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Growth of the Facultative Anaerobe *Shewanella putrefaciens* by Elemental Sulfur Reduction

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The growth of bacteria by dissimilatory elemental sulfur reduction is generally associated with obligate anaerobes and thermophiles in particular. Here we describe the sulfur-dependent growth of the facultatively anaerobic mesophile *Shewanella putrefaciens*. Six of nine representative *S. putrefaciens* isolates from a variety of environments proved able to grow by sulfur reduction, and strain MR-1 was chosen for further study. Growth was monitored in a minimal medium (usually with 0.05% Casamino Acids added as a growth stimulant) containing 30 mM lactate and limiting concentrations of elemental sulfur. When mechanisms were provided for the removal of the metabolic end product, H_2S, measurable growth was obtained at sulfur concentrations of from 2 to 30 mM. Initial doubling times were ca. 1.5 h and substrate independent over the range of sulfur concentrations tested. In the cultures with the highest sulfur concentrations, cell numbers increased by greater than 400-fold after 48 h, reaching a maximum density of 6.8×10^8 cells · ml^{-1}. Yields were determined as total cell carbon and ranged from 1.7 to 5.9 g of C · mol of S^0 $consumed^{-1}$ in the presence of the amino acid supplement and from 0.9 to 3.4 g of C · mol of S^{0-1} in its absence. Several lines of evidence indicate that cell-to-sulfur contact is not required for growth. Approaches for the culture of sulfur-metabolizing bacteria and potential ecological implications of sulfur reduction in *Shewanella*-like heterotrophs are discussed.

That certain microbes reduce elemental sulfur to H_2S has been known for many years (50), yet generally, this metabolism has been considered to be of dubious physiological or environmental importance (46). The first generic description of a dissimilatory sulfur-reducing bacterium appeared in 1976 with that of the obligately anaerobic mesophile *Desulfuromonas acetoxidans* (43). Numerous sulfur-reducing isolates have been characterized subsequently, and while phylogenetically diverse, virtually all have proven to be obligate anaerobes (1, 47, 58). Sulfur reduction is a unifying characteristic of the hyperthermophilic archaea (1, 47), which together with certain methanogens (1, 52) constitute the archaeal sulfur reducers. Eubacterial representatives include at least one thermophilic taxon, the *Thermotogales* (20), and a variety of mesophiles. Especially well studied have been members of the δ-purple proteobacteria, including aptly named genera like *Desulfuromonas* (43), *Desulfurella* (8), and *Desulfuromusa* (28) as well as *Pelobacter* (30), *Geobacter* (12), and certain sulfate reducers (4). Additionally, some spirilloid bacteria (27), including the rumen isolate *Wolinella succinogenes* (32), reduce S^0. Other eubacterial groups that facultatively reduce sulfur, but not as a primary means of energy generation, include the *Beggiatoa* spp. (39), members of the family *Thiorhodaceae* (54), and certain filamentous cyanobacteria (40, 49).

The first account of sulfur reduction by a facultative anaerobe appeared in 1985 with Balashova's description of a hydrogen-oxidizing *Pseudomonas mendocina*-like isolate (2), which apparently has not been studied further. Preliminary descriptions of sulfur reduction by various strains of the γ-purple proteobacterium *Shewanella putrefaciens* have appeared recently (35, 38, 42). Here we report growth and yield data for *S. putrefaciens* MR-1 as well as approaches for the cultivation of sulfur-metabolizing bacteria and methods for biomass determination in the presence of insoluble substrates such as sulfur. Coupled with recent observations from the environment, these data suggest a potentially significant but underestimated ecological role for sulfur-reducing facultative anaerobes.

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MATERIALS AND METHODS

Bacterial strains. Eubacteria of the *S. putrefaciens* group are γ-purple proteobacteria (31) that grow with most or all of a considerable variety of metabolic electron acceptors, including oxygen, nitrate, nitrite, trimethylamine *N*-oxide, dimethyl sulfoxide, thiosulfate, fumarate, metal oxides (36), and elemental sulfur (see references 38 and 42 and this work). The sources of the isolates employed for this study are listed in Table 1. The strains designated iron reducers A, B, and C were identified as relatives of *S. putrefaciens* by use of the BIOLOG (Hayward, Calif.) bacterial identification system.

Culture conditions. *Shewanella* strains were maintained aerobically on LB (33) agar. Inocula were grown aerobically overnight in LB liquid medium at 30°C with shaking. Prior to inoculation into mineral medium (M1 medium; pH 7.4) (36), cells were harvested by centrifugation and resuspended in sterile M1 medium. In plating experiments, the cells were either streaked for isolation or poured in top agar over the surface of aged (see below) sulfur plates. Liquid cultures (50 ml) were shaken in 125-ml Erlenmeyer flasks open to the anaerobic chamber atmosphere. A sterile 0.5-cm glass bead was added to each to grind the sulfur (43), which otherwise tended to clump. All anaerobic manipulations were performed in an anaerobic glove chamber (Coy Laboratory Products, Grass Lake, Mich.) with an atmosphere maintained at ca. 2% H_2 (the balance in N_2). To prevent sulfide damage to the platinum-palladium catalyst packs in our anaerobic chambers, activated carbon (6/14 mesh; Fisher Scientific) was integrated into the gas recirculators and a 2-liter flask containing 1 liter of 500 mM zinc acetate was placed on a rotary shaker inside the chamber.

Unless otherwise noted, lactate was employed as a carbon and energy source and added to 30 mM from an autoclaved 1 M lactic acid stock (pH adjusted to 7.2 with 10 N NaOH). Formate, when tested as an unfermentable substrate, was added at 50 to 100 mM from an autoclaved 1 M sodium formate stock (pH 7.4). Casamino Acids (Difco, Inc., Detroit, Mich.) were sometimes included as a growth stimulant to a 0.05% final concentration from a 10% autoclaved stock solution. Elemental sulfur was precipitated directly into the growth media from a 2.25 M (in terms of elemental sulfur recoverable upon acidification) alkaline polysulfide stock solution prepared as described previously (58). Briefly, this stock solution was made by mixing 7.2 g of sulfur flowers and 24 g of $Na_2S \cdot 9H_2O$ into boiling water for a final volume of 100 ml and then maintaining the boil for an additional 15 min with stirring. The resulting transparent, reddish solution was assumed to be sterile for our purposes and stored in a sterile glass bottle with minimal headspace, where it remained stable for at least several months. The high pH of this stock solution initially exceeded the buffering capacity of M1 medium when added to a concentration greater than ca. 10 mM, but the original pH poise of liquid medium could be restored with overnight

TABLE 1. *S. putrefaciens* strains used

Strain	Environmental source	Reference[a]
MR-1	Anoxic sediment, Oneida Lake, N.Y.	36
MR-4	Marine water column, Black Sea	37
MR-7	Marine water column, Black Sea	37
200	Petroleum field fluids	48
Iron reducer A	Estuarine sediment	Unpublished[b]
Iron reducer B	Estuarine sediment	Unpublished[b]
Iron reducer C	Estuarine sediment	Unpublished[c]
8071	Rancid butter	ATCC
8073	Rancid butter	ATCC

[a] The unpublished strains were provided by R. Blakemore. ATCC, American Type Culture Collection, Rockville, Md.

[b] The isolates denoted iron reducers A and B were identified as *S. putrefaciens* by the BIOLOG automated microbial identification system (see Materials and Methods).

[c] Not positively identified by the BIOLOG microbial identification system. The closest match was *S. putrefaciens.*

shaking in air, which allowed for the dispersal of sufficient sulfide to permit the precipitation of sulfur globules. Sodium sulfide (added to 1 mM final concentration from a freshly prepared 1 M stock in deaerated H_2O) was added at the time of inoculation to initiate the polysulfide-generating reaction (53) and scavenge residual oxygen. The preparation of solid media was comparatively simple provided that plates were allowed to age overnight in air prior to inoculation. In this case, the polysulfide stock solution was added to yield a final S^0 concentration of 40 mM immediately prior to the pouring of molten 1.5% agar in M1 or LB medium.

To demonstrate that the sulfur clearing typically associated with growth was not due to the accumulation of polysulfide, the following qualitative assay was performed. Cells and residual elemental sulfur were removed from stationary-phase cultures grown in liquid M1 medium on sulfur and lactate by centrifugation at 6,000 × *g* for 15 min. The resulting supernatant was titrated with an excess of 6 N HCl, since polysulfide becomes unstable and precipitates as elemental sulfur at a pH below 7.0 (53). In controls, the cloudiness caused by freshly precipitated elemental sulfur was easily detectable by eye at 100 μM.

Epifluorescence microscopy. Direct microscopic examination was routinely employed to monitor changes in cell number (13, 44). Cultures were fixed by dilution into 0.5% glutaraldehyde and stained with 1.0 μg of DAPI (4′,6-diamidino-2-phenylindole; Sigma Chemical Co.) per ml. Stained cell suspensions (50 to 500 μl, depending on density) were further diluted into 10 ml of potassium phosphate buffer (100 mM, pH 7.2) and vacuum collected on 25-mm Poretics (Livermore, Calif.) black polycarbonate membranes. Fluorescing cells were counted under UV illumination with an Axiolab microscope (Carl Zeiss, Inc., Thornwood, N.Y.). Generally, 30 to 60 fields (21) from duplicate cultures were counted, but in some cases, the nonrandom distribution of cells necessitated counting additional fields (up to 120 in practice). Prior to counting, slides were randomized and identified numerically to eliminate operator bias. Growth yields, when expressed as total cell number, were normalized by subtracting the inoculated cells from those present at the end of the experiment.

Total carbon determination. Bacterial growth was also measured in liquid culture as particulate carbon retained on filters by using an adaptation of an approach typically employed to analyze suspended biomass in natural waters and continuous culture (19, 24). Portions of a growing culture (1 to 40 ml, depending on cell density) were collected under light vacuum (<12 lb/in^2) on 2.5-cm glass microfiber filters (GF/F; Whatman). A single wash with 10 ml of potassium phosphate buffer (100 mM, pH 7.2) was employed to remove residual noncell carbon. Filters were dried at 60°C for 30 min, and 0.4-cm subsamples were collected with a filter punch, crushed within ultralight tin capsules (8 by 5 mm; Elemental Microanalysis Ltd., Manchester, Mass.), and combusted for analysis in an elemental analyzer (Carlo Erba Instruments, Milan, Italy). Cell carbon was quantitated on the basis of a standard curve generated for each instrument run with acetanilide (C_8H_9NO; Eastman Kodak, Rochester, N.Y.). Yields expressed as total cell carbon were normalized by subtracting the carbon value at time zero from that measured at the end of the experiment.

RESULTS

Sulfur reduction as a species characteristic. Table 2 indicates the prevalence of sulfur reduction (and growth on sulfur) as a metabolic trait from among representative *S. putrefaciens* isolates as determined by an agar plate assay. Within the 7-day time course of this experiment, six of the nine strains tested visibly cleared sulfur in M1 medium with 0.05% Casamino Acids as well as in the richer LB medium, both containing lactate as an electron donor.

TABLE 2. Growth-associated sulfur reduction by *S. putrefaciens* strains

Strain	M1 medium[a]		LB medium[a]	
	Growth[b]	Clearing[c]	Growth	Clearing
MR-1	+++	++++	+++	+
MR-4	++++	++++	+++	+
MR-7	+++	+++	+++	++
A	++	+++	+++	+
B	+	++	+++	+
C	−	−	++	−
8071	++	++	+++	+
8073	−	−	++	−
200	−	−	+++	−[d]

[a] Both media were supplemented with 0.05% Casamino Acids, lactate at 30 mM, and elemental sulfur at 40 mM. As controls, M1 media containing Casamino Acids, lactate, or elemental sulfur alone were tested. All remained negative for growth and clearing over the time course of this experiment.

[b] For growth assessment, the size of colonies ranged from barely visible after 7 days (+) to >1 mm in diameter (++++). −, no growth.

[c] Symbols: +, any visible clearing; ++, greater clearing but not through to the bottom of the plate; +++, clearing barely through to the bottom of the plate; ++++, extensive clearing; −, no clearing.

[d] With longer incubation time (2 to 3 weeks), strain 200 displays significant sulfur clearing on LB medium.

Qualitative assessment of sulfur-associated growth on solid medium. As shown in Fig. 1 and 2 and described in Table 2, for the majority of the *Shewanella* strains considered here, growth on the surface of M1 agar plates was correlated with sulfur clearing. However, with incubations of greater than roughly 10 days, some growth was noted on many of the no-sulfur control plates containing Casamino Acids alone or in combination with lactate; this did not interfere with growth scoring on the shorter time scales utilized here. With the rich medium (i.e.,

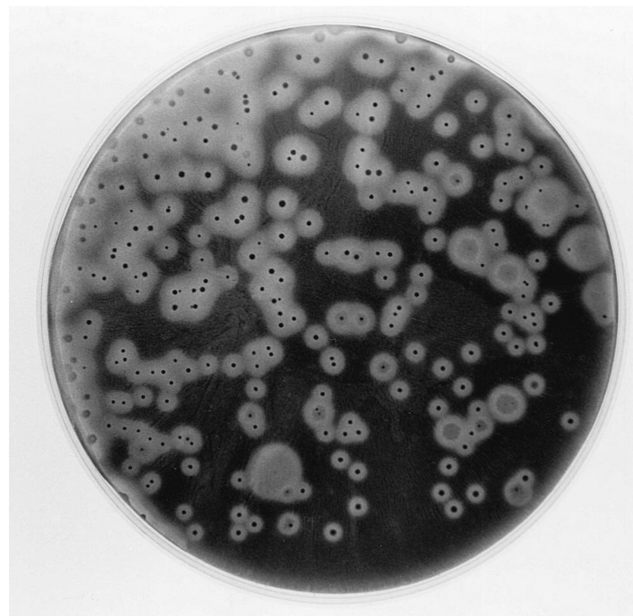

FIG. 1. Strain MR-1 distributed by top agar overlay and growing on minimal medium containing 30 mM lactate and 40 mM elemental sulfur with 0.05% Casamino Acids. The sulfur, while in reality white, appears dark as a result of back-illumination. Light areas are zones of sulfur clearing. The extremely large, diffuse colonies are at the surface of the agar.

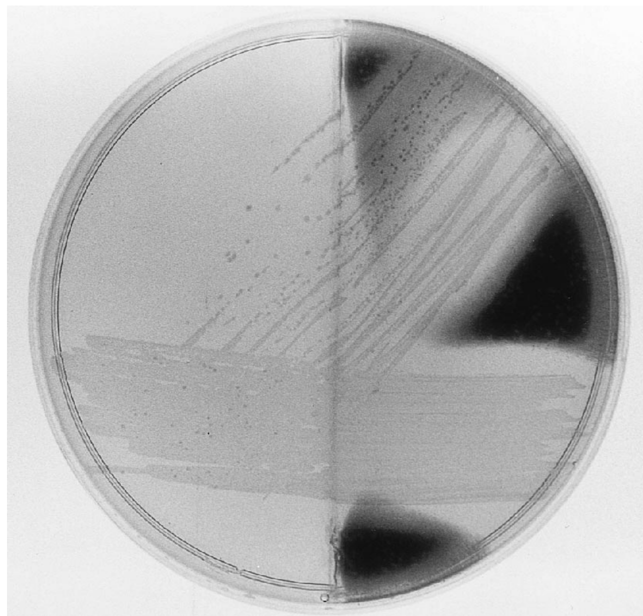

FIG. 2. The effectively soluble nature of the sulfur substrate. The right half of the plate contains 40 mM elemental sulfur and 30 mM lactate; the left half of the plate contains 30 mM lactate only. Uniform growth throughout indicates that the electron acceptor utilized here was functionally soluble.

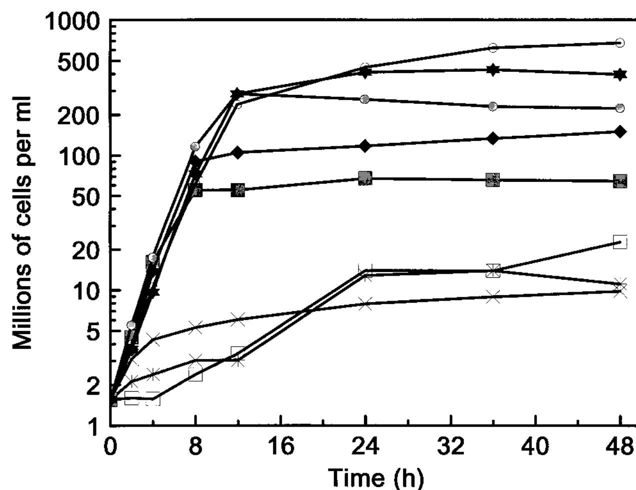

FIG. 3. Growth dynamics of strain MR-1 with limiting concentrations of elemental sulfur. The figure includes data generated from two separate experiments, whose growth curves were essentially interchangeable, justifying this approach and verifying the reproducibility of the results. Cells were grown in M1 medium containing (in addition to elemental sulfur) 30 mM lactate and 0.05% Casamino Acids. Symbols: ■, 2 mM sulfur; ◆, 5 mM sulfur; ●, 10 mM sulfur; ★, 20 mM sulfur; ○, 30 mM sulfur. Controls: ×, 30 mM elemental sulfur only; $*$, 30 mM lactate only; □, 0 mM sulfur (30 mM lactate, 0.05% Casamino Acids).

LB medium; also containing S^0 and lactate), growth was noted for all isolates, but appreciable sulfur clearing occurred only for those that grew with and cleared sulfur on the minimal medium. In this regard, a possible exception was strain 200, which displayed relatively weak but significant sulfur reduction after ca. 3 weeks on LB medium.

Figures 1 and 2 provide an indication that direct cell-to-sulfur contact is not required for sulfur reduction. The cleared zones in Fig. 1 extend well beyond the colony boundaries. Furthermore, the left half of the plate shown in Fig. 2 contained no sulfur prior to inoculation, yet the growth noted was approximately equal to that directly over the right half, which contained 40 mM sulfur.

Sulfur-associated growth of *S. putrefaciens* in liquid culture. Figure 3 illustrates the growth characteristics of strain MR-1 in M1 medium amended with 0.05% Casamino Acids and a presumed excess of lactate and limited for elemental sulfur as the major electron acceptor. As was the case with solid media, any visible clearing of particulate sulfur was associated with increases in measured bacterial biomass. When subinhibitory concentrations of Na_2S were added at the time of inoculation, these cells displayed apparently lag-free growth. Considerable growth lags (of up to several days) were typical in the absence of added sulfide (34). At all substrate concentrations tested, initial growth rates were effectively equal, with doubling times of ca. 1.5 h noted until the particulate sulfur had cleared. To address the possibility that at least some of the cleared sulfur might be present as polysulfide, spent supernatants from stationary-phase cultures were acidified and observed for the appearance of cloudiness (see Materials and Methods), none of which was ever noted.

Yield estimates. Approximate cell number yields for strain MR-1 grown in amino acid-supplemented M1 medium over a range of limiting sulfur concentrations at 48 h can be derived from Fig. 3. The highest cell concentration noted at the termination of this experiment was 6.8×10^8 cells $\cdot$ ml^{-1} (corresponding to 30 mM elemental sulfur cleared). A strong correlation ($r^2 = 0.99$) between initial sulfur concentration and cell density was evident after 48 h, indicating that sulfur availability limited growth potential. Figure 4 displays yield data for cells cultured under conditions identical to those of the experiment illustrated in Fig. 3 but incubated for an additional 3 days to ensure that growth was complete.

Figure 4 indicates that strain MR-1 grew on sulfur and lactate in both the presence and absence of amino acid supplementation. Molar growth yields calculated from the data in Fig. 4 and expressed as cell production per mole of sulfur consumed ranged from 4×10^{13} to 1×10^{14} when Casamino Acids were provided and from 2×10^{13} to 6×10^{13} in their

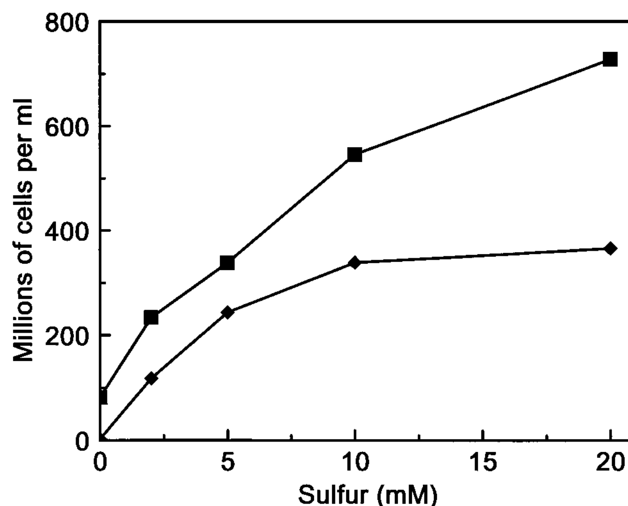

FIG. 4. Effect of amino acid supplementation on sulfur-dependent growth yield after 5 days. Lactate was added to 30 mM for all, and sulfur was added as indicated. Symbols: ■, growth in the presence of 0.05% Casamino Acids; ◆, growth in the absence of added Casamino Acids.

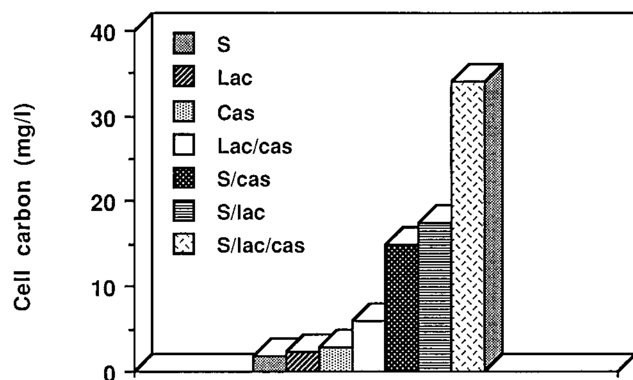

FIG. 5. Growth yields of controls for the experiment illustrated in Fig. 4 (expressed as milligrams of particulate carbon · liter^{-1}). S, 20 mM S^0; lac, 30 mM lactate; cas, 0.05% Casamino Acids. Combinations are indicated by a slash.

absence. Over the range of sulfur concentrations tested, Casamino Acids increased overall growth efficiency (per unit of sulfur consumed) by approximately a factor of two. Biomass increases for the same cultures in terms of particulate carbon were also measured. Curves derived from these data (34) were virtually identical to those generated from the cell number data presented in Fig. 4, indicating that cell carbon content represents a viable parameter with which to track growth in the presence of elemental sulfur. When these two data sets were compared, a mean conversion factor of 19.8 million cells · mg of C^{-1} (standard deviation, 1.4 million cells · mg of C^{-1}) was noted. When expressed as total carbon, molar growth yields for sulfur ranged from 1.7 to 5.9 g of C · mol S^0 consumed^{-1} in the presence of Casamino Acids and 0.9 to 3.4 g of C · mol of S^{0-1} in their absence.

The carbon measurements in Fig. 5 indicate the growth of MR-1 after 5 days under conditions identical those used to generate the data in Fig. 4. The highest yields were noted for cells grown on 20 mM S^0 with a combination of both 30 mM lactate and 0.05% Casamino Acids. In the presence of like concentrations of elemental sulfur, lactate, or Casamino Acids alone, growth was at or below the limit of detection. With extended incubations, however, growth was noted for Casamino Acids or lactate alone (data not shown), and MR-1 displayed measurable growth within the time frame of this experiment when provided with the combination of lactate and Casamino Acids even in the absence of sulfur. Final cell biomass estimates were three times those of the other controls in this case. Growth from the reduction of S^0 with 0.05% Casamino Acids acting as the sole electron donor and carbon source was nearly equal to that obtained with 30 mM lactate and sulfur (Fig. 5).

DISCUSSION

Presented here are the results of what may be the first detailed investigation into the heterotrophic growth patterns of a facultative anaerobe cultivated under sulfur-reducing conditions. Six of nine *S. putrefaciens* isolates from a variety of sources displayed growth-associated elemental sulfur clearing on agar plates (Table 2), and from among these, strain MR-1 was chosen for further study. Some growth characteristics of MR-1 provided elemental sulfur, lactate, and a light dose of Casamino Acids (0.05%) in liquid are illustrated in Fig. 3. The strong correlation ($r^2 = 0.99$) between initial sulfur concentration and growth after 48 h indicates that sulfur acts as a limiting substrate under these conditions. The apparently lag-free nature of growth is consistent with the report by Perry et al. that sulfide production from S^0 is measurable at 10 min for *S. putrefaciens* MR-4 (42). Our observed growth rate (doubling time, 1.5 h) compares favorably with that of 1.2 h reported for *W. succinogenes* grown on sulfur and formate (32).

Improvements in culture and analytical technique proved instrumental to obtaining and tracking the growth of MR-1 on sulfur. In our hands (34, 38, 42) and as reported by others (12, 30), an accumulation of H_2S in sealed batch culture invariably restricts growth potential under sulfur-reducing conditions. Several authors have utilized headspace gas exchange to remove H_2S and thus stimulate (32) or reinitiate (3) growth. For this work, we incubated cultures open to the anaerobic chamber atmosphere, which allowed for the diffusive release of H_2S (and possibly the availability of H_2 as an accessory electron donor). Yields of sealed batch cultures grown by sulfur reduction have been typically reported as 10- to 15-fold increases in cell number coincident with the accumulation of H_2S to ca. 2 mM (12, 30, 38, 42). In this study, *S. putrefaciens* demonstrated a greater than 400-fold cell number increase, which corresponded with the clearing of up to 30 mM elemental sulfur.

Throughout these experiments, a certain amount of variability in growth rate or even the initiation of growth and sulfur clearing was unavoidable in liquid. Ultimately, we found that the addition of Na_2S at a subinhibitory concentration (1 mM) at the time of inoculation largely exacerbated this effect, most likely by priming the chemical production of polysulfide. This soluble species is formed from elemental sulfur in the presence of H_2S (53) and considered the probable metabolic substrate for thermophilic and obligately anaerobic sulfur reducers (5, 26). The presence of cleared zones surrounding colonies in Fig. 1 and the plate-wide distribution of colonies irrespective of particulate elemental sulfur availability on the plate shown in Fig. 2 indicate that cell-to-sulfur contact is not required for growth. Also in support of this hypothesis is the observation that strain MR-1 utilizes particulate sulfur isolated behind 8,000-molecular-weight cutoff dialysis membranes or embedded within 3% agar (34). When truly insoluble substrates such as metal oxides are used, reduction rates and presumably growth are directly coupled to substrate concentration and, in particular, available surface area (10). The concentration-independent initial growth rates displayed by MR-1 on sulfur (Fig. 3) are thus consistent with a functionally soluble substrate.

Casamino Acids in even low concentrations were stimulatory to growth on sulfur and readily utilized as the sole source of carbon and energy (Fig. 5). It has been proposed that amino acids enhance the solubility of sulfur (5); thus, any growth effect may not be entirely nutritional. In addition, the comparatively low but significant growth of MR-1 on lactate and amino acids in the absence of an added electron acceptor suggests the use of amino acids as fermentable substrates or terminal electron acceptors. Amino acids or mixtures of peptides have been employed as electron donors for *S. putrefaciens* growing by iron reduction (29). *S. putrefaciens* has long been associated with fish pathologies and the spoilage of proteinaceous foods (14, 41, 51). Thus, amino acids may play an unappreciated role in the anaerobic metabolism of these bacteria.

That *S. putrefaciens* grows in defined media with only lactate and elemental sulfur as substrates (Fig. 4 and 5) is not necessarily indicative of respiratory metabolism, since the possibility of a sulfur-facilitated lactate fermentation has yet to be resolved. Indeed, even after repeated attempts with different strains, substrate concentrations, media, and culture condi-

tions, we have been unable to demonstrate more than an approximate doubling of *S. putrefaciens* on elemental sulfur provided with formate as the sole carbon source. It should be noted, however, that *S. putrefaciens* cells on the surface of agar plates very effectively mediate the clearing of elemental sulfur when provided 50 to 100 mM formate (34), apparently indicating that formate can serve as an electron donor for sulfur reduction.

The environmental relevance of microbial sulfur reduction remains unclear. Being only partially oxidized, S^0 contains much of the energy liberated by other processes and may participate in energy transfer between anaerobic and oxygenated zones (57). Found in most anoxic environments (55), elemental sulfur is formed by either the biological (45) or chemical (11, 17) oxidation of H_2S. S^0 represents a potentially dynamic sulfur cycle intermediate which undergoes rapid transformations in natural environments. King et al. documented $^{35}S^0$ turnover times of as little as 2 days (25), and Cutter and Velinsky reported the loss of 3.2 mmol of $S \cdot liter^{-1} \cdot day^{-1}$ in salt marsh sediments (15). Jorgensen et al. noted an accumulation rate of 2 to 5 mmol of $S \cdot m^{-2} \cdot day^{-1}$ in a stratified lake (23). Bonch-Osmolovskaya reported biological sulfur reduction rates of up to 4,160 $mg \cdot liter^{-1} \cdot day^{-1}$ in a thermophilic cyanobacterial mat (6) and 5,737 $mg \cdot liter^{-1} \cdot day^{-1}$ in shallow water marine hot vents where sulfur reduction was described as the major terminal reductive process (7).

Some sedimentary environments display annual variations in their elemental sulfur content, suggesting that bacterial reduction, stimulated by episodic spikes in carbon input, may act to mobilize S^0 (15, 55). It is possible that sulfur-driven, microbially mediated redox cycles functionally analogous to those known for iron and manganese may focus around sulfur-rich redoxclines. For such a model to operate, soluble intermediates or reaction products are required. Polysulfide is readily reduced by microbes (5, 26, 56) and freely soluble in water (53) and has been shown to dominate partially reduced sulfur pools in at least some laminated systems (56). Pronounced elemental sulfur peaks have been located at the surface of marine sediments (55) or within stratified water columns at the top of the sulfide zone. Midwater S^0 peaks have been noted at the chemoclines of both the Cariaco Trench (18) and the Black Sea (280 and 103 m, respectively [18, 22]). Spikes in the local abundance of *S. putrefaciens* have been noted at a corresponding depth (105 m) in the Black Sea (up to $10^5 \cdot ml^{-1}$) (37) and a comparable interface in the Baltic (the Gotland Deep, where *S. putrefaciens* constituted 32 to 80% of the total culturable bacteria) (9).

The data presented here establish that elemental sulfur reduction can support significant growth in this group of facultative anaerobes. While measured environmental S^0 concentrations in nature are often low (200 nM to 100 μM [18, 22]) in aqueous systems, sulfur atoms are known to rapidly cycle between intermediate pools without any change in their relative concentrations (16). This may represent a mechanism by which the concentration of S^0 available to microbes may be functionally higher than environmental measurement would appear to indicate. Further efforts will be required to establish the prevalence of this metabolism among *S. putrefaciens* strains and the environmental role of sulfur-reducing facultative anaerobes.

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REFERENCES

1. **Adams, M. W. W.** 1994. Biochemical diversity among sulfur-dependent hyperthermophilic microorganisms. FEMS Microbiol. Rev. **15:**261–277.
2. **Balashova, V. V.** 1985. The use of molecular sulfur as an agent oxidizing hydrogen by the facultative anaerobic *Pseudomonas* strain. Mikrobiologya (USSR) **54:**324–326.
3. **Belkin, S., C. O. Wirsen, and H. W. Jannasch.** 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. Appl. Environ. Microbiol. **51:**1180–1185.
4. **Biebl, H., and N. Pfennig.** 1977. Growth of sulfate-reducing bacteria with sulfur as electron acceptor. Arch. Microbiol. **112:**115–117.
5. **Blumenthals, I. I., M. Itoh, G. J. Olson, and R. M. Kelley.** 1990. Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56:**1225–1262.
6. **Bonch-Osmolovskaya, E. A.** 1991. Sulfur-reducing bacteria and their activity in natural environments. Zh. Obshch. Biol. **52:**453–463.
7. **Bonch-Osmolovskaya, E. A., M. L. Miroshnichenko, E. V. Pikuta, D. Yu, Y. I. Sorokin, and B. B. Namsaraev.** 1993. Bacterial sulfur reduction in shallow-water hot vents of the southwestern part of the Pacific Ocean. Microbiology **62:**348–352.
8. **Bonch-Osmolovskaya, E. A., T. G. Sokolova, N. A. Kostrikina, and G. A. Zavarzin.** 1990. *Desulfurella acetivorans* gen. nov. and sp. nov.—a new thermophilic sulfur reducing eubacterium. Arch. Microbiol. **153:**151–155.
9. **Brettar, I., and M. G. Hofle.** 1993. Nitrous oxide producing heterotrophic bacteria from the water column of the central Baltic: abundance and molecular identification. Mar. Ecol. Progr. Ser. **94:**253–265.
10. **Burdige, D. J., S. P. Dhakar, and K. H. Nealson.** 1992. Effects of manganese oxide mineralogy on microbial and chemical manganese reduction. Geomicrobiol. J. **10:**27–48.
11. **Burdige, D. J., and K. H. Nealson.** 1986. Chemical and microbiological studies of sulfide-mediated manganese reduction. Geomicrobiol. J. **4:**361–387.
12. **Caccavo, F., Jr., D. J. Lonergan, D. R. Lovely, M. Davis, J. F. Stolz, and M. J. McInerney.** 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. **60:**3752–3759.
13. **Clesceri, L. S., A. E. Greenberg, and A. D. Eaton (ed.).** 1989. Standard methods for the examination of water and wastewater, p. 9-39–9-40. American Public Health Association, Washington, D.C.
14. **Curtiss, C. F.** 1931. Bacteriology of butter. IV. Bacteriological studies on surface taint butter. Research bulletin no. 145. Agricultural Research Station, Iowa State College of Agriculture and Mechanic Arts, Ames.
15. **Cutter, G. A., and D. J. Velinsky.** 1988. Temporal variations of sedimentary sulfur in a Delaware salt marsh. Mar. Chem. **23:**311–327.
16. **Fossing, H., S. Thode-Anderson, and B. B. Jorgensen.** 1992. Sulfur isotope exchange between ^{35}S-labeled inorganic sulfur compounds and anoxic marine sediments. Mar. Chem. **38:**117–132.
17. **Goldhaber, M. B., and I. R. Kaplan.** 1974. The sulfur cycle, p. 569–655. *In* E. D. Goldberg (ed.), The sea. John Wiley & Sons, Inc., New York.
18. **Hastings, D., and S. Emerson.** 1988. Sulfate reduction in the presence of low oxygen levels in the water column of the Cariaco Trench. Limnol. Oceanogr. **33:**391–396.
19. **Hecky, R. E., P. Campbell, and L. L. Hendzel.** 1993. The stoichiometry of carbon, nitrogen, and phosphorous in particulate matter of lakes and oceans. Limnol. Oceanogr. **38:**709–724.
20. **Jannasch, H. J., R. Huber, S. Belkin, and K. O. Stetter.** 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic eubacterial genus *Thermotoga*. Arch. Microbiol. **150:**103–104.
21. **Jones, J. G.** 1979. A guide to methods for estimating microbial numbers and biomass in fresh water, p. 31. Freshwater Biological Association scientific publication no. 39. Titus Wilson and Son, Kendal, United Kingdom.
22. **Jorgensen, B. B., H. Fossing, C. O. Wirsen, and H. J. Jannasch.** 1991. Sulfide oxidation in the anoxic Black Sea chemocline. Deep Sea Res. **38:**S1083–S1103.
23. **Jorgensen, B. B., J. G. Kuenen, and Y. Cohen.** 1979. Microbial transformations of sulfur in a stratified lake (Solar Lake, Sinai). Limnol. Oceanogr. **24:**799–822.
24. **Kepkay, P. E., and K. H. Nealson.** 1987. Growth of manganese oxidizing *Pseudomonas* sp. in continuous culture. Arch. Microbiol. **148:**63–67.
25. **King, G. M., B. L. Howes, and J. W. H. Dacey.** 1985. Short-term end products of sulfate reduction in a salt marsh: formation of acid volatile sulfides, elemental sulfur, and pyrite. Geochim. Cosmochim. Acta **49:**1561–1566.
26. **Klimmek, O., A. Kroger, R. Steudel, and G. Holdt.** 1991. Growth of *Wolinella succinogenes* with polysulfide as terminal electron acceptor of phosphorylative electron transport. Arch. Microbiol. **155:**177–182.
27. **Laanbroek, H. J., L. J. Stal, and H. Veldkamp.** 1978. Utilization of hydrogen and formate by *Campylobacter spec.* under aerobic and anaerobic conditions. Arch. Microbiol. **119:**99–102.
28. **Liesack, W., and K. Finster.** 1994. Phylogenetic analysis of five strains of gram-negative obligately anaerobic, sulfur-reducing bacteria and description

of *Desulfuromusa* gen. nov., including *Desulfuromusa kysingii* sp. nov., *Desulfuromusa bakii* sp. nov. and *Desulfuromusa succinoxidans* sp. nov. Int. J. Syst. Bacteriol. **44:**753–758.
29. **Lovely, D. R., E. J. P. Phillips, and D. J. Lonergan.** 1989. Hydrogen and formate oxidation coupled to dissimilatory reduction of iron and manganese by *Alteromonas putrefaciens*. Appl. Environ. Microbiol. **55:**700–706.
30. **Lovely, D. R., E. J. P. Phillips, D. J. Lonergan, and P. K. Widman.** 1995. Fe(III) and S^0 reduction by *Pelobacter carbinolicus*. Appl. Environ. Microbiol. **61:**2132–2138.
31. **MacDonell, M. T., and R. R. Colwell.** 1985. Phylogeny of the Vibrionaceae, and the recommendation for two new genera, *Listonella* and *Shewanella*. Syst. Appl. Microbiol. **6:**171–182.
32. **Macy, J. M., I. Schroder, R. K. Thauer, and A. Kroger.** 1986. Growth of *Wolinella succinogenes* on H_2S plus fumarate and on formate plus sulfur as energy sources. Arch. Microbiol. **144:**147–150.
33. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Moser, D. P.** Unpublished data.
35. **Moser, D. P., and K. H. Nealson.** 1995. Elemental sulfur respiration in a facultative aerobe: *Shewanella putrefaciens*, abstr. N-70, p. 344. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
36. **Myers, C. R., and K. H. Nealson.** 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science **240:** 1319–1321.
37. **Nealson, K. H., C. R. Myers, and B. Wimpee.** 1991. Isolation and identification of manganese reducing bacteria, and estimates of microbial manganese reducing potential in the Black Sea. Deep Sea Res. **38:**S907–S920.
38. **Nealson, K. H., D. Saffarini, and D. Moser.** Anaerobic respiration of *Shewanella putrefaciens*: potential use of solid electron acceptors for pollutant removal and bioremediation. *In* M. DeLuca (ed.), Bioremediation in anoxic environments, in press. Rutger's University Press, Rutgers, N.J.
39. **Nelson, D. C., and R. W. Castenholz.** 1987. Use of reduced sulfur compounds by *Beggiatoa* sp. J. Bacteriol. **147:**140–154.
40. **Oren, A., and M. Shilo.** 1979. Anaerobic heterotrophic dark metabolism in the cyanobacterium *Oscillatoria limnetica*: sulfur respiration and lactate fermentation. Arch. Microbiol. **122:**77–84.
41. **Parker, L. L., and R. E. Levin.** 1983. Relative incidence of *Alteromonas putrefaciens* and *Pseudomonas putrefaciens* in ground beef. Appl. Environ. Microbiol. **45:**769–799.
42. **Perry, K. A., J. E. Kostka, G. W. Luther III, and K. H. Nealson.** 1993. Mediation of sulfur speciation by a Black Sea facultative anaerobe. Science **259:**801–803.
43. **Pfennig, N., and H. Biebl.** 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. **110:**3–12.
44. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**948–951.
45. **Robertson, L. A., and J. G. Kuenen.** 1992. The colorless sulfur bacteria, p. 385–413. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
46. **Roy, A. B., and P. A. Trudinger.** 1970. The biochemistry of inorganic compounds of sulfur, p. 274–275. Cambridge University Press, Cambridge.
47. **Schauder, R., and A. Kroger.** 1993. Bacterial sulfur respiration. Arch. Microbiol. **195:**491–497.
48. **Semple, K., and D. Westlake.** 1987. Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. Can. J. Microbiol. **33:**366–371.
49. **Stal, L. J., H. Heyer, S. Bekker, M. Villbrandt, and W. E. Krumbein.** 1989. Aerobic-anaerobic metabolism in the cyanobacterium *Oscillatoria limosa*, p. 266. *In* Y. Cohen and E. Rosenberg (ed.), Microbial mats: physiological ecology of benthic microbial communities. American Society for Microbiology, Washington, D.C.
50. **Starkey, R. L.** 1937. Formation of sulfide by some sulfur bacteria. J. Bacteriol. **33:**545–571.
51. **Stenstrom, I.-M., and G. Molin.** 1990. Classification of the spoilage flora of fish, with special reference to *Shewanella putrefaciens*. J. Appl. Bacteriol. **68:**601–618.
52. **Stetter, K. O., and G. Gaag.** 1983. Reduction of molecular sulfur by methanogenic bacteria. Nature (London) **305:**309–311.
53. **Teder, A.** 1971. The equilibrium between elementary sulfur and aqueous polysulfide solutions. Acta Chem. Scand. **25:**1722–1728.
54. **Then, J., and H. G. Truper.** 1984. Utilization of sulfide and elemental sulfur by *Ectothiorhodospira halochloris*. Arch. Microbiol. **139:**295–298.
55. **Troelson, H., and B. B. Jorgensen.** 1982. Seasonal dynamics of elemental sulfur in two coastal sediments. Estuarine Coastal Shelf Sci. **15:**255–266.
56. **Visscher, P. T., J. W. Nijburg, and H. van Gemerden.** 1990. Polysulfide utilization by *Thiocapsa roseopersicina*. Arch. Microbiol. **155:**75–81.
57. **Whitcomb, J. H., R. D. DeLaune, and W. H. Patrick, Jr.** 1989. Chemical oxidation of sulfide to elemental sulfur: its possible role in marsh energy flow. Mar. Chem. **26:**205–214.
58. **Widdel, F., and N. Pfennig.** 1992. The genus *Desulfuromonas* and other gram-negative sulfur-reducing eubacteria, p. 3379–3389. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.