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Phylogenetic Characterization of the Endosymbionts of the Deepest-living Vesicomyid Clam, *Calyptogena fossajaponica*, from the Japan Trench

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Abstract: The bacterial endosymbionts of the vesicomyid clam *Calyptogena fossajaponica*, collected from the hadal zone in the Japan Trench, were characterized. Transmission electron microscopic (TEM) observations showed numerous bacteria in epithelial cells of the gill tissues of *C. fossajaponica*. Sulfur content and isotope analyses of this clam also suggested the presence of sulfur-oxidizing bacteria (thioautotrophs). Phylogenetic analysis based on the 16S ribosomal RNA gene (rDNA) sequence indicated that the bacteria was related to sulfur-oxidizing endosymbionts of other vesicomyid clams from deep-sea hydrothermal vents and cold seeps. The symbionts of *C. fossajaponica* formed a monophyletic group with the symbionts of *C. magnifica*, *C. phaseoliformis* and *Calyptogena* sp. (Florida Escarpment). These 4 species live relatively deeper than other vesicomyid clams whose symbionts have been phylogenetically characterized. Vertical distribution patterns of vesicomyid clams are likely influenced by their symbionts.

Keywords: Thioautotrophic symbiosis, Vesicomyid clam, Cold seeps, Japan Trench, Vertical distribution

Introduction

Vesicomyid clams live near sulfide-rich hydrothermal vents and cold seeps, harboring symbiotic sulfur-oxidizing bacteria in their gill tissue (Fisher, 1990; Sibuet & Olu, 1998; Tunnicliffe *et al.*, 1998). They rely on the primary production of their symbionts for nutrition (reviewed by Fisher, 1990). At least fourteen living species of vesicomyid clams have been described from Japanese waters (Fujikura *et al.*, 2000). *Calyptogena fossajaponica* was recently described from the Japan Trench (Okutani *et al.*, 2000). A dense aggregation of the clam was discovered at 6,809 m in the Japan Trench, the deepest record for vesicomyid clams (Okutani *et al.*, 2000). They had fleshy gills, and both the clams and the surrounding sediments had a hydrogen sulfide smell, suggesting *C. fossajaponica* harbored thioautotrophic symbiotic bacteria.

The phylogenetic analysis of the 16S ribosomal RNA gene (16S rDNA) sequence is a useful tool for examining the existence of thioauto- and methanotrophic symbionts in deep-sea species, because none of them have been cultured to date. The phylogeny of bacterial symbionts from many host species has been examined (Distel *et al.*, 1988; Eisen *et al.*, 1992; Cary, 1994; Distel & Cavanaugh, 1994; Distel *et al.*, 1994; Distel *et al.*, 1995; Dubilier *et al.*, 1995; Durand & Gros, 1996; Durand *et al.*, 1996; Feldman *et al.*, 1997; Peek *et al.*, 1998; Dubilier *et al.*, 1999; Di Meo *et al.*, 2000; Fujiwara *et al.*, 2000). However, such examination for vesicomyid clams living around

Japan has been limited. *Calyptogena soyoae* collected in Sagami Bay and *C. phaseoliformis* collected in the Japan Trench are the only species whose symbionts have been characterized using molecular phylogenetic analysis (Kim *et al.*, 1995; Peek *et al.*, 1998).

In order to characterize the symbionts of *C. fossajaponica*, epithelial cells of gill tissues were observed using transmission electron microscopy (TEM) and the nearly complete bacterial 16S rDNA sequences were determined. The sulfur content and $\delta^{34}S$ values of soft tissues from the clams and surrounding sediment were also measured.

Materials and methods

Specimen collection

Calyptogena fossajaponica specimens were collected from 6,809 m in the Japan Trench (40°06'N, 144°14'E) during dive #114 of the ROV *Kaiko* (JAMSTEC) in 1999. Upon recovery, the clams were immediately transferred to fresh, chilled (~4 ℃) seawater.

Treatment for TEM observations

Small pieces of gill tissue were fixed with 2.5 % glutaraldehyde in filtered seawater at 4 ℃ for one week. Blocks were rinsed 10 times with 0.05M phosphate buffer (pH 7.8) for 10 minutes at room temperature and post fixed in 1 % OsO_4 in 0.05M phosphate buffer (pH 7.8) at 4 ℃ for 2 hours. Then, tissues were dehydrated and embedded in EPON 812 resin (TAAB, Aldermaston, UK). 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.

Sulfur content and isotope analyses

Details of the analytical procedures employed in the present study have been given by Mizota *et al.* (1999). Soft tissues from twenty-three individuals of *C. fossajaponica* were dissected and divided into gill, foot, mantle and adductor muscle. Each tissue was dialyzed against deionized water overnight at 5 ℃ to eliminate seawater sulfates in the tissues. The salt-free materials were then freeze-dried. The dry materials (200 to 500 mg) were combusted under a high-pressure oxygen bomb (Parr Bomb #1108) to convert all the sulfur compounds into sulfates. Core sediments (from 2 to 6 cm deep) were collected from the site where the clams were collected. Seawater sulfate in the sediment was removed as described above. Non-sulfate sulfur compounds in the sediment were recovered by treatment with warm hydrogen peroxide. All the solution sulfates were converted into $BaSO_4$. The dry $BaSO_4$ was mixed with V_2O_5 and silica mixture (1:1) to yield SO_2 by thermal decomposition. The $^{34}S/^{32}S$ ratios were determined on the SO_2 with a SIRA 10 mass spectrometer (VG Isogas, Cheshire) at the Institute for Study of the Earth's Interior, Okayama University. $^{34}S/^{32}S$ ratios were shown by a conventional $\delta^{34}S$ notation, a per mil (‰) variation relative to Canyon Diablo Troilite (CDT). The overall analytical error is less than 0.2 ‰.

DNA preparation

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

PCR amplification

Bacterial 16S rDNA was amplified by PCR using the Ex Taq PCR kit (TaKaRa, Kyoto). Two

oligonucleotide primers (1 μM each) and < 1 μg of DNA template were added to the reaction mixtures. Thermal cycling was as follows: denatured at 96 ℃ for 20 seconds, annealed at 55 ℃ for 45 seconds, and extended at 72 ℃ for 2 minutes 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 S (Nippon Gene, Toyama) gel electrophoresis.

Cloning of amplified 16S rDNAs

Amplified 16S rDNAs were extracted from the agarose gel slices sequentially with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and precipitated with ethanol (Takai and Sako 1999). After centrifugation, DNA pellets were resuspended in sterile distilled water. The purified 16S rDNAs were inserted into the pCR-TOPO vector using the TOPO TA cloning kit (Invitrogen, San Diego). The inserted vector was transduced into One Shot TOP10 *Escherichia coli* Competent Cells (Invitrogen). Blue/white screening was performed to select colonies of *E. coli* having vectors with an inserted 16S rDNA sequence.

Restriction fragment length polymorphism (RFLP) analysis of inserts

Each white colony was directly used as a template for the PCR amplification using the Insert Check-Ready-Blue kit (Toyobo, Osaka). The PCR products containing appropriately sized inserts were identified by 1.2 % (w/v) agarose gel electrophoresis. The appropriately sized inserts were used as template for PCR amplification using Bac27F and 1492R and the PCR products were subjected to RFLP analysis using restriction enzymes which recognize a 4-bp restriction site. Two such restriction enzymes were used, *Msp*I (C'CGG) and *Rsa*I (GT'AC). The restriction enzyme reaction products were electrophoresed through a 3 % Agarose X (Nippon Gene, Toyama) gel.

Sequencing of amplified 16S rDNAs

DNA sequencing of the amplified 16S rDNAs was performed using the BigDye Terminator Cycling Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, USA). Nine universal 16S rDNA-specific primers were used in sequencing reactions (Kato *et al.*, 1997). Sequencing was performed using an ABI PRISM 310 genetic analyzer. The sequences reported here have been deposited in the DDBJ database under accession numbers as follows: AB044744.

Sequence and phylogenetic analyses

Nearly complete sequences of the 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 approximately 1,500 bp were used to run the similarity analysis. The database used for similarity analysis was the non-redundant nucleotide sequence database from GenBank. Sequences were manually aligned and phylogenetic analysis was restricted to nucleotide positions that were unambiguously alignable in all sequences. The genetic distance between operational taxonomic units (OTUs) was calculated by Kimura's two-parameter method (Kimura, 1980). Neighbor-joining analysis were accomplished using the CLUSTAL X software package (Thompson *et al.*, 1997). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

Results

TEM observations

Numerous bacteria were observed in the epithelial cells of gill tissue of *C. fossajaponica* using

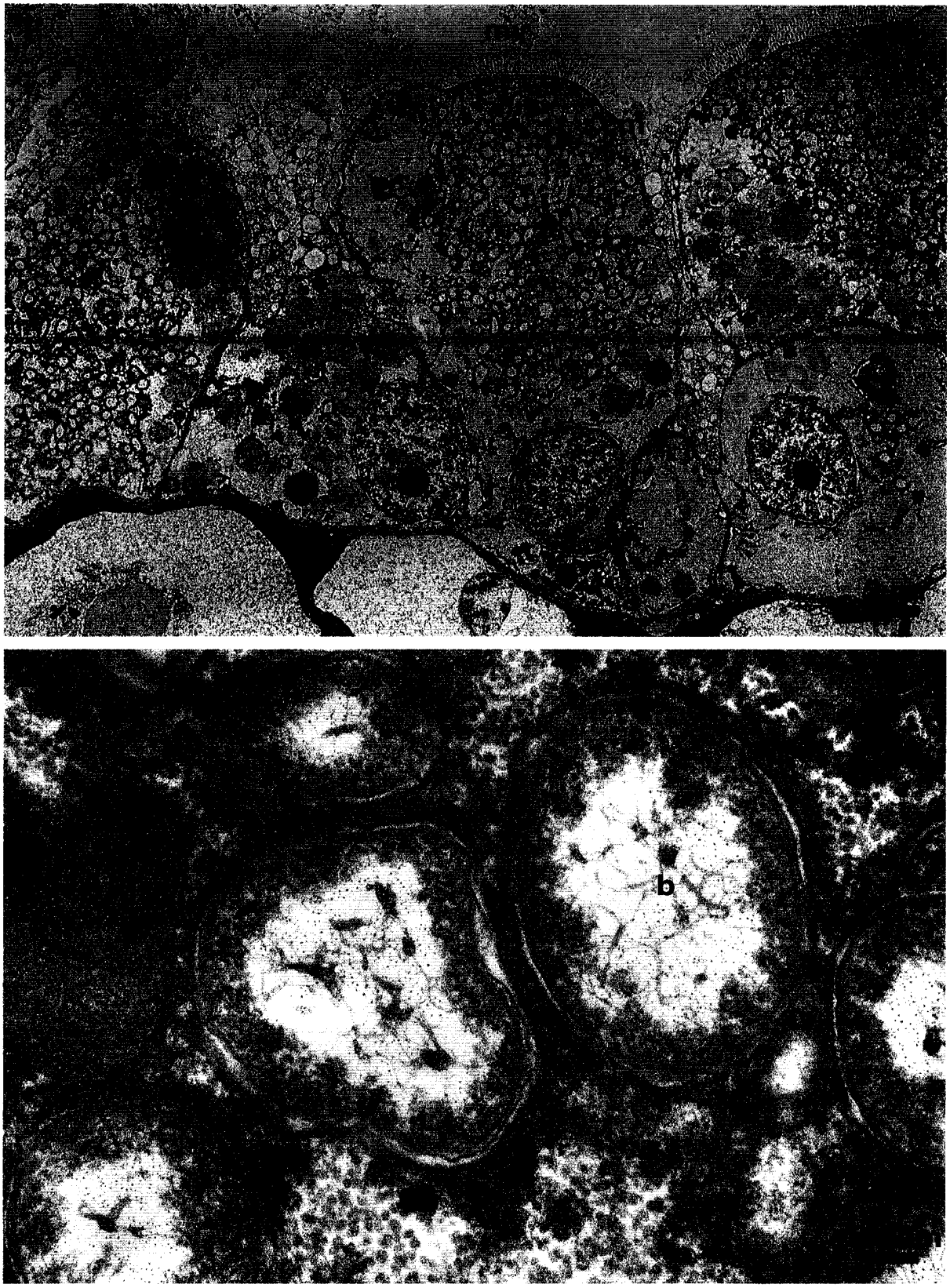

Fig. 1. *Calyptogena fossajaponica*. TEM photo of a transverse section of a gill filament showing intracellular gram-negative bacterial symbionts (arrowheads) without stacked internal membranes. bm: basal membrane; cm: cytoplasmic membrane; g: grains; mv: microvilli; n: nucleus.

Fig. 2. *Calyptogena fossajaponica*. High magnification of TEM photo of intracellular bacterial symbionts within a bacteriocyte of epithelium of host gill. The symbionts were gram-negative and contained no stacked internal membranes. b: bacteria.

TEM (Fig. 1). Mono-morphological bacteria were visible and contained within vacuoles of the host cells. A trilamellar cell envelope, typical of gram-negative bacteria, surrounded the bacteria (Fig. 2). Divisional stages of the bacteria were observed (data not shown). The bacteria lacked internal membranes and were small cocci or short rods that averaged 0.79 μm (SD = 0.14, n = 37) in diameter. Except for the apical part, the gill filaments were composed of a unicellular row of bacteriocytes (primarily), filled with bacteria. Some intermediate cells free of bacteria appeared among the bacteriocytes (Fig. 1). The external surface of each bacteriocyte was fringed by well developed microvilli. Electron-dense grains (85 nm in diameter) were also present and were accumulated next to the bacteriocytes in the inner region of the filament.

Sulfur content and isotope analyses

Results of sulfur content and stable isotope analyses for each soft tissue of *C. fossajaponica* and surrounding sediment are summarized in Table1. A high concentration of sulfur (15.2 %) was observed in the gill tissue compared with 0.8 – 1.0 % in other soft tissues. The δ^{34}S values of the soft tissue varied from –4.0 (adductor muscle) to –0.5 ‰ (foot) and the value of the sediment was –4.0 ‰.

RFLP analysis and 16S rDNA gene sequences

RFLP analysis was performed on 85 appropriately sized inserts which were amplified from 3 specimens of *C. fossajaponica* using Bac27F and 1492R as primers. Three distinct RFLP patterns appeared. The emergence ratio of these patterns was 83 : 1 : 1. Partial sequences (≈ 500 bp) of bacterial 16S rDNA from three distinct RFLP patterns of inserts were homogeneous. Nearly complete sequences of 16S rDNA from 4 clones that included the three patterns were also homogenous (99.4 – 99.5 % identical between the 4 sequences), and the lengths of the inserts were 1,467 bp.

Phylogenetic analysis of 16S rDNA sequences

Phylogenetic analysis performed by the neighbor joining method placed the 16S rDNA sequence of the *C. fossajaponica* symbionts within the γ subdivision of Proteobacteria containing the majority of thioautotrophic symbionts in marine invertebrate hosts (Fig. 3). The symbionts were associated with thioautotrophic bacteria and formed a monophyletic group with the symbionts of other vesicomyid clams supported by a bootstrap value of 100 %. The symbionts were sibling bacteria of the *C. magnifica* symbionts (98.1 % sequence identity in 1,409 bp overlapped) and formed a monophyletic group with the symbionts of *C. magnifica*, *C. phaseoliformis* and *Calyptogena* sp. (Florida Escarpment), supported by a bootstrap value of 83 %.

Table 1. Sulfur content and isotopic composition of different soft tissues of *Calyptogena fossajaponica* and surrounding sediment

Materials	Sulfur content (%)*	δ^{34}S (‰)
Gill	15.2	–0.6
Foot	1.0	–0.5
Mantle	0.9	–1.5
Adductor muscle	0.8	–4.0
Surrounding sediment	1.0	–4.0

* 105 ℃ dry-matter basis

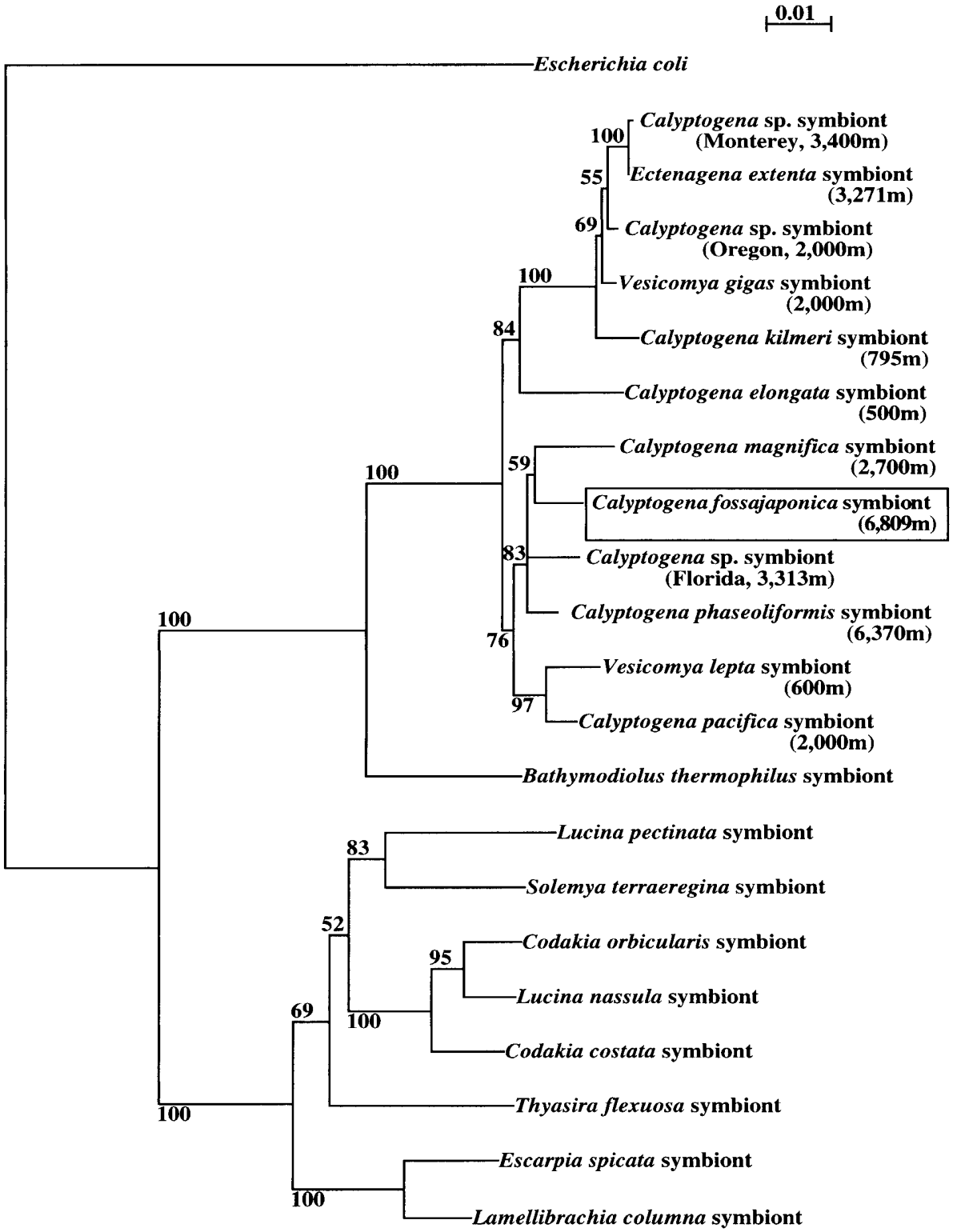

Fig. 3. Phylogenetic tree inferred by neighbor joining analysis of 1,357 homologous positions of 16S ribosomal RNA gene sequences of the symbionts of *Calyptogena fossajaponica* and representative free-living and symbiotic bacteria within the γ subdivision of Proteobacteria. Scale bar represents 0.01 nucleotide substitutions per sequence position. The percentage of 1000 bootstrap resamplings is indicated. Bootstrap values for nodes supported in greater than 500 of 1000 trees are shown. Symbiont of the clam examined in this study is highlighted. The accession numbers used for this study were as follows: AF035719, AF035720, AF035721, AF035722, AF035723, AF035724, AF035725, AF035726, AF035727, AF165908, E05133, L01575, L25708, L25710, L25712, M99445, U62131, U77481, X84979, X84980, X95229.

Discussion

This is the deepest record of chemosynthetic symbiosis to date for vesicomyid clams. Morphological observations, sulfur content and isotope analyses, and phylogenetic results suggest unanimously the bacteria of this symbiosis are thioautotrophic. Morphological features of the gill tissue of this clam are consistent with previous studies of other vesicomyid clams (reviewed by Fisher, 1990). The large size and the structure of the gill tissues clearly indicated that this should be the primary organ involved in nutrition. TEM observations showed that the structure of the gill tissue was similar to that of other vesicomyid clams (Fiala-Médioni & Métivier, 1986; Fiala-Médioni & Le Pennec, 1989). Numerous bacteria were visible in the epithelial cells of the gill tissue (Fig. 1), with divisional stages indicating active reproduction. The bacteria were gram-negative and had no stacked internal membranes. These features coincide with those of other thioautotrophic symbionts found in marine invertebrates (Fisher, 1990). The size of the bacteria (0.79 μm mean diameter) was in the range of that of other vesicomyid clam symbionts (Fisher, 1990).

The 16S rDNA sequence analysis of the symbionts of *C. fossajaponica* suggested that they were phylogenetically related to thioautotrophic symbionts from other vesicomyid clams. This was consistent with our TEM observations. The symbionts of *C. fossajaponica* formed a monophyletic group with the symbionts of *C. magnifica*, *C. phaseoliformis* and *Calyptogena* sp. (Florida Escarpment). These four host species live in relatively deeper zones than other vesicomyid clams. Differences in the vertical distributions of each vesicomyid clam species have been reported (Olu *et al.*, 1996; Kojima & Ohta, 1997; Fujikura *et al.*, 2000). Olu *et al.* (1996) suggested that different physiological tolerance to pressure might result in different depth distributions. Since no chemotrophic symbiont has been cultured to date, it is difficult to distinguish the influence of the host and symbiont physiology on the distribution of clams. It is possible that the symbionts influence host distributions, because such symbionts play an essential role in host nutrition, similar to chloroplasts in plants. Deep-sea mussels containing chemoautotrophic and/or methanotrophic symbionts are one of several conspicuous groups at hydrothermal vents and cold seeps, and are distributed under various chemical conditions (Fujiwara *et al.*, 2000). However, the distributions of mussels containing only methanotrophic symbionts are restricted to areas of high methane concentration (Fujiwara *et al.*, 2000).

A high concentration of sulfur in the gill tissue of *C. fossajaponica* also supports the presence of thioautotrophic symbiosis in this clam. Similar high concentrations have been observed in *Calyptogena soyoae* (Mizota & Maki, 1998), which is known to contain thioautotrophs (Kim *et al.*, 1995). The yellowish white color of the fresh gill indicates the presence of elemental sulfur (Fiala-Médioni & Le Pennec, 1989). The δ^{34}S values of the soft tissues of the clams (–4.0 to –0.5 ‰) were similar to the value of sulfides in the sediment (–4.0 ‰). These values were lower than those of seawater, indicating sulfur reduction in the substratum (Mizota, 1997). These results suggest that *C. fossajaponica* uses hydrogen sulfide as the major source of nutrition, and assimilates it into body tissues. The nature and role of electron-dense grains in the internal part of the filament next to the bacteriocytes is not clear. Such grains were also found in the gill tissue of *C. magnifica* and were suggested to be metallic concretions, which might represent energetic reserves for the bacteria (Fiala-Médioni & Métivier, 1986).

This study characterized the deepest chemosynthetic symbiosis in a vesicomyid clam. Results were similar morphologically, phylogenetically, and isotopically to other thioautotrophic symbioses in marine bivalves. Symbiont likely influence host distributions, especially for clams living in relatively deep areas. Although no chemoautotrophic symbionts have been cultured to date, their influence should be clarified by physiological studies of symbionts under different pressure condi-

tions, in addition to host physiology studies.

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日本海溝産オトヒメハマグリ科ナラクシロウリガイ *Calyptogena fossajaponica* の鰓中に共生する化学合成細菌の系統学的特徴

藤原義弘・小島茂明・溝田智俊・牧陽之助・藤倉克則

要　旨

日本海溝の超深海域より採集したオトヒメハマグリ科ナラクシロウリガイ *Calyptogena fossajaponica* の共生細菌の性状を明らかにするため，形態観察，硫黄含有量および同位体組成分析，分子系統解析を行った。透過型電子顕微鏡観察により，この二枚貝の鰓上皮細胞中に多数の細菌を確認した。また，この二枚貝の軟体部の硫黄含有量と硫黄同位体組成の解析により，硫黄細菌の存在を示唆する結果を得た。更に，この細菌の 16S リボソーム RNA 遺伝子配列を決定し，系統解析を行ったところ，この細菌は深海の熱水噴出域，冷水湧出域に生息する他のオトヒメハマグリ科二枚貝の共生硫黄細菌と近縁であった。この共生細菌はガラパゴスシロウリガイ，ナギナタシロウリガイおよびフロリダ海底崖産シロウリガイ類の 1 種の共生細菌と単系統群を形成した。これら 4 種の二枚貝は他の多くのシロウリガイ類に比べて大深度に分布していた。シロウリガイ類は種ごとに垂直分布が異なることが知られているが，このような垂直分布様式の違いはそれぞれの共生細菌の影響を受けているのかもしれない。

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