

Methods and materials not specifically discussed above, such as composition of other media, addition of carbon sources and catalase, initiation of growth experiments, and determinations of growth rates and yields have been described previously (20).

RESULTS

Qualitative observations: sulfur granules and growth. Clone 75-2a was capable of growth on medium A agar which contained no organic compounds other than NTA and those present in Difco agar (20). In fact, stock cultures of similar clones have been maintained on this medium at room temperature with monthly transfers for 5 years. Experiments were undertaken to determine whether clone 75-2a was growing on trace impurities in mineral-agar medium (medium A). Though not conclusive, the results suggested that this was the case (D. C. Nelson, Ph.D. thesis, University of Oregon, Eugene, 1979).

If sodium thiosulfate (0.40 or 2.0 mM) was added to medium A agar, the *Beggiatoa* filaments became filled with granules of elemental sulfur (20). That these granules were S^0 was supported by a number of observations which matched perfectly with published criteria for identifying S^0 granules (30). Evidence included: (i) the granules in *Beggiatoa* sp. were highly refractile when viewed with phase-contrast microscopy and had a dark ring around the periphery when bright-field optics were employed; (ii) the granules did not stain with Sudan Black B; (iii) they were produced only when the cells were exposed to sulfide or thiosulfate; and (iv) they could be extracted and recrystallized from wet cells with pyridine.

These S^0 granules did not reach their maximum concentration inside the cells until the agar surface was completely covered by *Beggiatoa* sp.; this occurred about 1 week after an agar plate was inoculated with a central streak. The S^0 granules seemed to remain at about the same size and frequency as long as the filaments re-

mained intact on the agar medium (4 to 6 additional weeks). When viewed macroscopically, growth on medium A agar supplemented with thiosulfate appeared more luxuriant than growth on the equivalent medium lacking thiosulfate. However, when viewed at ×30 on a trans-illuminated dissecting microscope, both types of plates appeared to contain about the same density of filaments. The illusion of greater growth on the plates containing thiosulfate was apparently due to the increased opacity of the filaments containing S^0.

Yield increment due to sulfur deposition. Clone 75-2a behaved like a classical *Beggiatoa* sp. strain, depositing granules of S^0 when supplied with soluble sulfide in the presence of air (Fig. 2). For this experiment, 250 ml of stationary-phase culture grown on 3.7 mM acetate was concentrated about 10-fold by centrifugation and added to approximately 400 ml of medium A contained in a bubbler vessel. A sterile sample of a sodium sulfide solution was added to the vessel at h 17, and the concentration of sulfide in the vessel was monitored at frequent intervals thereafter. Total filterable dry weight and the percentage of that dry weight represented by S^0 were also measured over the course of the experiment. Microscopic examination showed that sulfide addition caused S^0 to be deposited both within and outside the cells, but it also caused significant lysis (Fig. 2). To quantify the percentage of cell weight which was attributable to "internal" S^0 granules in this experiment, it was necessary to take advantage of the habit of *Beggiatoa* sp. of growing in tufts several millimeters in diameter (20). These tufts were taken up in a Pasteur pipette and passed through several rinses of liquid medium to free them from external S^0 granules. The percentage of S^0 contained in these tufts was then determined in the usual manner. At a maximum, S^0 represented more than 20% of the total cell weight.

In contrast to the above results, when clone 75-2a was grown in medium containing thiosulfate and acetate, S^0 granules were found within the cells almost exclusively (except in senescent cultures). When grown in liquid medium A containing 3.7 mM acetate and 2.0 mM thiosulfate, this clone deposited internal granules of S^0 which accounted for 21% of the dry weight in a stationary-phase culture. When this S^0 increment was subtracted, the remaining dry weight compared very closely with a yield of 81.5 mg/liter for growth on the same medium (3.7 mM acetate) without thiosulfate. These data are presented along with comparable data for yields on (i) a different concentration of acetate with and without thiosulfate and (ii) acetate plus malate with and without thiosulfate (Table 1). For the three organic substrate-thiosulfate combinations, the percentage of dry weight attributable to S^0 ranged from 21 to 27%. In all cases, the observed yield on the organic substrate alone compared very well with the yield on the same substrate(s) plus thiosulfate if the S^0 contribution was subtracted.

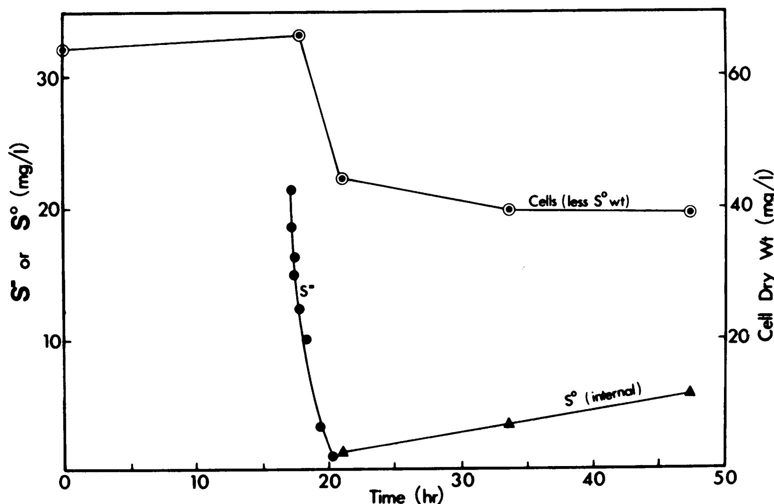


FIG. 2. *Deposition of internal S^0 granules from soluble sulfide. Beggiatoa sp. used was grown in medium A containing 3.7 mM acetate, concentrated by centrifugation, and resuspended in 400 ml of medium A (lacking acetate) in a bubbler vessel. Sterile line air was used in the experiment. Sulfide was added at h 17 from a sterile stock of sodium sulfide. Open circles, dry weight of cells after subtracting weight of total S^0. Solid circles, sulfide concentration. Triangles, S^0 determined to be internal to the cells by methods discussed in the text. All values represent averages of duplicate samples.*

TABLE 1. *Yields in organic media with and without thiosulfate and determination of S° contributions to yields*

Substrate	Thiosulfate concn	Yield[a]			
		Substrate + thiosulfate (mg/liter)	% Due to S°	Substrate + thiosulfate − S° wt (mg/liter)	Substrate without thiosulfate (mg/liter)[b]
0.735 mM acetate	0.403 mM	21.9 (±3.0)	22	17.0 (±12)	17.1 (±3.2)
3.68 mM acetate	2.02	101 (±1.4)	21	80.0 (±1.4)	81.5 (±1.6)
3.68 mM acetate + 1.86 mM L-malate	2.02	190 (±32)	27	139 (±11)	137 (±17)

[a] Yield (± confidence limit) is given.
[b] Separate control flasks lacking thiosulfate.

A number of other organic compounds were tested with and without thiosulfate to determine whether thiosulfate would significantly promote growth in cases where the organic compound alone was insufficient. For these experiments, no quantification was made of the S^0 present in yields with thiosulfate media. However, the increase in dry weight due to the presence of thiosulfate always showed a value close to 20% (Table 2), which was about the value assignable to S^0 when this increment was directly measured. This same percentage S^0 increment appears to occur even in instances in which the organic compound tested was not capable of supporting growth. In these cases, "yield" may represent the result of growth on organic compounds carried over, in the medium or as internal stores, from growth on medium B agar (two transfers earlier), or it may represent utilization of the organic chelator, NTA (see below).

Time course of S^0 deposition: acetate plus thiosulfate. The time course of S^0 deposition accompanying growth was studied in further detail in medium containing acetate and thiosulfate. Several experiments, performed with thiosulfate and acetate supplemented medium NC, showed a general increase in S^0 and sulfate and a decrease in thiosulfate. In those experiments, the concentration of thiosulfate was adjusted so that all of that compound was consumed by *Beggiatoa* sp. This adjustment was made to facilitate measurements of the sulfate concentrations at the ends of the experiments by minimizing the interference due to excess thiosulfate. The results of one such experiment with medium containing 3.7 mM acetate and 0.68 mM thiosulfate are shown (Fig. 3). It appears that the S^0 deposition and thiosulfate consumption did not commence until growth had almost ceased. A more detailed study of the exponential growth phase confirmed that there was virtually no S^0 deposition during exponential growth of clone 75-2a on acetate plus thiosulfate medium (Nelson, Ph.D. thesis).

The value of the sulfate concentration measured shortly before thiosulfate disappearance is fairly reliable. Given such a value and a second reliable determination of the sulfate concentration made soon after the thiosulfate concentration became immeasurably low, it is possible to determine the concentration of sulfate when the thiosulfate had first disappeared. This interpolation was aided by the fact that the thiosulfate decrease was linear with time (Fig. 3). Since the initial concentration of sulfate in medium NC was undetectably low, all of the sulfate measured in this experiment was presumably a result of the metabolic activity of *Beggiatoa* sp.

A number of similar experiments were performed at different initial concentrations of thiosulfate. When the resultant concentrations of sulfate corresponding to the points where thiosulfate had just disappeared were plotted against the initial concentration of thiosulfate, it was clear that there was essentially a 1:1 molar relationship between thiosulfate consumed and sulfate produced (Fig. 4). On the other hand, concentrations of S^0 at the time of thiosulfate exhaustion were only slightly more than one-half of the initial molar concentrations of thiosulfate. The stoichiometric relationship between that concentration of S^0 and the initial concentration of thiosulfate also seems more variable than the ratio of sulfate produced to thiosulfate consumed.

There is evidence (Fig. 3) that after the concentration of thiosulfate had become zero, the quantity of S^0 in the system decreased and the quantity of sulfate continued to increase. The interpretation of these results (especially for the decrease in measurable S^0) is confounded by the fact that the total dry weight in the experiment also decreased (possibly by lysis). To explore this matter further, cells were grown to stationary phase in medium NC containing 3.7 mM acetate and 0.81 mM thiosulfate and then con-

TABLE 2. *Effect of thiosulfate on various substrate yields*

Substrate	Supports growth alone	Yield (mg/liter)[a] on: Substrate	Substrate + 2 mM thiosulfate	Estimation of S^0 contribution to yield (%)[b]
1.0 g of YE per liter[c]	Yes	12.2 (±0.9)[d]	17.6 (±2.0)	31
1.5 g of YE per liter	Yes	14.7 (±0.8)[e]	20.0 (±2.4)[d]	26
0.74 mM acetate	Yes	17.3 (±0.2)	21.2 (±1.7)	18
4.2 mM succinate	No	5.3 (±1.0)	6.9 (±2.2)	23
Same	No	4.1 (±0.8)	5.1 (±2.7)	20
6.6 mM glycolate	No	1.7 (±3.3)	2.0 (±0.9)	17
3.4 mM glutamate	No	2.6 (±0.4)	3.2 (±1.6)	19
10.9 mM formate	No	2.2 (±0.9)	2.7 (±1.1)	17

[a] Yield (± confidence limit) is given.
[b] Calculated as (column 4 − column 3)/column 4.
[c] YE, Difco yeast extract.
[d] Average of three experiments.
[e] Average of seven experiments.

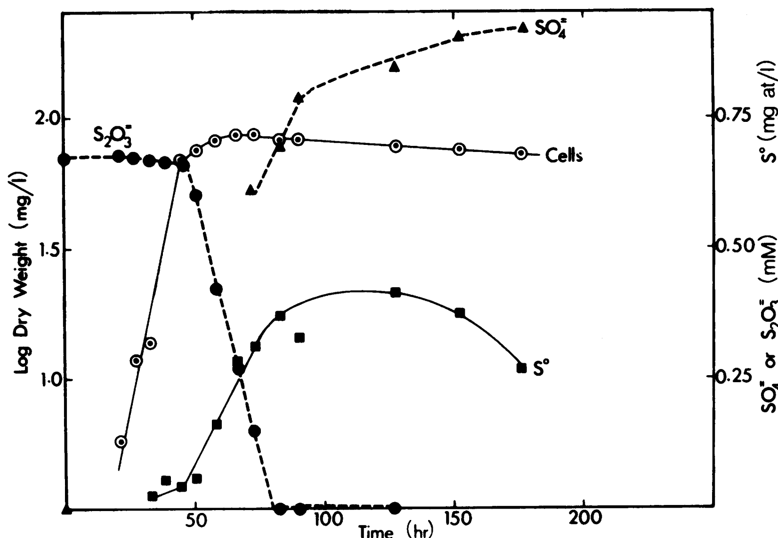


FIG. 3. *Time course of growth, consumption of thiosulfate, and production of S^0 and sulfate. Medium NC supplemented with 3.7 mM acetate and 0.68 mM thiosulfate was employed in a vessel bubbled with sterile line air. Sulfate could not be determined until thiosulfate was almost consumed (see text). All data points represent single values.*

centrated aseptically by centrifugation to remove unconsumed traces of thiosulfate. The cells were resuspended in a bubbler vessel containing medium NC with no additional organic compounds or thiosulfate. Dry weight, sulfate, and S^0 were then monitored over time (Fig. 5). Dry weight appeared to have declined by about 20% over the course of 5 days in that experiment, and S^0 declined in parallel fashion so that the percentage of dry weight attributable to S^0 remained steady or increased slightly. As shown in Fig. 3, the increase in sulfate concentration over the course of the experiment was more than sufficient to account for the S^0 decrease.

Attempts to demonstrate autotrophy. A number of additions were made to medium A under aerobic and semiaerobic conditions in attempts to demonstrate any hidden autotrophic abilities of clone 75-2a. Various combinations of thiosulfate, catalase, vitamins, and bicarbonate were used in medium A, usually with control experiments lacking thiosulfate. The results of these studies argue against autotrophic growth of clone 75-2a (Table 3). As may be seen from these experimental summaries, very small yields obtained under these conditions were probably due to the presence of the organic chelator, NTA, in the medium. NTA supports the growth of some bacteria (11). Any yield increments due to the addition of thiosulfate or catalase were

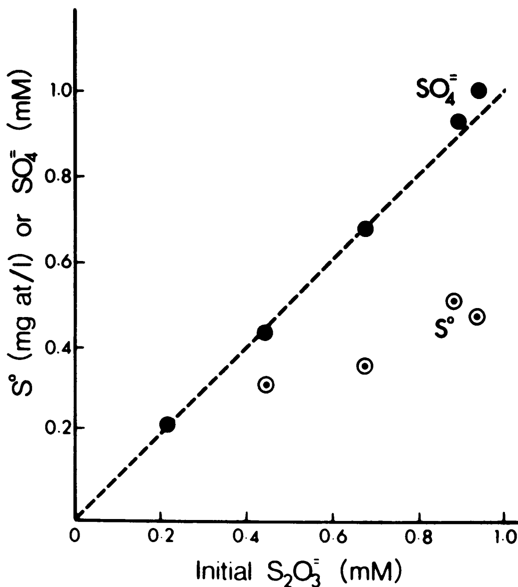


FIG. 4. *Relationship between S^0 and sulfate produced and thiosulfate consumed by Beggiatoa sp. Results are from experiment of Fig. 3 and similar time course experiments with different initial concentrations of thiosulfate. S^0 and sulfate were determined at the time when thiosulfate had just disappeared. Dashed line represents a 1:1 molar ratio between sulfur species consumed and produced.*

entirely consistent with enhancement of yield by impurities in catalase (20) or by the contribution of S^0 dry weight as noted earlier or both.

In spite of the fact that autotrophic growth could not be demonstrated from growth yield data, [^{14}C]bicarbonate was used under a variety of conditions to determine the amount of carbon "fixed" per hour per milligram of *Beggiatoa* sp. This figure was then converted to the percentage of existing cell carbon fixed from inorganic carbon per hour (Table 4), with the assumption that about 50% of the dry weight of cells is carbon (19). The maximum hourly increment of 1.7% came from an experiment with exponential-phase cells grown in a medium containing 0.74 mM acetate and 4.0 mM thiosulfate. For that experiment, the hourly increment was essentially maximal, regardless of whether thiosulfate, acetate, or nothing was added to the incubation vial, but was severely depressed for aerated cells in the presence of sulfide. Experiments with stationary-phase cells always gave values of less than one-half of the maximum value for log-phase cells. Maximum uptake for stationary-phase cells always occurred in incubation vials supplemented with acetate.

As a control, clone 763 (a strain of *Beggiatoa* sp. which deposits S^0 from sulfide but not from thiosulfate) was tested for [^{14}C]bicarbonate uptake. Cells from log phase, grown in medium A plus 3.7 mM acetate, gave a maximum hourly increment from inorganic carbon of 1.6% with the same concentration of acetate. Values in unsupplemented medium or medium containing only thiosulfate were only slightly less, but as before, uptake in the presence of sulfide was severely depressed.

For clone 75-2a, a number of incubation conditions were examined in time course experiments to determine whether ^{14}C uptake was linear with time. In some cases, saturation of incorporation occurred within 2 h. A cause of saturation could not always be determined because the number of controls was limited for a given experiment; however, whenever such an assignment could be made, saturation of ^{14}C incorporation correlated with the depletion or lack of acetate.

Anaerobic metabolism and the reduction of S^0. In preliminary experiments, *Beggiatoa* sp. was grown aerobically under conditions which caused the cells to fill with S^0 granules (acetate and thiosulfate present), and the effects of subsequent transfer of these cells to anaerobic conditions were studied. Large quantities of cells transferred to rigorously anaerobic conditions in bubbler vessels containing medium A supplemented with 3.7 mM acetate increased in dry weight by a factor of 2.5, an increase which was about one-fourth of that which would have resulted from aerobic growth on the same amount of acetate. This dry weight increase was fairly continuous over a 5-day period at 32°C. Controls lacking acetate showed significant lysis and a loss in dry weight over the same 5-day period. S^0-filled cells incubated anaerobically in acetate-containing medium appeared to loose much of the stored sulfur by day 5. Concomitantly, sulfide was produced and was collected by a zinc acetate trap connected to the exhaust hose from the bubbler vessel. The amount measured accounted for about 20% of all of the S^0 present in the cells at the start of the anaerobic incubation.

In experiments with S^0-loaded cells in acetate-containing medium, increases in dry weight did not occur beyond about 5 days under anaerobic conditions since cell lysis then became pronounced. *Beggiatoa* sp. lacking S^0 granules showed some gain in dry weight when transferred into anaerobic conditions in acetate-containing medium, but the yield increment was only about one-half as great as that of S^0-containing cells incubated under similar conditions (unpublished data).

Reduced sulfur compounds and catalase. It has been suggested (5) that sulfide or S^0 oxidations or both served a function in *Beggiatoa* sp. similar to that of the enzyme catalase—

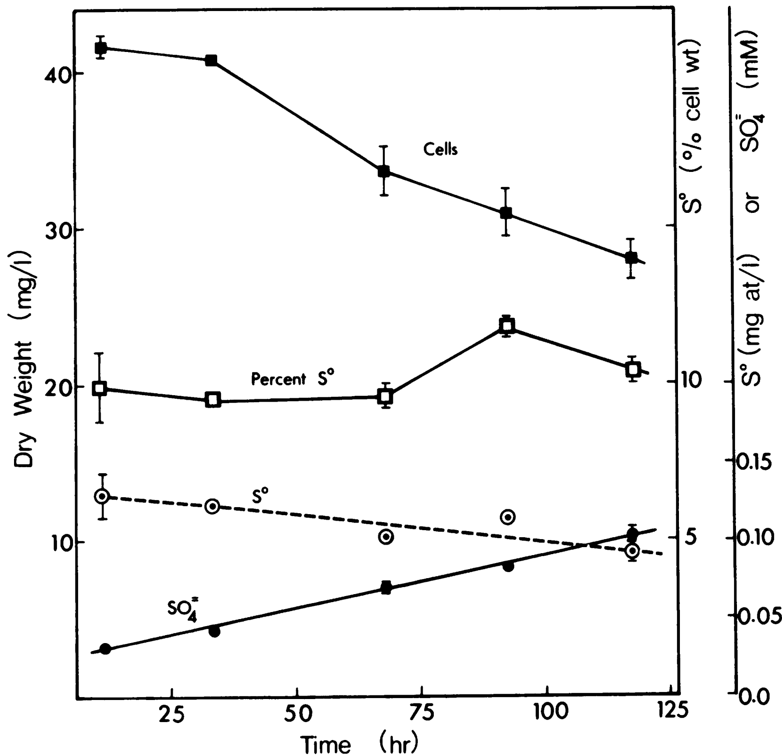


FIG. 5. *Time course of S^0 consumption, sulfate production, and dry weight loss in a stationary-phase culture. Cells were grown in medium NC containing 3.7 mM acetate and 0.81 mM thiosulfate, centrifuged, and resuspended in a bubbler vessel containing medium NC (lacking organic and sulfur compounds). The vessel was bubbled with sterile line air. Dry weight of cells includes weight attributable to S^0. S^0 represents total elemental sulfur collected on the filter; however, at first two sample times, internal S^0 was also determined and found to account for all of the S^0. Error bars represent the range of duplicate determinations. Where no bars are visible, range was less than width of data symbol.*

decomposition of harmful peroxides. In testing this with clone 75-2a, it was discovered that catalase itself probably had no influence on growth yields in a variety of media tested (20). However, in certain media, catalase greatly decreased the growth lag and possibly increased growth rate. In medium A with 1.5 g of yeast extract per liter, the addition of catalase (20 Sigma units per ml [20]) to medium inoculated with blended filaments of *Beggiatoa* sp. at about 1% of their final yield resulted in full yields much more rapidly than occurred in cultures containing no catalase. Thiosulfate (2.0 mM) reduced the lag almost as much (Fig. 6). It was clear that catalase or thiosulfate greatly reduced the lag time for growth when these small inocula were used. The exponential growth rates also seemed slightly higher (Nelson, Ph.D. thesis).

If the inoculum for a similar experiment was grown for several days on medium containing thiosulfate in addition to yeast extract, the inoculum contained cells full of S^0 and probable traces of thiosulfate. Much of the lag for growth on medium A plus 0.15% yeast extract (control flasks) was then eliminated (Table 5). Even if the inoculation culture was rinsed twice in sterile medium A to remove the traces of thiosulfate, most of the lag was still eliminated when growth took place on yeast extract medium lacking thiosulfate and catalase (Table 5). Finally, the addition of both thiosulfate and catalase to medium containing 0.15% yeast extract produced growth patterns identical to those of catalase addition alone.

Clone 75-2a, growing on yeast extract and thiosulfate, deposited S^0 during the exponential phase at a rate parallel to growth (Nelson, Ph.D. thesis). This is in contrast to medium containing acetate and thiosulfate, in which no S^0 deposition occurred until growth had essentially ceased (Fig. 3). Unlike the experiments with yeast extract, the growth pattern from small inocula in acetate-containing medium was unaffected by catalase or thiosulfate (Table 5).

TABLE 3. *Summary of experiments exploring autotrophic possibilities of clone 75-2a*

Vessels	Medium tested	Gasses	Control medium	Results
Flasks	A + $S_2O_3^{2-}$ (0.4 and 4 mM)	Air	A	Yield poor. No difference except as attributable to S° weight.
Flasks	A + 2 mM $S_2O_3^{-2}$	Air	A	Yields poor, but does grow up to level of previous transfer from 1% inoculum through four serial transfers. Yields in controls about the same as with thiosulfate.
Flasks	A (without NTA)[a] + 2 mM S_2O_3	Air	None	No filaments found after four serial transfers with 1% inoculum.
Flasks	A + additional NTA	Air	A	Yields poor, but increment due to NTA addition.
Flasks	A + 0.7 mM bicarbonate + 2 mM $S_2O_3^{2-}$	Air	Test without $S_2O_3^{2-}$	Yields poor. No differences except as attributable to S° weight increment.
Flasks	A + 2 mM $S_2O_3^{2-}$ + catalase (20 Sigma units per ml)	Air	A	Yields poor. Differences slight and consistent with S° deposition and catalase impurities (20).
Bubblers	A + $S_2O_3^{2-}$ (2 and 10 mM) + 10 mM bicarbonate	Air-CO_2 (99:1)	None	No growth.
Bubblers	A + 2 mM $S_2O_3^{2-}$ + 10 mM bicarbonate + vitamins[b] + catalase (40 Sigma units per ml)	N_2-air-CO_2 (89:10:1)	Test without $S_2O_3^{2-}$	Yields poor. Vessel with thiosulfate had typical 20% more *Beggiatoa* sp. weight, probably assignable to S°.

[a] Medium A (without NTA) requires that D medium stock (20) be made omitting NTA, sodium chloride, and calcium sulfate. Using this stock, proceed as for making medium A but add calcium chloride and sodium chloride from separate sterile stocks after the medium is autoclaved.

[b] Several vitamins were added according to Scotten and Stokes (28). In addition, B_{12} (0.5 mg/ml) and thiamine (0.0025 mg/ml) were added.

TABLE 4. *Maximum hourly increment in cell carbon attributable to [^{14}C]bicarbonate uptake for various incubation conditions (temp = 32°C)*

Growth phase of incubated cells[a]	Increment (%) in cell carbon with additions to experiment vials							
	None	2 mM $S_2O_3^{2-}$	10 mM S_2O_3	Acetate		2.0 mM $S_2O_3^{2-}$ + 3.7 mM acetate	Sulfide	
				3.7 mM	18 mM		0.062 mM	0.31 mM
Exponential	1.6	1.7		1.4			0.44	0.22
Stationary	0.20	0.25	0.37	0.70	0.59	0.66	0.25	0.18

[a] Incubated cells were recently harvested from growth on medium A containing various concentations of acetate (with and without thiosulfate). Cells had been grown at 32°C. The entries in the table represent the highest values from a number of experiments using cells grown on different combinations of acetate and thiosulfate.

DISCUSSION

The model shown below accounts for the generation of S^0 and sulfate from thiosulfate, with the oxidation of one of the sulfur atoms linked to the reduction of the other. It is the simplest model consistent with the stoichiometries between thiosulfate consumed, sulfate produced, and S^0 granules produced, assuming the involvment of oxygen. Similar mechanisms for thio-

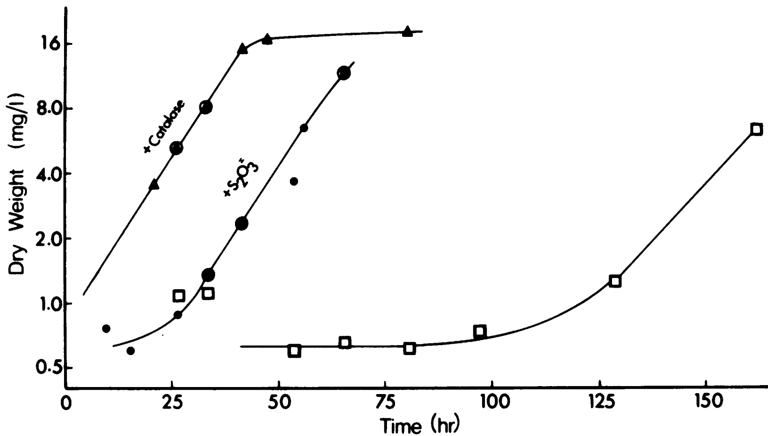
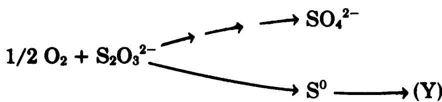


FIG. 6. *Effect of thiosulfate or catalase on yeast extract-supported growth. Experiment was initiated with small, equal inocula of Beggiatoa sp. in replicate flasks. Inoculation culture was grown in medium A supplemented with 1.5 g of yeast extract per liter. Experiments used the same medium unsupplemented (squares) or supplemented with 2.0 mM thiosulfate (circles) or 20 Sigma units of catalase per ml (triangles). A circle around a data symbol indicates that duplicate flasks were harvested. All other points represent single values.*

sulfate utilization have been proposed for *Thiobacillus* sp. (1, 15) and for *Chromatium* sp. (31).



There are undoubtedly a number of intermediates between thiosulfate and sulfate, as indicated by the multiple arrows. However, the 1:1 molar relationship between sulfate produced and thiosulfate consumed (Fig. 4) implies that these intervening reactions are rapid and without substantial accumulation of intermediates. On the other hand, the smaller proportional representation of S^0 in the same figure indicates a significant accumulation of (Y) to account for the deficiencies. If the above model is correct, (Y) cannot represent sulfate, but it may represent an intermediate in the conversion of S^0 to sulfate. (Y) may also represent a form of S^0 which is not retained by the filtration step (0.2-μm pore size) which precedes quantification. There are two lines of evidence supporting the idea that some S^0 could have gone undetected in this analysis. (i) newly formed sulfur granules are small enough that, if released from cells, they could pass through such a filter. (ii) Pfennig (23) cites evidence that biologically produced S^0 can exist in a "soluble form" without the appearance of S^0 granules.

It would appear that regardless of whether (Y) represents lost S^0 or some other chemical species, a significant amount of S^0 is eventually converted to sulfate as well (Fig. 3 and 5). Significant cell lysis in those experiments, however, made it difficult to determine whether the oxidation of S^0 was biologically mediated.

Energetically, the deposition of S^0 from thiosulfate is not likely to be a costly process. Since growth yields on media containing organic substrates and thiosulfate (when corrected for the weight of the S^0) were identical to yields on the corresponding organic controls (Table 1), the energy involved in S^0 deposition must be negligible.

Autotrophy. It should be noted that the results of Keil (14) are the only data which seriously support the contention that *Beggiatoa* sp. can grow autotrophically. His assertion that *Beggiatoa* sp. would grow autotrophically in the presence of ammonium but not nitrate ions is curious, since no other culture of *Beggiatoa* sp. grown on a defined carbon source has been found with such a requirement, but this does not invalidate his results. The findings of Winogradsky (36, 37) and Kowallik and Pringsheim (16), because of the flaws already discussed, do little to prove that *Beggiatoa* sp. is a potential autotroph.

There is no evidence in this study to support the notion that clone 75-2a is capable of autotrophic growth, using reduced sulfur compounds. There is considerable evidence against such a possibility, including the results in all of the ^{14}C uptake experiments (Table 4) and the evidence discussed below. However, there are several reasons why the general question of *Beggiatoa* autotrophy must be left open. First, the

TABLE 5. *Relative ability of S^0 and thiosulfate to mimic catalase relief of time lags in yeast extract-supported growth*[a]

Medium of inoculation culture	Growth lag (h)	
	With added thiosulfate (2.0 mM)[b]	In control flasks[c]
A + 0.15% yeast extract	26	128
A + 0.15% yeast extract + 2.0 mM thiosulfate	7	12
A + 0.15% yeast extract + 2.0 mM thiosulfate (rinsed)[d]	7	27
A + 3.7 mM acetate	0	0

[a] The first row of entries are from the experiment shown in Fig. 6. The rest of the information is from three related experiments.

[b] Lag is measured relative to flasks containing medium A, catalase (20 Sigma units per ml), and the same organic carbon source and concentration as the inoculation culture. Catalase-stimulated growth requires a certain amount of time to reach a dry weight value equal to 25% of its maximum. Lag is defined as the additional time it took for cultures in which the catalase had been eliminated or replaced by thiosulfate to reach that same yield. The 25% value was chosen because the cells for all parts of the four experiments were in exponential growth phase at that point.

[c] Control flasks contained the same organic compound as the inoculating culture (at the same concentration) but lacked thiosulfate and catalase.

[d] Experiment identical to that of previous row except that the inoculation culture was twice centrifuged and rinsed in sterile medium A just before the replicate flasks were inoculated.

experiments of Keil (14) contain a strong, unstated note of caution, since he claimed autotrophic growth for his strain only under a very restricted and undefined range of gas mixtures. None of the more recent studies (5, 8, 26–28, 33) showing negative results were as thorough as Keil's in exploring the ranges of environmental parameters. Their negative results may be explained by postulating that their strains were perhaps capable of facultative autotrophy but not under the conditions employed. A second reasonable explanation for the failure of all recent studies to detect autotrophic growth in *Beggiatoa* sp. is that there may be distinct autotrophic and heterotrophic strains (5). The appearance of only the latter type in all recent studies would be reasonable since these strains were enriched for and/or isolated on organic media. The clone employed in this study was isolated on a medium (20) which contained thiosulfate and very low concentrations of organic compounds (traces in the agar, plus the chelator, NTA). Thus, clone 75-2a was isolated under conditions which were probably more selective for autotrophic *Beggiatoa* sp. than any other culture conditions employed within the last 50 years.

The [^{14}C]bicarbonate incorporation data for clone 75-2a seem to argue against an autotrophic metabolism. The maximum uptake rate recorded (1.7% cell carbon added per h) corresponds to a doubling time of about 41 h. That rate is not far out of line with the observation that enrichment cultures of "autotrophically" grown *Beggiatoa* sp. had doubling time of approximately 1 day (36). However, incorporation rates very close to 1.7% per h were also obtained with clone 763 or clone 75-2a grown and tested in acetate medium lacking a reduced sulfur source. The maximal rates of bicarbonate incorporation with clone 75-2a were only observed in cells which had been in exponential growth phase on acetate medium just before the uptake experiments were initiated.

Stationary-phase cells, full of S^0 and with their organic substrates exhausted, are cells which one might expect to show autotrophic growth. These cells, however, invariably showed lower incorporation rates than did rapidly growing cells, and the rates again appeared to be dependent on the presence of acetate in the medium. Cells showing maximum [^{14}C]bicarbonate uptake rates (1.7% per h) had, just before those experiments, been growing with a doubling time of 7 h (10.5% per h) in acetate medium (32°C). Thus, [^{14}C]bicarbonate uptake represents, at maximum, about 16% of the total carbon incorporated into the cells, and this uptake appears to be linked to normal heterotrophic metabolism. This rate of fixation in heterotrophically grown *Beggiatoa* sp. is not surprising since a significant number of CO_2 incorporation reactions are known in obligate heterotrophs (38). Such reactions are not autotrophic since the necessary energy and reductants come from organic compounds.

Mixotrophy. Our findings argue also against mixotrophy in clone 75-2a. The strongest evidence is that no combination of organic substrates and thiosulfate could be found which resulted in enhancement of the yield beyond that accounted for by the additional weight of S^0 (Tables 1 and 2). In this study, visual estimates of the yields of S^0-containing filaments frequently gave evidence of mixotrophy. However, after quantitative comparisons with controls, the evidence always vanished. *Beggiatoa*

filaments containing S^0 are much more opaque and the tufts are "fluffier," producing illusions of greater quantity. Thus, previous reports of increased yields on organic media in the presence of sulfide (all made on the basis of visual estimates) are open to question.

Mixotrophy of *Beggiatoa* sp. is invoked most commonly to explain an apparent enhancement by sulfide of yields with acetate (26, 33). All tests for autotrophic growth of those particular strains were negative. It is difficult to imagine that these reportedly mixotrophic strains are capable of providing a substantial portion of cell carbon by autotrophic means while still maintaining a need for an external source of acetate. Therefore, it is reasonable to assume that any mixotrophy involved would be litho-heterotrophy. In such mixotrophic growth, the increase in yield by the action of a reduced sulfur compound should be at most twofold. Cells growing heterotrophically on organic substrates apportion only about 40% of the organic carbon toward energy generation (21). Hence, replacing organic energy generation with an inorganic source would "free" enough organic carbon to almost double the growth yield. With that in mind, and given the problems in visualizing *Beggiatoa* abundance, a quantitative approach is indispensable if mixotrophy is to be proven for *Beggiatoa* sp. When sulfide was added to a medium containing low concentrations of acetate, great apparent increases in growth yield were reported (26, 33). If these increases really do represent more than a twofold increase, some explanation other than mixotrophy must be evoked.

Possible role of S^0 in anaerobic environments. It is clear that some process dependent on organic compounds (such as acetate) allows *Beggiatoa* sp. to survive and increase in dry weight for several days under rigorously anaerobic conditions. As noted, if cells were loaded with S^0 before that anaerobic incubation, significant S^0 loss and production of sulfide were detected. Anaerobic respiration, utilizing S^0 as an electron acceptor, has been demonstrated for other bacterial types (4). Whether this anaerobic sulfide production has metabolic significance for *Beggiatoa* sp. remains to be demonstrated. For an organism, such as *Beggiatoa* sp., which is apparently forced into an anaerobic environment for a portion of every day by a negative light response (D. C. Nelson and R. W. Castenholz, manuscript in preparation), the ability to utilize S^0 for respiration would prove extremely valuable. Our experiments suggest that *Beggiatoa* sp. can store S^0 in quantities which cannot be exhausted by even several consecutive days of anaerobic reduction. Field and laboratory observations also suggest that *Beggiatoa* sp. spends a portion of every 24 h in aerobic conditions which may allow S^0 replenishment.

Catalase substitute role. Prior quantitative demonstrations of benefits of reduced sulfur compounds to *Beggiatoa* sp. are lacking. The evidence here that thiosulfate or S^0 can almost completely replace the beneficial effect of catalase is the first such demonstration (Fig. 6 and Table 5). That peroxides are actually contained, or produced, in medium A supplemented with yeast extract has not been demonstrated spectrophotometrically because other compounds in the medium absorb strongly in the same region of the ultraviolet spectrum. Burton and Morita (5) utilized a different yeast extract-containing medium with *Beggiatoa* sp. and determined that the concentration of peroxide-like substances increased in the absence, but not in the presence, of active catalase. Apparently, they did not test soluble sulfide or thiosulfate in this fashion, even though they postulated that sulfide could serve the same function as catalase. Their findings and those of others (25) argue that the peroxide-like substances are products of *Beggiatoa* metabolism in yeast extract medium. Since these peroxides seem to represent a case of autotoxicity rather than simply a component of the medium itself, the adaptive significance of detoxification via reduced sulfur compounds in complex media is emphasized.

Spectrophotometric evidence has been obtained which shows that soluble sulfide can react non-biologically with hydrogen peroxide (Nelson, unpublished data). This is in accordance with the assertion that sulfide can react nonenzymatically with peroxides (17), and this speculation has been carried a step further. Burton and Morita (5) believed that peroxides produced in localized areas of oxidation could react with soluble sulfide and result in the formation of S^0 granules. On the other hand, Kuenen reasoned that it was likely that enzymes were necessary for any potential interaction of peroxides with S^0 granules (17). Data presented here strongly support the idea that S^0 and peroxides can interact.

With yeast extract medium, S^0 granules were deposited during the exponential phase of growth, and they appeared to provide protection against peroxides during that phase (Fig. 6 and Table 5). *Beggiatoa* sp. in exponential growth phase in medium A containing 3.7 mM acetate is catalase negative (20). Since catalase did not enhance growth on this medium (Table 5), it appears that peroxides are not formed during exponential growth with acetate. Perhaps significantly, no thiosulfate oxidation (and hence no S^0 deposition or oxidation) occurs during exponential growth on medium containing 3.7 mM acetate and 0.68 mM thiosulfate (Fig. 3).

This lack of S^0 deposition, in conjunction with the apparent lack of peroxide production, supports the idea that S^0 deposition may be regulated by some process related to peroxide formation. This, however, leaves unanswered the question of why S^0 is deposited after cessation of growth on acetate. Perhaps, some metabolic change accompanying the end of exponential growth (such as the oxidation of poly-β-hydroxybutyrate granules) results in the production of peroxides, which in turn triggers the deposition of S^0 granules.

Finally, why do cells in yeast extract medium, lacking catalase, thiosulfate, and S^0 (Fig. 6), eventually begin exponential growth? For many hours there was no growth at all, but once a few very small tufts had formed, growth proceeded rapidly. The microenvironment inside a tuft is probably quite different from that in the rest of the culture, and this more anaerobic environment would provide some protection against peroxides or peroxide formation. If true, the long growth lag in these control flasks would represent the time necessary for the random encounters that eventually produce aggregations large enough to protect the majority of the filaments within them. This hypothesis is in concordance with the other available data. The controls in medium A plus 0.15% yeast extract, when inoculated with filaments containing S^0 granules, showed a much shorter lag than those lacking S^0 (Table 5). The amount of S^0 in the filaments presumably afforded protection sufficient to allow some growth and tuft formation before exhaustion of these internal stores. At that point, it is again postulated that the environment of the tufts provided protection against peroxides. The significance of aggregations of bacteria as an adaptation for dealing with oxygen toxicity has been commented on previously (24).

Beggiatoa filaments are frequently found singly as well as in large aggregations in the field (13). The possible protective function of the tufts, therefore, does not rule out an additional need for protection of single filaments by reduced sulfur compounds. It has been stated that the development of protection against toxic effects of oxygen (such as peroxide reactions) was a necessary evolutionary step for all organisms in order to live in an aerobic environment (24). It is possible that two of the most interesting features of *Beggiatoa*—the ability to metabolize reduced sulfur compounds and the tendency to grow in tufts—may represent unique ways of dealing with oxygen and its products.

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