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ORIGINAL ARTICLE

Diversity and methane oxidation of active epibiotic methanotrophs on live *Shinkaia crosnieri*

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Shinkaia crosnieri **is a galatheid crab that predominantly dwells in deep-sea hydrothermal systems in the Okinawa Trough, Japan. In this study, the phylogenetic diversity of active methanotrophs in the epibiotic microbial community on the setae of *S. crosnieri* was characterized by reverse transcription-polymerase chain reaction (RT-PCR) of a functional gene (*pmoA*) encoding a subunit of particulate methane monooxygenase. Phylogenetic analysis of *pmoA* transcript sequences revealed that the active epibiotic methanotrophs on *S. crosnieri* setae consisted of gammaproteobacterial type Ia and Ib methanotrophs. The effect of different RNA stabilization procedures on the abundance of *pmoA* and 16S rRNA transcripts in the epibiotic community was estimated by quantitative RT-PCR. Our novel RNA fixation method performed immediately after sampling effectively preserved cellular RNA assemblages, particularly labile mRNA populations, including *pmoA* mRNA. Methane consumption in live *S. crosnieri* was also estimated by continuous-flow incubation under atmospheric and *in situ* hydrostatic pressures, and provided a clear evidence of methane oxidation activity of the epibiotic microbial community, which was not significantly affected by hydrostatic pressure. Our study revealed the significant ecological function and nutritional contribution of epibiotic methanotrophs to the predominant *S. crosnieri* populations in the Okinawa Trough deep-sea hydrothermal systems. In conclusion, our study gave clear facts about diversity and methane oxidation of active methanotrophs in the epibiotic community associated with invertebrates.**

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Introduction

Many species of invertebrates that dwell in deep-sea hydrothermal vents are known to host bacteria (epibionts) that adhere to the surface of specialized tissues such as the dorsal setae of the polychaete *Alvinella pompejana*, the gill chambers of the shrimp *Rimicaris exoculata* and the setae of the galatheid crabs *Shinkaia crosnieri*, *Kiwa hirsuta* and *Kiwa puravida* (Polz and Cavanaugh, 1995; Cary *et al.*, 1997; Goffredi *et al.*, 2008; Watsuji *et al.*, 2010; Thurber *et al.*, 2011). The epibiotic microbial communities associated with these hosts were thought to have chemolithoautotrophic and methanotrophic productivity and to provide continuous nutrition to their hosts (Goffredi *et al.*, 2008; Grzymski *et al.*, 2008; Watsuji *et al.*, 2010; Thurber *et al.*, 2011; Ponsard *et al.*, 2012). The autotrophic productivity of epibiotic communities has been denoted by detection of the activity of key carbon fixation enzymes such as ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO) (Wirsen *et al.*, 1993), the occurrence and expression of a gene for ATP citrate lyase (Campbell *et al.*, 2003) and the incorporation of ^{13}C-labeled bicarbonate into epibiotic cells (Watsuji *et al.*, 2010). Energy metabolism in these epibiotic communities was primarily investigated by analysis of their genomic DNA, leading to the identification of genes with putative functions in energy metabolism (Goffredi *et al.*, 2008; Grzymski *et al.*, 2008; Hugler *et al.*,

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2011). Recently, several studies have determined actual functional activity in the epibiotic communities and combined phylogenetic characterization of epibionts with functional activity (Watsuji *et al.*, 2010, 2012; Ponsard *et al.*, 2012).

Experiments using ^{13}C-labeled bicarbonate in the presence of thiosulfate and ferrous iron have found that *R. exoculata* epibionts are capable of chemolithoautotrophic growth by oxidizing reduced sulfur compounds and reduced iron (Ponsard *et al.*, 2012). Tracer experiments, microscopic fluorescence *in situ* hybridization (FISH) and nanoscale secondary ion mass spectrometry have indicated that epibiotic *Sulfurovum* cells present on the *S. crosnieri* setae assimilate inorganic carbon in the presence of thiosulfate (Watsuji *et al.*, 2012), recognizing that these predominant epibionts are thioautotrophs. In contrast, ^{13}C-bicarbonate incorporation into *S. crosnieri* epibionts was not enhanced by molecular hydrogen (Watsuji *et al.*, 2010).

The methanotrophic productivity of the epibiotic community on *S. crosnieri* setae has been indicated by the incorporation of $^{13}CH_4$ into the epibionts when dissected setae were incubated with $^{13}CH_4$ as the sole energy and carbon source (Watsuji *et al.*, 2010). In addition, potential methanotrophic epibionts related to the gammaproteobacterial type I methanotrophs were identified as a morphotype of thin short filaments by FISH analysi targeting 16S rRNA (Watsuji *et al.*, 2010). These findings point to the possible relevance of gammaproteobacterial type I methanotrophs in the productivity of epibiotic microbial communities, although convincing evidence has not yet been obtained. Recently, epibionts affiliated with type I methanotrophs have also been detected in the gill chamber of *R. exoculata* by FISH, but the methanotrophic productivity in the *R. exoculata* epibionts has not been verified (Guri *et al.*, 2012; Ponsard *et al.*, 2012). Thus, it is still unresolved whether methanotrophic productivity in the epibiotic microbial communities of deep-sea vent invertebrates is driven by the gammaproteobacterial type I methanotrophs or other candidates.

The Iheya North field represents a typical deep-sea hydrothermal system in the Okinawa Trough. Its hydrothermal fluid chemistry is highly influenced by the presence of thick terrigenous sediments in the trough basin and is characterized by high concentrations of CH_4 and CO_2 and a relatively low concentration of iron and molecular hydrogen (Kawagucci *et al.*, 2011). High-temperature vent emissions around enormous hydrothermal mineral deposits provide numerous diffusing hydrothermal fluid flows (Nakagawa *et al.*, 2005a) that support widespread dense colonies of *S. crosnieri* (Watsuji *et al.*, 2010). Among the Okinawa Trough hydrothermal systems examined so far, the Iheya North field has been shown to contain *S. crosnieri* populations with epibiotic microbial communities that display dual thiotrophic and methanotrophic productivity (Watsuji *et al.*, 2010, 2012). Here we characterized the phylogenetic affiliation of the functionally active methanotrophs present in the epibiotic community of *S. crosnieri* from the Iheya North field. To accomplish this, we examined transcripts of *pmoA*, which encodes a subunit of the particulate methane monooxygenase (pMMO) essential for aerobic methane-oxidizing metabolism and identified active epibiotic methanotrophs based on the deduced PmoA amino-acid sequences. We also estimated methanotrophic activity in the epibiotic community by quantifying the number of *pmoA* transcripts and by directly measuring methane consumption of *S. crosnieri* individuals under atmospheric and *in situ* hydrostatic pressure conditions. Moreover, we report that the newly introduced *in situ* RNA fixation method, which is performed immediately after sampling, effectively preserves epibiotic RNA assemblages, particularly labile mRNA populations.

Materials and methods

Collection of S. crosnieri from the deep-sea hydrothermal field

S. crosnieri individuals were collected from the Iheya North hydrothermal field in the Okinawa Trough, Japan, during dive no. 1447 on 25 October 2012 (27°47.44′N, 126°53.80′E, depth 1001 m) using the JAMSTEC remotely operated vehicle '*HyperDolphin*'. Individuals were collected using a suction sampler and stored in a box of chilled seawater attached to remotely operated vehicle. Immediately after onboard recovery, the individuals were washed with sterile artificial seawater. These procedures were performed below 5 °C because the ambient temperature of *S. crosnieri* in their natural habitats ranges from 4 to 6 °C. Immediately after recovery (within several hours), the methane oxidation activity of three living *S. crosnieri* individuals was measured under *in situ* hydrostatic (12.0 MPa) and atmospheric pressure (0.1 MPa) (described below).

Fixation

Several *S. crosnieri* individuals were collected using another suction sampler from the same colony as that during dive no. 1447 and stored in a 3.5-l sampling box. After collection at the seafloor, several individuals in the sampling box were immersed in an RNA Stabilization Reagent (RNAlater; Qiagen, Tokyo, Japan) colored in yellow of phenol red filling in the sampling box (*in situ* fixation). The system consisted of the sampling box, a flexible 6-l plastic bag (Sekisui Chemical, Osaka, Japan) containing 5 l of the fixation solution and a silicon tube (φ9 mm; Togawa, Fuchu, Japan) with a valve used to connect the bottom part of the sampling box and the plastic bag. The high-density fixation solution in the flexible bag was poured into the sampling box by lifting the flexible bag above the

sampling box using a manipulator. When all the seawater in the box containing the *S. crosnieri* individuals had been completely replaced by the fixation solution, the valve was closed and fixation continued. After onboard recovery, the setae were dissected from the fixed *S. crosnieri* individuals and gently resuspended in the same fixation solution (*in situ* fixation samples). In addition, the setae samples from *S. crosnieri* individuals collected using the normal suction sampler were dissected and gently suspended in the fixation solution (onboard fixation samples). After the suspensions were preserved at 5 °C overnight, the harvested setae were stored at −80 °C for further analyses. The setae that were not treated with the fixation solution were also dissected and stored at −80 °C as a reference (nonfixed samples). These setae samples were derived from *S. crosnieri* individuals with similar carapace lengths of 53–57 mm.

Total RNA preparation
Total RNA was extracted from the epibiont communities associated with the dissected setae (stored at −80 °C) using the RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA extract was treated with 0.5 U DNase I (Qiagen) for 10 min at room temperature to remove any contaminating DNA. The treated RNA was recovered using the RNeasy Mini Kit (Qiagen). The RNA quantity was determined using the Quant-iT RNA Assay Kit (Life Technologies, Tokyo, Japan).

Complementary DNA synthesis and PCR amplification
Complementary DNA (cDNA) was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Tokyo, Japan) according to the accompanying instruction manual. In total, 200 ng of the extracted total RNA was used as a template for reverse transcription, and the mixture was stored at −20 °C for subsequent PCR amplification. To verify the absence of DNA contamination of the RNA extracts, control reactions were prepared without reverse transcriptase. PCR amplifications were performed in 50-μl (total volume) volumes in 0.3-ml tubes. Each PCR mixture consisted of 1 × PCR buffer (Takara, Otsu, Japan), 200 mM of each dNTP (Takara), 400 nM each of forward and reverse primers, 0.05% (w v^{-1}) bovine serum albumin (Sigma-Aldrich, Tokyo, Japan), 2.5 U of *Taq* DNA polymerase (Takara) and 10 ng of cDNA from the epibiotic community fixed *in situ*. A partial fragment of *pmoA* was amplified with the primers A189f/mb661r (Costello and Lidstrom, 1999) and primers A189f/A682r (Holmes *et al.*, 1995). The amplification was performed with an initial denaturation step at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, at different annealing temperatures as required for 1 min and at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. The annealing temperature was 55 °C for primers A189f/mb661r (Dumont *et al.*, 2011) and 56 °C for primers A189f/A682r (Holmes *et al.*, 1995).

Quantitative PCR for pmoA mRNA and 16S rRNA
Quantitative PCR (qPCR) of *pmoA* was performed using the 7500 Real-Time PCR System (Applied Biosystems). All data analyses were performed with 500 System SDS software (version 1.4.0.25; Applied Biosystems). A partial fragment of *pmoA* was amplified with the primers A189f/mb661r. The reaction mixture contained 1 × Platinum SYBER Green SuperMix (Invitrogen, Tokyo, Japan), 1 × ROX, 400 nM of each primer, 0.05% (w v^{-1}) bovine serum albumin, 1 mM $MgCl_2$, 10 ng of cDNA and double-distilled H_2O to a final volume of 50 μl. Reactions for each sample were performed in triplicate. The plasmid DNA cloning a *pmoA* cDNA sequence ihePR661_206, which was amplified by primers A189f/mb661r, was used as a qPCR standard. The *pmoA* cDNA sequence cloned into the pCR2.1-TOPO vector (Invitrogen) was purified with a Plasmid Mini Kit (Qiagen). The recombinant plasmid DNA was quantified by Quant-iT dsDNA BR Assay Kit (Life Technologies). An external standard curve was generated with serial dilutions of the plasmid DNA ranging from 1.0×10^2 to 1.0×10^7 copy number of the *pmoA* cDNA/reaction. The amount of total RNA extracted from all setae of a *S. crosnieri* individual should be equivalent to that of total RNA of an epibiotic population of a *S. crosnieri* individual, although the amount will depend on extraction efficiency. Because total RNA of epibiotic communities was obtained by total RNA extraction from all setae of a *S. crosnieri* individual, quantification of the *pmoA* cDNA was normalized as the abundance of *pmoA* mRNA per epibiotic population of a *S. crosnieri* individual, which was estimated using the external standard curve and the amount of total RNA extracted from all setae of a *S. crosnieri* individual. The qPCR of *pmoA* was performed with an initial denaturation step at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Fluorescence data were acquired during the extension procedure. The qPCR of whole microbial 16S rRNA using the cDNA libraries was performed as described previously (Takai and Horikoshi, 2000). Abundance of 16S rRNA per epibiotic population of a *S. crosnieri* individual was also estimated using external standard curve and the amount of total RNA extracted from all setae of a *S. crosnieri* individual.

Sequencing and phylogenetic analysis
The RT-PCR-amplified products of *pmoA* mRNA were sequenced as described previously (Watsuji *et al.*, 2010). The sequences were determined on

both strands. The partial PmoA amino-acid sequences deduced from the cDNA sequences were compared first among all the PmoA amino-acid sequences obtained from the *S. crosnieri* epibiont cDNA libraries tentatively to identify the representative PmoA sequences (showing >97–99% amino-acid sequence similarity), and then were compared with the similar sequences in public databases by BLAST searches. The resulting sequences were manually aligned with similar sequences downloaded from the public databases, and the phylogenetic relationships were analyzed using ARB software package (Ludwig *et al.*, 2004). The aligned sequence length for the PmoA amino-acid sequences was 157 residues. Distance matrix trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), and the topology of the trees was evaluated by bootstrapping with 1000 resamplings (Felsenstein, 1985). Phylogenetic trees using the PmoA amino-acid sequences were also reconstructed by Bayesian method using the program MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The analysis was conducted as six chains for one million generations. Sample frequencies were 100 generations, and the burn-in was the first 2500 samples.

Methane oxidation activity of live S. crosnieri

Methane oxidation of epibionts associated with *S. crosnieri* individuals under *in situ* hydrostatic pressure was examined using a previously described high-pressure continuous-flow incubation apparatus (Watsuji *et al.*, 2012). This ensured a continuous supply of a certain concentration of dissolved methane and oxygen to a live *S. crosnieri* individual confined to a glass cylinder (Watsuji *et al.*, 2012) under a hydrostatic pressure of 12.0 MPa. On the other hand, the methane oxidation of *S. crosnieri* individuals under atmospheric hydrostatic pressure was examined using a continuous-flow apparatus that incubated a live *S. crosnieri* individual in the same glass incubation vessel but under a hydrostatic pressure of 0.1 MPa and with a continuous supply of similar concentrations of dissolved methane and oxygen. The continuous-flow apparatus comprised a peristaltic pump (MasterFlex L/S model 7524-50, pump head model 7518-10; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and glass incubation vessels (Horiguchi Ironworks Co., Kobe, Japan) directly connected to hydraulic tubes for transporting the liquid supplied by the peristaltic pump. All the hydraulic tubing used for transporting the liquid was manufactured using MasterFlex silicone tubing (Cole-Parmer Instrument Co.; size 25).

Three *S. crosnieri* individuals were used to measure methane oxidation under hydrostatic pressures of both 0.1 and 12.0 MPa, with the first measurement being made under 0.1 MPa and the next under 12.0 MPa. Each individual was incubated at 5 °C in a glass cylinder filled with artificial seawater (25 g l^{-1} NaCl, 4.2 g l^{-1} $MgCl_2 \cdot 6H_2O$, 3.4 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} KCl, 0.7 g l^{-1} $CaCl_2 \cdot 2H_2O$, 14 mg l^{-1} K_2HPO_4; adjusted to pH 6.8) filtered using a 0.22-μm-pore membrane under air. All the artificial seawater contained dissolved methane at a final concentration of approximately 20 μM. The dissolved methane was manufactured as follows: 2.5 ml of methane was added to 1 l of artificial seawater in a 1150-ml glass bottle (Duran, Berlin, Germany) sealed with a butyl rubber stopper, and this was incubated at 5 °C for 2 days to equilibrate the methane gas. In the experiment, the artificial seawater was supplied at a flow rate of 3 ml min^{-1} for 70 min at 5 °C under a hydrostatic pressure of 0.1 or 12.0 MPa. Pressurization to 12.0 MPa took 1–2 min. Thereafter, 5 ml of influent and effluent seawater was sampled by syringe at 5-min intervals during incubation. These samples were then injected into a 69-ml vacuum vial (V-50; Nichiden-Rika Glass Co., Kobe, Japan) with a butyl rubber stopper and frozen at −20 °C. At the onshore laboratory, the vacuum-extracted methane in the vial was assayed on a gas chromatograph (GC-4000; GL Science, Tokyo, Japan) with a pulsed discharge detector and a column packed with Molecular Sieve. The wet weight of the three specimens (specimens 1, 2 and 3) used for measuring methane oxidation was 12.5, 14.2 and 10.4 g, respectively. The dry weight of the setae from the three specimens (specimens 1, 2 and 3) was 73.4, 57.3 and 59.0 mg, respectively.

Results and discussion

There are two known forms of methane monooxygenases: soluble methane monooxygenase and pMMO. Soluble methane monooxygenase is found only in certain methanotrophs and typically expressed only under low-copper conditions (Murrell *et al.*, 2000). In contrast, pMMO is found in all known methanotrophs, except in species of the genus *Methylocella* (Dedysh *et al.*, 2000). Thus, *pmoA*, which encodes the transmembrane subunit of pMMO, is an established molecular marker of methanotrophic activity potential. In fact, the occurrence of *pmoA* has been identified in the epibiotic community of *R. exoculata* (Zbinden *et al.*, 2008). FISH analysis targeting 16S rRNA also detected cells phylogenetically related to type I methanotrophs in the epibiotic communities associated with *S. crosnieri* and *R. exoculata* (Watsuji *et al.*, 2010; Guri *et al.*, 2012), although sulfur- and iron-oxidizing autotrophic productivity was suggested by ^{13}C-tracer assimilation experiments in *R. exoculata* epibionts (Ponsard *et al.*, 2012). The detection of functional genes in DNA assemblages isolated from an epibiotic community does not necessarily indicate that the encoded function is operative because DNA can be stable in dormant and dead cell populations (Lindahl, 1993). Furthermore, the detection of 16S rRNA gene sequences closely

related to gammaproteobacterial type I methanotrophs and the 16S rRNA-targeting FISH analyses do not ensure the presence of methanotrophic activity. An adequate level of certainty requires the combined use of nanoscale secondary ion mass spectrometry or stable isotope probing to specify the cellular level of uptake of labeled CH_4 (Hery *et al*., 2008; Watsuji *et al*., 2012).

In this study, uncloned cDNA libraries were constructed from total RNA extracted from epibiotic communities associated with three *S. crosnieri* individuals fixed *in situ*. Because none of the PCR products derived from *pmoA* transcripts was obtained from cDNA libraries prepared without reverse transcriptase, it was confirmed that the amplified *pmoA* genes do not originate from contaminating genomic DNA. A total of 138 *pmoA* cDNA clones amplified using two primer sets were sequenced, and 18 representative PmoA amino-acid sequences were identified (Table 1). This report describes the first detection of *pmoA* transcripts in RNA assemblages extracted from the epibiotic community associated with a deep-sea vent invertebrate, *S. crosnieri*, by RT-PCR. The RT-PCR amplification of *pmoA* transcripts is more tightly coupled with methane-oxidizing metabolism in functionally active methanotrophic populations owing to the susceptibility of RNA to degradation in inactive cells. This along with results from previous studies strengthens the presumption that there is a significant population of functionally active methanotrophs in these epibiotic communities.

Table 1 Composition of representative reverse-transcribed *pmoA* clones in the epibiotic community associated with *S. crosnieri*

Group represent ID	*INSD accession no.*	*Number of clones*		*PmoA sequence identity (%) among each other*
		A189/ mb661r	*A189/ A682r*	
Type Ia				
ihePR682_243	AB794881	3	4	98–100
ihePR682_350	AB794882	34	28	97–100
ihePR661_145	AB794867	1	1	99
ihePR682_216	AB794870	0	1	—
ihePR682_157	AB794879	0	1	—
ihePR661_313	AB794873	2	1	99–100
ihePR682_112	AB794874	2	8	99–100
ihePR661_111	AB794866	15	6	99–100
ihePR661_218	AB794871	1	0	—
ihePR682_135	AB794876	6	6	97–100
ihePR682_364	AB794883	3	1	99–100
ihePR661_206	AB794868	4	1	99–100
ihePR661_215	AB794869	1	0	—
ihePR661_216	AB794870	1	0	—
ihePR661_222	AB794872	1	0	—
Type Ib				
ihePR682_134	AB794875	0	4	100
ihePR682_147	AB794878	0	1	—
ihePR682_136	AB794877	0	1	—

Abbreviation: INSD, International Nucleotide Sequence Databases.

Next, the representative sequences were subjected to phylogenetic analysis (Table 1). It has been pointed out that the phylogenetic relationship of PmoA amino-acid sequences deduced from *pmoA* sequences is congruent with the phylogeny based on 16S rRNA gene sequences (Heyer *et al*., 2002; Kolb *et al*., 2003). It has also been accepted that the characterization of *pmoA* transcripts can be an important molecular tool for assessing the diversity of active methanotrophic communities in various habitats (Chen *et al*., 2008). All the PmoA amino-acid sequences identified in the RNA assemblages from epibiotic communities were classified as gammaproteobacterial type I methanotrophs. They were further divided into type Ia and type Ib subgroups of methanotrophs in the phylogenetic trees using neighbor-joining and Bayesian methods (Figure 1 and Supplementary Information). The PmoA amino-acid sequences identified as type Ia and Ib methanotrophs, using the neighbor-joining method, showed similar patterns using the Bayesian method (Figure 1 and Supplementary Information). Therefore, the active epibiotic methanotrophs were composed mainly of type Ia and Ib methanotrophs, although a larger number of sequencing analyses may reveal a greater diversity of PmoA sequences, and therefore active methanotrophs, in the epibiotic communities of *S. crosnieri*. In addition, *S. crosnieri* has been nutritionally supported by methanotrophic production from methanotrophs associated with the host animal, and the host-associated methanotrophs have been regarded as active epibiotic methanotrophs contributing to methanotrophic productivity, because ^{13}C-methane tracer experiment using live *S. crosnieri* revealed that the labeled methane was incorporated into host *S. crosnieri* as well as its epibionts (Watsuji *et al*., 2010). Thus, the results of our previous and present studies suggested that the active type Ia and Ib methanotrophs in the epibiotic community could have a significant role in the nutrition of *S. crosnieri*, although a potential contribution of other methanotrophs was not completely excluded (Watsuji *et al*., 2010).

Most of the epibiotic PmoA sequences affiliated with the type Ia methanotrophic PmoA, except for ihePR661_222 and ihePR682_216, shared >93% sequence similarity with the PmoA sequence of the *Methylococcaceae* bacterium isolate SF-BR (Figure 1). It has been pointed out that similarity of 93% in PmoA amino-acid sequence corresponds to that of 97% in 16S rRNA gene sequence (Degelmann *et al*., 2010). The microbiological characteristics of the methanotrophic strain SF-BR are completely unknown and only the *pmoA* and 16S rRNA gene sequences have been deposited in public databases. In our previous study, phylogenetic analysis of 16S rRNA gene sequences obtained from DNA extracts of *S. crosnieri* epibiotic communities revealed that

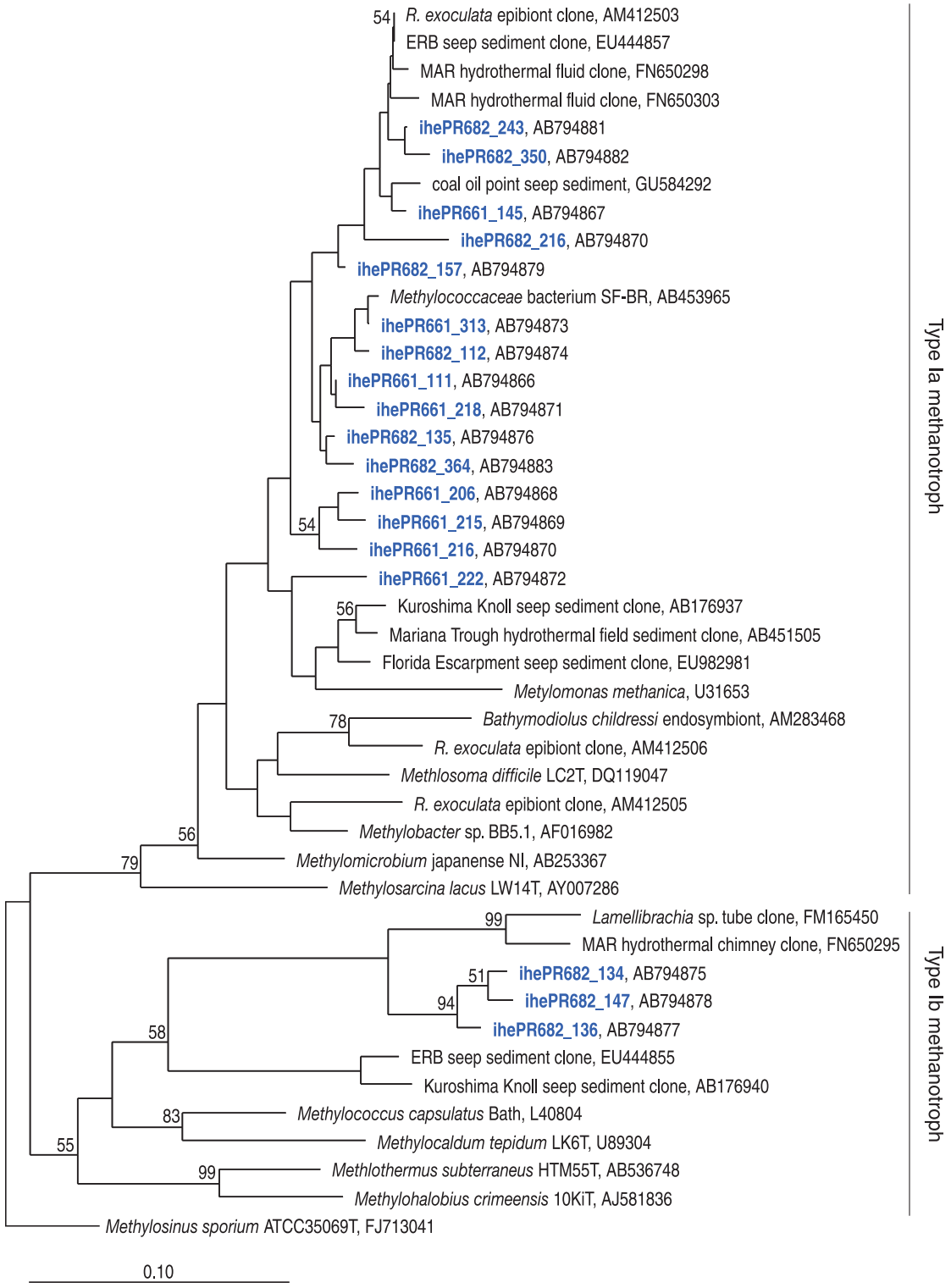

Figure 1 Phylogenetic tree based on the partial amino-acid sequences of the pMMO subunit A, deduced from the *pmoA* transcript sequences. The tree was constructed by neighbor-joining analysis using 157 amino-acid residues. Sequences obtained from the epibiotic community on *S. crosnieri* are shown in colored letters. Bootstrap analysis was performed with 1000 resampled data sets. Bootstrap values >50% are shown at branch points. Scale bar indicates 0.1 substitutions per site.

only two 16S rRNA gene sequences (defined with a >97% similarity threshold in 16S rRNA gene sequence) were related to the gammaproteobacterial type Ia methanotrophs and that the one sequence, IHE0_1191 (accession no. AB476240), was the most abundant ribotype in this group (Watsuji *et al.*, 2010). This 16S rRNA gene sequence also had 97% similarity with the 16S rRNA gene sequence of the

strain SF-BR (Watsuji *et al.*, 2010). Because both the abundant 16S rRNA gene (e.g., IHE0_1191) (Watsuji *et al.*, 2010) and the *pmoA* transcript sequences in the epibiotic community are closely related to the 16S rRNA and *pmoA* gene sequences of the strain SF-BR, these sequences could be derived from genetically similar epibionts. Previous microscopic FISH analysis targeting type Ia methanotrophic 16S rRNA, including sequence IHE0_1191, indicated the abundance of potentially viable type Ia methanotrophic cells in the epibiotic community (Watsuji *et al.*, 2010). RT-PCR amplification of *pmoA* mRNA probably confirmed the presence of active epibiotic type Ia methanotrophs in this study. These results strongly suggest that the methanotrophic productivity of the *S. crosnieri* epibiotic community is, in a large part, sustained by the methane oxidation of type Ia methanotrophs closely related to strain SF-BR. To validate the substantial contribution of the type Ia methanotrophs closely related to strain SF-BR to methanotrophic productivity, FISH analyses specifically targeting both their 16S rRNA and *pmoA* mRNA will be conducted for the *S. crosnieri* epibiotic community, similar to previous experiments on the role of methanotrophic endosymbionts in the gills of vent mussels (Wendeberg *et al.*, 2012). In addition, the substantial contribution to methanotrophic productivity made by potentially abundant methanotrophic populations will be further substantiated by a combination of FISH analysis targeting the 16S rRNA sequences and incorporation of ^{13}C-labeled methane by nanoscale secondary ion mass spectrometry or ^{14}C-labeled methane using microscopic autoradiography.

The epibiotic PmoA sequences classified as type Ib methanotrophic PmoA were not closely related to any of the previously known PmoA sequences but were phylogenetically associated with sequences from the tube-adhesive microbial community of the tubeworm *Lamellibrachia* sp. (90–91% sequence similarity) (Figure 1). In our previous study, transmission electron microscopic observation of the epibiotic community on an *S. crosnieri* individual from the Iheya North field revealed the presence of two different morphotypes (short filaments and rods) of cells. These morphotypes represent an evident intracytoplasmic membrane structure (Watsuji *et al.*, 2010) known as a typical morphological characteristic of type I methanotrophs (Jensen and Corpe, 1991; Heyer *et al.*, 2005). Microscopic FISH analysis specific to the 16S rRNA of the type Ia methanotrophs only revealed a short filament morphotype (Watsuji *et al.*, 2010). It seems likely that the epibiotic type Ib methanotrophs identified through the sequencing of *pmoA* transcripts may be rod-shaped morphotypes with an intracytoplasmic membrane structure, although FISH analysis specific to the *pmoA* mRNA of the type Ib methanotroph will be needed to determine the morphology of the epibiotic type Ib methanotrophic cells.

The abundance of *pmoA* transcripts in the total RNA assemblages retrieved from the epibiotic communities of three *S. crosnieri* individuals fixed *in situ* was estimated by RT-qPCR using the primer set A189f/mb661r. We also compared the abundance of *pmoA* mRNA and 16S rRNA in the epibiotic communities of three *S. crosnieri* individuals collected using different sampling procedures (*in situ* fixation, onboard fixation and nonfixed samples). In each of the fixation procedures, the epibiont RNA assemblages from three host individuals were used for the quantification of the abundance of *pmoA* mRNA and 16S rRNA. RT-qPCR quantification was performed in triplicate for each epibiont RNA assemblage from each individual. The standard errors of the RT-qPCR quantifications were estimated to be within 4.1% and 7.7% of the average amounts of *pmoA* mRNA and 16S rRNA, respectively. The mean and standard deviation values obtained from the epibiont RNA assemblages from three host individuals are shown in Figure 2a.

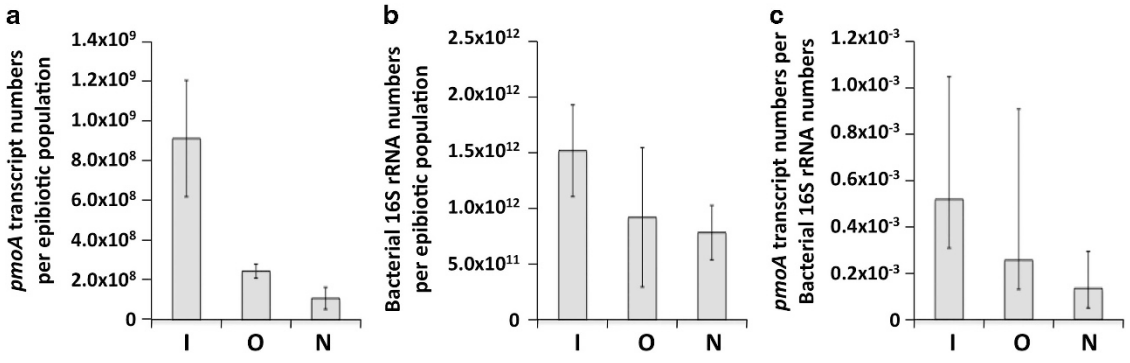

Figure 2 The number of *pmoA* transcripts and 16S rRNAs quantified by RT-qPCR using RNA extracted from the epibiotic communities on *S. crosnieri* individuals by different sampling procedures. (**a**) The abundance of *pmoA* transcripts per epibiotic population of a *S. crosnieri* individual. (**b**) The abundance of bacterial 16S rRNA per epibiotic population of a *S. crosnieri* individual. (**c**) The abundance (copy number) ratio of *pmoA* transcripts and 16S rRNA in the epibiotic population of a *S. crosnieri* individual. I indicates the *in situ* fixation samples, O indicates the onboard fixation samples and N indicates the nonfixed samples. Three different *S. crosnieri* individuals were assessed for each process, and the data are expressed as the mean ± s.d.

The most abundant *pmoA* mRNA was obtained from the epibiotic communities on *S. crosnieri* individuals fixed *in situ*. On average, 9.5×10^8 copies per epibiotic population of a *S. crosnieri* individual were estimated by the abundance of *pmoA* mRNA in total RNA extracted from all setae of a *S. crosnieri* individual (Figure 2a). The amounts of *pmoA* mRNA obtained from the epibiotic communities using onboard fixation and no fixation were 2.6×10^8 and 1.1×10^8 copies per epibiotic population of a *S. crosnieri* individual, respectively (Figure 2a). Thus, the relative abundance of *pmoA* transcripts in the epibiotic community fixed *in situ* was 3.7- and 8.6-fold higher than that of the community sampled by onboard fixation and no fixation, respectively, and these differences were statistically significant (unpaired *t*-test, $P<0.05$) (Figure 2a). In general, fixation timing after sampling of a planktonic microbial community in the *in situ* habitat is critical for determining the abundance and pattern of *in situ* cellular gene expression (Frias-Lopez *et al.*, 2008). The relatively greater abundance of *pmoA* mRNA in the epibiotic community fixed *in situ* clearly indicates the effective stabilization of labile mRNA in the epibiotic cells by the addition of the fixative immediately after sampling. Using the remotely operated vehicle, the recovery and sampling of *S. crosnieri* individuals from their deep-sea colonies in the Iheya North field (the water depth is approximately 1000 m) required at least 2 h. In addition, the recovery from the deep-sea colonies and the transfer to the onboard laboratory was accompanied by a drastic change in the physical and chemical conditions of the seawater in the sampling box for the *S. crosnieri* individuals as well as their epibionts. The drastic environmental changes could be inhi bitory and even lethal and lead to the degradation of labile mRNA, including the *pmoA* transcripts in the epibiont cells. Indeed, it has been demonstrated that the abundance of a subunit of ammonia monooxygenase gene (*amoA*) transcripts in the planktonic microbial community of the central Baltic Sea (70–120 m depth) fixed *in situ* was 6- to 30-fold higher than that in the planktonic microbial community that was not treated with a fixative (Feike *et al.*, 2012).

We also estimated the abundance of whole bacterial 16S rRNA in the epibiotic community by RT-qPCR analysis. As observed with the *pmoA* mRNA, the most abundant 16S rRNA was obtained from the epibiotic community on *S. crosnieri* individuals fixed *in situ* (Figure 2b). In the case of 16S rRNA, the difference in abundance among the various fixation procedures was not statistically significant (unpaired *t*-test, $P>0.05$). However, the abundance (copy number) ratio of *pmoA* mRNA and whole bacterial 16S rRNA in the epibiotic population of a *S. crosnieri* individual was significantly influenced by the presence or absence of the RNA fixation procedure and the timing of fixation after the sampling (Figure 2c). The ratios were 5.4×10^{-4} in the *in situ* fixation samples, 2.7×10^{-4} in the samples fixed onboard and 1.4×10^{-4} in the non-fixed samples, respectively (Figure 2c). These results strongly suggest that the *pmoA* mRNA in the epibiotic community degrades faster than the whole bacterial 16S rRNA. It has been reported that the 16S rRNA abundance of planktonic microbial communities is not significantly influenced by different fixation processes (Deutscher, 2006; Feike *et al.*, 2012). Thus, our quantitative data with epibiotic *pmoA* mRNA and whole bacterial 16S rRNA may result from differences in degradation susceptibility between mRNA (for methanotroph *pmoA*) and rRNA (for the whole epibiotic bacteria 16S rRNA). Otherwise, some specific bacterial populations, in this case gammaproteobacterial type I methanotrophs, may be more sensitive to the environmental changes during sampling and recovery, and thus both the *pmoA* mRNA and 16S rRNA of type I methanotrophs may degrade faster than those of other predominant epibiotic populations. These possible explanations will be clarified by future quantitative metatranscriptomic analysis (a number of sequence analysis) of RNA assemblages, including whole bacterial rRNAs and mRNAs obtained from the epibiotic communities of three *S. crosnieri* individuals under the different fixation procedures. Nevertheless, our quantitative data clearly demonstrate the necessity of using the *in situ* fixation procedure for subsequent transcriptomic analysis of epibiotic communities associated with deep-sea vent invertebrates in their natural environments. In addition, epibiotic metatranscriptomics will become more important to understand actual functional activities because functional gene analyses of epibiotic communities associated with *R. exoculata*, *A. pompejana* and *Kiwa hirsuta* have already been performed (Goffredi *et al.*, 2008; Grzymski *et al.*, 2008; Hugler *et al.*, 2011).

Methane oxidation activity was estimated using the same three *S. crosnieri* individuals under both atmospheric and elevated hydrostatic pressures via a continuous-flow incubation of dissolved methane and oxygen (Table 2). We confirmed the viability of the *S. crosnieri* individuals throughout the atmospheric and *in situ* pressure incubation experiments by visual observation of their movement and behaviors. A relative decrease in the dissolved methane concentration of the effluent seawater compared with that of the influent seawater was also detected in the negative control experiments where no *S. crosnieri* individual was present. This was probably due to the diffusion of methane gas through the silicone tube and the formation of methane gas bubbles in the flow lines (Table 2). However, much greater methane consumption rates were obtained from the experiments with a living *S. crosnieri* individual (Table 2). The net methane consumption rates were quite similar between the experiments under atmospheric and *in situ*

Table 2 Net methane consumption rates of live *S. crosnieri* individuals at hydrostatic pressures of 0.1 and 12.0 MPa during continuous-flow incubation

Specimen no.	*Pressure (MPa)*	*Methane consumption rate (μmol h^{-1})*		*Net methane consumption rate*		
		With individual[a]	*Without individual*[a]	*(μmol h^{-1} per individual)*	*(μmol h^{-1} g^{-1} of wet individual)*	*(μmol h^{-1} g^{-1} of setae)*
1	0.1	3.12 ± 0.10	1.01	2.11 ± 0.10	0.17 ± 0.008	28.7 ± 1.4
	12.0	2.24 ± 0.16	0.22	2.02 ± 0.16	0.16 ± 0.013	27.5 ± 2.2
2	0.1	2.46 ± 0.13	1.01	1.45 ± 0.13	0.10 ± 0.009	25.3 ± 2.3
	12.0	1.86 ± 0.01	0.22	1.64 ± 0.01	0.12 ± 0.001	28.6 ± 0.2
3	0.1	2.64 ± 0.36	1.01	1.63 ± 0.36	0.16 ± 0.035	27.6 ± 6.1
	12.0	1.84 ± 0.09	0.22	1.62 ± 0.09	0.16 ± 0.009	27.5 ± 1.5

[a]All methane consumption rates were measured at 5-min intervals during incubation and determined using steady-state data. They are expressed as the mean ± s.d.

hydrostatic pressure (Table 2), and no statistically significant differences between the independent experiments were observed, although these used three different specimens (unpaired *t*-test, $P > 0.05$).

It has been suggested that *S. crosnieri* ingest the epibionts because of frequently observed behaviors *in situ* and when reared in the laboratory. *S. crosnieri* comb out setae that are densely covered with epibionts using their third maxillipeds and bring the maxilliped to their mouths (Watsuji *et al.*, 2010). As feeding of epibionts could affect the activity measurements, the potential feeding behaviors were visually observed during the experiments. The behaviors did barely occur during the incubation time of 70 min. In addition, even if the epibionts were frequently ingested by the potential feeding behaviors, the extant biomass of the epibiotic community was much larger than the amount ingested by the host *S. crosnieri*, and the reproduction rate of the epibiotic community was probably higher than the ingestion rate of the host *S. crosnieri*. Thus, the potential feeding behaviors of the host individuals seem to have little effect on the metabolic activity of the epibionts during the experiments, such as the sulfur oxidation activity in the previous study (Watsuji *et al.*, 2012) and even the methane oxidation activity in this study.

The methane oxidation activities of epibiotic methanotrophic populations under atmospheric pressure have been previously measured using the shell surface of a limpet (*Lepetodrilus fucensis*) and the tube of a tubeworm (*Ridgeia* sp.) from deep-sea hydrothermal vent fields (de Angelis *et al.*, 1991b). The methane oxidation rate of the *S. crosnieri* individuals studied here was more than 30 000 times higher than that of the epibionts on these animal tissues (0.0551 nmol h^{-1} per *L. fucensis* shell and 0.0033 nmol h^{-1} per tube of *Ridgeia* sp.). The much greater methane oxidation potential of *S. crosnieri* is likely consistent with the nutritional significance of epibiotic methanotrophic productivity to the host *S. crosnieri* individuals, as suggested previously (Watsuji *et al.*, 2010). The methane oxidation activity of a cold seep mussel (*Bathymodiolus* sp.) that contains a gammaproteobacterial type I methanotroph as an endosymbiont has been measured at a hydrostatic pressure of 0.54 MPa (Kochevar *et al.*, 1992). The methane oxidation rates per unit (1 g wet-individual weight) of *S. crosnieri* individuals were two- to sixfold lower than the rates per unit (1 g shell-free wet-individual weight) of *Bathymodiolus* sp. individuals at the same dissolved methane concentration of 20 μM, and at similar temperatures (5 °C in this study and 6 °C in the previous study) (Kochevar *et al.*, 1992). The comparison may simply reveal that the average methane oxidation rate of a *S. crosnieri* individual is lower than that of a cold seep *Bathymodiolus* sp. and its endosymbionts. However, the methane oxidation rates of cold seep *Bathymodiolus* sp. individuals are standardized by the shell-free wet weights, and the cold seep *Bathymodiolus* sp. has only the single phylogenetic group of methanotroph as the endosymbiont, as opposed to the heterologous phylogenetic and metabolic compositions of the *S. crosnieri* epibionts (Kochevar *et al.*, 1992). Thus, the comparison may not simply be interpreted as a result of different methane oxidation rates per individual between *S. crosnieri* and the cold seep *Bathymodiolus* sp. Rather, it may be an important finding that the methane oxidation rates per individual between the *S. crosnieri* and the cold seep *Bathymodiolus* sp. are within the same order of magnitude.

The methane oxidation rates of the three different individuals were not statistically significantly different when the experiments were performed under *in situ* hydrostatic and atmospheric pressure. Indeed, the net methane consumption rates per an amount of setae were very similar (Table 2), and the differences among the six values were not statistically significant (unpaired *t*-test, $P > 0.05$). These results indicate that the methane oxidation activity of *S. crosnieri* individuals is dependent on the

amount of setae, that is, the biomass of the epibiotic community and that the epibiotic methane-oxidizing function is not significantly affected by hydrostatic pressure. Using the planktonic microbial communities in the hydrothermal plume and the ambient deep-sea waters at the Endeavor segment field of the Juan de Fuca Ridge, de Angelis *et al.* (1991a) demonstrated that $^{14}CH_4$ incorporation into microbial cells was elevated under *in situ* hydrostatic pressure (21–62%) compared with that under atmospheric pressure. Because this study estimated $^{14}CH_4$ assimilation into cellular compounds but not methane oxidation (consumption) in the planktonic microbial communities (de Angelis *et al.*, 1991a), the results are not directly comparable with the effect of hydrostatic pressure on the methane oxidation activity of the *S. crosnieri* epibiotic methanotrophs. In addition, the planktonic microbial communities were collected from a water depth of approximately 2200 m (de Angelis *et al.*, 1991a), whereas the *S. crosnieri* individuals and their epibionts are distributed at a water depth of 1000 m. Both the samples were also decompressed once during sample recovery. Thus, it remains unclear whether hydrostatic pressure substantially affects *in situ* methanotrophic producti vity and methane oxidation by deep-sea vent microbial populations, and *in situ* labeled-tracer experiments or temperature- and pressure-preserved sampling and onboard measurement tools are necessary to better estimate these parameters. Nevertheless, our measurement of the methane oxidation activity provides the first rough estimation of the methane oxidation potential of *S. crosnieri* individuals and their epibiotic communities and provides insight into the biogeochemical and ecological significance of the dominant *S. crosnieri* populations in the large-scale carbon cycle of the methane-dominant Okinawa Trough deep-sea hydrothermal systems.

The previous study clearly showed that the epibiotic community of *S. crosnieri* in the Iheya North field exhibited methanotrophic and thioautotrophic production (Watsuji *et al.*, 2010). The ^{13}C-labeled bicarbonate and methane incorporation into the epibiotic cellular compounds in the presence of thiosulfate and methane as the energy sources, respectively, suggested that the carbon fixation was approximately 1.9-fold greater via methane (methanotrophy) than bicarbonate (thioautotrophy) (Watsuji *et al.*, 2010). This result seems to indicate that methanotrophic productivity in the epibiotic community dominates thioautotrophic productivity. However, as the incorporation experiments were conducted under aerobic conditions for a relatively longer time (48 h) (Watsuji *et al.*, 2012), the thiotrophic productivity of the O_2-sensitive filamentous *Sulfurovum* members (Nakagawa *et al.*, 2005b; Macalady *et al.*, 2008), as the predominant sulfur-oxidizing epibiont population, would be significantly inhibited by exposure to O_2. On the other hand, the aerobic oxidation activity of reduced sulfur compounds of *S. crosnieri* individuals from the Iheya North field were estimated to be 550–580 μmol sulfide $h^{-1} g^{-1}$ dry weight of setae under both *in situ* and atmospheric pressures using the same continuous-flow incubation method used in this study (Watsuji *et al.*, 2012). In this study, the aerobic methane oxidation activity of *S. crosnieri* individuals from the Iheya North field was determined to be 28–29 μmol methane $h^{-1} g^{-1}$ dry weight of setae under both *in situ* and atmospheric pressures. These results seem to indicate that the sulfur oxidation activity of the epibiotic community potentially outcompetes the methane oxidation activity as the preferred mode of energy metabolism. However, these activity measurements may be conducted under different kinetic conditions of the metabolic functions (such as different substrate concentrations). It has been reported that the methane oxidation rate of cold seep *Bathymodiolus* sp. endosymbionts was highly variable with varying CH_4 concentrations (up to 300 μM)(Kochevar *et al.*, 1992), and the sulfide oxidation rate of tubeworm *Riftia pachyptila* endosymbionts under high hydrostatic pressure was also affected by increasing sulfide concentrations (up to 600 μM) (Girguis and Childress, 2006). Therefore, the concentration ranges of dissolved reduced sulfur compounds and CH_4 in *in situ* colonies are quite important to estimate properly the *in situ* metabolic potentials for CH_4 and sulfur oxidation in the *S. crosnieri* populations with their epibionts, although it is very difficult to determine the highly fluctuating concentrations of these substrates in the widespread *S. crosnieri* colonies of the Iheya North field. In the previous studies, it has been indicated that long, thick filamentous *Sulfurovum* epibionts on *S. crosnieri* were thioautotrophs and that long, thin filamentous gamma-proteobacterial epibionts on *S. crosnieri* were potential thioautotrophs (Watsuji *et al.*, 2010, 2012). This study suggests that short, thin filamentous morphotype of epibionts would be active type Ia methanotroph (Figure 1). It was already demonstrated by the FISH analysis targeting whole *Epsilonproteobacteria*, *Gammaproteobacteria* and specific type I methanotrophic *Gammaproteobacteria* that the cell abundances of long filamentous *Sulfurovum* and gamma-proteobacterial epibionts were significantly greater than the cell abundance of short filamentous potentially methanotrophic epibionts (Watsuji *et al.*, 2010). Thus, based on the comparison of extant biomass abundances, it seems likely that the thioautotrophic productivity dominates the methanotrophic productivity in epibiotic community associated with *S. crosnieri*. As *S. crosnieri* is one of the predominant chemosynthetic macrofaunal populations in the Okinawa Trough deep-sea hydrothermal systems, the *in situ* metabolic rates and kinetic properties of thioautotrophy and methanotrophy of episymbiosis in the *S. crosnieri* populations will be key clues to

understanding the biogeochemical cycles of carbon, nitrogen and sulfur associated with the hydrothermal activity. The metatranscriptomic quantification of various key energy and carbon metabolisms using *in situ*-fixed specimens shown in this study and reported by Sanders *et al.,* (2013) will be one possible approach (Sanders *et al.*, 2013). In addition, *in situ* continuous-flow or batch incubation experiments to estimate metabolic rates and kinetic properties will be featured in our future investigations.

Conflict of Interest

The authors declare no conflict of interest.

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