Molecular characterization of a deep-sea methanotrophic mussel symbiont that carries a RuBisCO gene

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Abstract: In our previous investigation on the genes of 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) in deep-sea chemoautotrophic and methanotrophic endosymbioses, the gene encoding the large subunit of RuBisCO form I (*cbbL*) had been detected in the gill of a mussel belonging to the genus *Bathymodiolus* from a western Pacific back-arc hydrothermal vent. This study further revealed the symbiont source of the RuBisCO *cbbL* gene along with the genes of 16S ribosomal RNA (16S rDNA), particulate methane monooxygenase (EC 1.14.13.25; *pmoA*) and ATP sulfurylase (EC 2.7.7.4; *sopT*). The 16S rDNA sequence analysis indicated that the mussel harbors a monospecific methanotrophic Gammaproteobacterium. This was confirmed by amplification and sequencing of the methanotrophic *pmoA*, while thiotrophic *sopT* was not amplified from the same symbiotic genome DNA. Fluorescence *in situ* hybridization demonstrated simultaneous occurrence of the symbiont-specific 16S rDNA, *cbbL* and *pmoA*, but not *sopT*, in the mussel gill. This is the first molecular and visual evidence for a methanotrophic bacterial endosymbiont that bears the RuBisCO *cbbL* gene relevant to autotrophic CO_2 fixation.

INTRODUCTION

Endosymbiotic interaction between bacteria and host mussels plays a key role in the ability of mussels to colonize hydrothermal vents that favor chemoautotrophic (CO_2-assimilating) and methanotrophic (methane-assimilating) bacteria as primary producers (Felbeck et al., 1981; Van Dover et al., 2002). The common phylogenetic tool that characterizes mussel symbiosis is ribosomal RNA gene (rDNA) sequence analysis. The 16S rDNA-based phylogeny indicates that deep-sea mussels possess a symbiotic complexity that is not seen in tubeworms and clams. Most of these mussels harbor thiotrophic Gammaproteobacteria in specialized cells, i.e. bacteriocytes, located in the large gill organ. On the other hand, methanotrophic endosymbionts have been described in mussels from several deep-sea habitats such as the Gulf of Mexico (Cavanaugh et al., 1987), sediments in the Skagerrak (Schmalijohann and Fugel, 1987), and a Mid-Okinawa Trough hydrothermal vent (Fujiwara et al., 2000). Moreover, some deep-sea mussels of the genus *Bathymodiolus* carry thiotrophic and methanotrophic endosymbionts simultaneously (Pernthaler and Amann, 2004; Duperron et al., 2005).

Three key enzymes constitute the backbone of symbiont trophic processes in deep-sea animals. The first and dominant one is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which characterizes most known autotrophs. RuBisCO is the key to the Calvin-Benson Bassahm reductive pentose phosphate cycle and is responsible for the fixation of CO_2 into organic carbon. RuBisCO exists in several forms, namely I, II, III (archaeal RuBisCO) and IV (archaeal-like bacterial RuBisCO) (Tabita, 1999). Form I, the dominant form in nature, consists of large and small subunits (L_8S_8) encoded by the genes *cbbL* and *cbbS*, respectively (Tabita, 1999). The form I large subunits have been further classified into four types, IA-ID, based on inferred amino acid homology (Watson and Tabita, 1997).

The genes of RuBisCO forms IA and II have been studied in bacterial endosymbionts within several deep-sea gutless animals. Thiotrophic endosymbionts in different taxa of deep-sea molluscs and some pogonophorans often bear form I type IA (Stein et al., 1990; Kimura et al., 2003; Schwedock et al., 2004). In contrast, vestimentiferan tubeworms, the vent polychaete Alvinellid, vent shrimp *Rimicaris exoculata* and the clam *Calyptogena* harbor symbionts that carry only the RuBisCO form II (Robinson et al., 1998; Elsaied et al., 2002).

The second key enzyme is methane monooxygenase (MMO). MMO oxidizes methane to methanol as the first step in the assimilation of methane-derived carbon (Hanson and Hanson, 1996). MMO is found in two forms, particulate (pMMO) and soluble (sMMO) (Hanson and Hanson, 1996). pMMO characterizes almost all discovered methanotrophs including discovered deep-sea methanotrophic symbionts (Pernthaler and Amann, 2004). pMMO consists of three consecutive classes of proteins, C, A and B, which are encoded by a gene cluster of three consecutive genes designated *pmoC*, *pmoA* and *pmoB*, respectively (McDonald and Murrell, 1997).

Methanotrophs are classified based on metabolic biochemical pathways, ultrastructure and 16S rRNA gene-based phylogeny into three types. Type I and X methanotrophs belong to Gammaproteobacteria, while Type II methanotrophs belong to Alphaproteobacteria (Trotsenko and Khmelenina, 2002). A species of Type X is characterized by its carrying of RuBisCO form I type IA (Baxter et al., 2002). The phylogenetic diversity of the MMO genes has been studied in free-living deep-sea methanotrophs (Wang et al., 2003; Elsaied et al., 2005). The *pmoA* transcript was recently detected in the gills of the mussel *Bathymodiolus azoricus*, which is

characterized by dual thiotrophic/methanotrophic symbiosis (Pernthaler and Amann, 2004).

The third key enzyme is ATP sulfurylase (ATPS) or sulfate adenyl transferase, which is the key enzyme in energy-generating sulfur oxidization as well as in several reversible sulfur metabolisms in wide range of prokaryotes and eukarya (Friedrich et al., 2001; Kappler and Dahl, 2001). The gene encoding ATPS has various synonyms with different abbreviations such as *sat* in photothiotrophic bacteria (Hipp et al., 1997), *cysD* and *cysN* in *Escherichia coli* (Leyh et al., 1992), *MET3* in *Saccharomyces cerevisiae* (Mountain and Korch, 1991) and *sopT* in deep-sea vestimentiferan endosymbionts (Laue and Nelson, 1994).

Frequently, a RuBisCO gene if found in a deep-sea mussel is an indicator of existence of a thiotrophic symbiont. However, no methanotrophic symbiont that carries a RuBisCO gene has been reported so far. Although the RuBisCO activity was earlier detected physiologically in a mytilid species that characterized by carrying methanotrophs in a Louisiana seep (Distel and Cavanaugh, 1994), but no evidence confirmed that the source of this RuBisCO is a methanotrophic symbiont. We have detected only one copy of the RuBisCO form I type IA gene *cbbL* in the bulk gill DNA of a mussel species collected from a methane hydrothermal vent at Mid-Okinawa Trough (Elsaied and Naganuma, 2001). It was not investigated whether this RuBisCO gene came from a methanotrophic symbiont, as the rRNA and other functional autotrophy-related genes of this symbiont were never characterized before.

In this study, we focused on the symbiont source of the RuBisCO *cbbL* gene in this mussel by linking the 16S rRNA gene-based phylogeny with autotrophy-related genetic analyses. In addition to the RuBisCO gene *cbbL*, two functional genes, *pmoA* and *sopT*, which are powerful markers in the detection of deep-sea methanotrophs and thiotrophs (Laue and Nelson, 1994; Elsaied et al., 2005) respectively, were applied in this symbiosis investigation. The phylogenetic analyses were also visualized by co-localization of the target genes in the symbiont-carrying mussel gill tissue by fluorescence *in situ* hybridization.

MATERIALS AND METHODS

DNA Analysis

Specimens of the *Bathymodiolus* mussel were collected from a hydrothermal vent in Mid-Okinawa Trough and part of mussel gill was pretreated for DNA analysis as previously described (Elsaied and Naganuma, 2001). The other gill part fixed in Davidson's fixative (10% glycerol, 8% formaldehyde, 28.5% ethanol, 10% acetic acid and 30% seawater) (Jowett, 1997) and kept at room temperature for *in situ* hybridization.

All the current target genes were tested for PCR amplification from the bulk mussel gill DNA that used previously for the amplification of the RuBisCO gene *cbbL* (Elsaied and Naganuma, 2001). To identify the mussel endosymbiont, the bacterial 16S rRNA gene was amplified from the bulk gill DNAs using the bacterial universal primers 27F and 1492R, which yielded a product of about 1484 bp (Lane et al., 1985). To minimize potential bias during PCR amplification of the bacterial 16S rDNA (Suzuki and Giovannoni, 1996), the amplification procedure was done multiple times using fewer PCR cycles, such as 22-26 cycles, and then the PCR products were combined for cloning.

To increase the amplification efficiency and specificity of the primers to the gene *pmoA* from methanotrophic symbionts, the primers *pmoA*-189 and *pmoA*-682 that are universal for both target (*pmoA*) and non-target (ammonia-oxidizing related gene, *amoA*) (Holmes et al., 1995), were modified based on the alignment of the *pmoA* sequences of free-living methanotrophs in the hydrothermal vent plume around the MOT-mussel colony (Elsaied et al., 2005). The modified primers *pmoA*-189f (5′-GGCGACTGGGACTTCTGGAC-3′) and *pmoA*-682r (5′- GAACGCYGARAAGAACGCAG -3′) could amplify 530-bp fragment of *pmoA* from the mussel bulk gill DNA using a PCR mixture and conditions according to Elsaied et al., (2005).

Primers for amplification of the *sopT* gene were newly designed based on the alignment of ATPS gene sequences from several photo- and chemo-thiotrophic bacteria. Alignment included the relevant gene sequences of the *Riftia pachyptila* endosymbiont (L26897), *Allochromatium vinosum* (U84759), *Synechocystis* sp. PCC 6803 (D90913), *Synechococcus* sp. WH 8102 (BX569689), *Chlorobium tepidum* (AE012853), *Gloeobacter violaceus* PCC 7421 (AP006571), *Thermosynechococcus elongatus* BP-1 (AP005372), *Nostoc* sp. PCC 7120 (AP003598) and *Prochlorococcus marinus* MIT9313 (BX572100). The degenerated PCR primers *sopT*-1218f (5′-TTY CAG ACC CGC AAC CCS ATG CAC CGC GCY-3′) and *sopT*-1744r (5′-CAC YTC RGG RCG GGA GAA CTC MGG SGG-3′) were used to amplify 555 bp encoding the catalytic domain of the ATPS of the *Riftia pachyptila* endosymbiont (Laue and Nelson 1994). The PCR mixture contained 20 ng of genomic DNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each of dNTPs, 50 pmol each of the primers, and 2.5 units of ExTaq polymerase (TaKaRa Bio Inc, Otsu, Japan). Amplification was initiated by DNA denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, optimum annealing at 55 °C for 2 min and extension at 72 °C for 3 min followed by final extension at 72 °C for 10 min to allow 3'-A overhangs for the amplified PCR product to facilitate TA cloning. The primers could amplify *sopT* from the genomic DNAs of positive controls of a known mussel thiotrophic symbiont collected from hydrogen sulfide hydrothermal vent, Suiyo Seamount, Japan (accession no. AB178052), the tubeworm *Lamellibrachia* sp. thiotrophic symbiont (Naganuma et al., 1997) and the common thiotroph *Thiobacillus denitrificans* (data not shown). Although the

amplification efficiency of the *sopT* primers is directed to thiotrophs, the gene *sopT* could not be detected in the bulk gill DNA of the studied mussel.

The amplified products, which were 16S rRNA and *pmoA* genes, were cloned using the TOPO TA-cloning Kit (Invitrogen, California, USA) according to the manufacturer's instructions. Randomly selected clones that have the target inserts were directly analyzed by sequencing using the Dye-terminator Cycle-sequencing Kit on an ABI automated sequencer model 373A (Perkin-Elmer, Illinois, USA). The resulting sequences were compared and the similarity is calculated using the program FASTA at the DNA Database Bank of Japan (DDBJ: www.ddbj.nig.jp).

The phylogenetic trees were constructed using the neighbour-joining method and visualized using the program TREEVIEW (Page, 1996). The branching patterns of the constructed phylogenetic trees were confirmed by reconstruction of the phylogenies using the methods of maximum parsimony and maximum likelihood, contained within the PHYLIP package (Felsenstein, 1989).

The 16S rDNA phylotype MOT-mussel(16S)-1 and the *pmoA* sequence MOT-mussel (*pmoA*)-1 obtained in this study were deposited in the DNA database bank DDBJ under accession numbers AB056868 and AB062137, respectively.

Fluorescence *In Situ* Hybridization

The 16S rRNA-targeted oligonucleotide probe specific for the current endosymbiont was designed by alignment with those from other related species in databases using ClustalW (DDBJ). The probe MOT-475R (5′-GAGACGAGAGATTATCTTGCCT-3′) corresponded to positions 453 to 475 of the *E. coli* 16S rRNA gene. Mismatching of the probe target regions was checked by comparison with sequences from related species using the programs BLAST and PROBE MATCH. The sequence region of the specific 16S rRNA oligonucleotide probe MOT-475R showed the minimal nucleotide mismatches of 4 nucleotides with that of the closely related endosymbiont of *Bathymodiolus platifrons* (Fujiwara et al., 2000).

The universal bacterial-domain-EUB338 probe was used as a positive control to visualize all possible bacterial species within the gill tissue of the study mussel. The bacterial 16S rRNA universal and specific oligonucleotide probes were both chemically synthesized with a DNA synthesizer provided by Roche Diagnostics Co. (Tokyo, Japan).

The RuBisCO *cbbL* polynucleotide probe was prepared by amplification of 150-nucleotide-antisense fragment from the total 800 bp plasmid insert (Elsaied and Naganuma, 2001), and represented a highly mismatched sequence (34 mismatches) with other deep-sea endosymbiont RuBisCO *cbbL*s (Stein et al., 1991; Schwedock et al., 2004). The probe region corresponded to positions 1175 to 1323 of the *cbbL* of the chemoautotrophic symbiont of the deep-sea clam *Solemya velum* (Schwedock et al., 2004). The 150-nucleotide-antisense *pmoA* probe was amplified from the plasmid *pmoA* insert and represented the sequence positions 2612-2762 of the *pmoA* sequence of the methanotroph *Methylococcus capsulatus* (Stolyar et al., 1999). This *pmoA* sequence region contains the maximum nucleotide mismatches (88 mismatches) with the closely related *pmoA* sequences recovered from deep-sea hydrothermal vent plumes (Elsaied et al., 2005).

The length of each of the *cbbL* and *pmoA* polynucleotide probes (150 nucleotides) used in this study was shorter than that we used previously (Elsaied et al., 2002) in order to increase the specificity of the polynucleotide probes to their gene targets. Also, the selection of the sequences that contained the maximum nucleotide mismatches with the closets sequences recorded in the databases came to support this specificity. This was clear in the *pmoA* probe, which contains almost 58.7% mismatches of the total length of the probe. Although the RuBisCO probe contains almost 22.7% mismatches of total length of the probe, the hybridization signal specificity of the polynucleotide probes was controlled by co-hybridization with the specific 16S rRNA oligonucleotide probe as indicated in Elsaied et al., (2002).

The *sopT* probe was prepared by amplification and sequencing of 555-bp *sopT* fragment (AB178052) from bulk gill DNA of the positive control hydrothermal vent thiotroph-dependent mussel using the *sopT* primers described above. To minimize the mis-visualization of minor species of thiotrophs in the gills of the studied mussel, 150-nucleotide-antisense symbiont probe was amplified from the 555-bp positive control *sopT* fragment and represented the conserved sequence (average, 95% nucleotide similarity) with those of the *Riftia pachyptila* (Laue and Nelson, 1994) and *Lamellibrachia* sp. (AB177532, Elsaied and Naganuma, unpublished data) thiotrophic symbionts. This *sopT* probe covered positions 1375 to 1523 of that of *Riftia pachyptila* endosymbiont.

For fluorescence *in situ* hybridization (FISH), both the oligonucleotide and polynucleotide probes were labeled with fluorescent dyes Alexa Fluor-546 and Alexa Fluor-488 (cat. no. U-21652 and U-21650; Molecular Probes Inc, USA). Methods of labeling and calculating the labeling efficiency of the probes were in accordance with the manufacturer's instructions.

FISH was performed on consecutive paraffin-fixed 5 μm-thick cross-sections of gill filament according to Distel et al., (2002) and Pernthaler and Amann, (2004) with modifications. The gill sections were deparaffinized by immersion in two changes of xylene (10 min each), followed by re-hydration through a descending series of 100%, 95%, 80%, 70% and 50% ethanol. The sections were then incubated for 10 min in 0.2 M HCl and rinsed briefly in 0.2 M Tris with 50 mM EDTA (pH 7.8). To

allow permeation of the probes, the gill sections were partially digested with proteinase K (7 μg ml^{-1}) (Wako Chemical Co., Osaka, Japan) for 10 min at room temperature. Sections were then postfixed in 3.7% formalin in 0.02 M Tris-HCl followed by washing with TE buffer (pH 8). To reduce tissue background fluorescence, the sections were incubated three times for 10 min each in a freshly prepared solution of 50 mM sodium borohydride and 50 mM Tris (pH 7.8). They were then rinsed in distilled water and dehydrated in an ascending series of 70%, 95% and 100% ethanol for 1 min at each concentration and air-dried.

In situ hybridization was performed on gill tissue sections using a hybridization buffer (1 M NaCl, 0.05% SDS, 100 mM Tris, 5 mM EDTA, 40% formamide, pH 7.5). A total of 100 μl of the hybridization buffer was mixed with 50 ng of each probe and incubated for 5 min at 80 °C for probe denaturation followed by chilling in ice. The probe-hybridization buffer mixture was added to pre-treated sections, which were then covered with parafilm coverslips. Hybridization temperatures from 42 to 46 °C were determined according to the Tm value of each probe in the presence of 40% formamide. The sections were incubated overnight in humidity chambers at the desired temperatures. The hybridized sections were washed three times for 5 min each in 0.2 x SET (30 mM NaCl, 200 nM EDTA, 4 mM Tris-HCl, pH 7.5) at 50 °C. They were then air-dried, mounted with anti-fading medium (Molecular Probes Inc, Eugene, USA) and visualized with an epifluorescence microscope equipped with the appropriate filter set for Alexa Fluor-546 and Alexa Fluor-488 fluorescent dyes (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

Occurrence of only Monophyletic Methanotrophic Endosymbiotic Species within the gills of *Bathymodiolus* sp. based on 16S rDNA sequence analysis.

One hundred clones containing an approximately 1484-bp insert of the bacterial 16S rDNA showed >99% nucleotide similarity, indicating the existence of a single bacterial 16S rDNA phylotype. Although the predominance of a single 16S rDNA sequence in the amplification products could have been the result of bias in amplification efficiencies, the controlling of 16S rDNA PCR amplification beside the results of the functional gene sequencing followed by *in situ* hybridization confirmed that this was not the case. Bootstrap values from the constructed trees strongly supported that the phylotype, MOT-mussel(16S)-1, is placed in the cluster of deep-sea methanotrophic mussel endosymbionts (Figure 1). MOT-mussel(16S)-1 was monophyletic and deeply rooted with the cluster of Gammaproteobacterial cultivable Type I and X methanotrophs. MOT-mussel(16S)-1 was phylogenetically distinct from the RuBisCO-carrying methanotroph *Methylococcus capsulatus*, showing 82% nucleotide similarity.

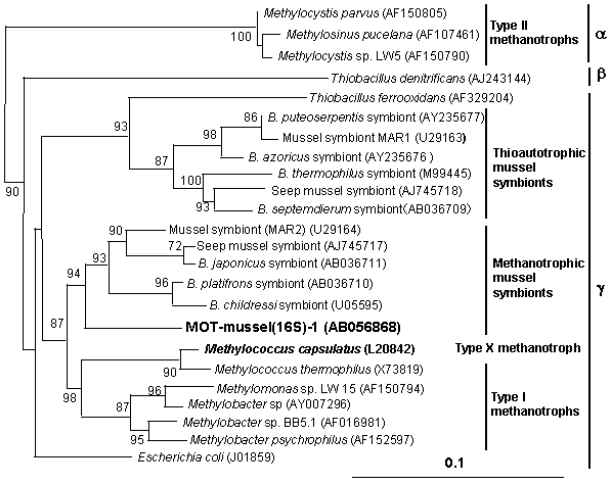

Figure 1. Phylogenetic trees based on the 16S rDNA sequences. The tree shows the phylogenetic localization of the studied mussel Bathymodiolus symbiont MOT-mussel(16S)-1 among other Bathymodiolus symbionts and cultured thio-/methanotrophs, with a typical Gammaproteobacterium Escherichia coli. Tree topography and evolutionary distances were determined by the neighbor-joining method. Bootstrap values, calculated from 1000 replicates, are expressed as percentages and only values of more than 50% are shown at the nodes of the tree. Accession numbers are shown in parentheses. Abbreviations: α, Alphaproteobacteria; β, Betaproteobacteria; and, γ, Gammaproteobacteria. Scale bar, 0.1 substitutions per site.

Occurrence of a single *pmoA* gene along with the previously recorded RuBisCO *cbbL* in the gills of *Bathymodiolus* sp.

The gene *pmoA* was positively PCR amplified along with the RuBisCO *cbbL* sequence MOT-mussel(*cbbL*)-1 that was previously recorded from the analysis of fifty PCR clones (Elsaied and Naganuma, 2001). Also, a fixed number of fifty clones from *pmoA* clone library was screened by direct sequence to obtain an equilibrium distribution for the diversity of functional genes in the studied mussel gill DNA. However, this number of analyzed clones is sufficient to expect total diversity of functional gene in the clone library as described by Elsaied and Naganuma, (2001). Only, one *pmoA* sequence designed as MOT-mussel(*pmoA*)-1 was obtained from the sequence analysis. In contrast, the thiotrophic *sopT* was not amplified with same genomic DNA, indicating that thiotrophic symbionts are absent in the studied mussel and that the *cbbL* source is a methanotroph.

The RuBisCO *cbbL* phylogenetic tree (Figure 2) placed the RuBisCO MOT-mussel(*cbbL*)-1 in the IA branch where those of *M. capsulatus* and thiobacilli species were also placed. The difference in phylogenetic localization of current mussel symbiont in the 16S

rDNA- and *cbbL*-trees was due probably to lateral gene transfer of RuBisCO genes across species boundaries (Delwiche and Palmer, 1996). Although the existence of this multiple lateral gene transfer through the RuBisCO phylogney, the RuBisCO genes keep conservation among wide range of phylogenetic taxa in order to keep its function stable (Delwiche and Palmer, 1996). This was obvious in the current symbiont *cbbL* gene, which showed similarity with that of *M. capsulatus* and thiobacilli (Figure 2) (Elsaied and Naganuma, 2001).

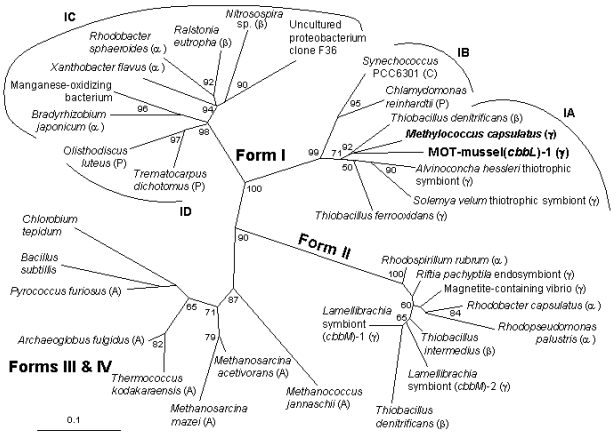

Figure 2. Phylogenetic tree based on the deduced amino acid sequences of RuBisCO large subunit genes (*cbbL*) from selected representatives of all identified RuBisCO forms. The tree was reconstructed from that of Elsaied and Naganuma, (2001) by addition of new discovered RuBisCO *cbbL* genes from the data bases in order to show the exact phylogenetic position of the MOT-mussel(*cbbL*)-1. Tree topography and evolutionary distance were determined by the neighbor-joining method. Bootstrap values, calculated from 1000 replicates, are expressed as percentages and only values of more than 50% are shown at the nodes of the tree. Letters in parentheses represent the expected classification from 16S rDNA-based phylogentics and other studies: α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria; C, Cyanobacteria; P, eukaryotic plastids; and, A, archaea. Scale bar, 0.1 substitutions per site.

The symbiont MOT-mussel(*pmoA*)-1 and the cloned *pmoA* from the vent plume around the mussel colony (Elsaied et al., 2005) formed a monophyletic group (Figure 3), consistent with the 16S rDNA-tree that distinguish our methanotrophic symbiont from all previously examined symbionts in hydrothermal vent and hydrocarbon seep organisms (Fujiwara et al., 2000). Similarly, the present data suggest that this endosymbiont differs considerably from cultivable Gammaproteobacterial methanotrophic species with respect to both its metabolic capabilities and physiological characteristics. The occurrence of *pmoA* in the endosymbionts of *Bathymodiolus azoricus* from the Mid-Atlantic Ridge (Pernthaler and Amann, 2004) and the present MOT-mussel, as well as in free-living hydrothermal plume methanotrophs (Elsaied et al., 2005), suggests the dominance of Gammaproteobacterial methanotrophs in hydrothermal vent habitats, in contrast to the detection of Alphaproteobacterial methanotrophs in deep-sea non-vent sediment off China (Wang et al., 2003). Moreover, these results support the hypothesis of the universal existence of *pmoA* in all types of methanotrophs, including those from deep-sea methanotrophic symbionts (Semrau et al., 1995).

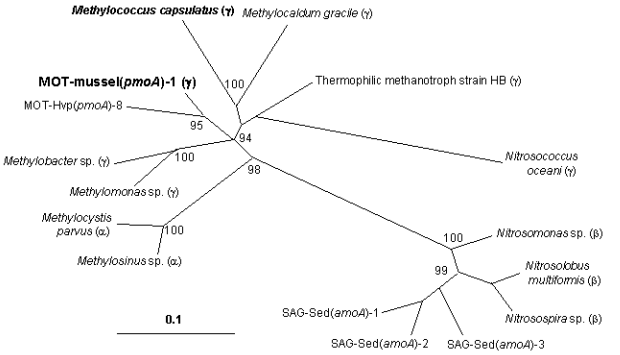

Figure 3. Phylogenetic tree based on the deduced amino acid sequences of particulate methane monooxygenase gene (*pmoA*) of the hydrothermal vent mussel symbiont MOT-mussel(*pmoA*)-1 and a clone from the same hydrothermal vent plume, along with cultured methanotrophs. Tree topography and evolutionary distance were determined by the neighbor-joining method. Bootstrap values, calculated from 1000 replicates, are expressed as percentages and only values of more than 50% are shown at the nodes of the tree. Ammonia monooxygenase genes (*amoA*) of selected ammonia-oxidizing bacteria and environmental clones from methane seep sediment in Sagami Bay, Japan, were used as an out-group. The letters in parentheses represent the expected classification from the 16S rRNA gene-based phylogenetics: α, Alphaproteobacteria; β, Betaproteobacteria; and, γ, Gammaproteobacteria. Scale bar, 0.1 substitutions per site.

Fluorescence *In Situ* Hybridization.

FISH was done to link phylogenetic identification and thio/methanotrophic function of the mussel symbiont. Success of FISH depends on the specificity of the designed probes. This hybridization specificity was clearly visible in the identical hybridization signals for both the specific oligonucleotide probe MOT-475R and the other *cbbL* and *pmoA* polynucleotide probes (Figure 4).

Dual-probe FISH in mussel gill tissue demonstrated that the genes amplified from the mussel bulk gill DNA contained the same target sequences in the endosymbiont within the gill. This finding confirms that the amplified sequences of 16S rDNA, *cbbL* and *pmoA* originate from

the symbiont genome rather than from any epibionts or contaminants. Five groups of dual-probe hybridizations were done on five consecutive mussel gill sections (Figure 4). The symbiotic bacteria are distributed in the form of bundles with high cell concentration, approximately, $3x10^9$ bacterial cells per gram of mussel gill filament, looking like culture of bacterial cells along the peripheries of the gill filaments (Salerno et al., 2005). This was clear in the highly intensity of hybridization signals showing these bacterial bundles. FISH demonstrated two pieces of visual evidence for the occurrence of monospecific endosymbiont in the gill tissue of the studied mussel. The first evidence was indicated by the typical signal patterns for both the 16S rRNA universal and specific probes (Figure 4A).

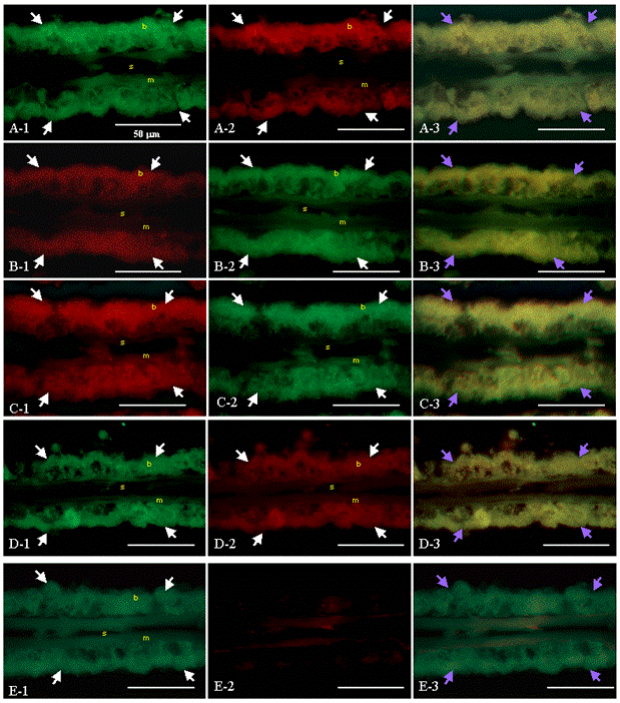

Figure 4. Dual-probe hybridizations on five consecutive sections of the mussel gill. (A1-A3) Section hybridized with the 16S rRNA bacterial-domain-probe EUB338 labeled with Alexa-fluor 488 (A-1, green) and the specific probe MOT-475R labeled with Alexa-fluor 546 (A-2, red). The overlap of the two signals is illustrated in pattern A-3. (B1-B3) Hybridization section patterns for the probes MOT-475R labeled with Alexa-fluor 546 (red) and RuBisCO *cbbL* probe labeled with Alexa-fluor 488 (green) followed by overlap of the two signal patterns (orange). (C1-C3) Hybridization section patterns for the probes MOT-475R labeled with Alexa-fluor 546 (red) and *pmoA* probe labeled with Alexa-fluor 488 (green) followed by overlap of the two signal patterns (orange). (D1-D3) Hybridization section patterns for the *pmoA* probe labeled with Alexa-fluor 488 (green) and RuBisCO *cbbL* probe labeled with Alexa-fluor 546 (red) followed by overlap of the two signal patterns (orange). (E1-E3) Hybridization section patterns for the *pmoA* probe labeled with Alexa-fluor 488 (green) and *sopT* probe labeled with Alexa-fluor 546 (red) followed by the overlap of the positive hybridized *pmoA* pattern with that negatively hybridized with the *sopT* probe (only green). The white arrows show the signal patterns for a single probe. The violet arrows show the overlap of the hybridization patterns for two probes. The letters **b, m** and **s** show the localizations of symbiont-containing bacteriocytes, symbiont-free basement membrane and blood sinus, respectively. Scale bar, 50 µm.

The second evidence was visualized in gill tissue sections by dual-probe hybridization of the 16S rRNA-specific probe MOT-475R and the functional gene probes for *cbbL* and *pmoA* (Figure 4B-D).The most interesting result represented here is the visual existence of the RuBisCO gene *cbbL* in the methanotrophic endosymbiont, demonstrated in three co-hybridization sets (Figure 4B-D). Coupling of the dual signals for the probes was observed in the same bacterial bundles (not in different bundles) along the periphery of the gill filament. This indicated by the orange color resulted from coupling of the red signal from the 16S rRNA-specific probes and the green signals from the respective *cbbL* or *pmoA* probes. This identical overlapping of FISH signal patterns was distinguished from the separate signal patterns in the dual methano-/thiotrophic symbioses of other *Bathymodiolus* sp. (Pernthaler and Amann 2004; Duperron et al. 2005). The lack of detection of *sopT* in the current mussel by PCR or by *in situ* hybridization (Figure 4E) refuted our primary expectation that the mussel gills may harbor a RuBisCO-carrying thiotrophic endosymbiont (Elsaied and Naganuma, 2001).

Novel Mussel Symbiont

Co-localization of symbiont specific 16S rRNA, *cbbL* and *pmoA* confirmed that the vent mussel harbours only a methanotrophic endosymbiont that carries the RuBisCO gene *cbbL*. Occurrence of a RuBisCO gene in methanotrophs is known only in the Type X methanotrophs such as *M. capsulatus*; however, 16S rDNA sequences of the mussel symbiont and *M. capsulatus* were not closely related (Figure 1). In contrast, the *cbbL* sequences of the mussel symbiont and *M. capsulatus* showed a close relationship within the form IA branch (Figure 2). These contrastive relationships suggest that: 1) the "Type X" is a phylogenetically incoherent group of methanotrophs; and, 2) certain classes of *cbbL* such as form IA more easily transferable than other RuBisCO genes.

Deep-sea mussels from different localities have been variously shown to harbor a thiotroph only (Won et al., 2003), a methanotroph only (Fujiwara et al., 2000), both as dual symbioses (Pernthaler and Amann, 2004; Duperron et al., 2005), or a methanotroph, which carries dual-character methanotrophy and autotrophy (present

study). This may suggest that mussel-bacterial symbioses have multiple origins and lineages. This is in contrast with all known deep-sea vestimentiferan tubeworms, which harbor only chemoautotrophic endosymbionts (Elsaied et al., 2002).

Occurrence of methanotrophic symbiont that carries the RuBisCO form IA gene *cbbL* may be correlated with vent characteristics. Mid-Okinawa Trough vents are characterized by high concentrations of methane (about 16 mM) and carbon dioxide (about 200 mM) (Kataoka et al., 2000), favoring the abundance of both free-living and symbiotic methanotrophs (Elsaied et al., 2005). If the *cbbL* gene of the methanotrophic symbiont is indeed expressed and the enzyme functions actively, this would represent a unique trophic strategy, namely that a single endosymbiont possesses dual pathways for carbon assimilation. In fact, RuBisCO *cbbL* activity has been recorded in the presence of methane and CO_2 during autotrophic growth of the common methanotroph *M. capsulatus* (Baxter et al., 2002). Hence, the co-existence of autotrophy- and methanotrophy-related genes in one endosymbiont is realistic, and would be highly advantageous adaptive behavior to a habitat characterized by high concentrations of methane and CO_2. This dual-trophy in a single endosymbiont might be a consequence of the natural selection of a host-symbiont relationship, which enables survival and flourishing in variable hydrothermal vent conditions.

The present phylogenetic data confirm the existence of a novel deep-sea mussel methanotrophic symbiosis. We are now investigating the physiology and enzymology of this unique symbiosis to evaluate the diversity, evolution, biogeography and biological production of deep-sea mussel-bacterial symbiosis.

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