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## Evidence for Methylo-trophic Symbionts in a Hydrothermal Vent Mussel (*Bivalvia: Mytilidae*) from the Mid-Atlantic Ridge†

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Symbioses between chemolithoautotrophic bacteria and the major macrofaunal species found at hydrothermal vents have been reported for numerous sites in the Pacific Ocean. We present microscopical and enzymatic evidence that methylo-trophic bacteria occur as intracellular symbionts in a new species of mytilid mussel discovered at the Mid-Atlantic Ridge hydrothermal vents. Two distinct ultrastructural types of gram-negative procaryotic symbionts were observed within gill epithelial cells by transmission electron microscopy: small coccoid or rod-shaped cells and larger coccoid cells with stacked intracytoplasmic membranes typical of methane-utilizing bacteria. Methanol dehydrogenase, an enzyme diagnostic of methylo-trophs, was detected in the mytilid gills, while tests for ribulose-1,5-bisphosphate carboxylase, the enzyme diagnostic of autotrophy via the Calvin cycle, were negative. Stable carbon isotope values ( $\delta^{13}\text{C}$ ) of mytilid tissue ( $-32.7$  and  $-32.5\text{‰}$  for gill and foot tissues, respectively) fall within the range of values reported for Pacific vent symbioses but do not preclude the use of vent-derived methane reported to be isotopically heavy relative to biogenically produced methane.

The discovery of thriving invertebrate communities surrounding deep-sea hydrothermal vents in the Atlantic and Pacific oceans (17, 33) has focused our attention on the use of both alternative energy sources and metabolic strategies in the deep sea. Rather than depending on photosynthesis as the base of the food chain, these communities appear to exist through bacterial chemosynthesis utilizing reduced energy sources in the hydrothermal fluids for autotrophic fixation of  $\text{CO}_2$ . In addition to being the main source of food for vent filter-feeding and grazing organisms, chemosynthetic bacteria also occur in symbiotic associations with the major species of macrofauna typically found at vents in the Pacific, i.e., vestimentiferan tube worms, vesicomyid clams, and mytilid mussels. Considerable evidence indicating that these symbionts are chemolithoautotrophs, utilizing reduced sulfur compounds such as hydrogen sulfide available in the vent fluids as an energy source for the fixation of carbon dioxide as their primary source of carbon, has been presented (for reviews, see references 10 and 22). These procaryotic symbionts, occurring intracellularly within the tissues of these animals, thus provide their hosts with an internal source of nutrition while the hosts supply the bacteria with the inorganic substrates required for chemosynthesis.

This work was followed by the discovery of symbiotic associations between methylo-trophic bacteria and certain deep-sea invertebrates (for a review, see reference 11). So far, all animals harboring methylo-trophs are found in reducing sediments at cold seeps: namely, two new species of mytilid mussels from the base of the Florida Escarpment (12) and hydrocarbon seeps in the Gulf of Mexico (7, 14) and the tube worm *Siboglinum poseidoni* (phylum Pogonophora)

from the central Skagerrak (34). On the basis of a variety of evidence, including the presence of intracytoplasmic membranes, these symbionts are believed to be methanotrophic, i.e., capable of using methane as their primary carbon and energy source.

In 1985, an active vent site at 23°N (Snakepit site) was discovered on the Mid-Atlantic Ridge (MAR). The first submersible dive to this hydrothermal site was made in 1986 (37). The invertebrate populations of the MAR vent sites are distinctly different from those found at Pacific vents and are dominated by a single species of shrimp (38). A few mussels of a new species of the family Mytilidae (as yet unnamed [37a]) were observed and collected at the Snakepit site in 1986 (37). Since the water chemistry of the MAR vent emissions is very similar to that of Pacific vents (8), it could be assumed that these mytilids would also harbor sulfur-oxidizing chemoautotrophs within their gill tissues. Instead, ultrastructural features, as well as enzymatic and biochemical evidence, suggest that in contrast to the Pacific hydrothermal vent bivalves, the MAR hydrothermal vent mytilid harbors methylo-trophic symbionts within its gills.

### MATERIALS AND METHODS

**Organisms.** Mytilids were collected in January 1990 from the MAR 23°N hydrothermal vent site (Snakepit; depth, 3,476 m) by using DSRV (deep-submergence research vehicle) *ALVIN*. The area of collection on the southwest mound showed shimmering water in much of the region around the mytilids. Four animals were collected with a dip net, put into an insulated container while the submarine was on the ocean floor, transported to the surface, and transferred to chilled seawater (4°C) before being processed. Specimens were either dissected aboard the ship and fixed for electron microscopy or frozen whole at  $-70^\circ\text{C}$ . The latter specimens

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were transported to the laboratory on dry ice and stored at  $-40^{\circ}\text{C}$ .

**Methylobacterium extorquens** AM1, a free-living methylotrophic bacterium used as a positive control for enzyme assays, was grown for 48 h on methanol in ammonium mineral salts (NMS) medium (23). Cells were pelleted at  $12,000 \times g$ , washed in chilled medium, repelleted, and frozen at  $-70^{\circ}\text{C}$  or used immediately.

**Microscopy.** Pieces of gill tissue dissected aboard the ship were stored fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 3 weeks until they were returned to the laboratory. Tissues were then dehydrated through an alcohol series and embedded in Spurr's. Thin sections were stained with lead citrate and uranyl acetate and examined with a Hitachi 7000 transmission electron microscope operating at an accelerating voltage of 60 kV.

**Enzyme assays.** Mytilid gill and mantle tissues were tested for activities of enzymes which are diagnostic of autotrophy and methylotrophy. Cell extracts were assayed for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) by using the procedure of Beudeker et al. (4) as modified by Nelson and Jannasch (30). Methanol dehydrogenase (MeDH) was assayed by the method of Anthony and Zatman (3).

Tissues from two whole frozen mytilids were dissected, weighed ( $\sim 0.5$  to  $0.75$  g), and homogenized in 4 to 5 volumes of the appropriate assay buffer. Spinach, serving as a positive control for RuBisCO, was treated similarly. *M. extorquens* AM1, a positive control for MeDH, was resuspended in 2.0 ml of NMS medium without methanol. Tissue homogenates and the *M. extorquens* AM1 cell suspension were run through a chilled French press ( $9,000$  lb/in $^2$ ), and cell extracts were prepared by collecting the supernatant after centrifugation for 4 min at  $14,000 \times g$ . Both crude homogenates and cell extracts were tested in initial experiments; activities were comparable, so subsequent experiments used only cell extracts to reduce absorbance by cell debris. For each specimen, activities were determined at three different concentrations (ranging from 35 to 300  $\mu\text{l}$ ) of cell extract. Protein was determined by the Coomassie brilliant blue dye binding technique (6) with the Bio-Rad Laboratories assay kit.

**Stable isotopes.** Stable isotope ratios, an indication of possible food sources, were determined by standard methods (5, 39) in the laboratories of Brian Fry (Ecosystems Center, Marine Biological Laboratory, Woods Hole, Mass.) and Joseph Montoya (Harvard University). Dried samples of gill, foot, and mantle tissues were combusted in sealed tubes by the Dumas method and analyzed on an isotope ratio mass spectrometer. Isotope values are calculated relative to the standards Pee Dee Belemnite and atmospheric nitrogen by using the standard delta notation  $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$ , where  $X = {}^{13}\text{C}$  or  ${}^{15}\text{N}$  and  $R = {}^{13}\text{C}:{}^{12}\text{C}$  or  ${}^{15}\text{N}:{}^{14}\text{N}$ .

## RESULTS

**Microscopy.** As in other deep-sea mytilid-bacterium symbioses, the gills of the MAR mytilid were thick and fleshy compared with those of symbiont-free species such as *Mytilus edulis* and *Modiolus demissus*. Examination with transmission electron microscopy revealed other overall similarities. The gills comprised epithelial cells (bacteriocytes) containing numerous subcellular inclusions resembling procaryotic cells, interspersed with symbiont-free intercalary cells (Fig. 1). The symbionts, as the bacterium-like inclusions will be referred to, had gram-negative cell enve-

lopes and were contained within vacuoles bound by a membrane presumed to derive from the host cell. Myelin-like inclusions were typically observed in the basal region of the bacteriocyte, suggesting possible lysosomal digestion of symbionts in this region.

The MAR mytilid, unlike other hydrothermal vent bivalves, appeared to have two distinctly different types of symbionts based on ultrastructure, one large and one small (Fig. 2). The large symbionts averaged  $1.2 \mu\text{m}$  in diameter, were round to oval, and contained stacked intracytoplasmic membranes. The small symbionts averaged  $0.4 \mu\text{m}$  in diameter, appeared rod or coccoid shaped in cross section, and completely lacked internal membranes.

**Enzyme assays.** Activities of the Calvin-Benson cycle enzyme, RuBisCO, were not detectable in cell extracts of the two gill samples tested but were high in the spinach control.

Activities of MeDH, the dissimilatory-pathway enzyme which catalyzes the oxidation of methanol to formaldehyde, were detectable in the gill tissues of both MAR mytilids tested, averaging 4 and 81  $\text{nmol min}^{-1} \text{mg of protein}^{-1}$  (Table 1). Activity was not detected in symbiont-free mantle tissue extracts. The initial methanol oxidation rate increased proportionally with increasing amount of cell extract and was completely abolished by a 5-min boiling, indicating that the observed oxidation was enzyme mediated.

**Stable isotopes.** Stable carbon and nitrogen isotope values for MAR mytilid tissues are shown in Table 2. Stable isotope ratios were similar for symbiont-containing gill and symbiont-free mantle and foot tissues for a given specimen. Overall,  $\delta^{13}\text{C}$  values ranged from  $-32.5$  to  $-35.6\text{‰}$  and  $\delta^{15}\text{N}$  values ranged from  $-4.2$  to  $-10.5\text{‰}$ .

## DISCUSSION

The MAR mytilid is somewhat unusual in terms of its occurrence compared with bivalve populations at Pacific vent sites. Very few specimens were observed only at one location of the 23 $^{\circ}\text{N}$  site (Snakepit). To date, the reported bacterial symbionts of hydrothermal vent bivalves, including mytilids and vesicomyid clams, are all sulfur-oxidizing chemolithoautotrophs (for a review, see reference 22). We were therefore surprised to find bacteria containing stacked intracytoplasmic membranes in the gill tissue of a mytilid found at the MAR hydrothermal vents. Such membranes are not present in sulfur oxidizers but are characteristic of ammonia- and nitrite-oxidizing autotrophs as well as methanotrophs, a group of methylotrophic bacteria that utilize methane (26).

While we cannot rule out the presence of RuBisCO in the gills of this animal, the enzyme data indicate that at least some of the symbionts observed are methylotrophic. MeDH, an enzyme diagnostic of methylotrophs, was measurable in the MAR mytilid gill extracts at activities comparable to those found in the gills of seep mytilids (Table 1). Assuming that bacterial symbiont protein equals 10 to 20% of the total extract protein, the MAR mytilid MeDH activities fall well within the range for free-living methylotrophs (Table 1). We did not test for methane utilization in these specimens because of the limited supply of tissue, the duration of frozen storage (21 months), and the extreme lability of methane monooxygenase, the enzyme catalyzing the oxidation of methane to methanol, to freezing (11, 12, 32). However, the co-occurrence of MeDH activity and stacked internal membranes suggests that the larger symbionts are methanotrophs.

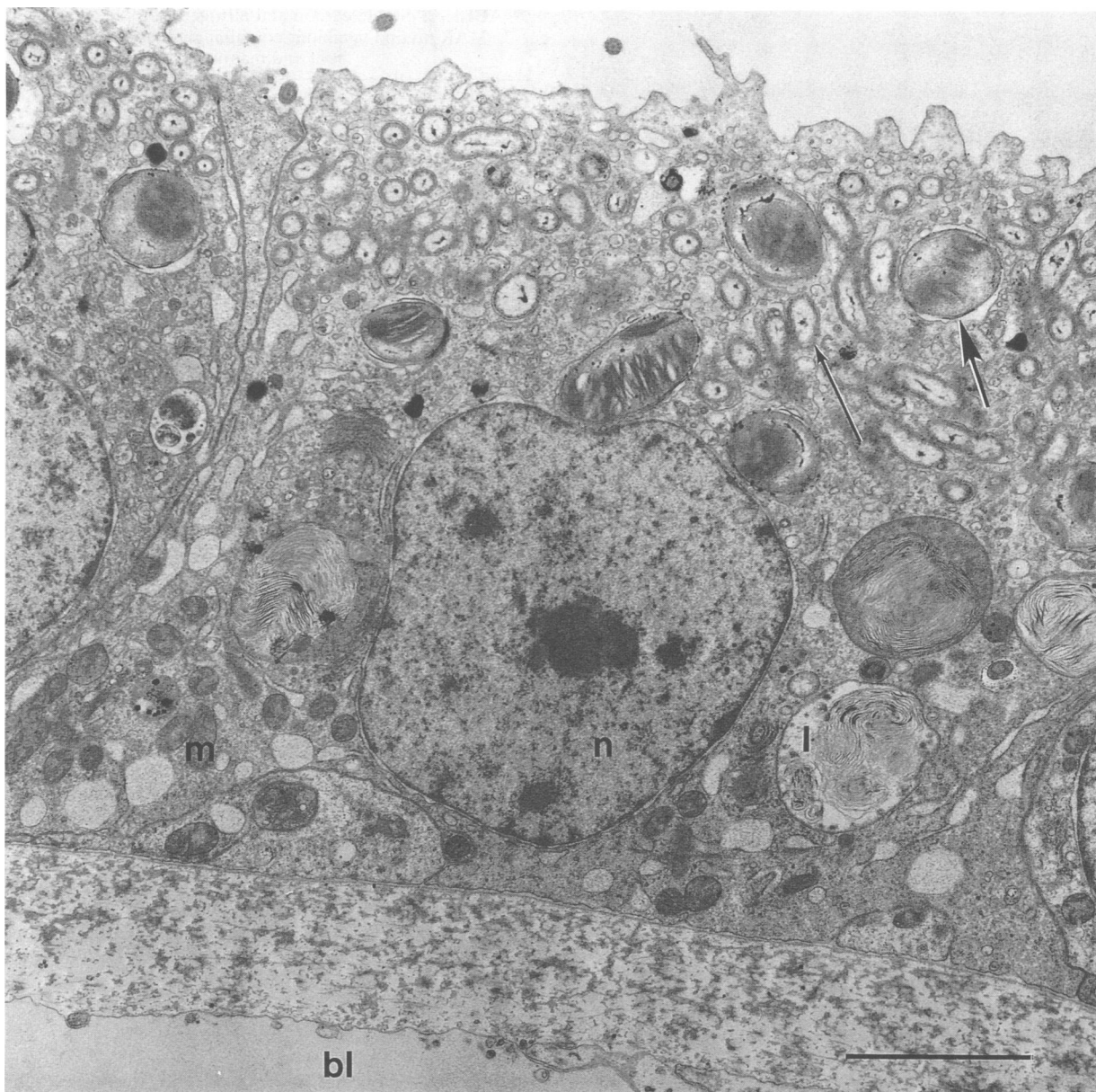


FIG. 1. Transmission electron micrograph of a gill filament of the MAR mytilid showing bacteriocytes containing two different size classes of procaryotes: larger coccoid cells containing stacked intracytoplasmic membranes (thick arrow) and smaller rod- or coccoid-shaped cells (thin arrow). Lysosome-like residual bodies suggest possible digestion of symbionts in the basal region of the cell. Bar = 3  $\mu$ m. Abbreviations: bl, blood space; l, lysosome-like residual body; m, mitochondria; n, bacteriocyte nucleus.

Observation of two types of symbionts in the MAR mytilid (Fig. 1 and 2), distinguished by both size and presence of intracytoplasmic membranes, parallels transmission electron microscopy observations of the Florida Escarpment seep mytilid symbiosis (12). It is not known for either of these mytilids whether the smaller symbionts are a different species or represent a developmental stage of a single symbiont. The latter is possible since methanotrophs may not develop stacked intracytoplasmic membranes when growing on methane under certain culture conditions (15, 32). In support of this, only a single type of dominant symbiont has been shown to exist in all invertebrate-chemoautotroph symbioses on the basis of 16S rRNA sequence analysis (20, 21).

However, phylogenetic analysis of 16S rRNA sequences indicates that there are two symbionts present in the Florida Escarpment mytilid (19a). The coexistence of two different species of bacteria in a single eucaryotic host cell is not common for metazoan-procaryote symbioses, although it is well documented among protist-procaryote symbioses (see, for example, references 25 and 36). Pending fresh material collected from the MAR site, further characterization of the MAR mytilid at the subcellular level, using immunological detection of autotrophic or methylotrophic enzymes and in situ localization of symbiont 16S rRNA, will allow us to address this issue.

Stable carbon and nitrogen isotope analyses have been



FIG. 2. Transmission electron micrograph of the MAR mytilid at a greater magnification than that used for Fig. 1. Two different symbiont morphotypes with cell envelopes typical of gram-negative bacteria are shown. Symbionts are contained within membrane-bound vacuoles (arrows). Note stacks of intracytoplasmic membranes in the large symbiont. Bar = 0.5  $\mu$ m.

used extensively to examine trophic relationships between organisms and to establish the dependence of vent species on organic material produced via chemosynthesis or methanotrophy (for a review, see reference 16). Unlike the other invertebrate-methanotroph symbioses (7, 31, 34), the stable carbon isotope signature of the MAR mytilid provides few clues as to the symbiont carbon source. The MAR mytilid  $\delta^{13}\text{C}$  values, ranging from  $-32.7$  to  $-35.6\text{‰}$  (Table 2), show no unusual depletion indicative of a biogenic or thermogenic methane source but rather are similar to those reported for Pacific vent bivalve-chemolithoautotroph symbioses (16). These values do not preclude the use of vent-derived methane, which is likely to be similar to the isotopically heavy hydrothermal-fluid methane ( $\delta^{13}\text{C} = -15$  to  $-17\text{‰}$ ) reported for the 21°N East Pacific Rise (40). Furthermore, the isotopic composition ( $\delta^{13}\text{C}$ ) of methane from deep-earth natural-gas samples is reported to range from  $-25$  to  $-80\text{‰}$  (35).

TABLE 1. MeDH activities in mytilid symbiont-containing gills and in free-living methylotrophs

| Organism(s)                     | Enzyme activity <sup>a</sup> (n) | Reference  |
|---------------------------------|----------------------------------|------------|
| <b>Mytilids</b>                 |                                  |            |
| MAR                             | 4.4, 81 (2)                      | This study |
| Florida Escarpment <sup>b</sup> | 4–32 (2)                         | 12         |
|                                 | $163 \pm 43^c$ (4)               | 9          |
| Louisiana slope                 | 2.7–4.4 <sup>c</sup> (3)         | 7          |
| <i>M. extorquens</i> AM1        | 173                              | This study |
| All methylotrophs               | 60–600 <sup>d</sup>              | 2          |

<sup>a</sup> Expressed as nanomoles minute<sup>-1</sup> milligram of protein<sup>-1</sup>.

<sup>b</sup> Lower values are for mytilids frozen for 1.5 years; higher values are for freshly collected mytilids.

<sup>c</sup> Assuming protein accounts for 15% of tissue (wet weight).

<sup>d</sup> Usual range.

TABLE 2. Stable carbon and nitrogen isotopic composition of MAR mytilid symbiont-containing gill and symbiont-free foot and mantle tissues

| MAR mytilid | Tissue | $\delta^{13}\text{C}$ (‰) | $\delta^{15}\text{N}$ (‰) |
|-------------|--------|---------------------------|---------------------------|
| 1           | Gill   | $-32.7$                   | $-4.2$                    |
|             | Foot   | $-32.5$                   | $-4.6$                    |
| 2           | Gill   | $-35.6$                   | $-8.7$                    |
|             | Mantle | $-34.0$                   | $-10.5$                   |
| 3           | Gill   | $-35.1$                   | $-9.9$                    |
|             | Mantle | $-34.6$                   | $-9.9$                    |

The stable nitrogen isotope values of the MAR mytilid tissues, ranging from  $-4.2$  to  $-10.5\text{‰}$  (Table 2), fall within the range of those reported for symbiont-containing vent and seep bivalve tissues ( $\delta^{15}\text{N} = -12.0$  to  $+1.8\text{‰}$ ) (16). These isotope ratios are quite depleted compared with those of nonvent deep-sea invertebrates ( $\delta^{15}\text{N} = +9.8$  to  $+16.5\text{‰}$ ) (16) and with  $\delta^{15}\text{N}$  values for potential deep-ocean nitrogen sources (nitrogen  $+1\text{‰}$ ; nitrate,  $+4.5$  to  $+7\text{‰}$ ; ammonia,  $+7\text{‰}$ ; and organic nitrogen,  $-1$  to  $+6\text{‰}$ ) (1, 28, 29). Information on the availability and isotopic composition of nitrogen sources at the MAR vent site and on the symbiosis nitrogen assimilation pathways is needed to explain these depleted values.

While reduced inorganic sulfur, and not methane, is the electron donor for animal-bacterium symbioses reported for Pacific hydrothermal vent sites (22), the potential for the use of methane as an energy source at these sites nevertheless exists. Methane oxidation has been measured at the Juan de Fuca hydrothermal vent site for bacteria both in water samples and on the surfaces of invertebrates (18, 19). Methane is present in end member fluids at strikingly similar concentrations (range, 50 to 100  $\mu\text{M}$ ) at the MAR Snakepit site (13, 24) and East Pacific Rise sites (27, 40). Further studies are required to determine the biochemical versatility of the symbionts (one or two types) present as well as environmental conditions that may be specific for the MAR habitat.

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