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Physiological Characteristics of *Thiomicrospira* sp. Strain L-12 Isolated from Deep-Sea Hydrothermal Vents†

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Growth of the obligately chemolithotrophic *Thiomicrospira* sp. strain L-12, isolated from a hydrothermal vent at a depth of 2,550 m in the Galapagos Rift region, was optimal at pH 8 and required 200 mM Na^+ and divalent ions (Ca^{2+} and Mg^{2+}). The organism was microaerophilic and tolerated 300 μM sulfide without a decrease in the rate of CO_2 incorporation. Growth and CO_2 incorporation occurred within the temperature range of 10 to 35°C, with both optimal at 25°C. At the in situ pressure of 250 atm, the rate of CO_2 incorporation was reduced by 25% relative to that measured at 1 atm; it was entirely suppressed at 500 atm. The results of this physiological characterization suggest that *Thiomicrospira* sp. strain L-12 can be an active autotroph in the hydrothermal environment.

Dense assemblages of benthic invertebrates have recently been found in association with hydrothermal vents of East Pacific ocean-floor spreading zones at a depth of 2,550 m (3, 6, 21). This discovery has led to studies on non-photosynthetic sources of organic carbon as a primary food supply for these unusually massive concentrations of animals, which are located at a depth where the biomass is normally very sparse as a result of the remoteness of the productive surface waters (18).

Warm water (up to ca. 20°C) discharging from the vents contains geothermally reduced inorganic compounds, of which hydrogen sulfide is among the most abundant (4, 5). This, coupled with evidence of dense microbial populations in the venting water (3, 8, 11), has led to the hypothesis that sulfur-oxidizing, chemoautotrophic bacteria function as primary producers for the vent ecosystem (8, 14). Enrichment cultures based on sulfide diffusion gradients and inoculated with vent water samples collected in the Galapagos Rift region have led to the repeated isolation of obligately chemolithotrophic bacteria that were described as belonging to the genus *Thiomicrospira* (17). Representatives of this genus were originally isolated from estuarine environments by Kuenen and Veldkamp in 1972 (13). Further information on their physiology was subsequently published by Timmer-ten Hoor (22), Kuenen et al. (12), and Cohen et al. (2).

We examined physiological characteristics of *Thiomicrospira* sp. strain L-12, one of 12 isolates that were essentially indistinguishable from one another (17). We focused on the effects of hydrostatic pressure, temperature, and sulfide concentration on growth and CO_2 incorporation in an effort to relate the occurrence of these organisms in a highly specific deep-sea environment to particular physiological traits.

MATERIALS AND METHODS

Organism. *Thiomicrospira* sp. strain L-12 was isolated from a sulfide diffusion gradient enrichment (17) inoculated with water and pieces of mussel periostracum collected at a vent in the Rosegarden area of the Galapagos Rift region (0° 45.25'N, 86° 13.48'W) at a depth of 2,550 m (3). Eleven other essentially indistinguishable strains were obtained from similar enrichments or direct platings of samples collected from this and a second vent area (Mussel Bed).

Culturing. A supplemented artificial seawater solution (ASW) based on that of Kuenen and Veldkamp (13) was generally used. It contained 430 mM NaCl, 7.6 mM $(NH_4)_2SO_4$, 6.1 mM $MgSO_4 \cdot 7H_2O$, 3.1 mM KH_2PO_4, 2.4 mM $NaHCO_3$, and 2.0 mM $CaCl_2 \cdot H_2O$, with 20 mM Tris-hydrochloride buffer, adjusted to 8.0, and 1 ml of trace element solution (24) per liter. Thiosulfate-supplemented ASW (T-ASW) contained 10 mM thiosulfate.

A thiosulfate-limited continuous culture of *Thiomicrospira* sp. strain L-12 was maintained at 25°C and served as a source of inocula for batch cultures incubated on a rotary shaker. The continuous culture was grown on T-ASW and remained at a density of 2.4 $\times 10^8$ to 3.0 $\times 10^8$ cells per ml at a dilution rate of 0.03 h^{-1}. Cell density was monitored by epifluorescence microscopy after staining with acridine orange (7). A density of 10^8 cells per ml was equivalent to 4.2 μg of total cell carbon per ml as analyzed with a Perkin-Elmer 240 elemental analyzer (17).

Analytical. Oxygen was monitored with a Rank Brothers continuously recording oxygen electrode at 21°C. Thiosulfate concentration was determined by

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the method of Sorbo (20). The production of acid equivalents (H^+) was estimated by periodic titration to the initial pH (8.3) with a 1 M solution of NaOH by use of a continuously recording Radiometer Titrator pH electrode.

The incorporation of CO_2 into cell material was measured as previously described (17). Samples of cultures grown in media containing [^{14}C]bicarbonate were filtered through 22-mm-diameter membrane filters (0.2-μm pore size Amicon Corp.). Filters were washed twice with 10 ml of seawater and placed in a desiccator with fuming HCl for 15 to 20 min to volatilize residual bicarbonate. Filters were transferred to vials and aired for 1 h before addition of 10 ml of Bray's solution for liquid scintillation counting. Corrections for quenching were made by the channels ratio method.

Growth studies. The effect of salts on the growth of *Thiomicrospira* sp. strain L-12 was determined in a basal medium containing 50 mM Tris-hydrochloride, pH 7.7, 9.5 mM NH_4Cl, 5 mM KCl, 0.16 mM KH_2PO_4, and 100 mg of ferric ammonium citrate per liter. To this were added, singly or in combinations, 100 mM NaCl, 0.2 mM $MgSO_4$, and 0.05 mM $CaCl_2$, 100 mM KCl, or 150 mM sucrose. Growth in the presence of different concentrations of Na^+ was examined in modified ASW media containing between 0 and 400 mM added NaCl. Potassium tetrathionate (13 mM) was included as the energy source. The cultures were incubated at 25°C for 3 days with periodic determination of cell density by epifluorescence direct count.

The effect of pH on the growth rate of strain L-12 was determined in cultures inoculated in T-ASW buffered to pH 6.0, 6.5, or 7.0 with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) or to pH 7.5, 8.0, or 8.5 with 50 mM Tris-hydrochloride buffer. Growth at 20°C was monitored for 2 days by epifluorescence direct count, and the growth rates were calculated.

CO_2 incorporation studies. A freshly prepared solution of 1 M Na_2S, adjusted to pH 8.5 with HCl, was diluted to various concentrations in ASW containing [^{14}C]bicarbonate. After inoculation, samples were removed at 5, 10, and 20 min. The samples were quickly filtered, and CO_2 incorporation was determined by scintillation counting. Rates of incorporation were calculated from these data.

For the study of pressure effects, three media were used: T-ASW, ASW plus 100 μM sulfide, and (as a control) ASW without addition of a reduced sulfur source. Each medium was inoculated with cells and, after the addition of [^{14}C]bicarbonate, was used to completely fill 42 polyethylene tubes, which were then sealed with serum stoppers. For each pressure tested, a set of triplicate tubes of each of the three media (nine tubes per set) was placed into seven pressure vessels and incubated at the test pressure. Seven identical sets of tubes were left at 1 atm. At about 2-h intervals, a pressure vessel was opened, the contents of its tubes were immediately filtered, and the amount of CO_2 incorporated was determined. At the same time, a set of 1-atm tubes was filtered. The values measured in the ASW controls were subtracted from those of the thiosulfate- and sulfide-containing cultures. They usually amounted to less than 5% of the experimental values.

Excretion of acid-stable products of CO_2 fixation was examined in an exponentially growing culture in T-ASW containing [^{14}C]bicarbonate. Viability of the cultures was judged by comparing epifluorescence direct counts with thiosulfate-soft agar plate counts (13). At intervals of several hours, samples were filtered, and the amount of label incorporated into cellular material was determined. The filtrate was acidified to pH 4 with 2 N H_2SO_4 to remove residual bicarbonate. After passing a stream of N_2 over the filtrate for 5 min, 0.1- and 0.5-ml samples were placed into 10 ml of Bray's solution plus 0.1 ml of hyamine hydroxide, and the acid-stable labeled organic compounds in the filtrate were counted.

RESULTS

Effects of salt and pH. *Thiomicrospira* sp. strain L-12 did not grow at NaCl concentrations below 80 mM, and about 200 to 400 mM was required for maximum cell density (data not shown). The presence of divalent ions (in the form of a $MgSO_4$-$CaCl_2$ combination) was also necessary for maximum growth. In the absence of either NaCl or divalent ions, growth was less than 1% of the control. Neither KCl nor an osmotically equivalent concentration of sucrose could satisfy the specific requirement for Na^+.

Growth occurred at a significant rate (greater than 40% of maximum) between pH 6.0 and 8.5. The optimal growth rate was at pH 8.0, which is close to the pH of seawater (8.1 to 8.3).

Effects of substrate (thiosulfate) and oxygen concentration. Upon addition of thiosulfate to a washed cell suspension of strain L-12, O_2 was consumed at a constant rate of 70 μmol/h per 10^{10} cells until the concentration reached about 20 μM (Fig. 1). Below this oxygen concentration the rate of consumption decreased and at about 2 μM reached an imperceptible level. The same cell suspension incorporated CO_2 after thiosulfate addition at a rate of 6 μmol/h per 10^{10} cells (Fig. 1, inset). Thus, about 12 mol of oxygen was consumed per mol of CO_2 incorporated, which compares well with the average ratio of 11.6 reported by Baalsrud and Baalsrud (1) for two species of *Thiobacillus*. About 1.6 mol of H^+ equivalents (as determined by titration) was produced per mol of thiosulfate utilized during balanced growth (data not shown). This suggested an 80% conversion of thiosulfate to sulfate.

Effects of sulfide, temperature, and hydrostatic pressure. Incorporation of CO_2 occurred over a broad range of sulfide concentrations from 10 to 2,000 μM. Figure 2 shows rates determined during the first 20 min in two separate experiments. Rates were linear over that period, indicating that sulfide was not depleted. Optimal CO_2 incorporation rates of 6 to 8 μmol/h per 10^{10} cells (at 20 to 21°C) were observed between 100 and 300 μM sulfide. These rates were equivalent to those observed with thiosulfate as the energy source. Pregrowing cells on either sulfide or

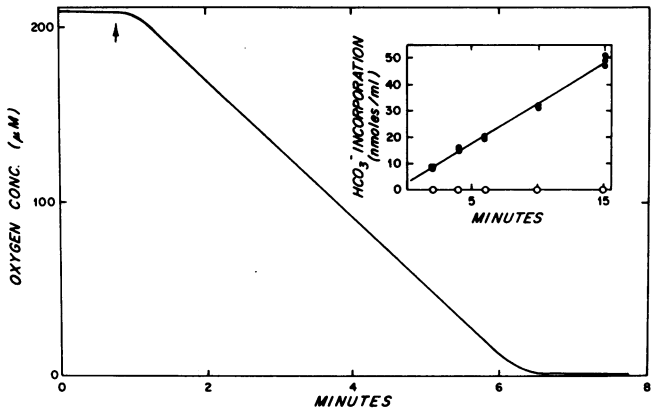

FIG. 1. Thiosulfate-initiated oxygen consumption by *Thiomicrospira* sp. strain L-12 at 21°C. Oxygen concentration was monitored with a continuously recording oxygen electrode. The arrow indicates the addition of 10 mM thiosulfate. Inset: Linear incorporation of CO_2 into cell material by the same culture at 21°C. Washed cells were incubated in ASW containing 111 μCi of [^{14}C]bicarbonate per ml either with (●) or without (○) 10 mM thiosulfate.

thiosulfate did not lead to a lag in the utilization of the other substrate.

During balanced growth the incorporation of CO_2 paralleled the increase in cell density (data not shown); the rates of both were measured to determine the effect of temperature on growth. Growth occurred over a temperature range of 10 to 35°C and was optimal at 25°C (Fig. 3). Under the conditions used the maximum rates of CO_2 incorporation and cell proliferation were equivalent to a doubling time of 190 min. The data indicated a rather narrow temperature range for optimal growth.

Cultures incubated at a hydrostatic pressure above 100 atm exhibited decreased CO_2 incorporation rates relative to those of 1-atm controls. For example, at a pressure of 300 atm the activity was reduced by about 35% (Fig. 4). Virtually the same results were obtained with either sulfide or thiosulfate as the substrate. Beyond a pressure of 100 atm, the rate of CO_2 incorporation declined linearly, and at 500 atm no CO_2 incorporation was detected (Fig. 5).

Excretion of fixed carbon. The filtrate of a highly viable (>95%) culture in balanced growth contained a significant amount of acid-stable fixed carbon (Fig. 6). The rate of appearance of excreted labeled carbon was similar to the rate of CO_2 incorporation into cells and accounted for an average of 9% of the total carbon fixed. The excretion of organic compounds has been

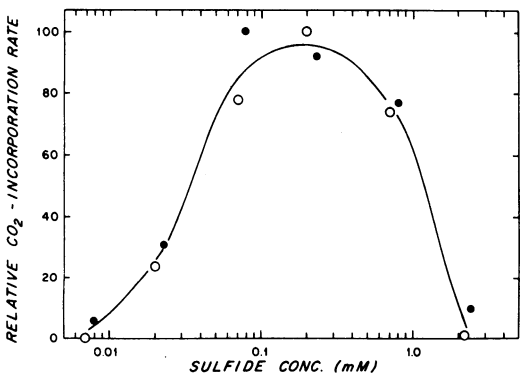

FIG. 2. Effect of initial sulfide concentration on the rate of CO_2 incorporation by *Thiomicrospira* sp. strain L-12. Data for two separate experiments (○, ●) are given. Cultures contained between 8×10^6 and 9×10^6 cells per ml and 140 μCi of [^{14}C]bicarbonate per ml. Maximum incorporation rates were 1,200 to 1,300 cpm/20 min.

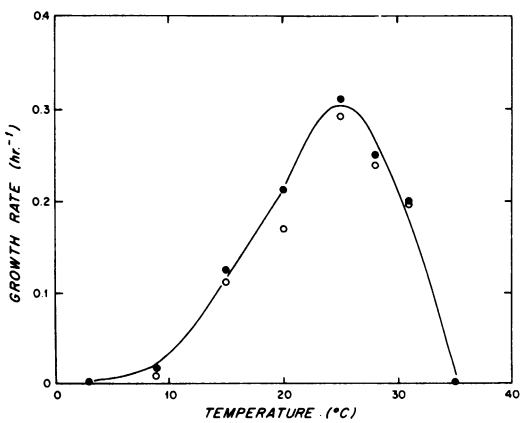

FIG. 3. Growth rate of *Thiomicrospira* sp. strain L-12 as a function of temperature. Growth was determined by the increase in cell number per hour (○) and the increase of CO_2 incorporation per hour (●). Cultures of T-ASW at pH 7.5 containing 5.3 μCi of [^{14}C]bicarbonate per ml and 2.3×10^6 cells per ml were maintained in water baths at the desired temperature.

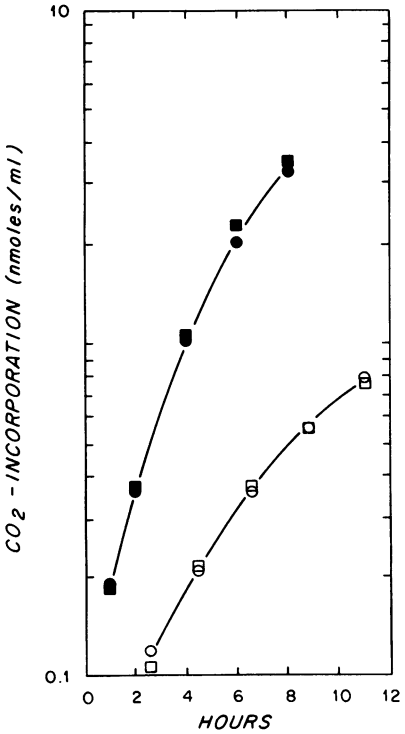

FIG. 4. Effect of hydrostatic pressure on CO_2 incorporation by *Thiomicrospira* sp. strain L-12 at 23°C. Cultures containing 8×10^5 cells per ml and 9 μCi of [^{14}C]bicarbonate per ml were incubated at 1 atm (solid symbols) and 300 atm (open symbols) with thiosulfate (●, ○) or sulfide (■, □) as substrates.

reported for *Thiobacillus* species and *T. pelophila* (2, 19) and depended on culture conditions.

DISCUSSION

The requirements for Na^+ and divalent ions found with *Thiomicrospira* sp. strain L-12 were similar to those reported for types of bacteria commonly found in the sea (15, 16) and are consistent with an existence in the marine environment. Although the optimal pH for growth was near that of seawater, significant rates of growth also occurred at pH 7.3, the estimated value for vent water (5). This preference for pH values above 7 differs from the more acidic pH optima of *T. pelophila* (13) and of the genus *Thiobacillus* (23) (except for the mixotroph, *T. novellus*, pH 7.8 to 9.0). Most marine strains of sulfur-oxidizing bacteria have been isolated from marsh muds or coastal sediments in which microniches or microzones of low pH are likely to exist (10). Because we had no access to the inside of the deep-sea vents, where the bulk of growth is believed to occur, physicochemical

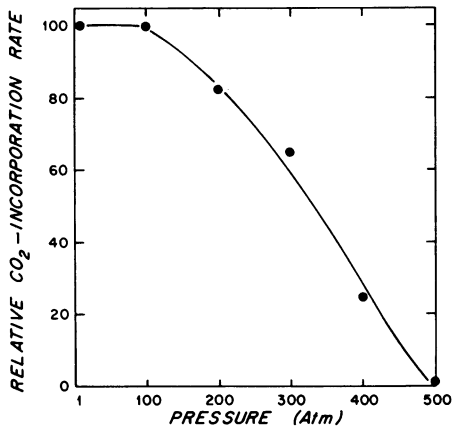

FIG. 5. Relative rates of CO_2 incorporation by *Thiomicrospira* sp. strain L-12 at hydrostatic pressures between 1 and 500 atm. Rates were calculated by linear regression of data from thiosulfate-containing cultures during the first 6 to 10 h of incorporation.

characteristics of possible microzones are not yet known.

The absence of a specific pressure adaptation in *Thiomicrospira* sp. strain L-12 is not surprising in the light of other, similar findings of barotolerant growth responses by deep-sea heterotrophic isolates (9, 25). At a hydrostatic pressure equivalent to that found at the vents

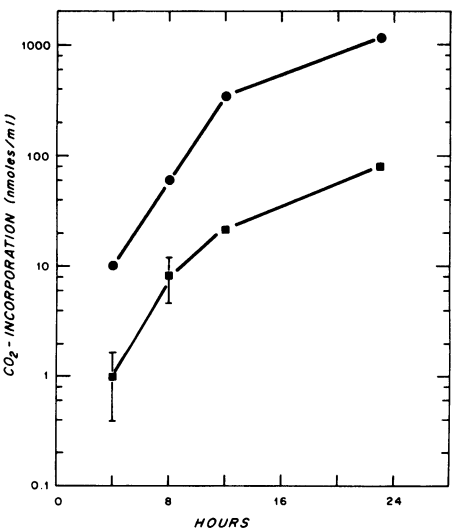

FIG. 6. CO_2 fixation by *Thiomicrospira* sp. strain L-12 into cell material (●) and into acid-stable dissolved organic compounds (■). Cultures in T-ASW containing 3.5×10^6 cells per ml and 76 μCi of [^{14}C]bicarbonate per ml were incubated at 23°C. Each measurement was performed in triplicate; 1 standard deviation error bars (if larger than the symbol) are shown.

(250 atm), the rate of CO_2 incorporation by strain L-12 was still 75% of its maximum value (Fig. 5). This maximum rate of CO_2 incorporation required sulfide concentrations of 100 to 300 μM and a temperature of 25°C. The values measured in water collected near a vent opening were 160 μM sulfide and 17°C (5). Sulfide concentration and temperature can be expected to increase with depth in the vents, where the rates of CO_2 incorporation and growth of bacteria like *Thiomicrospira* sp. strain L-12 would likely be the greatest. *Thiomicrospira* sp. strain L-12, like some other sulfur-oxidizing chemoautotrophs (2, 19), not only produces particulate biomass during cell growth, but also excretes a significant portion of its fixed carbon products as dissolved organic compounds. This activity may contribute to the increased concentrations of dissolved organic compounds reported in water samples collected close to the vents (P. M. Williams, K. L. Smith, E. M. Druffel, and T. W. Linick, Nature [London], in press).

Whereas the capacity for nitrogen fixation (17) and thermophilic behavior (J. Baross, personal communication) was observed among the thiobacillus-like isolates obtained from the vents, the *Thiomicrospira* sp. strain described in this paper exhibited neither of these traits.

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