APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1987, p. 1940–1943
0099-2240/87/081940-04$02.00/0

Vol. 53, No. 8

NOTES

Fractionation of Stable Carbon Isotopes during Chemoautotrophic Growth of Sulfur-Oxidizing Bacteria†

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Received 3 February 1987/Accepted 7 May 1987

Laboratory-grown strains of chemoautotrophic *Thiomicrospira* sp. strain L-12 and *Thiobacillus neapolitanus* produced cell carbon that was 24.6 to 25.1 ppt (24.6 to 25.1 mg/g) lower in ^{13}C isotope abundance than the ambient source of carbon dioxide and bicarbonate. This degree of ^{13}C isotope depletion was comparable to that found in organic material produced in deep-sea hydrothermal-vent communities.

Photosynthetic organisms are responsible for the bulk of organic carbon and cellular energy generated in the biosphere; recently, however, it has been recognized that, in certain environments, the contribution of bacterial chemosynthesis may be of great significance (15–17). In particular, chemoautotrophic sulfur-oxidizing bacteria are believed to play a role in recycling energy and reduced carbon within marsh sediments (23), to act as nutritional symbionts in a variety of marine invertebrates (2, 30, 34), and to be the major primary producers of organic carbon in deep-sea hydrothermal-vent communities (18, 19). At least some portion of all of these environments is characterized by both the absence of significant photosynthetic activity and the presence of sulfur-oxidizing bacteria plus the substrates (H_2S and O_2) required for their chemosynthetic growth.

It is often difficult to identify the source(s) of organic carbon or to follow its flow through such complex ecosystems as those mentioned above, but trophic studies have been aided by comparisons of stable-carbon-isotope ratios in ambient carbon dioxide or bicarbonate with the ratios in tissues of organisms at different trophic levels (12). The most significant degree of fractionation of carbon isotopes occurs during the initial steps of phototrophic reduction of inorganic carbon (i.e., carbon dioxide and bicarbonate) (8) and results in a decreased $^{13}C/^{12}C$ isotope ratio in the organic product relative to that of the source carbon (22). Subsequent utilization of the organic carbon leads to small but detectable increases in the $^{13}C/^{12}C$ isotope ratio as the material passes through the food chain. Thus, the relative degree of isotope depletion has been used in a number of studies to infer a relationship between photosynthetic primary producers and the organisms that depend upon them for organic carbon (12, 25).

Application of this approach to studies of trophic interactions in environments where chemosynthesis is believed to be the sole or major source of organic carbon has been severely limited by the lack of data on the extent of carbon isotope fractionation that characterizes chemosynthetic carbon fixation. The isotopic composition of a mat of a sulfur-oxidizing *Beggiatoa* sp. collected near a marine oil seep has been reported (31); however, no measurement of the source carbon composition was made, and the net degree of fractionation could not be calculated.

There is a similar absence of information about both the conditions of growth and the source-carbon-isotope ratio in a report on the isotopic composition of laboratory-grown cells of a marine nitrifying bacterium, *Nitrosocystis* (renamed *Nitrosococcus* [35]) *oceanus* (7). Nevertheless, these studies have suggested that the reduction of inorganic carbon by chemoautotrophs may lead to a significantly greater depletion of ^{13}C than that resulting from photosynthetic fixation (7). Thus, because tissues of certain hydrothermal-vent invertebrates also demonstrate unusually high ^{13}C depletion, it has been suggested that chemosynthesis, rather than photosynthesis, is the major source of reduced carbon in both hydrothermal-vent communities and symbiotic vent invertebrates (24, 34).

It is inherently difficult, however, to interpret the information provided by ratios of stable isotopes in naturally occurring organisms. Such ratios are the additive result of an often complex series of chemical and biological processes that are characteristic of a particular environment during carbon fixation. Although the step at which the major fractionation of carbon occurs is commonly assumed to be the initial fixation event, even this process is rarely measurable in the natural environment.

The extent of fractionation of stable carbon isotopes, as well as of stable sulfur isotopes, is affected by rates of metabolism (7, 10, 11, 14, 26), by temperature, and possibly by other growth parameters (3, 7). Therefore, in order to substantiate the significance of isotope enrichments or depletions as indicators of chemoautotrophic or chemolithotrophic metabolism, studies under controlled growth conditions are necessary. Furthermore, knowledge of the isotope composition of the source carbon (or sulfur) is an absolute requirement. To draw attention to this need is one reason to communicate the following preliminary data on the carbon isotope fractionation by two chemoautotrophic sulfur-oxidizing bacteria: *Thiomicrospira* sp. strain L-12, a strain with no assigned specific epithet that was isolated from a hydrothermal vent (27), and a strain of *Thiobacillus*

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† Contribution no. 6405 of the Woods Hole Oceanographic Institution.

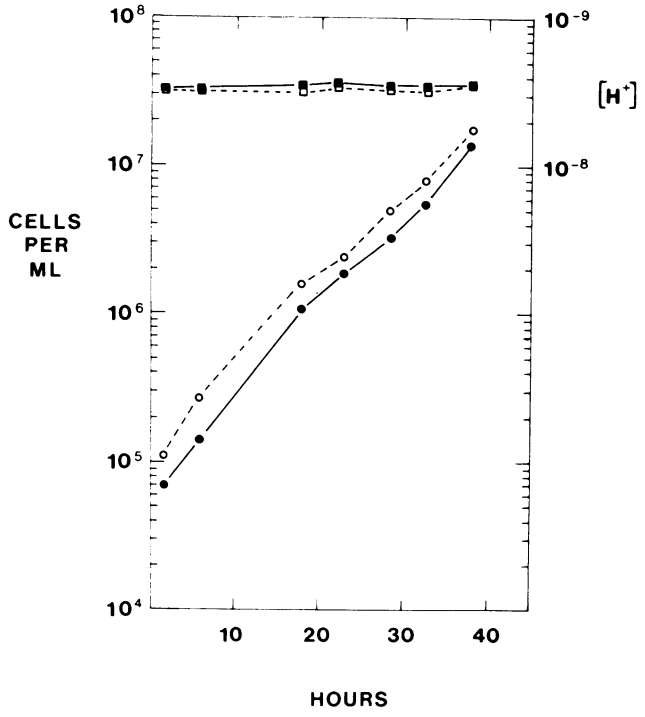

FIG. 1. Growth of *Thiomicrospira* sp. strain L-12 in artificial seawater containing thiosulfate. Cell densities in two parallel growth experiments (○ and ●) were determined by fluorescence microscopy after staining with acridine orange (27). Medium pH (□ and ■) was determined on culture portions by using a pH electrode. The cultures were maintained at a temperature of 20 to 22°C and were purged constantly with a stream of bottled, compressed air.

neapolitanus, isolated from a small marsh near Woods Hole, Mass.

Thiomicrospira sp. strain L-12 (27, 28) was grown in a supplemented artificial-seawater solution similar to that used by Kuenen and Veldkamp (20). It contained 417 mM NaCl, 7.6 mM $(NH_4)_2SO_4$, 6.1 mM $MgSO_4$, and 2.7 mM $CaCl_2$ plus 50 mM Tris hydrochloride buffer, adjusted to pH 8.35, and 2 ml of trace element solution (32) per liter. After being autoclaved, filter-sterilized solutions of $Na_2S_2O_3$ (16 mM), $NaHCO_3$ (7.1 mM), and KH_2PO_4 (0.1 mM) were added to achieve the concentrations indicated in parentheses. Identical glass culture vessels, each containing 10 liters of sterile growth medium, were fitted with silicone stoppers and tubing to allow constant aseptic sparging of the culture with bottled compressed air before and during cell growth. After equilibration with the sparging gas, the total concentration of inorganic carbon (dissolved carbon dioxide and bicarbonate) was between 2 and 4 mM, which is about that typical of seawater and is 35 to 50% of the concentration reported for some hydrothermal-vent water (6). Portions of the medium were removed by siphoning, with minimal exposure to the atmosphere.

When inoculated into growth media, duplicate cultures of *Thiomicrospira* sp. strain L-12 grew with a doubling time of about 5 h at 22°C (Fig. 1), a rate comparable to that previously reported for this strain when grown under optimal conditions (27). After 38 h the cultures were still increasing exponentially in cell number, whereas no decrease in medium pH was observed during the aproximately 200-fold increase in cell density. These data indicate that the cultures were exhibiting vigorous, balanced growth throughout the experiment.

TABLE 1. Fractionation of carbon by *Thiomicrospira* sp. strain L-12 during chemosynthetic growth[a]

Culture no.	Medium HCO_3^- concn (mmol/kg)	$\delta^{13}C$[b] of:		$\Delta\delta^{13}C$[c]
		Medium	Cells	
1 (at inoculation)	3.5	−9.1		
1 (at harvest)	2.8	−10.4	−34.8	−24.4
2 (at harvest)	3.5	−9.6	−34.5	−24.9

[a] Cells were grown for 38 h as described in the text. Where indicated, samples of medium or cells were taken for analysis either just before inoculation or at the time of harvest.

[b] Relative isotopic compositions are reported as $\delta^{13}C$ values, where $\delta^{13}C = [(^{13}C/^{12}C \text{ ratio of sample})/(^{13}C/^{12}C \text{ ratio of standard}) - 1] \times 10^{-3}$. The standard used for comparison is the Peedee belemnite standard (9).

[c] $\Delta\delta^{13}C = \delta^{13}C$ of cells − $\delta^{13}C$ of medium.

Samples of cell-free medium, as well as glass-fiber filters containing the total cell mass (6.3 mg of C) from 10 liters of the 42-h culture, were used to determine the isotopic composition of both the inorganic carbon in the medium and the cellular organic carbon by procedures similar to those described by Deuser and Hunt (9) and Craig (5), respectively. Analyses of the inorganic carbon in the medium revealed no major change in its total concentration or in its isotopic ratio during the course of cell growth (Table 1). In contrast, the isotopic composition of *Thiomicrospira* sp. strain L-12 cell carbon was considerably depleted in ^{13}C relative to that of the medium, indicating an unusually substantial and reproducible isotopic fractionation. A similar fractionation during an identical growth experiment was observed in a strain of *Thiobacillus neapolitanus* (Table 2), suggesting that such an unusually high degree of fractionation is characteristic of sulfur-oxidizing chemoautotrophs in general and not specific to hydrothermal-vent isolates.

There is no information on the isotopic composition of the inorganic carbon pool that served as the source for the symbiotic production of organic carbon in mussel tissue collected from the Galapagos hydrothermal-vent community (Table 2). However, if we assume that it is similar in

TABLE 2. ^{13}C values calculated for marine organic material[a]

Sample	$\delta^{13}C$ of:		$\Delta\delta^{13}C$
	Medium CO_2	Sample C	
Thiomicrospira sp. strain L-12	−10.0	−34.6	−24.6 ± 0.3[b]
Thiobacillus neapolitanus	−2.9	−28.0	−25.1 ± 0.6[b]
Mussel tissue[c]			
Muscle	—[d]	−33.3	−27.3[e]
Gonads	—[d]	−32.9	−26.9[e]
Marine plankton	+1 to −2[f]	−10 to −23[g]	−8 to −24

[a] Isotope ratios ($\delta^{13}C$) were calculated as described in footnote *b*, Table 1.

[b] Values are averages ± 1 standard deviation of two parallel cultures.

[c] Mussel tissue was dissected from specimens collected at the Galapagos Rift hydrothermal vent site during Atlantis II/Alvin Cruise no. 102 (January, 1979).

[d] —, Source carbon for mussel tissue is unknown.

[e] Based on a vent water bicarbonate $\delta^{13}C$ value of −6.0 (6).

[f] Composite data from references 8 and 23.

[g] Composite data from references 7 and 23.

composition to that reported for vent water (6), the calculated depletion of ^{13}C is very close to that observed for *Thiomicrospira* sp. strain L-12 and *Thiobacillus neapolitanus* and is significantly different from that characteristic of photosynthetically derived organic material found in marine plankton (Table 2). This conclusion supports the suggestion that chemosynthetically derived, rather than photosynthetically derived, organic carbon serves as the primary organic material supporting growth of mussels and certain other invertebrates surrounding hydrothermal vents.

Care must be exercised in drawing conclusions from a limited number of determinations of the extent of isotopic fractionation demonstrated by laboratory cultures. For example, although the extent of carbon isotope fractionation by the sulfur-oxidizing bacteria reported here was greater than that typical of photosynthetic organisms either found in natural samples or grown under controlled conditions (1, 23, 33), an equally great or greater extent of fractionation has also been observed in some photosynthetic bacteria (29), cyanobacteria (21), and green algae (21, 29). These data again indicate the importance of the particular growth conditions used in fractionation studies (7). This point is particularly critical in studies of symbiotic chemosynthetic associations. Both the bacterial symbiont's physiological state and the source carbon composition may be not only quite different from that of free-living chemosynthetic bacteria but also tightly regulated by the animal host.

The potential role of other chemosynthetic bacteria in the production of organic material in vent (17) and marsh (15) environments is also of interest. The possibility that microbially or geothermally produced methane may be utilized by marine invertebrates has been strengthened recently by the report on an apparent methanotrophic bacterial symbiosis in a marine mollusk (4). Because of the great carbon fractionation associated with methanogenesis (13), a significant input of microbial methane into the food chain would have a major effect on depressing the $^{13}C/^{12}C$ ratio in the associated heterotrophic biomass.

In conclusion, laboratory studies on microbial isotopic fractionations not only will provide indispensable background information for ecological interpretations of the isotope ratios observed in natural systems but also will help uncover the physiological and biochemical bases for the greater carbon isotope fractionation apparently characteristic of chemosynthesis.

We thank Carl Wirsen for supplying the mussel tissue carbon isotope fractionation data and Brian Fry for helpful discussions.

This work was supported by National Science Foundation grant OCE83-8631. For a portion of this study E.G.R. was supported by a postdoctoral fellowship from the Woods Hole Oceanographic Institution.

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