Diversity and Function of Epibiotic Microbial Communities on the Galatheid Crab, *Shinkaia crosnieri*

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The galatheid crab, *Shinkaia crosnieri* (Decapoda: Galatheidae), forms dense colonies in the Iheya North and Hatoma Knoll deep-sea hydrothermal fields and has numerous setae covered with filamentous epibiotic microorganisms. Molecular phylogenetic analyses revealed that the epibiotic communities in *S. crosnieri* consisted mainly of yet-uncultivated phylotypes within *Epsilonproteobacteria* and *Gammaproteobacteria* in both hydrothermal vent fields. Uptake experiments using 13C-labeled tracers clearly demonstrated that both H13CO3− and 13CH4 were assimilated into not only the epibiotic microbial communities associated with the setae, but also the epibiont-free tissue of living *S. crosnieri*. In addition, the incorporation of H13CO3− into the microbial cells was strongly stimulated by the presence of reduced sulfur compounds but not by H2. In conclusion, the uptake experiments suggested that sulfur-oxidizing chemolithoautotrophic and methanotrophic production by the epibionts provides the nutrition for *S. crosnieri*.

**Key words:** epibiont, carbon assimilation, methanotrophs, sulfur-oxidizing bacteria, nutritional ectosymbiosis

Many species of invertebrates dwelling in deep-sea hydrothermal vents and cold seeps are known to host bacteria in cells (endosymbionts) of specialized tissues and on the surface (epibionts) of specialized tissues. Although it has been clearly established that endosymbionts nutritionally support the growth of a variety of host invertebrates (8, 9, 26), the role of the epysymbiotic community in the host–symbiont interaction has not been evident. Previous studies disclosed that the dorsal epidermal expansions of *Alvinella pompejana* and the gill chamber of *Rimicaris exoculata* are predominantly covered by filamentous epibionts affiliated with the genus *Sulfurovum* within *Epsilonproteobacteria* (4, 27). Moreover, a metagenomic analysis of the epibiotic community in *A. pompejana* revealed the presence of genes involved in the complete reductive tricarboxylic acid (rTCA) cycle and oxidation of sulfur, suggesting the dominant epsilonproteobacterial epibionts to be capable of chemolithoautotrophic growth (12). Additionally, it has been suggested that the epibiotic communities nutritionally sustain *A. pompejana* and *R. exoculata* based on the stable carbon isotopic composition of host invertebrates’ tissues and the genetic characterization of epibiotic microbial communities (3, 6, 28). Nonetheless, neither the primary production nor the nutritional contribution by the epibionts in these hosts was clarified by tracer experiments using 14C-bicarbonate (1, 28). Rather, the abundant assimilation of 14C-labeled inorganic carbon in *R. exoculata* was found in the gut, probably involving the gut microbial communities (28).

The galatheid crab, *Shinkaia crosnieri*, is widely distributed in deep-sea hydrothermal vents in the Okinawa Trough, Japan (31). The most outstanding feature of hydrothermal systems in the Okinawa Trough is the extraordinary high concentrations of CO2 and CH4 in hydrothermal fluids (19, 23). *S. crosnieri* is one of the most dominant animal species colonizing habitats close to high-temperature hydrothermal emissions (31). In this study, since the microbial community associated with *S. crosnieri* was observed, we attempted to investigate the diversity and function of the epibiotic microbial communities on the setae of *S. crosnieri* living adjacent to gaseous carbon-enriched, hydrothermal vents.

**Materials and Methods**

*Collection of S. crosnieri from deep-sea vents*

Individuals of *S. crosnieri* were collected from two geographically distinct deep-sea hydrothermal fields: Hatoma Knoll (24°51.50’N, 123°50.49’E) and Iheya North field (27°47.46’N, 126°53.80’E) in the Okinawa Trough, Japan, by a manned submersible, ‘Shinkai 6500’, and a remotely operated vehicle (ROV), ‘HyperDolphin’ of JAMSTEC. The individuals collected, which were still alive after the rapid pressure and temperature change during their recovery, were immediately moved to shipboard tanks containing surface seawater at 4°C.

*Fixation of abdominal setae*

Immediately after the sampling of individuals of *S. crosnieri* from the deep sea, abdominal setae of the crabs with epibionts were fixed with 4% paraformaldehyde (for in situ hybridization) or 2.5% glutaraldehyde (for SEM) in artificial sea water (ASW, pH 7.4) at 4°C. The ASW did not contain ammonium chloride or sodium nitrate. After fixation overnight, the abdominal setae were carefully washed by decantation five times with ASW. The setae...
fixed with paraformaldehyde were resuspended with 50% (v/v) ethanol in ASW and stored at −20°C, while the setae fixed with glutaraldehyde were resuspended with ASW and stored at 4°C.

Nucleic acid extraction and 16S rRNA gene amplification

Genomic DNA of epibionts was extracted from the whole setae of *S. crosnieri* frozen at −80°C immediately after sampling from the sea. For the DNA extraction, an UltraClean soil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used following the manufacturer’s instructions. Bacterial and archaeal 16S rRNA genes were amplified from the extracted DNA by PCR using the oligonucleotide primers Bac27F and Uni1492R (20), or Arch21F and Uni1492R (5), respectively. A 20-μl volume of the PCR mixture containing 2.5-GC buffer I (Takara Bio, Otsu, Japan), 0.2 mM of each deoxynucleoside triphosphate, 0.4 μM of each primer, 2.5 U of *La Taq* polymerase (Takara Bio), and 50 ng of DNA, was prepared. The amplification was performed in a thermal cycler GeneAmp 9700 (PE Applied Biosystems, Carlsbad, CA, USA) with the following program for a total of 25 cycles: 96°C for 25 s, 53°C for 45 s, and 72°C for 75 s. The PCR products from several aliquots were purified by using the GelSpin DNA purification kit (Mo Bio Laboratories), and then cloned into the vector pCR2.1 with a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The insert 16S rRNA genes were directly amplified with M13 primer and treated with exonuclease I and shrimp alkaline phosphatase (GE Healthcare, Buckinghamshire, UK). The preparations were directly sequenced using a Big Dye Terminator Ver 3.1 cycle sequencing kit (PE Applied Biosystems) and ABI PRISM 3130xl sequencing analyzer (PE Applied Biosystems). The primer Bac27F was used for the initial single-strand sequencing of bacterial 16S rRNA gene sequences. Sequence similarity among all of the single-strand-read 16S rRNA gene sequences approximately 0.5 kb long was analyzed by the Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequences showing ≥97% identity on DNA sequencing were assigned to the same clone type (phyotype). A representative clone of each phyotype was further subjected to sequencing and an approximately 1.4 kb fragment of the 16S rRNA gene was determined from both strands. The phyotype sequences were subjected to a similarity search against the DDBJ/EMBL/GenBank databases with the FASTA3, BLAST, and Smith-Waterman Search programs. The sequences were subsequently imported into the ARB software. Bootstrap analyses for 1,000 replicates were performed to assign confidence levels to the tree topology.

Whole-cell FISH analysis

The setae cut from the hosts were fixed with 4% paraformaldehyde in seawater overnight at 4°C and then stored with 50% ethanol in seawater at −30°C until the FISH analysis. The fixated samples were spotted onto Teflon-coated slides and air-dried before dehydration by sequential washes in 50, 80, and 100% (v/v) ethanol for 3 hours at 4°C until the FISH analysis. The fixed samples were washed with distilled water and stained with 1% (v/v) aqueous uranyl acetate for 2 hours at 4°C. After rinsing with distilled water, the samples were dehydrated in a graded ethanol series, and embedded in Epon 812 resin (TAAB Laboratories Equipment, Berkshire, UK). Thin sections were cut with an ultramicrotome (Reichert Ultratome S; LEICA, Vienna, Austria). The thin sections were stained in 2% (v/v) uranyl acetate and lead citrate, and observed with TEM (JEM-1210; JOEL, Tokyo, Japan) at an acceleration voltage of 100 kV. For field emission-scanning electron microscopy (SEM), setae were fixed with 2.5% (v/v) glutaraldehyde in seawater overnight at 4°C. The samples were then washed in filtered seawater and post-fixed with 2% (v/v) OsO₄ in filtered seawater for 2 hours at 4°C. After setae were rinsed with distilled water, the conductive staining was performed by incubating 1% (v/v) aqueous tannic acid (pH 6.8) for 60 min. The samples were then washed with distilled water and treated with 1% (v/v) aqueous OsO₄ for 60 min. The setae were dehydrated in a graded ethanol series and critical point dried (JCPD-5; JOEL). The samples were coated with osmium by an osmium plasma coater (POC-3; Meiwafosis, Osaka, Japan) and observed by FE-SEM (JSM-6700F; JOEL) at an acceleration voltage of 5 kV.

Tracer experiments

Two living individuals (n = 1 for each condition) from the Iheya North field and the mixed setae cut from three individuals from the Hatoma Knoll immediately after capture were used for tracer experiments. The live specimens were incubated for 48 hours at 5°C in 2.45-L sealed tanks (GasPak system; BD, Maryland, USA) containing 1 L of artificial seawater (25 g L⁻¹ NaCl, 4.2 g L⁻¹ MgCl·6H₂O, 3.4 g L⁻¹ MgSO₄·7H₂O, 0.5 g L⁻¹ KCl, 0.7 g L⁻¹ CaCl₂·2H₂O, 35 mg L⁻¹ K₂HPO₄, 2.1 mg L⁻¹ NH₄Cl and 6.8 mg L⁻¹ NaNO₃; adjusted to pH 7.3) filtered with a 0.22-μm membrane. Moreover, the autotrophic substrate, 1 mmol NaH¹³CO₃ (99%¹³C), in the presence of 300 μmol sodium thiosulfate, or the methanotrophic substrate, 2 mmol NaH¹³CO₃ (99%¹³C), was added to the sealed tank. The mixed setae from three individuals were incubated for 48 hours at 5°C in 295-L sealed bottles containing 200 mL of the same artificial seawater. In this case, 200 μmol NaH¹³CO₃ in the presence or absence of each of 20 μmol sodium sulfide, 60 μmol sodium thiosulfate and 400 μmol molecular hydrogen, or 400 μmol NaH¹³CH₄ was included in the sealed bottle. All stable isotope-labeled chemicals were purchased from Cambridge Isotope Laboratories, Andover, MA, USA.

Stable carbon isotopic analyses of ¹³C-labeled and unlabeled *S. crosnieri* 1H DNA from two living individuals and the mixed setae from the ¹³C-labeled tracer experiments, and *S. crosnieri* individuals (n = 1 for each field) captured in both fields, were washed in artificial seawater without carbonates, frozen, and stored at −80°C. Not only the setae but also the walking leg muscle samples from the *S. crosnieri* were harvested, lyophilized, and ground into powder in separate microtubes. A total of thirteen microtubes were prepared as one sample for each type of analysis (Table 1). The opened microtubes were then placed for six hours in a desiccator containing
Table 1. Analysis of $^{13}$C-labeled and non-labeled $S$. crosnieri

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>incubation time (hours)</th>
<th>tracer and additional energy source</th>
<th>tissue</th>
<th>$\delta^{13}$C (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iheya North field</td>
<td>48</td>
<td>$^{13}$CO$_2$+thiosulfate</td>
<td>setae</td>
<td>1636±137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{13}$CH$_4$</td>
<td>leg muscle</td>
<td>−8.1±0.69</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>$^{13}$CO$_2$+mixed setae</td>
<td>setae</td>
<td>4185±84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>leg muscle</td>
<td>−12.1±1.8</td>
</tr>
<tr>
<td>Hatoma Knoll</td>
<td>48</td>
<td>$^{13}$CO$_2$</td>
<td>mixed setae</td>
<td>697±30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{13}$CO$_2$+sulfide</td>
<td>mixed setae</td>
<td>1247±8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{13}$CO$_2$+thiosulfate</td>
<td>mixed setae</td>
<td>1985±52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{13}$CO$_2$+hydrogen</td>
<td>mixed setae</td>
<td>663±51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{13}$CH$_4$</td>
<td>mixed setae</td>
<td>147±7</td>
</tr>
</tbody>
</table>

Mixed setae were pooled from three individuals and others were obtained from one individual. One sample for each type of analysis was measured in triplicate. Values are the mean±SD.

$\delta^{13}$C value of $S$. crosnieri colony water

Simultaneously with the sampling of $S$. crosnieri individuals from the deep-sea hydrothermal vent colonies in both fields, the seawater surrounding the colonies was collected using a gas-tight fluid sampler, “WHATS-II” (Water Hydrothermal-fluid Atsuryoku Tight Sampler II) (29), while the $in$ $situ$ temperature of the hydrothermal effluent was measured with a self-recording thermometer on the WHATS-II sampler. The colony-water was sampled into the WHATS-II gas-tight bottle (150 mL in volume). The bottle was processed on board using a high vacuum line within a few hours after the recovery. The extraction of dissolved gas components was based on Konno et al. (19). The extracted gases were stored in 50-mL stainless steel bottles. The stable carbon isotope composition of CH$_4$ and CO$_2$ in the extracted gas was analyzed by using a Mat-Finnigan 252 mass spectrometer coupled to a gas chromatograph (GC-IRMS system) as previously described (32). The analytical errors in these analyses were <0.1‰ and <0.4‰ for $\delta^{13}$C(CO$_2$) and $\delta^{13}$C(CH$_4$) in the GC-IRMS, respectively.

Results

Anatomical and microscopic observations

The $S$. crosnieri individuals possessed numerous flexible setae in the ventral aspect of the carapace, sternite, chelipeds and two pairs of anterior walking legs (Fig. 1). A series of successive images from the SEM revealed the colonization by filamentous microorganisms of the setae (Fig. 2A and B). The setae and associated epibiotic microbial communities of $S$. crosnieri from the Hatoma Knoll and Iheya North field showed similar morphological features. Most of the epibiotic microorganisms were filamentous, and could be classified into three morphotypes by diameter: 0.4–0.6 μm, 1.2–1.8 μm and 3.4–5.5 μm. However, the cross-sectional TEM revealed the presence of short filaments as well as rods that contained intracytoplasmic membranes only in the setae of $S$. crosnieri from the Iheya North field (Fig. 2C and D). Because the intracytoplasmic membrane is a morphological signature of type I gamma-proteobacterial methanotrophs (15, 17), the TEM observation suggested that methanotrophic epibionts might be present on the setae of $S$. crosnieri from the Iheya North field.

Phylogenetic analysis of bacterial 16S rRNA gene clone sequences

The setae were collected from several specimens from the Hatoma Knoll and Iheya North field to conduct the microbial community analysis using PCR amplification of the 16S rRNA gene. Although no archaeal 16S rRNA gene product was amplified from any of the setae, it was successfully amplified from the bacteria. The epibiotic bacterial 16S rRNA gene clones retrieved from the specimens of Hatoma Knoll and Iheya North field were referred to as HAT0 and IHE0, respectively. The numbers of clones sequenced and
Fig. 3. Phylogenetic tree of epibiotic phylotypes affiliated with the genus *Sulfurovum* within Epsilonproteobacteria and Marine Epibiotic Group I and methanotrophic group within Gammaproteobacteria obtained from setae. Colored HAT0 and IHE0 indicate the epibiotic bacterial 16S rRNA gene clones retrieved from the specimens of Hatoma Knoll and Iheya North, respectively. The tree was constructed by neighbor-joining analysis. Black bold indicates 16S rRNA gene clones obtained from setae. A bootstrap analysis was performed with 1,000 resampled data sets. Bootstrap values of >50% are shown at branch points. The bar indicates 0.1 change per nucleotide.

Fig. 4. Fluorescence microscopy of bacteria on central shaft of setae. Fluorescence microscopy is performed at the same position of seta(e) from Iheya North in the top (A, B, and C) and (D and E) panels, respectively. DAPI microscopy indicates total bacteria on setae (A). FISH with EP653 indicates specifically *Sulfurovum*-affiliated epibionts within Epsilonproteobacteria on setae (C). Light microscopy indicates total bacteria on setae (D and F). Image F expands the square in image D. FISH with MEG2 indicates specifically epibiotic type I methanotrophs on setae (E). The capital S in images indicates a seta. Scale bars: A, B and C = 50 μm, D and E = 10 μm, F = 2.5 μm.
specimen(s) analyzed were respectively \( n = 231 \) and \( 1 \) for HAT0, and \( n = 440 \) and \( 7 \) for IHE0.

The epibiotic bacterial 16S rRNA gene clone analysis revealed that the epibiotic phytype composition of the \( S. \) crosnieri individuals in two deep-sea hydrothermal fields was similar to each other. Most of the epibiont sequences were classified into two bacterial subdivisions, Epsilonproteobacteria and Gamma-proteobacteria, in both fields. All the epsilon-proteobacterial phylotypes formed a phylogenetic cluster related to members of the genus Sulfurovum (Fig. 3). The representative phylotype HAT0_536, which was found in the epibiotic 16S rRNA gene clone libraries from both fields, was closely related to the epibiont clone obtained from the setae of the hydrothermal vent crab \( Kiwa hirsuta \) (96% similarity) (11). Similar sequences for the phylotype HAT0_536 were retrieved from the epibiont clone libraries of the gastropod scaly snail (95% similarity) (10), the shrimp \( Rimoncaris exoculata \) (94% similarity) (27) and the polychaete annelid \( Alvinella pompejana \) (92% similarity) (14). In addition, the phylotype was related to the 16S rRNA gene sequence of \textit{Sulfurovum lithothrophicum} (93% similarity).

Gamma-proteobacterial phylotypes in the \( S. \) crosnieri setae were classified into a greater number of phylogenetic groups than epsilon-proteobacterial entities (Fig. 3 and Table S1). But many of the phylotypes belonged to a specific group (Marine Epibiotic Group I) consisting of many epibiont sequences retrieved from hydrothermal vent invertebrates (Fig. 3). The closest cultivated relative of the Marine Epibiotic Group I was \textit{Leucothrix mucor} (21), a filamentous marine heterotrophic bacterium known as an epibiont of benthic crustacea, fish eggs and algae (2, 18).

Among phylotypes affiliated with Gamma-proteobacteria, another important group was those closely related to type I methanotrophs, which were found in the epibiotic microbial communities from both hydrothermal fields (Fig. 3). The representative phylotype IHE0_1191 exhibited similarities with \textit{Methyllobacter marinus} (94% similarity) (13) and with a methanotrophic endosymbiont clone obtained from the hydrothermal vent mussel \textit{Bathymodiolus azoricus} (94% similarity) (7).

\textbf{Fluorescence in situ hybridization}

The fluorescenc \textit{in situ} hybridization (FISH) analysis of the epibiotic microbial communities revealed that the epsilon- and the gamma-proteobacterial cells were numerically abundant on the \( S. \) crosnieri setae from both fields (Fig. 4A, B and C). Both proteobacterial cells were filamentous whereas the epsilon-proteobacterial filaments were the thickest and longest in the epibiotic microbial communities on the setae (Fig. 4A, B and C). The components related to the epibiotic type I methanotrophs consisted of a part of the epibiotic community from the Iheya North field (Fig. 4D and E). The epibionts corresponding to FISH signals of potential methanotrophs formed dense colonies of depth and had thin and short filaments (Fig. 4D, E and F). On the contrary, in the setae from Hatoma Knoll, a clear FISH signal of epibiotic type I methanotrophs was not detected (data not shown). The FISH results indicated that the potential methanotrophs on the setae from the Iheya North field were more abundant than those from the Hatoma Knoll. No signal was detected in the epibiotic community hybridized with the antisense probe NON338 (data not shown).

\textbf{Stable isotope}

When the setae and walking leg muscle samples from \( S. \) crosnieri were harvested, lyophilized, and ground to decide the \( \delta ^{13} \)C composition, the setae themselves were not ground into a powder. However, because the epibiotic microbial cells on the setae were easily ground into a powder, powdered epibiotic cells were used to analyze the \( \delta ^{13} \)C composition of the cut setae. Using stable carbon isotope tracers with or without chemolithotrophic energy substrates, the capability of epibiotic microbial communities to assimilate inorganic carbons was investigated (Table 1). The \( ^{13} \)C-labeled bicarbonate in the presence of thiosulfate was incorporated into the epibionts (1636‰) and the leg muscle (−8.1‰) in the living \( S. \) crosnieri individuals from the Iheya North field (Table 1). Additionally, \( ^{13} \)C-labeled methane was also assimilated into both epibionts (4185‰) and leg muscle (−12.1‰) of the Iheya North individual (Table 1). Moreover, using the cut setae of the \( S. \) crosnieri from the Hatoma Knoll, the assimilation of \( ^{13} \)C-labeled carbons was investigated (Table 1). The incorporation of \( ^{1} \)H\textsubscript{3}CO\textsuperscript{−} into the epibiotic microbial community was enhanced with the addition of potentially thioautotrophic substrates such as sulfide and thiosulfate but not with the addition of molecular hydrogen (Table 1). The \( ^{13} \)C-labeled methane was assimilated into the epibiotic community from the Hatoma Knoll (−26.3‰ to 147‰), although making a comparison of mean values between the setae from a single individual and the mixed setae cut from three individuals requires statistical treatment. Furthermore, the enrichment (147‰) was much lower than that in the living individual from Iheya North (4185‰), although it is not easy to compare the mean values between the setae of the living \( S. \) crosnieri and the cut setae because there may also be some positive effects of the \( S. \) crosnieri on the epibionts; for example, \( S. \) crosnieri might enhance local water current around the epibionts, which could increase the availability of substrates for carbon assimilation (Table 1).

\textbf{Table 2. \( \delta ^{13} \)C values of \( \Sigma \)CO\textsubscript{2} and methane in the \( S. \) crosnieri colony-waters}

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>( \delta ^{13} )C (( \Sigma )CO\textsubscript{2}) (permil)</th>
<th>( \delta ^{13} )C (CH\textsubscript{4}) (permil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iheya North field</td>
<td>−3.6</td>
<td>−0.0</td>
</tr>
<tr>
<td>Hatoma Knoll</td>
<td>−3.6</td>
<td>−51.7</td>
</tr>
</tbody>
</table>

In this study, we obtained clear evidence that the epibiotic...
communities of *S. crosnieri* assimilated \(^{13}\)C-labeled bicarbonate (Table 1). These findings demonstrated for the first time that labeled bicarbonate was assimilated into the epibiotic microbial communities on the specialized tissues of the deep-sea invertebrates. Moreover, the incorporation of \(^{13}\)HCO\(_3^−\) into the epibiotic microbial communities was stimulated by the addition of sulfide and thiosulfate, but not by molecular hydrogen (Table 1), strongly suggesting that the epibiotic community of *S. crosnieri* would drive primary production by potential thiotrophic (sulfur-oxidizing) energy metabolism. The 16S rRNA gene clone analysis of the epibiotic communities indicated that many phylotypes were phylogenetically associated with the typical epibionts previously found in the microbial communities of the deep-sea invertebrates (Fig. 3). One of the most predominant epibiotic components from both fields was the *Sulfurovum*-affiliated epibionts within *Epsilonproteobacteria*, representing a thick filamentous morphotype (4, 11, 27). All the *Sulfurovum* strains isolated so far are capable of chemolithoautotrophic growth on a variety of redox couples such as hydrogen oxidation (coupled with elemental sulfur, oxygen or nitrate reduction) and thiosulfate oxidation (coupled with oxygen or nitrate reduction) (16, 23, 24). Because the epibiotic microbial community, including the *Sulfurovum* strains, intensely assimilated \(^{13}\)C-labeled bicarbonate in the presence of sulfide and thiosulfate, it was strongly suggested that at least *Sulfurovum*-affiliated epibionts in the microbial community would primarily drive thiotrophic production based on reduced sulfur compounds (Table 1). But incorporation of \(^{13}\)C-labeled bicarbonate into the epibiotic community was not stimulated by hydrogen (Table 1). This result suggests that the epibiotic microbial community may not include hydrogenotrophic components. Otherwise, since the hydrogenase activity in *Sulfurovum* sp. NBC37-1 was not detected when no molecular hydrogen (H\(_2\)) was fed to the culture (34), the absence of hydrogenotrophic production in the epibiotic community may be caused by a lower level of H\(_2\) for the induction of hydrogenotrophic production in the epibiotic microbial community. In addition, even without the addition of any energy source, the epibiotic communities showed inorganic carbon assimilation (Table 1). The H\(^{13}\)CO\(_3^−\) assimilation was probably sustained by the existing energy metabolism using the reduced sulfur-compounds that might be preserved inside and/or outside the epibiotic microbial cells on the setae.

We detected active methanotrophic production in the epibiotic communities of *S. crosnieri* (Table 1). The results indicated the existence of methanotrophs in epibiotic microbial communities on the setae from both fields. We also found potential gamma-proteobacterial type I methanotrophs in the epibiotic microbial community from the Iheya North field in the 16S rRNA gene clone library and FISH analysis (Fig. 3 and 4E). In addition, TEM observations of *S. crosnieri* setae from Iheya North revealed that thin and short filaments as shown in the FISH analysis formed an intracytoplasmic membrane structure (Fig. 2C and 4F). Therefore these findings strongly suggested that the potential methanotrophs found in the epibiotic microbial community from the Iheya North field were true methanotrophs. Recently, the existence of methanotrophic bacteria in the epibiotic microbial community has been reported in the deep-sea hydrothermal vent shrimp, *R. exoculata* (35). However, the conclusion was based only on analyses of TEM images and 16S rRNA gene sequences (35). Thus, the methanotrophs in the *S. crosnieri* setae are the first episymbiotic methanotrophs in marine invertebrates whose functions have been experimentally verified.

It is quite interesting that the natural stable carbon isotopic compositions of the *S. crosnieri* epibionts and host body (leg muscles) were quite different between the specimens from distinct hydrothermal fields (−35−37‰ for the Iheya North population and −26−29‰ for the Hatoma Knoll population) (Table 1). The different isotopic compositions of the epibionts and the host bodies were likely associated with the incorporation of different carbon sources for the *S. crosnieri* populations between the fields. Probably a highly \(^{13}\)C-depleted carbon source, namely CH\(_4\), would be more incorporated into the epibiotic microbial communities of the *S. crosnieri* populations in the Iheya North field than in the Hatoma Knoll field (Table 2). This result is consistent with a higher abundance of potential epibiotic methanotrophs and a greater contribution of methanotrophic activity to the epibiotic primary production of the *S. crosnieri* populations in the Iheya North field than in the Hatoma Knoll field (Table 1, S1 and Fig. 4). These results strongly suggest that the phylogenetic and metabolic diversity in epibiotic microbial communities would be variable between the habitats in different hydrothermal environments.

In this study, the most significant finding was the incorporation of H\(^{13}\)CO\(_3^−\) and \(^{13}\)CH\(_4\) into the *S. crosnieri* body as well as the epibiotic microbial communities (Table 1). The results indicated the potential nutritional transportation from the organic carbons produced by the *S*-oxidizing chemolithoautotrophic and methanotrophic bacteria associated with *S. crosnieri* to the host body as already clarified in the endosymbioses of invertebrates endemic to deep-sea hydrothermal vents (8, 9, 26). As the tracer experiments were carried out using thoroughly washed *S. crosnieri* individuals and sterile artificial seawater, the potential nutritional source would be provided by the epibionts on *S. crosnieri* that actually incorporated H\(^{13}\)CO\(_3^−\) and \(^{13}\)CH\(_4\). In addition, the natural stable isotope compositions of the epibiotic community and tissue of *S. crosnieri* individuals were similar to each other in each of the hydrothermal fields (Table 1). The result could also support that the epibiotic microbial populations were the potential nutritional source for *S. crosnieri*, although it was not completely excluded that *S. crosnieri* obtained nutrition from microbial communities other than the setae epibionts. Accordingly, it is not yet completely clarified whether the isotopically heavy inorganic carbon is transferred into the organic carbon of the animal body only via the setae epibiotic microbial communities or via other *S. crosnieri*-associated microbial communities such as the microbial community in the gut as previously suggested in *R. exoculata* (28). In fact, anatomical investigation clearly indicated that *S. crosnieri* had a gut (data not shown). However, a series of interesting behaviors by the *S. crosnieri* individuals have been very often observed in deep-sea habitats and in onboard rearing tanks (Supplementary Movie 1). The *S. crosnieri* individuals comb out the setae using their max-
illipped and then bring the maxilliped to their mouth. Thus we now hypothesize that the S. crosnieri in the Okinawa Trough may directly take in epibiotic microbial populations and digest them in their digestive organs. It will be important in future investigation to prove this hypothesis by in situ detection.

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References


