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RESEARCH ARTICLE

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A new bathymodioline mussel symbiosis at the Juan de Fuca hydrothermal vents

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Abstract Until recently, the only major hydrothermal vent biogeographic province not known to include bathymodioline mussels was the spreading centers of the northeast Pacific, but deep-sea dives using DSV Alvin on the Endeavor segment of the Juan de Fuca Ridge (47°56N 129°06W; ~2,200 m depth) in August 1999 yielded the only recorded bathymodioline mytilids from these northeastern Pacific vents. One specimen in good condition was evaluated for its relatedness to other deep-sea bathymodioline mussels and for the occurrence of chemoautotrophic and/or methanotrophic symbionts in the gills. Phylogenetic analyses of the host cytochrome oxidase I gene show this mussel shares evolutionary alliances with hydrothermal vent and cold seep mussels from the genus *Bathymodiolus*, and is distinct from other known species of deep-sea bathymodiolines, suggesting this mussel is a newly discovered species. Ultrastructural analyses of gill tissue revealed the presence of coccoid bacteria that lacked the intracellular membranes observed in methanotrophic symbionts. The bacteria may be extracellular but poor condition of the fixed tissue complicated conclusions regarding symbiont location. A single gamma-proteobacterial 16S rRNA sequence was amplified from gill tissue and directly sequenced from gill tissue. This sequence clusters with other mussel chemoautotrophic symbiont 16S rRNA sequences, which suggests a chemoautotrophic, rather than methanotrophic, symbiosis in this mussel. Stable carbon ($\delta^{13}C = -26.6\%$) and nitrogen ($\delta^{15}N = +5.19\%$) isotope ratios were also consistent with those reported for other chemoautotroph-mussel symbioses. Despite the apparent rarity of these mussels at the Juan de Fuca vent sites, this finding extends the range of the bathymodioline mussels to all hydrothermal vent biogeographic provinces studied to date.

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Introduction

To date, seven deep-sea hydrothermal vent biogeographic provinces have been described and the only province lacking bathymodioline mussels was Juan de Fuca in the northeast Pacific (Van Dover et al. 2002). Prominent fauna at the Juan de Fuca vents include alvinellid, polynoid, and ampharetid polychaetes, as well as copepods, limpets, snails, crabs, fish, and pycnogonids (Tunnicliffe 1988; Tunnicliffe et al. 1998; Tsurumi 2003). Symbiont-containing invertebrates at multiple sites at the Juan de Fuca vents include the vestimentiferan tubeworm (siboglinid polychaete), *Ridgeia piscesae* (Tunnicliffe et al. 1998), the limpet, *Lepetodrilus fucensis* (Bates et al. 2005), and occasionally the vesicomyid clam, *Calyptogena pacifica* (Tunnicliffe et al. 1998). In contrast, the East Pacific Rise (EPR), which was linked to the Juan de Fuca Ridge about 30 Mya, is habitat to dense populations of bathymodioline mussels, as well as three different species of vestimentiferan tubeworms and another vesicomyid clam, *C. magnifica* (Van Dover et al. 2002).

A research cruise on the Endeavor segment of the Juan de Fuca Ridge in 1999 yielded the first recorded bathymodioline mussel specimens. Two specimens were collected during the cruise from two different sites, and a mussel of the same size (~15–20 mm in length) and color was seen and reported by a submersible pilot (R. L.

Williams, personal communication) and by a geologist (V. Robigou, personal communication) on two different dives to yet a third site. While subsequent cruises have not observed additional bathymodioline mussels, the collection of this mussel raises questions regarding its evolutionary alliances with other deep-sea mussels as well as the presence, and identity, of any symbionts.

Mytilid mussels hosting bacterial endosymbionts within their gills are conspicuous members of communities at deep-sea hydrothermal vents and cold seeps in the Pacific, Atlantic, and Indian Oceans (Nelson and Fisher 1995; Dubilier et al. 1998; Van Dover et al. 2001). These deep-sea mussels belong to the genus *Bathymodiolus* which, along with the wood and whale fall endemic mussels from the genera *Idas, Adipicola, Myrina*, and *Benthomodiolus*, are included in the subfamily Bathymodiolinae (Distel et al. 2000). Distel et al. (2000) proposed the whale and wood fall habitats may serve as stepping stones for dispersal to hydrothermal vents. Resolving the phylogenetic relationships of species from both habitats is important for understanding the role of habitat in bathymodioline evolution.

Bathymodioline mussels are the most widespread of any hydrothermal vent or cold seep invertebrates that host chemoautotrophic or methanotrophic symbionts (Gustafson et al. 1998; Sibuet and Olu 1998; Van Dover 2000). Vent and seep mussels are unusual in the diversity of their endosymbiont composition. There are species that host sulfur-oxidizing chemoautotrophic bacteria (Cavanaugh 1983; Nelson et al. 1995), others that host methane-oxidizing (methanotrophic) bacteria (Childress et al. 1986; Barry et al. 2002), and yet others that host both types of bacteria within the same host cell (Cavanaugh et al. 1992; Fiala-Médioni et al. 2002). On the basis of the 16S rRNA gene sequences, these two symbiont types group in distinct clades, consistent with their metabolism, indicating separate evolutionary origins (Distel and Cavanaugh 1994). In terms of nutrition, there is evidence for carbon translocation via host digestion of the symbionts, as demonstrated in the methanotroph-hosting mussel, *Bathymodiolus childressi* (Fisher and Childress 1992; Kochevar et al. 1992; Streams et al. 1997), and by TEM observation of symbionts in "lysosomal bodies" in the basal region of gill epithelial cells of all bathymodioline mytilid species examined to date (Fiala-Médioni et al. 1990, 1994, 2002). The whale and wood fall mussels are also presumed to harbor chemoautotrophic symbionts, but only *Idas washingtonia* has been explicitly evaluated for the presence of chemoautotrophic symbionts (Deming et al. 1997). While host relatedness is not a proxy for symbiont characterization, the elevated levels of reduced sulfur compounds associated with the whale and wood falls where these mussels are observed support the potential for chemoautotrophic symbioses.

In the present study, the Juan de Fuca mussel was assessed for the presence, and nature, of bacterial symbionts, and the evolutionary alliances of the mussel and its symbiont(s). The collection of only two specimens in this relatively well-studied area raises questions regarding its presence at this hydrothermal vent. This preliminary characterization highlights the need to collect further specimens in order to understand the biogeography and phylogenetic affinities of all bathymodioline species.

Materials and methods

Specimen collection

Two individuals of an apparently new species of mussel were discovered in bulk invertebrate collections acquired with DSV Alvin in August 1999 on the Endeavor segment of the Juan de Fuca Ridge (47°56N 129°06W; ~2,200 m depth). The first individual was found in a partial collection of the community from an actively venting, diffuse flow chimney in the Mothra vent field. This individual had a shell length (SL) of approximately 16 mm and it was kept in chilled seawater until dissection. Gill tissue was either fixed for ultrastructural analyses or frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses. Foot tissue was dried for stable isotope analyses. A second individual with a SL of 22 mm was discovered after the cruise during the analysis of a quantitative collection (Govenar et al. 2002) of a type III chimney community from an actively venting, diffuse flow chimney in the main Endeavor vent field. Tissues from the second individual were not preserved appropriately for any of the analyses reported here, but the shape and size of the shell suggest it is likely the same species of bathymodioline mytilid (or yet another new species). Shell voucher has been deposited at the Museum of Comparative Zoology Mollusk Collection under accession number MCZ352840.

Transmission electron microscopy (TEM)

Small pieces of gill tissue were fixed for TEM in 3% glutaraldehyde in 0.4 M NaCl, 0.1 M cacodylate (pH 7.4) buffer and post-fixed in 1% osmium tetroxide for 1 h in the same buffer. The specimens were dehydrated through a graded ethanol series and embedded in Spurr's low viscosity resin. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and observed using a Zeiss 10CA transmission electron microscope.

Stable isotope analyses

Potential sources of carbon and nitrogen nutrition for this mussel were evaluated using stable isotope ratio analyses. Dried foot tissue samples were ground and then combusted to CO_2 and N_2 using standard methods

and analyzed on a mass spectrophotometer (Europa) at the Marine Science Institute Analytical Lab, University of California, Santa Barbara. Results are reported relative to Vienna PeeDee Belemite and atmospheric molecular nitrogen, for carbon and nitrogen standards, respectively.

PCR amplification and DNA sequencing

Genomic DNA was isolated from the symbiont-containing gill tissue for PCR amplification and sequencing. Gill tissue was digested overnight in a proteinase K digestion buffer and DNA was extracted by the standard phenol/chloroform technique (Ausubel et al. 1989). Given the lack of resolution seen with the 18S rRNA marker in the Distel et al. (2000) study, the cytochrome c oxidase subunit I (COI) gene was used for the host phylogeny. A ~700 base pair (bp) fragment of the COI gene was amplified with the LCO1490 and HCO2198 primers based on COI regions conserved in invertebrates (Folmer et al. 1994; Nelson and Fisher 2000). A ~1,500 bp portion of the 16S rRNA gene, used for the bacterial symbiont phylogeny, was amplified using universal bacterial primers, 27f and 1492r (Lane et al. 1985).

The COI PCR primers were used to sequence the COI amplicon in both directions. The 16S rRNA PCR product was sequenced with the two original PCR primers as well as an internal universal bacterial 16S rRNA primer, 530f (Lane et al. 1985). Sequences were obtained using an ABI 3100 automated sequencer.

Phylogenetic analysis

Nucleotide sequences were aligned using Pileup in the Genetics Computer Group (GCG) software package (Wisconsin Package, version 10.2). The alignments were also edited manually within GCG using inferred amino acid sequences for the host COI and known secondary structure features for bacterial 16S rRNA. Outgroup taxa for the host analysis included other mussels from the order Mytiloidea (*Mytilus edulis, M. trossulus*, and *Perna viridis)*, as well as mussels from the order Pinnoidea (*Atrina fragilis*) and order Pterioidea (*Pteria hirundo)*. For the symbiont phylogenetic analysis, free-living gamma proteobacteria (*Escherichia coli, Pseudomonas fluorescens, Thiomicrospira crunogena, T.* L-12, *T. thyasirae*, and *Methylobacter whittenburyi)* were used to root the tree. Phylogenetic analyses using maximum parsimony, conducted using PAUP 4.0 b10 (Swofford 2003), were based on 406 bp of COI and 1,456 bp of 16S rRNA; gaps were treated as missing data. Under the parsimony criterion, branch and bound searches using furthest addition sequence were performed on both datasets. To assess the robustness of the resulting topologies, 1,000 replicate bootstrap analyses were conducted under the same search parameters. The Juan de Fuca mytilid COI and symbiont 16S rRNA sequences have been deposited in GenBank under accession numbers DQØ77892 and DQØ77893, respectively.

Results

The morphology of the Juan de Fuca mussel is consistent with observations of other vent and seep mussels. The shell height was narrow with respect to length, like that of bathymodioline mytilids (Von Cosel and Metivier 1994; Gustafson et al. 1998; Von Cosel et al. 1999). Anatomically, the digestive system was reduced and the gills were fleshy and hypertrophied, as well as beige in color, like other bathymodiolines (Fiala-Médioni et al. 1986, 1987, 2002).

TEM

The gill tissue contained coccoid bacteria of uniform morphology that lack internal membranes (Fig. 1); a Gram-negative cell envelope could not be resolved given the quality of the tissue fixation. As in all other bathymodioline symbioses described to date, the bacteria appeared to be associated with gill cells (referred to as bacteriocytes) and were localized at the apical end, i.e., in the region where the gill surface is in contact with seawater in the mantle cavity. Due to the poor condition of the gill tissue, it is unclear whether the bacteria are intracellular and contained within membrane-bound vacuoles, as in other mussel symbioses, or whether they occur as epibionts. Interspersed with the symbionts are membrane-bound bodies that appear empty, potentially representing micelle-like bodies resulting from the bursting of the host membrane or symbionts whose cytoplasm was extracted during TEM preparation (C. Cavanaugh, personal observation).

Stable isotope analyses

The stable isotope values for mussel foot tissue, $\delta^{13}C = -26.6\%$ and $\delta^{15}N = +5.19\%$, are reported relative to values for other bathymodioline mussel symbioses (Table 1).

Sequence analyses

Each PCR yielded a single amplicon of expected size (COI, ~0.7 kb; 16S rRNA, ~1.5 kb) and each amplicon produced a single unambiguous sequence. A single most parsimonious tree resulted from the phylogenetic analysis of the COI gene sequences with the Juan de Fuca mussel falling out in the middle of the subfamily Bathymodiolinae deep-sea mussel clade (Fig. 2). Because some species included have multiple COI haplotypes (*Bathymodiolus japonicus, B. manusensis, B. platifrons, B. septemdierum*,

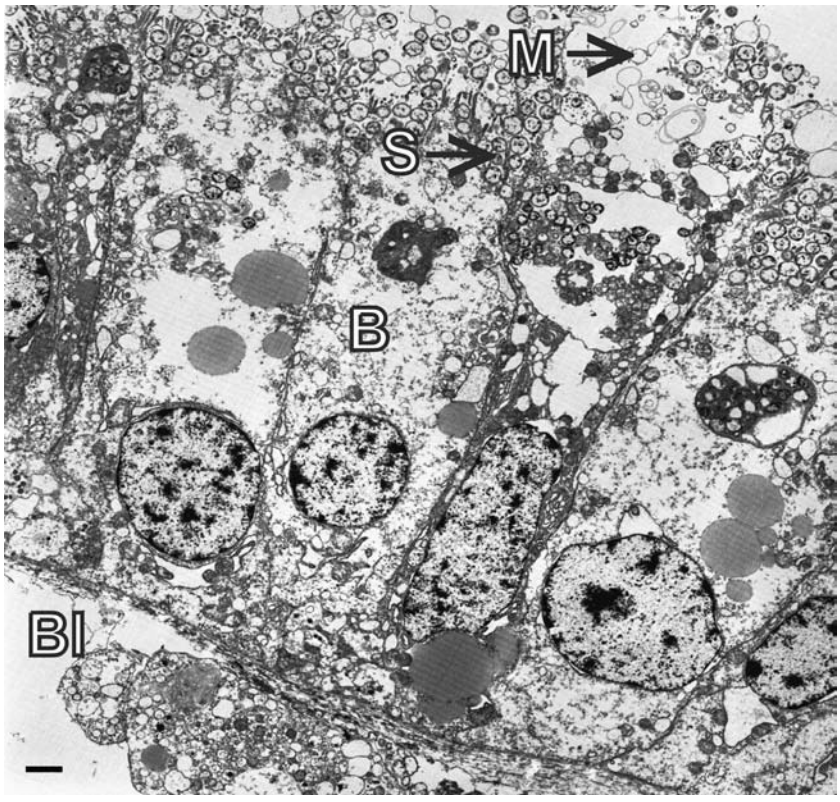

Fig. 1 *Bathymodiolus* sp. JdF. Transmission electron micrograph of a transverse section of a gill filament. Bacterial symbionts are localized at the apical surface of gill cells (referred to as bacteriocytes as in other bathymodioline symbioses) along with micelle-like bodies. Due to the poor condition of the fixed gill tissue, the exact location of the symbionts with respect to the host cell membrane is unclear. *B* bacteriocyte, *Bl* blood space, *S* symbiont, *M* Micelle-like body. *Scale bar* 1 μm

and *B. thermophilus*), three sequences for each were included in initial analyses and the same topology was recovered (results not shown). Since the focus of this study was relationships among species, only one haplotype was included in the final phylogeny. Bootstrap support for the subfamily Bathymodiolinae was 99%. Within this group, an undescribed species of *Bathymodiolus* from New Zealand was the most basal species. The remaining bathymodioline mussels formed three clades: the *Bathymodiolus* spp. hosting methanotrophic symbionts, the whale and wood fall species, and the *Bathymodiolus* spp. hosting chemoautotrophic or dual symbioses. The Juan de Fuca COI sequence is distinct from the other mussel COI sequences, suggesting this mussel is a new species, and is sister to *B. heckerae*, a dual symbiont-hosting mussel from the Gulf of Mexico cold seeps. The tree lacked strong bootstrap support for the internal nodes, with the exception of 100% support for the cluster of the western Pacific and Indian Ocean vent mussels hosting chemoautotrophic symbionts and the

Table 1 Stable carbon and nitrogen isotope ratios for bathymodioline mussel symbioses

Habitat/Species	Symbiont type(s)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Location
Hydrothermal vent				
Bathymodiolus sp. JdF[b]	C	−26.6	+5.19	Pacific Ocean
B. thermophilus[c, d]	C	−30.5 to −37.1	−8.1 to +9.6	Pacific Ocean
B. brevior[e]	C	−30.8 to −35.8	NA	Pacific Ocean
B. aff. *brevior*[f]	C	−20.0 to −30.8	−2.7 to −7.5	Indian Ocean
B. azoricus[g]	C, M	−21.3 to −32.6	−10.5 to +0.75	Atlantic Ocean
B. puteoserpentis[h]	C, M	−32.5 to −37.3	−17.2	Atlantic Ocean
Cold seep				
Tamu fisheri[c]	C[a]	−36.2 to −38.1	NA	Gulf of Mexico
B. childressi[c]	M	−37.5 to −67.1	−12.9 to +2.0	Gulf of Mexico
B. platifrons[i]	M	−67.5 to −68.1	NA	Japan Trench
B. brooksi[c]	C, M	−44.4 to −55.7	NA	Gulf of Mexico
B. heckerae[c]	C, M	−62.3 to −76.4	NA	Gulf of Mexico

C chemoautotroph, *M* methanotroph, *NA* not available
[a]This species' symbionts have not been fully characterized
[b]Present study;
[c]as reported in Nelson and Fisher (1995);
[d]Fisher et al. (1994);
[e]Dubilier et al. (1998);
[f]Van Dover (2002);
[g]Fiala-Médioni et al. (2002), Trask and Van Dover (1999);
[h]Robinson et al. (1998);
[i]Barry et al. (2002)

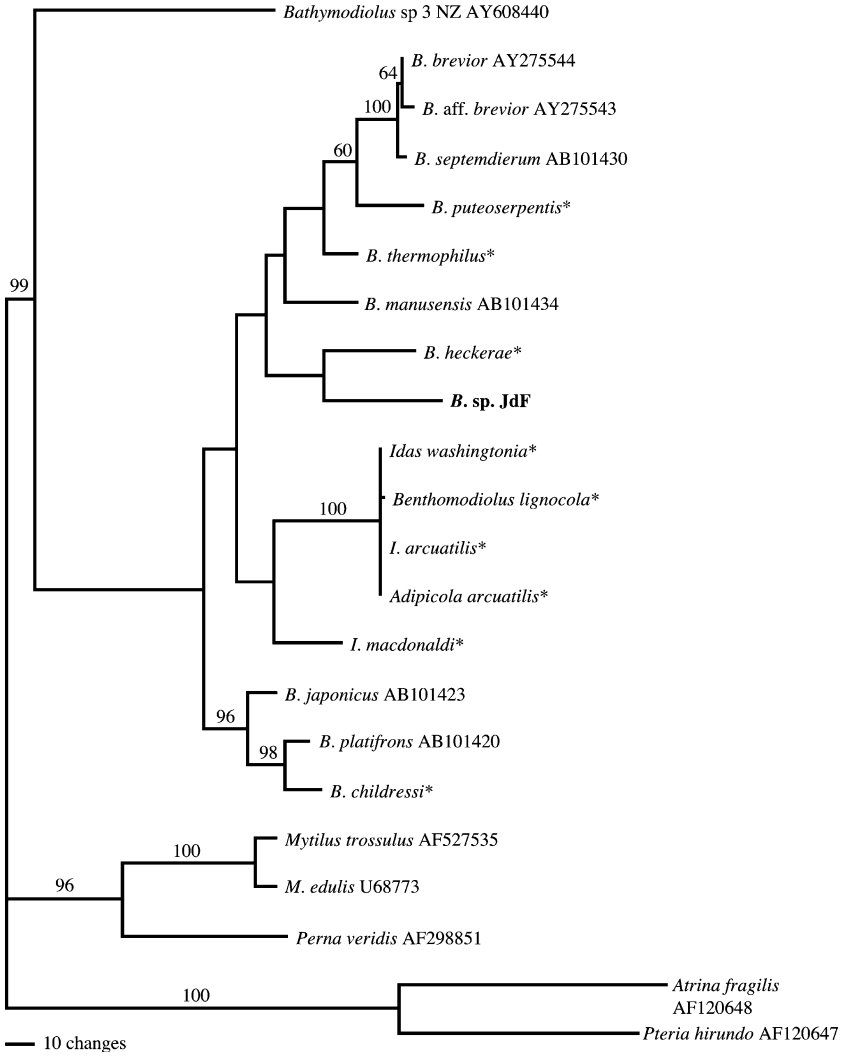

Fig. 2 Single most parsimonious tree showing phylogenetic relationships of *Bathymodiolus* sp. JdF with other bivalves (based on 582 bp of COI). Bootstrap values >50% indicated on tree. Mytilids in order Mytiloidea (*Mytilus edulis, M. trossulus*, and *P. viridis)*,Pinnoidea (*A. fragilis*), and Pterioidea (*P. hirundo)* were included as outgroups. Genbank accession numbers are included on the tree, *Asterisk* denotes unpublished sequences from Baco-Taylor (2002). Scale bar equals number of changes

cluster of the whale and wood fall mussels, excluding *Idas macdonaldi*.

Phylogenetic analyses of 16S rRNA sequences showed the putative symbiont of the Juan de Fuca mussel clustered with other bathymodioline mussel chemoautotrophic symbiont sequences, which were sister to the vesicomyid clam chemoautotrophic symbionts (Fig. 3). The clade including the clam and mussel symbionts had 100% bootstrap support. The methanotrophic mussel symbionts form a discrete clade with 100% bootstrap support, separate from the chemoautotrophic mussel symbionts.

Discussion

The data presented here indicate a new species of bathymodioline mussel, herein referred to as *Bathymodiolus* sp. JdF, has been discovered at the Juan de Fuca hydrothermal vents and it hosts a chemoautotrophic symbiosis. This finding further expands the geographical range inhabited by these mytilids and their symbionts.

The evolutionary relationships between this specimen and other deep-sea mussels, assessed using COI sequence analyses, suggest that *Bathymodiolus* sp. JdF is distinct from other species of bathymodioline mussel. As with phylogenetic analyses based on 18S rRNA (Distel et al. 2000), this COI phylogeny clearly supports evolutionary alliances between the vent and seep mussels and those of the whale/wood fall genera. There is not strong bootstrap support for the cluster containing *Bathymodiolus* spp. and *B.* sp. JdF, but this topology supports inclusion of the Juan de Fuca mussel in the subfamily Bathymodiolinae. While this tree shows an interesting trend of *Bathymodiolus* spp. grouping according to symbiont type, more taxa, as well sequence data from additional genes, are necessary to resolve the role of symbiont composition and habitat in bathymodioline evolution.

Like all of the other mytilid mussels inhabiting hydrothermal vents and cold seeps, *Bathymodiolus* sp. JdF harbors abundant bacteria in its gills. These symbionts are morphologically similar to the Gram negative coccoid-shaped chemoautotrophic symbionts of other mytilids, such as *B. thermophilus* (Le Pennec

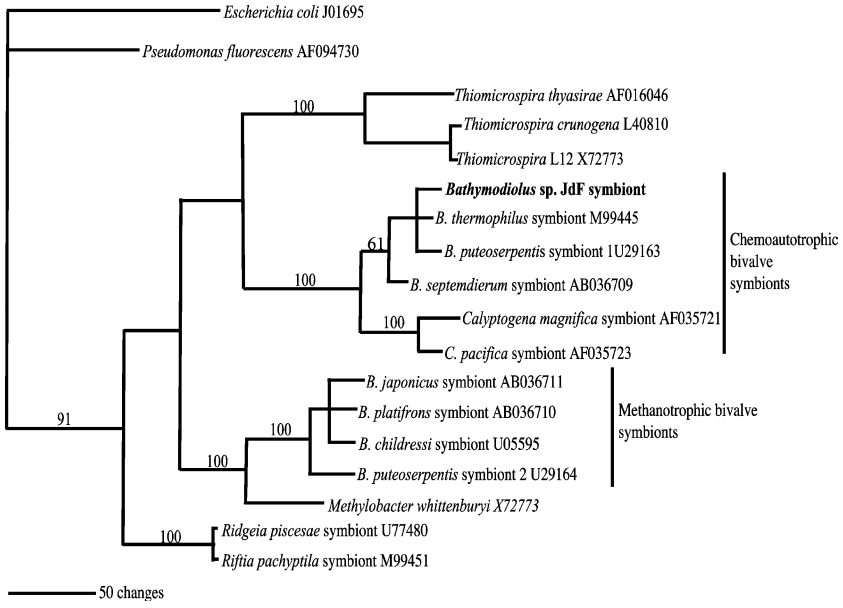

Fig. 3 Single most parsimonious tree showing phylogenetic relationship of the *Bathymodiolus* sp. JdF symbiont with symbiotic and free-living γ-Proteobacteria (based on 1,456 bp of 16S rRNA). Bootstrap values > 50% indicated on tree. Genbank accession numbers are noted on the tree. Scale bar equals number of changes

1984; Fiala-Médioni et al. 1986), *B. brevior* (Dubilier et al. 1998), and *B.* aff. *brevior* (McKiness and Cavanaugh 2005). They lack the intracytoplasmic membranes observed in methanotrophic symbionts of *B. childressi* (Cavanaugh et al. 1987; Fisher et al. 1987), *B. puteoserpentis* (Cavanaugh et al. 1992), and *B. azoricus* (Fiala-Médioni et al. 2002). Whether the symbionts are intracellular, i.e., contained within bacteriocytes as reported for other bathymodioline mussel symbioses, remains an open question due to the poor condition of the fixed gill tissue. Chemoautotrophic bacteria also occur as epibionts attached to or embedded in gill cells in other mollusk symbioses including another bathymodioline mussel, *Tamu fisheri*, found at Gulf of Mexico cold seeps (C. Fisher, personal observation), a limpet, *L. fucensis*, from northeast Pacific vents (de Burgh and Singla 1984), and thyasirid clams (Southward 1986; Fujiwara et al. 2001).

The 16S rRNA phylogenetic analysis demonstrated the symbiont of *Bathymodiolus* sp. JdF has a unique phylotype that definitively places it within the mussel chemoautotrophic symbiont clade, which is distinct from the clade containing mussel methanotrophic symbionts. While sequence verification was not possible due to lack of material, the fact a single PCR amplicon was successfully directly sequenced from gill tissue strongly supports the presence of a single bacterial 16S rRNA phylotype in the mussel gill tissue, as seen in other chemoautotroph-bathymodioline symbioses, e.g., *B. thermophilus* (Distel et al. 1988), *B. septemdierum* (Fujiwara et al. 2000), and *B.* aff. *brevior* (McKiness and Cavanaugh 2005).

The stable carbon isotope value ($\delta^{13}C$) for this mussel (−26.6%) is similar to those reported for other deep-sea mussels with symbionts and suggests that they derive little, if any, nutrition from photosynthetically derived carbon, which is typically more isotopically enriched (plankton, $\delta^{13}C = -19$ to −24%) (Peterson and Fry 1987; Van Dover and Fry 1989; Fisher 1995). Notably, depending on the methane source, mussels hosting dual symbioses, e.g., *Bathymodiolus heckerae* (Fisher et al. 1993), *B. puteoserpentis* (Robinson et al. 1998), and *B. azoricus* (Trask and Van Dover 1999), may have $\delta^{13}C$ values similar to mussels hosting only chemoautotrophic symbionts, e.g., *B. thermophilus* (Fisher et al. 1994), *B. brevior* (Dubilier et al. 1998), and *B.* aff. *brevior* (Van Dover 2002), or those mussels hosting only methanotrophic symbionts, e.g., *B. childressi* (Fisher 1993) and *B. platifrons* (Barry et al. 2002). The presence of methanotrophic symbionts in *B.* sp. JdF, even if minor, seems unlikely, given the isotopic signature of methane in high temperature fluid from the Endeavor segment is depleted, with an average $\delta^{13}C = \sim -50$ ‰ (Lilley et al. 1993). Further, *B. heckerae*, which hosts both symbiont types, has an even more depleted $\delta^{13}C$ value than *B. childressi*, which hosts only methanotrophic symbionts, collected from the same mussel bed (Fisher 1993). Thus, the relatively enriched tissue $\delta^{13}C$ value for this mussel is consistent with the absence of methanotrophic symbionts.

The tissue stable nitrogen isotope value is depleted ($\delta^{15}N = 5.19$), as seen with other vent and seep mussels which show lighter stable nitrogen ratios than non-vent and non-seep deep-sea fauna (Childress and Fisher 1992; Fisher 1995). Further interpretation of these values is complicated by differential contributions of nitrate and ammonia to host nutrition, as well as isotope fractionation via waste excretion. Combined, these ultrastructural, rRNA sequence, and isotope data indicate the host nutrition is consistent with

a deep-sea hydrothermal vent environment, typified by non-photosynthetically derived carbon and depleted nitrogen sources, and most likely chemoautotrophic in origin.

The collection of a new mussel symbiosis is particularly notable in the context of biogeography. While the apparent rarity of this species in the northeast Pacific vents may reflect sampling bias, it may also represent an initial colonization or an occasional settlement from a population that is not well adapted to these particular hydrothermal vent conditions, with potential sources of mussels including another site on the Juan de Fuca Ridge, an eastern Pacific cold seep (e.g., Monterey Bay), or a whale fall.

In sum, these data support the presence of chemoautotrophic bacterial symbionts in a new species of mussel recently discovered on the Endeavor segment of the Juan de Fuca Ridge. This finding extends the range of bathymodioline mussels to yet another hydrothermal vent biogeographic province. Additional samples will be critical to further characterizing the nature of the symbiosis in this mytilid, as well as formally describing the host species. This work not only extends our knowledge of the natural history of these unique habitats but offers additional insight into the nature and extent of chemoautotrophic symbioses as a means of ecological success and evolutionary innovation.

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