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Reduction of Sulfur by Spirillum 5175 and Syntrophism with *Chlorobium*

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A small spirillum, designated 5175, was isolated from an anaerobic enrichment culture for *Desulfuromonas* in which the major medium constituents were acetate and elemental sulfur. The organism grew only under anaerobic or microaerophilic conditions. Elemental sulfur was formed anaerobically in a malate-sulfide medium, and cell densities of 10^8 cells/ml were obtained. Hydrogen and formate were actively oxidized as substrates for growth under anaerobic conditions; S^0, SO_3^{2-}, or $S_2O_3^{2-}$, but not SO_4^{2-}, served as electron acceptors and were stoichiometrically reduced to sulfide. Malate or fumarate likewise served as electron acceptors and were reduced to succinate. Nutritional requirements were simple, no vitamins or amino acids being required. For growth in inorganic media where carbon dioxide was the only carbon source, the addition of acetate was required as a source of cell carbon. The organism is gram negative. Cells had a diameter of 0.5 μm and a wavelength of 5.0 μm. Cell suspensions exhibited an absorption spectrum indicative of a cytochrome with peaks in the reduced form at 552, 523, and 416 nm. Well growing syntrophic cultures with *Chlorobium* were established with formate as the substrate.

In recent years the important and dynamic role of hydrogen in anaerobic microbial habitats has become apparent (3, 4, 6, 16, 17). Hydrogen is rarely detectable in anaerobic environments where active microbial metabolism is in progress, since it is a highly desirable substrate. Studies by Wolin have stressed the importance of interspecies hydrogen transfer (17). Anaerobic oxidation of hydrogen may be coupled to a variety of electron acceptors, major acceptors being carbon dioxide, which is reduced to methane (1, 16), and sulfate, which is reduced to sulfide (2).

One of the poorly understood areas of environmental microbiology concerns the reduction of elemental sulfur in anaerobic environments. This aspect of the sulfur cycle has largely been ignored, since elemental sulfur may be reduced by a variety of organisms by mechanisms assumed not to be of physiological importance (13). In addition, the formation of elemental sulfur in anaerobic environments has been well documented only for photosynthetic bacteria (11, 15); *Thiobacillus denitrificans* is known to produce small amounts of S^0 when grown anaerobically with thiosulfate as substrate.

In this communication we present results of experiments with an organism which may produce as well as reduce elemental sulfur under defined anaerobic conditions in the dark. Evidence is presented that the organism can grow by the anaerobic oxidation of hydrogen and the reduction of S^0, SO_3^{2-}, or $S_2O_3^{2-}$ (but not SO_4^{2-}) according to the following equations (5):

	$\Delta G_0'$ (kcal/reaction)	
$3H_2 + SO_3^{2-} + 2H^+ \rightarrow H_2S + 3H_2O$	−38.6	(1)
$4H_2 + S_2O_3^{2-} + 2H^+ \rightarrow 2H_2S + 3H_2O$	−41.3	(2)
$H_2 + S^0 \rightarrow H_2S$	−6.5	(3)

We shall refer to the organism as spirillum 5175 until adequate taxonomic information is available.

MATERIALS AND METHODS

Organism and isolation procedures. Spirillum 5175 was isolated from an anaerobic enrichment culture in which *Desulfuromonas* was growing by the oxidation of acetate and reduction of elemental sulfur (11a). A sample of this culture was inoculated into a sterile medium which contained (in grams per liter of distilled water): KH_2PO_4, 1.0; NH_4Cl, 0.3; $MgCl_2 \cdot 6H_2O$, 4.0; $CaCl_2 \cdot 2H_2O$, 0.1. When these salts were dissolved, 10 ml of the trace element solution, SL4, of Pfennig and Lippert (12)

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and 2 ml of 2 M H_2SO_4 were added prior to sterilization. From sterile stock solutions, the following compounds were added aseptically to the sterile, cool medium (grams per liter of medium): $NaHCO_3$, 4.0; $Na_2S \cdot 9H_2O$, 0.3. The pH was adjusted to 7.2 with sterile solutions of dilute H_2SO_4 or Na_2CO_3, and the medium was dispensed aseptically into 50-ml screw-capped bottles, leaving a small air bubble in each bottle. To this basal medium were added substrates as desired from sterile 10% stock solutions. Sodium acetate or sodium pyruvate were added to a final concentration of 0.05%, sodium DL-malate being added at 0.2%. All cultures were incubated at 28°C. For experiments described in Fig. 3, 4, 5 and 6, the buffering capacity of the medium was increased by the addition of potassium phosphate (pH 6.8) to give a final concentration of 0.2%.

Pure cultures were obtained by serial dilution of a sample from the enrichment culture through agar shakes of the basal medium supplemented with 0.1% DL-malate, 0.05% acetate, and 0.05% pyruvate by the method described by Pfennig (10, 11a). Stock cultures were maintained in the same medium and stored at 4°C.

Growth studies. For growth studies, each 50-ml bottle of medium received an inoculum of 0.2 ml from an active culture. To sterilize elemental sulfur for use in certain experiments an aqueous suspension of flowers of sulfur was ground in a mortar to produce a fine suspension, which was autoclaved in a bottle for 30 min at 112 to 115°C; after sterilization the water was decanted. When elemental sulfur was used as an electron acceptor, it was added in excess, about 200 mg (dry weight) being added to 50 ml of medium. A sterile glass bead about 0.5 cm in diameter also was added to each bottle of S^0-containing medium. When hydrogen was used as the substrate, the standard screw cap of an inoculated 50-ml bottle of medium was aseptically replaced with a sterile rubber serum stopper, which was held in place by a metal screw-cap having an opening in the top. Hydrogen was added in the following manner. A sterile 10-cm^3 syringe with a 2-inch (about 5-cm), 20-gauge needle was lubricated with sterile water and flushed three times with hydrogen which had been filtered through sterile cotton. The syringe, which contained 10 cm^3 of hydrogen, was maintained in a vertical position, needle down, as the hydrogen was transferred to the bottle of medium. A sterile 20-gauge needle also was inserted through the rubber septum to serve as an exit for the medium, which was replaced as the hydrogen was forced from the syringe into the bottle. Each bottle was incubated on its side on a rotary shaker at about 180 cycles per min. To measure hydrogen consumption, a 10-cm^3 syringe with a glass piston was selected so that the piston floated freely, when lubricated with sterile water. The syringe was filled with hydrogen and handled as noted above. When the needle was inserted through the serum stopper of a bottle culture, the negative pressure within the bottle equilibrated with atmospheric pressure by addition of hydrogen from the syringe. The amount of hydrogen replaced was recorded. This technique was used successfully by S. Schoberth (personal communication) in the study of an organism which carried out a homoacetic fermentation from hydrogen and carbon dioxide (S. M. Schoberth and W. E. Balch, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, I90, p. 131). Readings were corrected for temperature and pressure variables by use of a control which consisted of an inoculated bottle of medium with 10 cm^3 of hydrogen but in which the electron acceptor was omitted. A second control consisted of the complete medium with 10 cm^3 of hydrogen but was not inoculated. Control bottles were handled in the same manner as the cultures.

Viable cells were estimated from colony counts, which were obtained by quantitative serial dilution of an aliquot from a liquid culture through a series of melted agar shakes by the method of Pfennig (10). Colonies were counted in tubes which contained between 30 and 80 colonies; results presented represent an average of two tubes for each dilution.

Assay techniques. A Zeiss DMR-21 spectrophotometer was used to determine the absorption spectrum of whole-cell suspensions, an integrating sphere being used to minimize light scattering. Sulfide was determined by the methylene blue method as described by Trüper and Schlegel (14). The presence of elemental sulfur was established in the manner used for cultures of *Chlorobium* (11). The base ratio (moles percent guanine plus cytosine [mol% G+C]) of the deoxyribonucleic acid (DNA) was determined by H. Hippe by use of the thermal denaturation method. Succinate was determined by D. Vollbrecht using quantitative gas chromatography (5% Reoflex 400 on Chromosorb GAW 60/80 mesh).

RESULTS

In agar-shake cultures designed for the isolation of freshwater strains of *Desulfuromonas acetoxidans* (11a), lens-shaped, yellowish colonies 1 to 3 mm in diameter were detected. Examination of colony suspensions in wet mounts by phase-contrast microscopy revealed actively motile spirilla and highly refractile globules of sulfur as noted in cultures of phototrophic green sulfur bacteria (11). As shown in Fig. 1, the spirillum has a cell diameter of 0.5 μm. Length of the spirals was found to vary depending upon culture conditions from 2.5 to 20 μm or more. Cells less than 2.5 μm in length may be formed. Cells were gram negative. In agar shakes of the malate-acetate-sulfide medium which were not layered with paraffin seals, isolated colonies deep in the agar reached a diameter of 5 mm in 6 days, showing that growth was stimulated under microaerophilic conditions. If the same medium without sulfide was prepared in tubes as a semisolid medium (agar 0.2%) and was inoculated at 40°C immediately after sterilization, cell growth localized in a narrow band about 1 cm below the surface; this band of growth expanded down the tube to a width of 2 cm or more in 2 weeks.

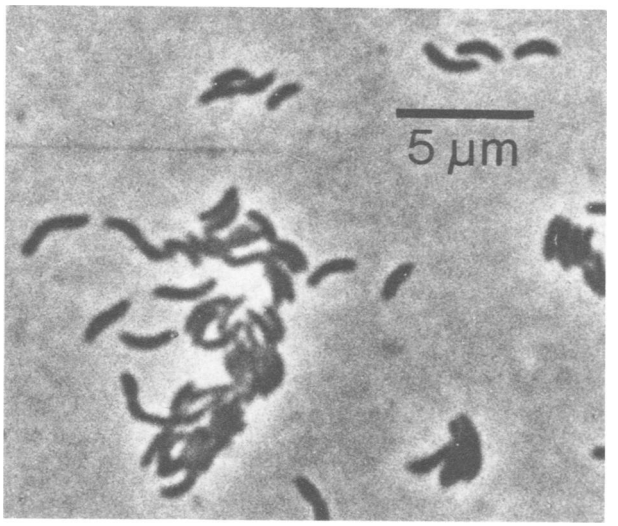

FIG. 1. *Phase-contrast photomicrograph of spirillum 5175.*

Cells remained actively motile. Growth under these conditions also was obtained when yeast extract or peptone (0.2%) was substituted for malate and acetate, indicating a lack of substrate specificity under microaerophilic conditions. Growth was never obtained aerobically on the surface of an agar plate, although a variety of media was tested.

Nutrition was found to be simple; addition of vitamins or amino acids was not required for growth in the basal medium with hydrogen or formate as substrate and with S^0, SO_3^{2-}, or $S_2O_3^{2-}$ as electron acceptor. However, under these conditions, the organism was unable to synthesize cell material solely from CO_2; acetate was required as a source of cell carbon, and pyruvate could be used instead of acetate. No growth occurred under these conditions in the absence of hydrogen or formate.

Although a variety of sugars and organic acids was tested, no evidence was obtained that the organism could be a fermentative anaerobe in the classical sense; that is, the organism could not produce an electron acceptor as a product of anaerobic metabolism. Addition of an electron acceptor was required. It is not surprising in view of these findings that whole-cell suspensions exhibited an absorption spectrum indicative of a cytochrome (Fig. 2). Peaks in the reduced form were observed at 552, 523, and 416 nm. Cells for spectral studies were grown anaerobically in the basal medium with malate, acetate, and sulfide.

The base ratio of the DNA was 38.4 mol% G+C.

Anaerobic oxidation of hydrogen. Sodium sulfite proved to be an excellent electron acceptor for the anaerobic oxidation of hydrogen by growing cells. The results (Fig. 3) were obtained from an experiment in which each of three culture bottles received a different level of sodium sulfite. Hydrogen consumption, sulfide formation, and final cell yield were measured for each vessel. The results are in excellent agreement with the stoichiometry of equation 1. The cell yield approximately doubled for each doubling of the amount of electron accep-

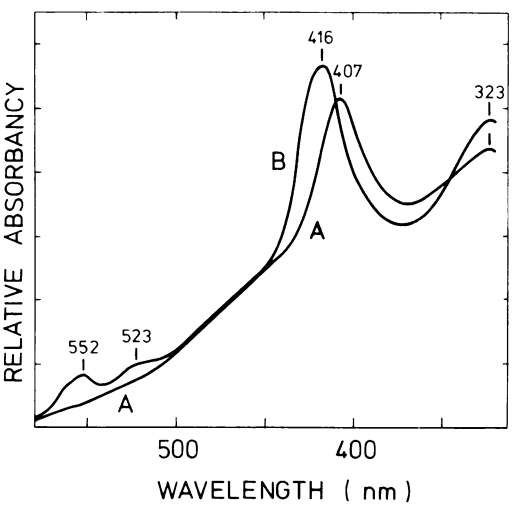

FIG. 2. *Absorption spectrum of whole cells of spirillum 5175. Curve A, Cells were suspended in water; curve B, cells were suspended in basal medium and exposed to a hydrogen atmosphere.*

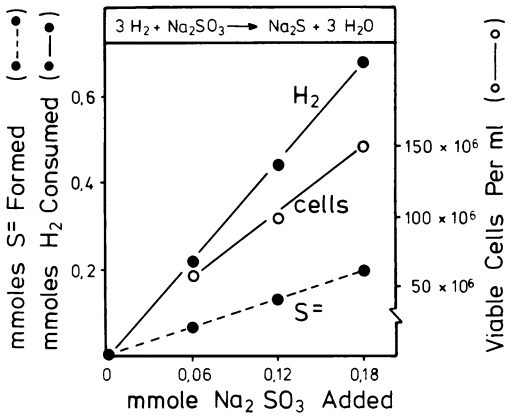

FIG. 3. *Relation of SO_3^{2-} added to hydrogen consumed, sulfide formed, and cell yield by growing cultures of spirillum 5175. Each culture bottle received 42 ml of basal medium (to which 16 mg of sodium acetate and 8 mg of sodium pyruvate had been added) and 10 cm³ of hydrogen. The electron acceptor, sodium sulfite, was added in the amounts indicated. Values for hydrogen consumed and sulfide formed represent final values per culture vessel. Hydrogen used by the culture was replaced at 24, 48, and 85 h. Growth period was 85 h.*

tor added. In a similar experiment, 0.32 mmol of sulfite was added; the culture produced 0.29 mmol of sulfide and consumed 0.86 mmol of hydrogen in agreement with the expected ratio of H_2:S^{2-} of 3:1. In this medium pyruvate could be used as a cell-carbon source, but when added at increasing levels in the presence of acetate, had no effect on cell yield or hydrogen consumption, indicating that it did not serve as an electron acceptor.

Results (Fig. 4) show values for the consumption of hydrogen and reduction of sodium thiosulfate to sulfide by a growing culture. The initial concentration of sodium thiosulfate was 0.16 mmol per bottle, and after 72 h the culture had consumed 0.67 mmol of hydrogen and produced 0.27 mmol of sulfide, with growth in proportion to hydrogen consumed and sulfide formed. Theoretical values would have been 0.64 mmol of hydrogen consumed and 0.32 mmol of sulfide produced. When sodium sulfate was substituted for sodium sulfite or sodium thiosulfate, no growth of the spirillum occurred and no sulfide was formed.

The organism was able to grow by the anaerobic oxidation of hydrogen and reduction of elemental sulfur. When malate-grown cells were inoculated into the basal medium which contained hydrogen, acetate, and elemental sulfur, considerable viability was lost in the first 36 h (Fig. 5). The culture recovered, growth following hydrogen consumption and sulfide formation. In a similar experiment, 1.8 mmol of hydrogen was consumed and 1.9 mmol of sulfide was detected. Cells were actively motile in this concentration of sulfide.

Anaerobic oxidation of formate. When formate was substituted for hydrogen, results for the reduction of SO_3^{2-}, $S_2O_3^{2-}$, and S^0 by the anaerobic oxidation of formate were similar to

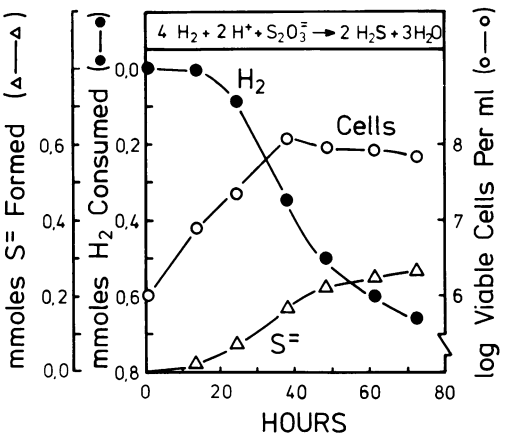

FIG. 4. *Relation of $S_2O_3^{2-}$ added to hydrogen consumed, sulfide formed, and cell yield by a growing culture of spirillum 5175. Culture conditions were similar to those for Fig. 3 except that pyruvate was omitted; 0.16 mmol of $S_2O_3^{2-}$ was added as the electron acceptor. At each time period indicated, hydrogen uptake was measured, and 0.2 ml of medium was removed, 0.1 ml being used for assay of viable cells and 0.1 ml being used for sulfide determination.*

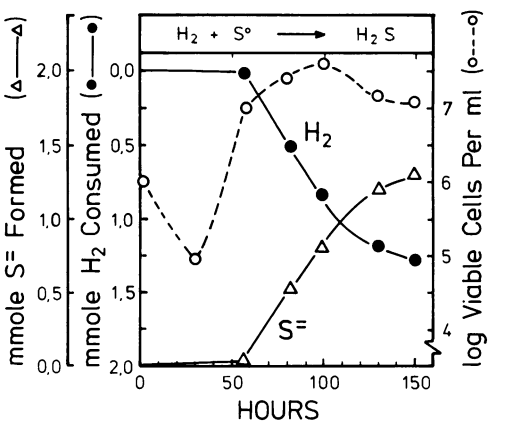

FIG. 5. *Relation of hydrogen consumed to sulfide formed and cell yield by growing cultures of spirillum 5175 in the presence of excess elemental sulfur. The experimental design was similar to that of Fig. 4 except that about 200 mg of elemental sulfur was added per bottle as the electron acceptor.*

those obtained for the anaerobic oxidation of hydrogen. For example, 1.47 mmol of sodium formate was added to an inoculated culture bottle which contained the basal medium plus acetate and excess elemental sulfur. After growth had ceased in 5 days, 1.42 mmol of sulfide was detected. Cells remained actively motile for many weeks at 25 or 4°C.

Syntrophism with *Chlorobium*. Since *Chlorobium limicola* does not use formate, it was possible to set up syntrophic mixtures with the spirillum. In this experiment, three culture bottles contained the basal medium with only formate and acetate added (Fig. 6A). The bottle labeled 9330 was inoculated only with *Chlorobium* strain 9330, and a small amount of growth occurred from the sulfide and acetate present in the basal medium. The bottle labeled 5175 received only the spirillum as inoculum; the medium remained water-clear, since no electron acceptor was provided for the anaerobic oxidation of formate. The culture bottle with both organisms showed heavy growth of *Chlorobium*. The bottles were fed formic acid every second day, and the pH was carefully monitored so that it did not rise above 7.3. A photomicrograph showing the syntrophic mixture of cells is shown in Fig. 6B. The proportion

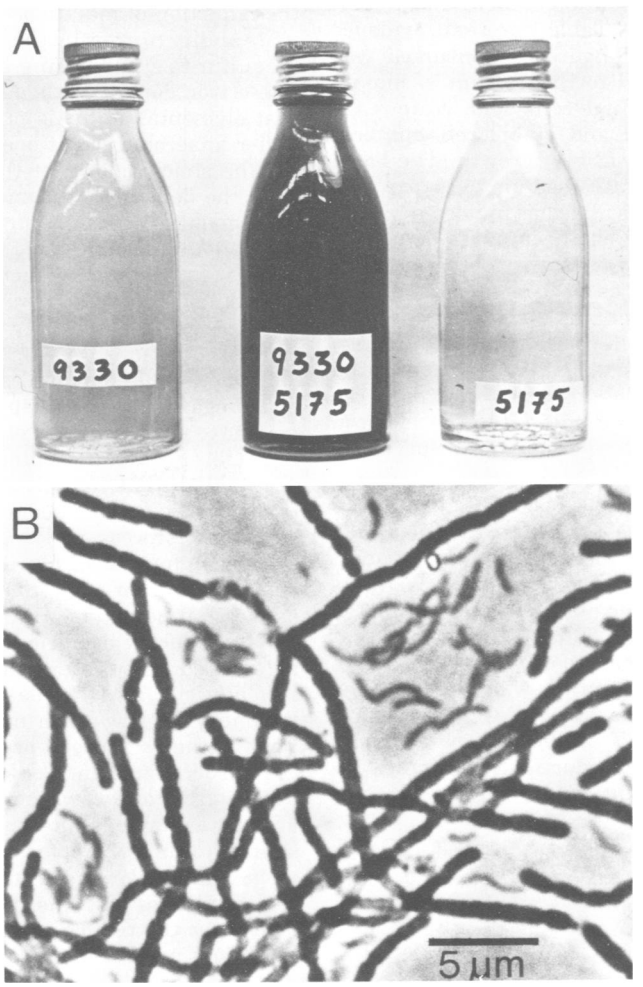

FIG. 6. *(A) Syntrophism between spirillum 5175 and Chlorobium limicola 9330. To each bottle sodium formate was added (from a 10% solution which had been adjusted to pH 6.7) to a final concentration of 0.2%. Sodium acetate was added as a carbon source at a final concentration of 0.02%. As formate was used the pH was adjusted to 6.8 by the addition of sterile formic acid. Time of incubation was 6 days in dim light (300 lux) at 25°C. (B) Phase-contrast photomicrograph of the syntrophic mixture showing the relative biomass of the two organisms. The culture was concentrated five times for the purpose of the photomicrograph.*

of the biomass of *Chlorobium* over that of the spirillum is apparent. Figure 7 presents the role of each organism in the syntrophic mixture and underscores the catalytic role of the limiting amount of sulfur in this system. Microscopic examination of such cultures revealed, when the oxidizable carbon substrate, formate, was depleted, since globules of elemental sulfur appeared. In cultures of *Chlorobium*, oxidation of S^0 to SO_4^{2-} is negligible as long as S^{2-} is present. Thus, S^0 served as an indicator of electron deficiency for the spirillum in syntrophic cultures.

Organic electron acceptors. Malate or fumarate also served as excellent electron acceptors for the anaerobic oxidation of molecular hydrogen by the spirillum. Table 1 presents evidence that the amount of hydrogen consumed was directly proportional to the amount of malate added and to the final cell yield. Values for hydrogen consumed and viable cell numbers were obtained after 72 h of growth. The basal medium contained 0.02% sodium pyruvate, and sodium L-malate was added as indicated. Succinate was identified as the product of malate reduction. Similar results were obtained when fumarate was added as electron acceptor. When hydrogen was present as the oxidizable substrate, the sulfide level in the medium remained constant as the malate concentration was increased. In the absence of hydrogen the organism grew, using sulfide as the electron donor for the reduction of malate to succinate.

DISCUSSION

To our knowledge spirillum 5175 is unlike any organism which has been reported. It most closely resembles *Vibrio succinogenes* in its ability to grow by the oxidation of formate and reduction of fumarate (18). It now appears that the reduction of fumarate as an electron acceptor is not unique and may be widespread among organisms, since recent studies show clearly that *Escherichia coli* is able to grow by oxidation of hydrogen and reduction of fumarate under anaerobic conditions (9). However, the

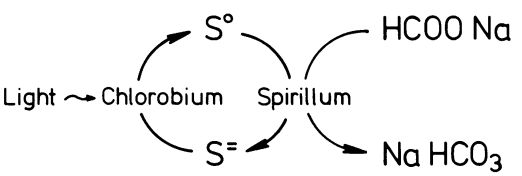

FIG. 7. *Syntrophism between Chlorobium limicola 9330 and spirillum 5175. The substrate formate is not metabolized by Chlorobium, and the limiting amount of sulfur is alternately reduced by the spirillum and oxidized by Chlorobium.*

TABLE 1. *Stoichiometry of hydrogen oxidation and malate reduction*

Bottle	L-Malate added (mmol)	Hydrogen consumed (mmol)	Sulfide detected (mmol)	Viable cells/ml
1	0.24	0.27	0.038	2.0×10^8
2	0.48	0.48		4.6×10^8
3	0.72	0.69	0.036	6.3×10^8

DNA base ratio is 49.2 mol% G+C for *V. succinogenes* and 38.4 mol% G+C for spirillum 5175. We prefer not to name the organism until other strains have been studied.

The new spirillum is unusual in its ability to anaerobically use H_2S as an electron donor for the reduction of malate or fumarate as well as in its ability to grow by the reduction of elemental sulfur to sulfide using hydrogen or formate as electron donors. Although we have observed that elemental sulfur is produced from sulfide under anaerobic conditions, we have not studied the stoichiometry of this process. Ecologically the detection of elemental sulfur may be analogous to the detection of hydrogen; it may not be found in anaerobic, dark environments because it is quickly metabolized. Spirillum 5175 differs from *Thiomicrospira* in being unable to grow aerobically under chemolithotrophic conditions (8).

Reduction of elemental sulfur to sulfide has been regarded as a nonspecific process which may have no significant role in cellular metabolism (13). Results presented in this paper (Fig. 5) establish that for spirillum 5175 the oxidation of hydrogen and reduction of S^0 to S^{2-} is a significant process, yielding energy for growth, even though the $\Delta G_0'$ for reaction 3 is only -6.5 kcal. In addition to S^0, SO_3^{2-} and $S_2O_3^{2-}$ serve as electron acceptors. However, in contrast to sulfate-reducing bacteria, spirillum 5175 is unable to reduce sulfate. It also differs from *Desulfovibrio* in its DNA base ratio as well as in its ability to grow under microaerophilic conditions in the presence of organic compounds.

The successful syntrophic culture of spirillum 5175 and *Chlorobium* reported here provides another example of interspecies sulfur transfer similar to the syntrophic culture of *Desulfuromonas* and *Chlorobium* studied recently (11a). The substrates now known to serve as electron donors for the reduction of S^0 under anaerobic conditions are H_2 and formate (spirillum 5175) as well as acetate, ethanol, propanol, and butanol (*Desulfuromonas*). In light of these results with syntrophic mixed cultures, we find it interesting to consider the report on the use of formate by a culture of "*Chloropseudomonas ethylica*" (7).

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