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Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami Bay, Japan

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Abstract. *Bathymodiolus platifrons,* a chemosynthetic mussel from cold seeps off Japan, relies for its nutrition on the productivity of methylotrophic or methanotrophic endosymbionts. High densities of bacterial symbionts appearing to be type I methanotrophs were observed in transmission electron micrographs of gill tissues. Methanol dehydrogenase activity in gill tissue from a single individual was positive compared to non-methanotrophic control samples, indicating a high potential for methanotrophy. Stable isotopic ratios of carbon in symbiont-containing gill tissue, as well as host tissues, were extremely depleted in ^{13}C, and similar to values reported for other methanotrophic species. TEMs of gill tissue showing symbionts in various stages of digestion support the hypothesis that carbon transfer from symbionts to *B. platifrons* occurs through intracellular digestion of the symbionts. Discovery of methane- or methanol-based symbioses in *B. platifrons* from cold seeps in Sagami Bay extends the range of such symbioses to include cold seeps and hydrothermal vents, and supports the idea that environmental methane levels control the distribution of these symbioses.

Additional key words: chemosynthesis, methanotrophy, methylotrophy, bivalve, biogeography

In chemoautotrophic bacterial-invertebrate symbioses, the animals receive much or all of their nutrition from the symbiotic bacteria, while the bacteria presumably benefit from a protected and stable physical and chemical environment favorable for carbon fixation. Such symbioses span various invertebrate phyla and are common among molluscs, especially bivalves inhabiting hydrothermal vents, cold seeps, and other sub-oxic sediments (Van Dover 2000 and references therein). Morphological and physiological specializations for chemosynthetic symbioses vary greatly among host species, ranging from those with epibiotic symbionts and few adaptations for nutritional integration (e.g., alvinellid polychaetes from hydrothermal vents) to species that lack feeding or digestive organs and are extremely specialized to enhance and exploit carbon fixation by bacterial symbionts (e.g., vestimentiferan worms). Molluscan hosts generally fall between these extremes, having enlarged gills containing high densities of endosymbiotic bacteria, and reduced conventional feeding and digestive structures (Fisher 1990; Childress & Fisher 1992; Nelson & Fisher 1995; Scott & Fisher 1995).

Although many reduced compounds are thought suitable to support carbon fixation by bacterial symbionts, sulfide oxidation is the basis for most known chemosynthetic symbioses (Cavanaugh 1985). The predominance of sulfur-based chemosynthesis in invertebrate-bacterial symbioses may be related to several factors. Some invertebrate hosts are able to bind sulfide, enabling them to elevate internal sulfide levels orders of magnitude above ambient environmental concentrations (Childress & Fisher 1992). Sulfide binding serves several functions, including reduced sulfide toxicity, storage of sulfide for use by symbionts as required, and partial physiological control over the supply of sulfide to symbionts.

Conversely, methane, though energetically comparable to sulfide in oxidation potential (Anthony 1982; Nelson & Hagen 1995), is less frequently the basis for chemosynthesis in invertebrate-bacterial symbioses. Because metazoans are unable to bind and store methane, species with endosymbiotic methanotrophic bacteria may thrive only in environments with stable and

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elevated methane levels. Methanotrophic species, obligate C_1 oxidizers that use methane as both a carbon source and an electron donor, are capable of oxidizing methane to methanol (catalyzed by methane monooxygenase), the first step in methane-based carbon assimilation. Methylotrophs do not oxidize methane, but can assimilate oxidized forms of methane such as methanol and other C_1 compounds, or other multi-carbon organic compounds (Anthony 1982). Because all sites where metazoans with methanotrophic or methylotrophic endosymbionts have been reported also have high environmental methane levels—which very likely represent the energy basis for chemosynthesis in these systems—we use methane-based symbioses to refer to invertebrate-bacterial symbioses involving methanotrophic, or possibly methylotrophic, symbionts.

Methanotrophic invertebrate-bacterial symbioses have been reported from the Atlantic Ocean for several species of mytilid mussels in the genus *Bathymodiolus* from methane-rich environments (Gulf of Mexico, Childress et al. 1986; Fisher et al. 1987; Barbados accretionary complex, Olu et al. 1996; Mid-Atlantic Ridge, Cavanaugh et al. 1992), a sponge-bacterial symbiosis in the Barbados accretionary complex (Vacelet et al. 1995, 1996), and a pogonophoran (*Siboglinum poseidoni*) in the Skagerrak (Schmaljohann & Flügel 1987). In the Pacific Ocean basin, methanotrophic symbionts have been reported recently from *B. platifrons* and *B. japonicus* from hydrothermal vents in the Okinawa Trough (Fujikura et al. 2000), and are possibly present in a snail (*Olgaconcha tufari*) from thermal springs in the Manus Basin (Gal'chenko et al. 1992). Methodological limitations concerning the analysis of *O. tufari* indicate that additional study is required to determine whether this species has methanotrophic symbionts.

In this paper, we document a methanotrophic or methylotrophic bivalve-bacterial symbiosis in the mussel *B. platifrons* from the Hatsushima cold seeps in Sagami Bay, Japan. Evidence is presented demonstrating the presence of methylotrophic or methanotrophic symbionts in gill tissues, activity of methanol dehydrogenase (MDH), a key enzyme in methane-oxidizing metabolic pathways, lysosomal features suggesting the dominant mode of carbon transfer from symbiont to host, and additional information indicating the nutritional reliance of *B. platifrons* on methane-based carbon fixation.

Methods

Study site—Hatsushima cold seeps

The Hatsushima cold seeps are located at a depth of 1100 m in Sagami Bay, Japan (34°59.9′N, 139°13.6′E) and have been described in detail by Hashimoto et al. (1989) and Tsunogai et al. (1996). Fluid seepage at these sites supports a diverse assemblage of chemosynthetic fauna dominated by vesicomyid clams (mainly *Calyptogena soyoae*), mytilid mussels (*Bathymodiolus platifrons* and other congeners), vestimentiferan worms (*Lamellibrachia* sp. and *Escarpia* sp.), and other less abundant chemoautotrophic species as well as numerous non-chemosynthetic benthic megafauna. Mytilid mussels are found commonly among rocky outcrops with partial sediment cover also inhabited by vestimentiferan worms, near sedimentary habitats with aggregations of vesicomyid clams.

Collection of specimens

Specimens of *Bathymodiolus platifrons* HASHIMOTO & OKUTANI 1994, *Calyptogena soyoae* OKUTANI 1957, and associated cold-seep fauna were collected from the Hatsushima cold seeps during dives of the Shinkai 2000 manned submersible operated by the Japan Marine Science and Technology Center (JAMSTEC). Specimens were collected from mussel aggregations on rock outcrops, placed in a sample basket, and covered with sediment to insulate the specimens from warm surface temperatures during recovery of the submarine. Following recovery, several individuals of *B. platifrons* and *C. soyoae* were frozen (−80°C) immediately. Additional specimens of both species were preserved for microscopy.

Electron microscopy

Samples of excised gill tissue from *B. platifrons* and *C. soyoae* were prepared for ultrastructural and morphological studies by preservation in cold 0.1 M cacodylate-buffered glutaraldehyde (2%) in filtered seawater. Samples for transmission electron microscopy (TEM) were also dehydrated and infiltrated using microwave techniques (Giberson et al. 1997).

Methanol dehydrogenase activity

Methanol dehydrogenase (MDH) catalyzes the oxidation of methanol to formaldehyde and is a key enzyme in all methanotrophic and methylotrophic organisms (Anthony 1982). Although the presence of methane mono-oxygenase, which catalyzes the conversion of methane to methanol, is a much more direct indication of methanotrophy, this enzyme degrades rapidly following the death of the individual and must be analyzed quickly (Fisher 1990). Because our analyses relied on frozen specimens, we were not able to discriminate between methanotrophy and methylotrophy with this technique.

Activity of MDH in tissues of *B. platifrons* was assayed from homogenates of frozen gill tissues. Approximately 0.5 g of gill tissue from a single individual of *B. platifrons* was suspended in 2.5 ml of chilled phosphate buffer (0.05 M, pH 6.0), ground in an ice-cold glass tissue grinder, and passed through a French pressure cell (15,000 psi). The supernatant (10,000 × g, 5 min) was assayed for MDH activity at 5°C by the method of Anthony & Zatman (1964). This procedure was repeated using homogenates from 3 individuals of *Calyptogena kilmeri* BERNARD 1974, a chemoautotrophic clam that is thiotrophic, rather than methanotrophic or methylotrophic, and thus should have no MDH activity and is a suitable negative control. Calculated rates are reported after subtracting the rate observed in the assay mixture without methanol. As a positive control, a species of *Hyphomicrobium* grown anaerobically on a methanol/nitrate medium at 30°C was assayed and found to have MDH activity of 95 nmol min^{-1} mg^{-1} protein, within the expected range. Protein was assayed by the Coomassie brilliant blue dye binding technique as described previously (Nelson et al. 1989).

Tissue isotopic analyses

Tissue samples of *B. platifrons* and *C. soyoae* were dissected from frozen specimens for analyses of stable carbon isotope ratios. After thawing to room temperature, samples of gill, foot, adductor muscle, and mantle tissues were dissected from individuals of each species, rinsed in filtered seawater, dried (60°C), and powdered in a mortar & pestle. Powdered samples were acidified with dilute HCl to remove inorganic carbonate, and combusted in a Finnagen mass spectrometer (R. Dunbar, Stanford University).

Results

Morphology and cytology

Micrographs (TEM) prepared from hypertrophied gill tissue of *Bathymodiolus platifrons* (Fig. 1) revealed high densities of coccoid or rod-shaped bacterial symbionts with distinctive, stacked intracytoplasmic membranes known only from type I methanotrophic bacteria (Brock 1974). Electron lucent inclusions in bacterial cells (Fig. 1C,D) are identical in appearance to poly-hydroxybutyrate-like inclusions reported from other methanotrophic bacteria (Anthony 1982).

The symbionts appear to represent several stages of degradation (Fig. 1C,D). A gradient in cell types from robust, intact bacterial symbionts with highly detailed type I methanotroph-like ultrastructure, to partially degraded cells, and finally, aggregations of membranous elements is evident in some TEM images. Secondary lysosomes, vacuoles filled with aggregations of whorled membranes or filaments, were most abundant adjacent to blood vessels. High densities of intact, apparently healthy symbionts of various sizes were common along the ciliated cell margin of bacteriocytes, where water flow would presumably provide locally high methane levels. This region of presumed symbiont development and growth contrasts with the margin of bacteriocytes adjacent to blood vessels, where membranous material and degraded symbionts were most common. Several stages of cell division within bacteriocytes, indicating *in situ* growth, were also observed.

TEMs of gill tissue of *C. soyoae* revealed high densities of sulfide-oxidizing bacteria.

Methanol dehydrogenase activity

MDH activity for the single individual tested was positive compared to control tissues, indicating that methanotrophic or methylotrophic symbionts, or both, were present in gill tissue. The MDH assay of gill tissue from *B. platifrons* yielded an activity of 3 nmol min^{-1} mg^{-1} protein. MDH activity in gills of *C. kilmeri,* which lacks methanotrophic or methylotrophic symbionts, was below the detection limit (0.1 nmol min^{-1} mg^{-1} protein) for all individuals assayed.

Carbon isotope ratios

Carbon isotopes analyzed from several tissues of *B. platifrons* were extremely depleted in ^{13}C (Table 1). Carbon was isotopically light in all tissues and was within the range ($\delta^{13}C < -50‰$) expected for nutritional reliance on methanotrophic or methylotrophic bacteria (Fisher 1990). Compared to tissues of *C. soyoae* ($\delta^{13}C = -35.6‰$), carbon in tissues of *B. platifrons* was considerably lighter isotopically, suggesting a difference in the source of organic carbon.

Discussion

Evidence for methane-based chemosynthesis

Several sources of information support the hypothesis that the bacterial symbiosis of *Bathymodiolus platifrons* in the Hatsushima cold seeps is based on methane oxidation. Symbionts with morphologies typical of type I methanotrophs (Anthony 1982) were found in gills of *B. platifrons.* The morphology of these symbionts is indistinguishable from methanotrophic symbionts reported recently in individuals of *B. platifrons* from hydrothermal vents in the Okinawa Trough (Fujiwara et al. 2000), and is very similar to methylo-

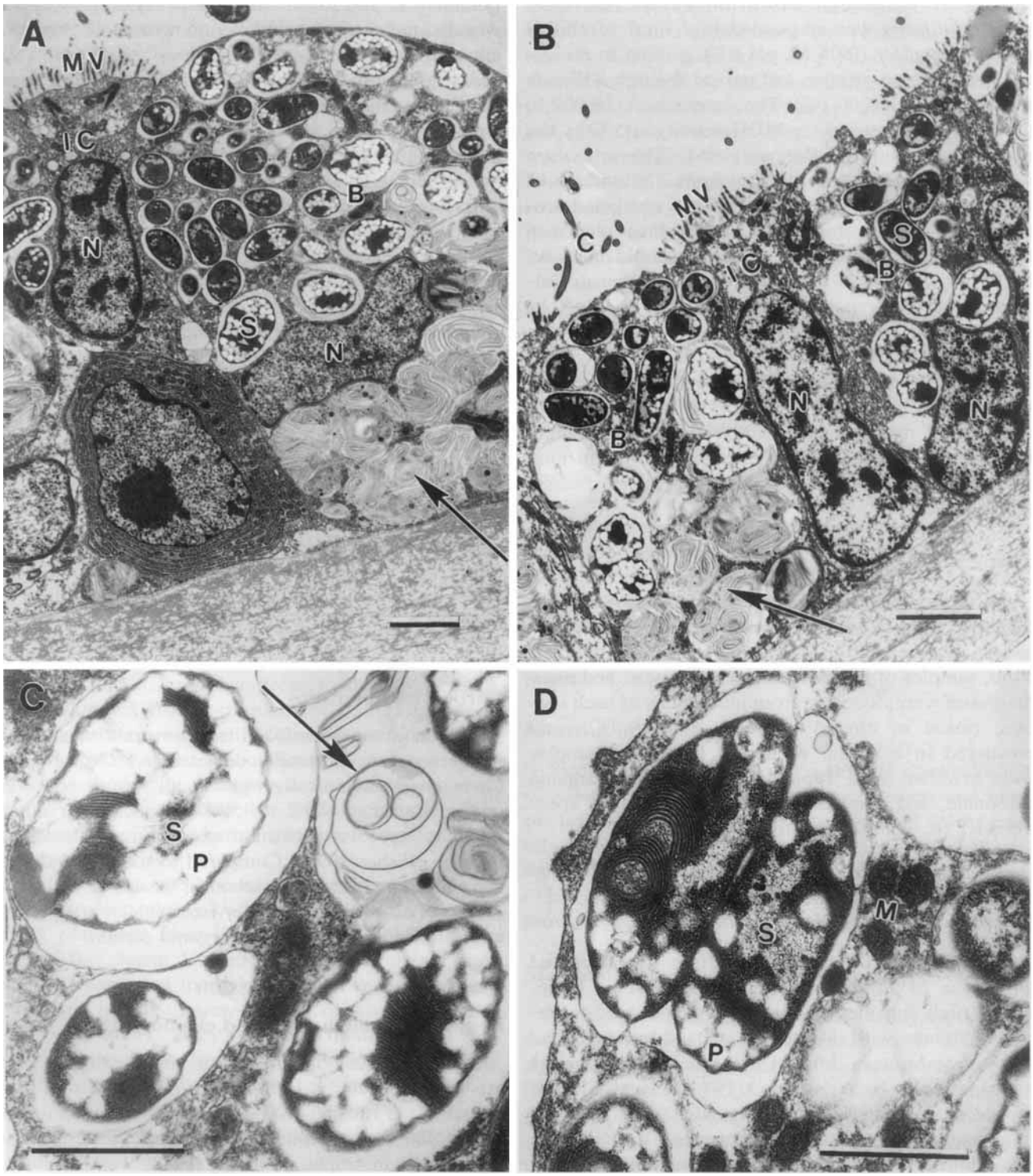

Fig. 1. Gill tissue from *Bathymodiolus platifrons*. TEM. **A, B.** Cross sections of gills showing symbiotic bacteria (S) in the bacteriocyte (B), intercalary cells (IC), their respective nuclei (N), microvilli (MV), cilia (C), and secondary lysosomal bodies (arrows). Water flow is above and blood vessel below. Scale bars, 2 μm. **C, D.** Detail of a symbiont (S) including stacked intracytoplasmic membranes, poly-hydroxybutarate inclusions (P), bacteriocyte mitochondria (M), and secondary lysosomal bodies (arrow) with residual membrane-like material. Scale bars, 1 μm.

Table 1. Stable carbon isotopic values for tissues for *B. platifrons*. Values for stable isotopic ratios are reported as ‰ relative to PeeDee belemnite (PDB). Data for each of two specimens of *B. platifrons* are given separately, followed by the mean of the two values.

Tissue	$\delta^{13}C$	$\delta^{13}C$	Mean $\delta^{13}C$
Foot	−63.77	−62.68	−63.22
Adductor	−64.01	−62.43	−63.22
Mantle	−67.64	−65.72	−66.68
Gill	−68.08	−67.54	−67.81

trophic or methanotrophic symbionts from species of *Bathymodiolus* in Atlantic seep and vent locations (Cavanaugh et al. 1992; Fisher & Childress 1992). As with *B. platifrons* from the Okinawa Trough, no other symbiont morphologies (i.e., sulfide-oxidizing bacteria, which lack stacked intracytoplasmic membranes) were observed.

All species of chemosynthetic mussels studied, including some with both methane-based symbionts and sulfide-oxidizing symbionts, have enlarged ctenidia and reduced digestive systems, representing adaptations for nutritional reliance on chemosynthesis rather than filter-feeding, even though the latter feeding mode remains possible for some species. The soft anatomy of *B. platifrons,* described in detail by Hashimoto & Okutani (1994), is similar to that of several congeners, with greatly thickened and elongated ctenidia, and only slight mantle fusion. The gut is short and straight, and lacks a recurrent loop found in most other mytilids. Species of *Bathymodiolus* that feed on particulate material have smaller and thinner ctenidia than *B. platifrons* and other methanotrophic mussels (Gustafson et al. 1998), further supporting a chemosynthetic nutritional mode for *B. platifrons.*

Although only a single individual of *B. platifrons* was analyzed, the positive assay for methanol dehydrogenase activity indicates the potential for oxidation of methanol to formaldehyde, an oxidative step found in both methanotrophs and methylotrophs. MDH activity measured for *B. platifrons* (3 nmol min^{-1} mg^{-1} protein) is within the range for bivalve gills known to contain methylotrophic symbionts (Cavanaugh et al. 1992). The much higher MDH activity in a culture of free-living methylotrophic bacteria (*Hyphomicrobium,* 95 nmol min^{-1} mg^{-1} protein), compared to that measured in *B. platifrons,* is not surprising and is consistent with methylotrophy or methanotrophy in both species. First, MDH activity was detected in *B. platifrons* and *Hyphomicrobium* sp., but was, as expected, undetectable (<0.1 nmol min^{-1} mg^{-1} protein) in all individuals of *C. kilmeri.* Second, temperature effects on MDH activity are partially responsible for the lower activity in symbionts of *B. platifrons* incubated near the temperature at the Hatsushima cold seeps (~5°C), compared to the *Hyphomicrobium* culture grown at 30°C. Assuming a Q_{10} of 2.0 for enzyme activity (Keeton 1980), the rate estimated for symbionts of *B. platifrons* at 30°C would be ~17 nmol min^{-1} mg^{-1} protein—closer, but still much lower than measured in the *Hyphomicrobium* culture. Third, MDH activity is scaled by protein content in the assay, which for tissue from *B. platifrons* includes protein from the symbionts and gill tissue, whereas the value for *Hyphomicrobium* is based on a pure bacterial culture. Because only the bacteria are expected to express MDH activity, the protein-specific rate calculated for *B. platifrons* is conservative.

We cannot, however, confirm whether symbionts of *B. platifrons* from Sagami Bay are methanotrophic or methylotrophic, even though conspecific mussels collected from hot vents in the Okinawa Trough, as well as several congeners from Atlantic sites have symbionts with 16S ribosomal RNA sequences that cluster phylogenetically among known methanotrophs (Distel et al. 1995; Robinson et al. 1998; Fujikura et al. 2000). Additional studies to measure methane uptake (e.g., Cary et al. 1989; Kochevar et al. 1992), coupled with enzyme assays (methane mono-oxygenase activity), lipid characterization, and genetic studies could all be used to discriminate between methylotrophic and methanotrophic pathways and determine whether *B. platifrons* from vents and seeps are solely methanotrophic.

The extremely light isotopic content of carbon in tissues of *B. platifrons* ($\delta^{13}C$ ~−65‰) and comparatively heavier carbon in tissues of sympatric vesicomyid clams (*Calyptogena soyoae,* $\delta^{13}C$ = −35.6‰) indicates a carbon source other than CO_2 for the former. Sulfide-oxidizing symbionts of vesicomyid clams use CO_2 as a carbon source, which in the top 21 cm of sediment at the Hatsushima cold seeps has an isotopic composition of $\delta^{13}C$ = −7 to −45‰ (Masuzawa et al. 1992), with heaviest CO_2 near the sediment surface. Because thiotrophic bacteria fractionate carbon isotopes by ~25‰ (Ruby et al. 1987), carbon in tissue of *C. soyoae* probably arose from a CO_2 source just below the sediment surface with values of $\delta^{13}C$ ~−11‰. This carbon source could not yield the isotopic composition measured for tissues of *B. platifrons,* even if this species had sulfide-oxidizing symbionts. Isotopically light, biogenic methane, as found at the Hatsushima seeps (Hattori et al. 1996) is a more likely carbon source for *B. platifrons.* Carbon values from $\delta^{13}C$ = −90 to −60‰ are generally considered to originate from biogenic methanogenesis, while val-

ues from −50 to −30‰ arise from thermogenic processes (reviewed by Martens et al. 1991). Tissues of *B. platifrons* ($\delta^{13}C$ = −65‰) should have an isotopic composition similar to its carbon source, because isotopic fractionation by methanotrophic or methylotrophic bacteria is considered minor (Fisher 1990) and fractionation via heterotrophy (i.e., filter-feeding) is very small (Parsons et al. 1984). Although its gill morphology is adapted for a chemosynthetic lifestyle (see above), *B. platifrons* retains an intact, though reduced, digestive system, and may also filter-feed. Any nutritional contribution from filtration must be minor, however, since filtered material would include, in addition to methanotrophic bacteria, isotopically heavier free-living thiotrophic and heterotrophic bacteria.

Organic carbon transfer from symbiont to host

Energy transfer between bacteria and host has been investigated for few invertebrate-bacterial symbioses. Transfer of fixed carbon is thought to occur by intracellular digestion of symbionts in host bacteriocytes or through translocation of dissolved metabolic products from intact symbionts to the host. Digestion of symbionts may be a primary mechanism for carbon transfer, or a consequence of "cellular housekeeping" in which the host digests aging symbionts (Fisher 1990), or both. The presence of primary and secondary lysosomes with accumulated membranous material from symbionts in various stages of digestion (Fig. 1C) is consistent with symbiont digestion as a mode of metabolite transfer. Similar ultrastructure has been reported for invertebrate-bacterial symbioses from several taxa (reviewed by Streams et al. 1997; Gustafson et al. 1998). Fisher & Childress (1992) and Streams et al. (1997) measured uptake and assimilation of ^{14}C-labeled methane by *Bathymodiolus childressi,* using pulse/chase radiolabel assays. Methane assimilation in *B. childressi* was rapid in gill tissues containing symbionts, but in tissue lacking symbionts, radiolabeled carbon did not accumulate for several days, supporting the hypothesis that carbon transfer occurred via symbiont digestion. Electron micrographs of gill tissues corroborated the carbon uptake measurements, showing evidence of intracellular symbiont digestion similar to that we observed for *B. platifrons.* Although additional studies are required to determine the dominant mode of carbon transfer, ultrastructural evidence (Fig. 1C,D) suggests that energy transfer occurs through intracellular digestion of symbionts.

We cannot exclude the possibility that some carbon transfer between *B. platifrons* and its symbionts occurs through translocation of intermediate carbon fixation products. Fisher & Childress (1986) detected the translocation of fixed radiocarbon from symbionts to non-symbiont containing tissues of *Solemya reidi* within several hours. Because carbon transfer via host digestion of symbionts is expected to require much longer, they concluded that carbon transfer in *S. reidi* occurs through the direct release of metabolites from symbiont to host. Excretion of succinate and glutamate has been measured from purified symbiotic bacteria from *Riftia pachyptila* (Felbeck & Jarchow 1998).

Distribution of methanotrophic symbioses

Congeners within the genus *Bathymodiolus* have been highly successful in exploiting chemosynthetic environments, including thiotrophic and methylotrophic or methanotrophic associations found in hydrothermal vents and cold seeps. Only *B. platifrons,* however, is now known to rely on methane-based symbioses at both cold seeps and hydrothermal vents. Of ~10 known methane-based symbioses, only 2 involve species outside the genus *Bathymodiolus:* a sponge-bacterial symbiosis in the Barbados accretionary complex (Vacelet et al. 1995, 1996) and a pogonophoran, *Siboglinum poseidoni,* in the Skagerrak (Schmaljohann & Flügel 1987).

Reports of methane-based chemosynthetic symbioses are much more common from the Atlantic Ocean than the Pacific (see, e.g., review by Sibuet & Olu 1998). In a survey of the literature on metazoan-bacterial symbioses, we counted 29 sulfide-based symbioses and 8 methane-based symbioses for the Atlantic, and 30 and 2 symbioses, respectively, for the Pacific (including this study). Is the putative rarity of methane-based invertebrate-bacterial symbioses in the Pacific a result of geochemical limits to the evolution and dispersal of such symbioses, or simply a consequence of limited sampling effort?

Although Barry et al. (1997) speculated that methanotrophic symbioses might be limited to seeps with methane-rich brines associated with evaporite deposits that are more common in the Atlantic Ocean than the Pacific, these symbioses are now known from seeps and vents in both ocean basins. In all cases, however, methane levels of venting or seeping fluids are high. Fujikura et al. (2000) reported that methane-based species were common only at sites with high (>2.6 mM) ambient methane concentrations, compared to those with thiotrophic or duel symbiont types, which were found in methane levels 10–100 times lower. Nonetheless, while all known methane-based symbioses are linked to high environmental methane, not all methane-rich sites have such symbioses, suggesting that other factors may limit their distribution. For example, methane-based invertebrate-bacterial symbioses have

not been observed near vents along the Juan de Fuca ridge in the Pacific where methane levels up to 3.9 mM have been measured (Lilley et al. 1993), or in Monterey Bay where methane levels up to ~8 mM have been measured. Broader exploration and investigation of the larval ecology and population dynamics of seep and vent species, coupled with identification of environmental constraints, will likely modify current views of the evolution and maintenance of chemosynthetic communities in the world ocean.

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