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Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions

Yoshihiro Fujiwara[1,*], Ken Takai[2], Katsuyuki Uematsu[3], Shinji Tsuchida[1], James C. Hunt[1], Jun Hashimoto[1]

[1]Marine Ecosystems Research Department, Japan Marine Science and Technology Center (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan

[2]Frontier Research Program for Deep-Sea Environment, Japan Marine Science and Technology Center (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan

[3]Marine Work Japan Co., 2-15 Natsushima, Yokosuka 237-0061, Japan

ABSTRACT: The bacterial endosymbionts of 3 hydrothermal vent mussels from Japanese waters were characterized by transmission electron microscopic (TEM) observation and phylogenetic analyses of 16S ribosomal RNA gene sequences. Endosymbionts of *Bathymodiolus septemdierum* were related to sulfur-oxidizing bacteria (thioautotrophs), while endosymbionts of *B. platifrons* and *B. japonicus* were related to methane-oxidizing bacteria (methanotrophs). This is the first report of deep-sea mussels containing only methanotrophs (lacking thioautotrophs) from hydrothermal vents. Comparison of methane and hydrogen sulfide concentrations in end-member fluids from deep-sea hydrothermal vents indicated that methane concentrations were much higher in habitats containing *Bathymodiolus* spp. which harbored only methanotrophs than in other habitats of hydrothermal vent mussels. The known distribution of other mussels containing only methanotrophs has thus far been limited to cold-seep environments with high methane concentrations from the interstitial water. These results suggest that the distribution of methanotrophic symbioses between deep-sea mussels and methanotrophs is strongly influenced by the methane or hydrocarbon concentrations provided from hydrothermal vent and cold-seep activities (or that methane concentration is a possible limiting factor that restricts the distribution of methanotrophy-dependent symbioses in the deep sea).

KEY WORDS: Methanotrophic symbiosis · *Bathymodiolus* · Phylogenetic analysis · Host distribution · Methane concentration · Hydrothermal vent

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INTRODUCTION

Deep-sea mussels of the genus *Bathymodiolus* are conspicuous species at deep-sea hydrothermal vent and cold-seep areas, which harbor symbiotic bacteria like other vent and seep species such as vesicomyid clams and vestimentiferan tubeworms (Fisher 1990). Deep-sea mussels rely primarily on symbionts for their nutrition, although they have other ways of obtaining nutrition as well (Fiala-Médioni et al. 1986, Le Pennec et al. 1988, 1992, 1995, Fisher 1990, Nelson & Fisher 1995).

The phylogeny of bacterial symbionts has been examined using 16S ribosomal RNA gene (16S rDNA) sequence analysis (see Distel et al. 1988), but studies on deep-sea mussel symbionts are wanting. To date, 13 species of *Bathymodiolus* have been described, with at least 10 more in the same family currently recognized (Kenk & Wilson 1985, Hessler & Lonsdale 1991, Cosel et al. 1994, 1999, Hashimoto & Okutani 1994, Paull et al. 1995, Cosel & Olu 1998, Dubilier et al. 1998, Gustafson et al. 1998, Tunnicliffe et al. 1998). Of these

*E-mail: fujiwara@jamstec.go.jp

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species, only 3 have had their bacterial symbionts examined using 16S rDNA sequence analysis: *B. thermophilus*, *B. puteoserpentis*, and an undescribed cold-seep mussel from the Louisiana Slope (Distel et al. 1988, 1995, Distel & Cavanaugh 1994). *B. thermophilus* has thioautotrophic endosymbiotic bacteria, the undescribed mussel has methanotrophic bacteria, and *B. puteoserpentis* has both.

Three species were examined in this study: *Bathymodiolus septemdierum*, *B. platifrons* and *B. japonicus*. *B. septemdierum* occurs near hydrothermal vents on 3 submarine volcanoes on the Izu-Bonin Arc, Japan (Hashimoto & Okutani 1994, Fujiwara et al. 1998). *B. platifrons* and *B. japonicus* live near hydrothermal vents in the Okinawa Trough and at cold seeps in Sagami Bay, Japan (Hashimoto & Okutani 1994). These 3 species have thick and enlarged ctenidia, and a short and straight gut, indicating that they are endosymbiont-harboring species.

In order to characterize the endosymbionts of these 3 species, we examined the morphology of the symbionts in gill tissue using transmission electron microscopy (TEM), as well as the nearly complete 16S rDNA sequences using domain-specific PCR primers. The results, along with a discussion of the relationship between symbiotic types of mussels and chemical factors in their habitats, are presented here.

MATERIALS AND METHODS

Specimen collection. *Bathymodiolus septemdierum* was collected from hydrothermal vents on the Myojin Knoll (32° 06.292′ N, 139° 52.185′ E, 1289 m depth) on the Izu-Bonin Arc during a dive of the submersible 'Shinkai 2000' in 1998. *B. platifrons* and *B. japonicus* were collected from hydrothermal vents at the North Knoll (27° 47.181′ N, 126° 53.985′ E, 1028 m depth) of the Iheya Ridge in the Okinawa Trough during a dive of the 'Shinkai 2000' in 1996. Upon recovery, the mussels were immediately transferred to fresh, chilled (~7°C) seawater.

Treatment for transmission electron microscopic observations. Small pieces of gill tissue were prefixed with 0.2% glutaraldehyde in seawater for 1 h at 4°C. Prefixed gills were dissected into 2 to 3 mm blocks using razor blades. After rinsing 10 times with 0.05 M phosphate buffer (pH 7.8) for 10 min at room temperature, additional fixation was performed for 3 h with 2.5% glutaraldehyde in 0.2 M phosphate buffer containing 4% tannic acid (pH 6.8) at room temperature. After rinsing 10 times with 0.05 M phosphate buffer (pH 7.8) for 10 min at room temperature, tissues were stored in 0.05 M phosphate buffer (pH 7.8) containing 10 mM sodium azide for about 1 to 3 wk at 4°C. Postfixation was conducted for 2 h in 1% OsO_4 in 0.05 M phosphate buffer (pH 7.8) at 4°C. Then, tissue was dehydrated and embedded in Epon 812 resin (TAAB). Ultra-thin sections of the specimens were stained with uranyl acetate and lead citrate, and were observed by a JEOL JEM-1210 transmission electron microscope at an acceleration voltage of 80 kV.

DNA preparation. DNA was extracted from gill tissue of each host species. To eliminate surface contaminants, each gill was thoroughly washed in autoclaved and filtered (0.22 µm) seawater. DNA extraction from tissue samples was followed by the physical disruption in liquid nitrogen and chemical lysis as described by Takai & Sako (1999).

PCR amplification. Bacterial 16S rDNA was amplified by PCR using the LA PCR kit (TaKaRa, Kyoto, Japan). Two oligonucleotide primers (1 µM each) and 1 ng ml^{-1} DNA template were added to the reaction mixtures. Thermal cycling was as follows: denatured at 96°C for 20 s, annealed at 55°C for 45 s, and extended at 72°C for 2 min for a total of 35 cycles. The oligonucleotide primer sequences used for bacterial 16S rDNA amplification were Bac27F and 1492R (Lane 1991). Molecular size of the PCR products was checked by 1.2% agarose gel electrophoresis.

Cloning and sequencing of amplified 16S rDNA. Amplified 16S rDNAs from 3 separate reactions were pooled and extracted from the agarose gel slices sequentially with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and precipitated with ethanol. After centrifugation, DNA pellets were resuspended in sterile distilled water. The purified 16S rDNA was cloned into the pCR II vector using the TA cloning kit (Invitrogen, San Diego, California). Clones containing appropriately sized inserts were identified by 1.2% (w/v) agarose gel electrophoresis. DNA sequencing of the inserts was performed by the colony direct-sequencing method (Ohno et al. 1991) using Taq DNA polymerase FS (Perkin Elmer). Universal 16S rDNA-specific primers (Lane 1991) and M13 forward and reverse primers (Sambrook et al. 1989) were used in sequencing reactions. Sequencing was performed using the ABI PRISM 373 and 310 genetic analyzers. The sequences reported here have been deposited in the database of the DNA data bank of Japan (DDBJ) under Accession Nos. AB036709, AB-036710, and AB036711.

Sequence and phylogenetic analyses. Nearly complete sequences of 16S rDNA were analyzed using the gapped-BLAST search algorithm (Altschul et al. 1997, Benson et al. 2000) to estimate the degree of similarity to other 16S rDNA sequences. Sequences of approx. 1500 bp were used to run the similarity analysis. The databases used for similarity analyses were the non-redundant nucleotide sequence databases from GenBank, European molecular biology laboratory (EMBL)

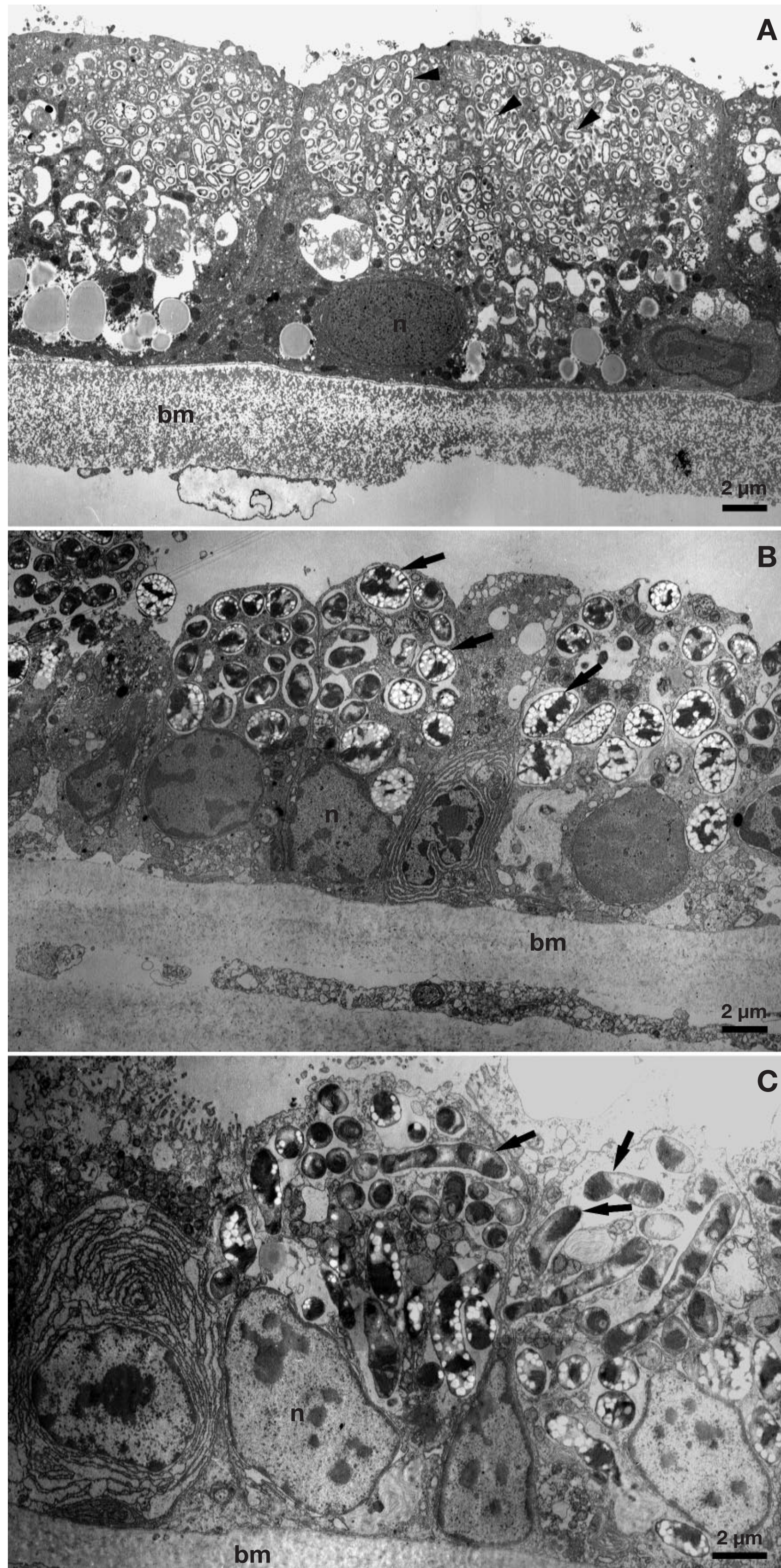

Fig. 1. (A) *Bathymodiolus septemdierum*, (B) *B. platifrons*, and (C) *B. japonicus*. Transmission electron micrographs of transverse sections of gill filaments showing intracellular gram-negative bacterial symbionts. Symbionts of *B. platifrons* and *B. japonicus* contained stacked internal membranes (arrows); *B. septemdierum* contained smaller symbionts that lack internal membranes (arroheads). bm: basal membrane; n: nucleus

and DDBJ. Sequences were manually aligned and phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences. Neighbor-joining (NJ) analysis was accomplished using the CLUSTAL X software package (Thompson et al. 1997). To provide confidence estimates for phylogenetic tree topologies inferred by the neighbor-joining analysis, bootstrap analyses were used. Maximum likelihood (ML) and maximum parsimony (MP) analyses were also performed using the PHYLIP package, Version 3.572 (obtained from J. Felsenstein, University of Washington, Seattle).

RESULTS

Electron microscopy

Numerous bacteria were observed in the epithelial cells of gill tissue from *Bathymodiolus septemdierum* (12 individuals, total cumulative area of 3 mm^2 observed), *B. platifrons* (4 individuals, total cumulative area of 1 mm^2 observed), and *B. japonicus* (5 individuals, total cumulative area of 1 mm^2 observed) using TEM (Fig. 1). The symbionts were contained in vacuoles within the host cells and most of them were localized in the apical region of the cells just below the surface. One distinct mono-morphological type of symbiotic bacteria was visible in electron micrographs in each of the 3 species. A trilamellar cell envelope, typical of gram-negative bacteria, surrounded each type of symbiont (Fig. 2). Dividing stages of each type of symbiont, indicating active reproduction, were observed (Fig. 2B). Secondary lysosomes containing the intermediate stages of symbiont digestion were also observed at the basal portion of the host bacteriocytes.

The *Bathymodiolus septemdierum* symbionts were small cocci or short rods, averaging 0.60 µm along the major axis (SD = 0.20, n = 40) without internal membranes (Fig. 2A), and resembling the thioautotrophic symbionts found in the gills of *B. thermophilus*.

The symbionts of *Bathymodiolus platifrons* and *B. japonicus* were larger than those of *B. septemdierum*, with an average major axis of 1.94 µm (SD = 0.55, n = 43) and 4.01 µm (SD = 2.00, n = 56), respectively (Fig. 2B,C). The *B. platifrons* symbionts were roughly coccoid (Fig. 2B), and the *B. japonicus* symbionts were strongly rod-shaped (Fig. 2C). Both contained complex, stacked, internal membranes.

16S rDNA gene sequences

Partial sequences (≈500 bp) of microbial 16S rDNA from the gill tissue of a single specimen of *Bathymodiolus septemdierum*, *B. platifrons*, and *B. japonicus*, were homogeneous (*B. septemdierum*, 22 clones; *B. platifrons*, 27 clones; *B. japonicus*, 7 clones). Nearly complete sequences of 16S rDNA from 2 clones of each species were examined. The sequence identities be-

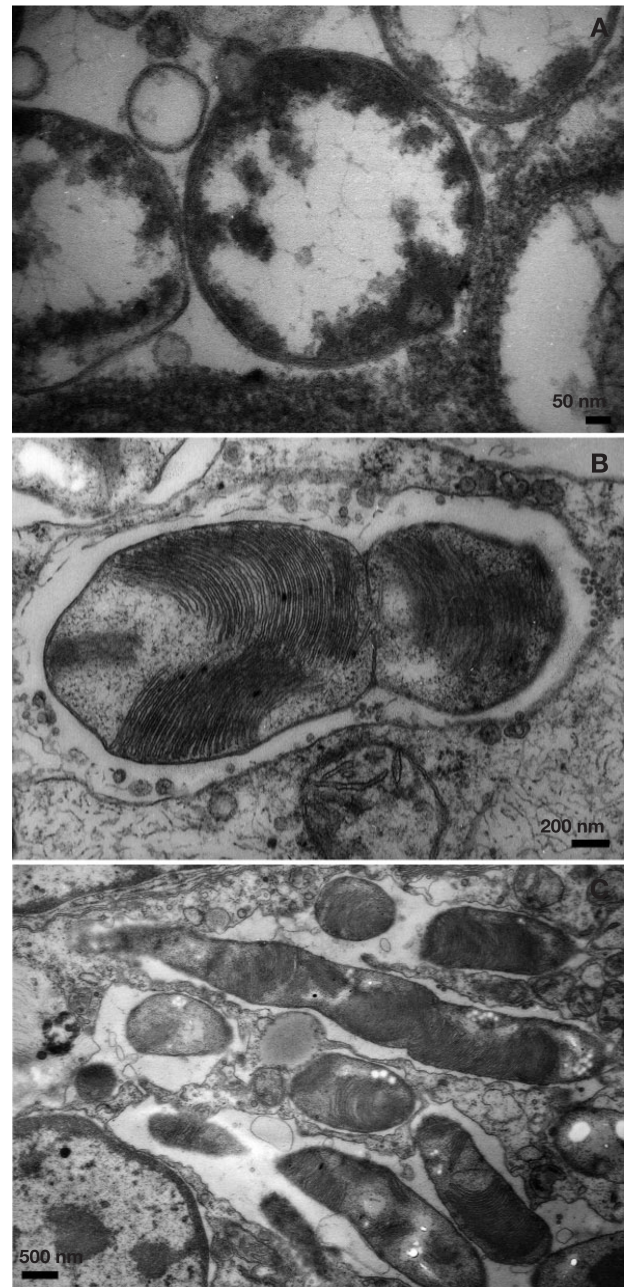

Fig. 2. (A) *Bathymodiolus septemdierum*, (B) *B. platifrons*, and (C) *B. japonicus*. High magnification of transmission electron micrographs of intracellular bacterial symbionts within bacteriocytes of epithelium of host gills. Gram-negative symbionts in (B) and (C) contained stacked internal membranes, but not those in (A)

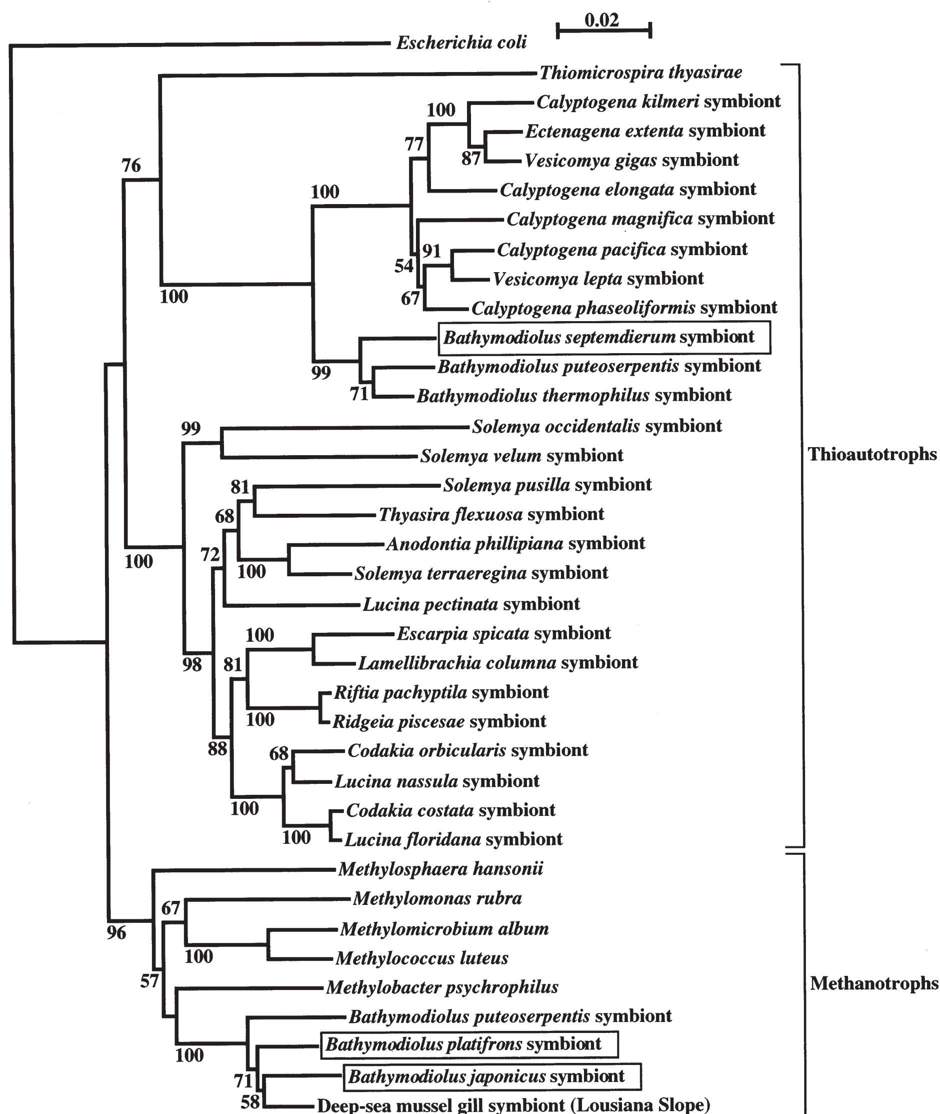

Fig. 3. Phylogenetic tree inferred by neighbor-joining analysis of 1335 homologous positions of 16S ribosomal RNA gene sequences of the symbionts of *Bathymodiolus septemdierum*, *B. platifrons* and *B. japonicus*, and representative free-living and symbiotic bacteria within the γ subdivision of Proteobacteria. Scale bar = 0.02 nucleotide substitutions per sequence position. Percentage of 1000 bootstrap resamplings is indicated on each branch. Bootstrap values are for nodes supported in >500 of 1000 trees. Symbionts of the 3 *Bathymodiolus* species examined in this study are framed

tween clones from the same species were 99.6% in *B. septemdierum* (1455 bp), 99.7% in *B. platifrons* (1464 bp), and 99.9% in *B. japonicus* (1462 bp).

Phylogenetic analyses of 16S rDNA sequences

Three phylogenetic trees created by the NJ, ML and MP methods yielded similar (but not identical) topologies. Those analyses placed the *Bathymodiolus septemdierum*, *B. platifrons*, and *B. japonicus* symbionts within the γ subdivision of Proteobacteria containing all previously studied *Bathymodiolus* spp. symbionts and the majority of other thioautotrophic symbionts associated with marine invertebrate hosts (the NJ tree is shown in Fig. 3). The symbiont of *B. septemdierum* was associated with thioautotrophic bacteria and formed a monophyletic group with the symbionts of *B. thermophilus* and *B. puteoserpentis* in the NJ and MP trees, supported by bootstrap values of 99% in the NJ tree. The symbionts of *B. platifrons* and *B. japonicus* were associated with methanotrophic bacteria. These symbionts were closely related to one another (97.5% sequence identity) and formed a monophyletic group with the symbionts of *B.*

puteoserpentis and the deep-sea mussel species from the Louisiana Slope in all 3 phylogenetic trees, supported by bootstrap values of 100% in the NJ tree.

DISCUSSION

In this study, we characterized symbionts of 3 *Bathymodiolus* species inhabiting Japanese waters. Although more than 20 species of deep-sea mussels are recognized at hydrothermal vents and cold seeps to date, this is the first record of hydrothermal vent mussels containing only methanotrophic symbionts (*B. platifrons* and *B. japonicus* had no thioautotrophs).

Morphological and phylogenetic data indicated that each of the 3 species had symbiotic bacteria in their gills, and relied nutritionally on thioautotrophic or methanotrophic carbon fixation. Numerous bacteria were visible in the epithelial cells of the gill tissue of each species, with divisional stages indicating active reproduction and intermediate stages of bacteria digestion indicating energy acquisition of the host from the symbionts (Le Pennec et al. 1988, Fisher & Childress 1992). The endosymbionts of these mussels were divided into thioautotrophs or methanotrophs based on their morphology. The complex, stacked, internal membrane is a prominent feature of methanotrophs and has not been observed in thioautotrophs (Cavanaugh et al. 1987). The symbiont of *Bathymodiolus septemdierum* had no internal membranes (Fig. 2B). In addition, the 3 phylogenetic analyses indicated that the 16S rDNA sequence of the symbiont was closely related with those of thioautotrophs (Fig. 3). Bootstrap analysis strongly supported the placement of the symbionts within a single monophyletic group with the symbionts of 2 other vent mussels. This group fell within a larger clade containing only thioautotrophic bivalve symbionts. The ML analysis did not group *Bathymodiolus* mussel thioautotrophic symbionts monophyletically, but it did show these symbionts and vesicomyid clam symbionts to be a single monophyletic group. This was consistent with the NJ and MP analyses as well as previous studies (Distel et al. 1988, 1995, Distel & Cavanaugh 1994). Conversely, the symbionts of *B. platifrons and B. japonicus* had complex, stacked, internal membranes (Fig. 2B,C). These symbionts formed a monophyletic group with the methanotrophic symbionts of 2 other mussels, supported by the 3 phylogenetic analyses (Fig. 3). This group fell within a larger clade containing only methanotrophs. Therefore, it is reasonable to conclude that the symbiont in *B. septemdierum* relies on sulfide oxidation for energy, while those in *B. platifrons and B. japonicus* rely on methane oxidation.

Our data coupled with previously published data suggest that high methane concentration is essential for the presence of invertebrate-bacterial symbioses based solely on methane (or hydrocarbon) oxidation as the energy source for primary production. The symbiont types in hydrothermal vent mussels along with methane and hydrogen sulfide concentrations in end-member fluids from host-species habitats are shown in Table 1. High methane anomalies were recorded in the Okinawa Trough (Chiba et al. 1993, Gamo 1995, Ishibashi et al. 1995). Four hydrothermal vent areas are currently known in the trough. All were dominated by *Bathymodiolus platifrons* or *B. japonicus* or both. Methane concentrations in end-member fluids from 3 of the sites were measured and were much higher than those typifying other vents where mussels occur. Methane concentration in the end-member fluid from the North Knoll of the Iheya Ridge (where *B. platifrons* and *B. japonicus* were collected for this study) was not measured. However, specimens of these mussels collected from another site in the trough and from a cold-seep site in Sagami Bay, where high methane concentration (2.5 mol kg^{-1}) was also measured (Tsunogai et al. 1996), were shown to have only methanotrophic symbionts using TEM (Y.F. unpubl. data). Additionally, most methanotrophic symbioses occurred at cold-seep environments where methane concentrations were higher in interstitial water than in venting fluids (Martens et al. 1991, Nix et al. 1995, Martin & Kastner 1996). These studies, along with the present study, strongly suggest that the distribution of methanotrophy-dependent symbioses was restricted to areas of high methane concentration.

However, high methane concentrations alone might not be sufficient to support methanotrophic symbioses. Methane concentrations of the end-member fluid from the Guaymas Basin and the Endeavour Ridge (12 to 16.5 and 0.5 to 1.4 mmol kg^{-1}, respectively) are 1 order of magnitude higher than those of most hydrothermal vent sites (Welhan & Lupton 1987, Lilley et al. 1989). Notwithstanding, no methanotrophic species have been reported from either site. High methane levels without the presence of methanotrophs were also found in seep sediments in Monterey Bay (J. Barry pers. comm.). Barry suggested that insufficient fluid flow might limit the biogeography of methanotrophic symbioses in seeps. Thus, it is possible that the same situation could occur at hydrothermal vents.

Although deep-sea mussels which contain only methanotrophs occur at hydrothermal vent sites with high methane concentrations, 3 species harboring both thioauto- and methanotrophs have been reported from hydrothermal vents with lower methane concentrations in the end-member fluids. These include 2 Mid-Atlantic Ridge mussels, *Bathymodiolus puteoserpentis* and *B. azoricus*, and the hydrothermal vent gastropod *Ifremeria nautilei* (Cavanaugh et al. 1992, Gal'chenko et al. 1992, Distel et al. 1995, Pond et al. 1998, Robinson

Table 1. *Bathymodiolus* spp. Hydrothermal vent mussels with thioautotrophic (T) and/or methanotrophic (M) symbionts. Symbiont types of deep-sea mussels and methane and hydrogen sulfide concentrations in vent fluids from host-species habitats are shown. Listed mussels were limited to those types for which symbionts were known or presumed. Chemical concentration values from some references were converted to mmol kg^{-1}. Proxy density value (1.025) was used for conversion because of absence of actual data. Source reference nos. (1–24) correspond to following papers: 1, Lilley et al. (1983); 2, Distel et al. (1988); 3, Von Damm et al. (1991); 4, Lilley et al. (1991); 5, Kim et al. (1984); 6, Bowers et al. (1988); 7, Merlivat et al. (1987); 8, Jean-Baptiste et al. (1991); 9, Distel et al. (1995); 10, Donval et al. (1994); 11, Pond et al. (1998); 12, Colodner et al. (1993); 13, Van Dover et al. (1996); 14, Ishibashi et al. (1994a); 15, Ishibashi et al. (1994b); 16, Dubilier et al. (1998); 17, J. Ishibashi pers. comm.; 18, Tsunogai et al. (1994); 19, Gamo (1995); 20, Ishibashi et al. (1995); 21, our preliminary results; 22, Ishibashi et al. (1990); 23, Sakai et al. (1990); 24, Chiba et al. (1993). *Error for concentrations was estimated as ±20%; EPR = East Pacific Rise; MAR = Mid-Atlantic Ridge

Species, Location	Symbiont type	Sulfide (mmol kg^{-1})	Methane (mmol kg^{-1})	Source
B. thermophilus				
Galápagos	T	–	0.083–0.29	1, 2
EPR 9–10° N		<63	0.29	3, 4
EPR 11° N		4.3–12	0.067–0.12	5, 6
EPR 13° N		2.8–8.0	0.028–0.055	6, 7
B. puteoserpentis				
MAR-Snake Pit	T & M	2.6	0.062	8, 9
B. azoricus				
MAR-Menez Gwen	T & M	<2	2.2	10, 11
MAR-Lucky Strike	T & M	<3.2	0.89	12, 13
B. brevior				
North Fiji Basin	T	2.0–4.0	0.03–0.44	14, 15, 16
B. sp. affinis *brevior*				
North Fiji Basin	T	2.0–4.0	0.03–0.44	14, 15, 16
B. septemdierum				
Myojin Knoll	T	<2.0	0.041	17, this study
Suiyo Sea Mount		1.4–1.7	000013–0.19	18
B. platifrons				
Iheya (North), Okinawa Trough	M	–	–	this study
Iheya (South), Okinawa Trough	M	15–24	3.1–4.9*	19, 20, 21
Izena, Okinawa Trough		12	7.6	22, 23
B. japonicus				
Iheya (North), Okinawa Trough	M	–	–	this study
Iheya (South), Okinawa Trough	M	15–24	3.1–4.9*	19, 20, 21
Minami Ensei, Okinawa Trough		1.6–2.4	2.6–7.0	24

et al. 1998). This might be due to flexibility in the method of nutritional acquisition in the hosts. The ratio of contribution of each symbiont to host nutrition was unknown, but site-specific variation in nutrition of *B. azoricus* has been reported, with specimens collected from 1 site being more dependent on methanotrophy than specimens collected from another site (Trask & Van Dover 1999).

This study showed no obvious evolutionary processes for the acquisition of symbionts by their host mussels. However, we believe that the phylogenetic trees published in Craddock et al. (1995) suggested that intracellular symbioses of deep-sea mussels began with methanotrophs and that the thioautotrophic symbionts were acquired later. In that study, 2 phylogenetic hypotheses were proposed. Both showed a group in which intracellular methanotroph-harboring seep mussels formed a basal paraphyletic group, while methano- and thioautotroph-harboring seep mussels were a monophyletic group in a higher position. However, the exact points at which thioautotrophs were acquired by mixotrophic species and methanotrophs were lost from thioautotroph-harboring species are not clear, because the 2 phylogenetic trees have different topologies at these points (Craddock et al. 1995).

The present study examined the phylogeny of 3 mussel symbionts and suggested the importance of symbiont type and chemical environment on host distribution, especially for methanotroph-harboring species. The distribution of methanotrophic symbioses between deep-sea mussels and their symbionts correlates with high methane concentrations at hydrothermal vents, as is the case for cold seeps. It is therefore likely that the distribution of methanotrophy-dependent symbiosis is greatly influenced by methane or hydrocarbon concentrations.

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