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ORIGINAL ARTICLE

Molecular evidence of digestion and absorption of epibiotic bacterial community by deep-sea crab *Shinkaia crosnieri*

Tomo-o Watsuji[1], Asami Yamamoto[1,2], Kaori Motoki[1,2], Kenji Ueda[2], Emi Hada[1], Yoshihiro Takaki[1], Shinsuke Kawagucci[1] and Ken Takai[1]

[1]*Department of Subsurface Geobiological Analysis and Research (D-SUGAR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Kanagawa, Japan and* [2]*Life Science Research Center, College of Bioresource Sciences, Nihon University, Fujisawa, Japan*

The hydrothermal vent crab *Shinkaia crosnieri* is considered to obtain nutrition from the epibiotic bacteria found on the setae, but previous studies have not shown how nutrients can be transferred from the epibionts to the host. In this study, microscopic observations of *S. crosnieri* intestinal components detected autofluorescent setae fragments and pigmentation derived from the digestion of epibionts in a dye-stained epibiont tracer experiment. An *in vitro* digestion experiment with epibiotic populations using an intestinal extract demonstrated the degradation of epibiotic cells by digestive enzymes. A phylogenetic analysis showed that many of the bacterial 16S ribosomal RNA gene sequences obtained from the intestine were closely related to the sequences of the epibionts, thus they were probably derived from the epibionts. A stable isotope tracer experiment also indicated that ^{13}C assimilated by the epibionts provided a carbon (nutrition) source for the host. Both activity measurements and isotope studies showed that chemosynthetic metabolism by the gut microbial components were inactive. Together with the feeding behaviour of living *S. crosnieri*, these results indicate that *S. crosnieri* ingests the epibionts using maxillipeds and assimilates them via its digestive organs as a nutrient source. The results of this study elucidate the mechanism of nutritional transfer in ectosymbiosis between chemosynthetic bacteria and deep-sea invertebrates.
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Introduction

Since the first discovery of a dense, unique and diverse ecosystem in deep-sea hydrothermal vents in 1977 (Corliss *et al.*, 1979), many studies have shown that deep-sea vent invertebrates such as tubeworms and *Bathymodiolus* mussels are sustained nutritionally by intracellular symbiotic bacteria (endosymbionts) as primary producers (Cavanaugh *et al.*, 1981, 1987; Felbeck, 1981; Childress *et al.*, 1986). In addition, several species of deep-sea vent invertebrates harbour bacteria (epibionts) that colonize the surfaces 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 *K. 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 are believed to have chemolithoautotrophic and methanotrophic products that support their hosts nutritionally, as well as the endosymbionts themselves (Goffredi *et al.*, 2008; Grzymski *et al.*, 2008; Watsuji *et al.*, 2010; Thurber *et al.*, 2011; Ponsard *et al.*, 2012). Radioisotope-labelled tracer experiments suggest that *R. exoculata* epibionts achieve chemolithoautotrophic production by oxidizing reduced sulphur compounds and ferrous iron (Ponsard *et al.*, 2012). A combination of ^{13}C tracer experiments, microscopic fluorescence *in situ* hybridization and nano-scale secondary ion mass spectrometry also indicate that the predominant *Sulfurovum*-affiliated epibiotic population engages in thioautotrophic productivity in the epibiotic community associated with *S. crosnieri* (Watsuji *et al.*, 2012). In addition, a combination of ^{13}C tracer experiments and transcriptomic analysis showed that gammaproteobacterial type Ia and Ib methanotrophs in the *S. crosnieri* epibiotic community also support primary production using CH_4 as energy and carbon sources (Watsuji *et al.*, 2010, 2012).

Correspondence: T-o Watsuji, Department of Subsurface Geobiological Analysis and Research (D-SUGAR), JAMSTEC, 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan.
E-mail: watsuji@jamstec.go.jp
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Although the community composition, productivity and metabolic activity of the epibionts associated with deep-sea vent invertebrates have been relatively well characterized, the nutritional relationship between the epibionts and their hosts requires further elucidation. Radioisotope-labelled tracer experiments using living *R. exoculata* demonstrated that labelled bicarbonate and organic compounds (^{14}C acetate and ^{3}H lysine) were incorporated into *R. exoculata* tissues as well as the epibiotic community (Ponsard *et al.*, 2012). In addition, these experiments demonstrated that the shrimps incorporated the labelled organic compounds across their integuments (Ponsard *et al.*, 2012). These results suggest that the products assimilated by the epibionts were transported nutritionally to the host body of *R. exoculata,* possibly via non-gastric tissues, such as the gill chamber integument, although there is no evidence of the transport of organic compounds released from the epibionts (Ponsard *et al.*, 2012). On the other hand, *R. exoculata* is known to possess a gut microbial community in its intestinal tract (Zbinden and Cambon-Bonavita, 2003; Durand *et al.*, 2009) and appreciable incorporation of labelled bicarbonate into the intestine containing the gut microbial community has been confirmed (Polz *et al.*, 1998). Thus, it has been suggested that primary production by the gut microbial community also contributes to nutrition in this shrimp.

^{13}C-labelled tracer experiments using living *S. crosnieri* individuals showed that ^{13}C-labelled bicarbonate and methane were incorporated not only into the epibiotic community but also into the muscle of *S. crosnieri* (Watsuji *et al.*, 2010). In addition, it has been reported that living *S. crosnieri* individuals frequently exhibit feeding-like behaviour (Watsuji *et al.*, 2010). For example, they comb the setae covered with dense epibionts using their third maxilliped and then bring the maxilliped to their mouth. Therefore, it has been predicted that nutrient acquisition by the host *S. crosnieri* is achieved by digesting chemosynthetic (thioautotrophic and methanotrophic) epibionts. However, no clear evidence has been obtained to support this hypothesis. Furthermore, the intestinal tract of *S. crosnieri* hosts a potential gut microbial community in addition to the epibiotic microbial community on its setae (Watsuji *et al.*, 2010). Therefore, it is unclear whether nutritional transfer to the host *S. crosnieri* is predominantly mediated by feeding on and digesting the epibionts, or via the gut-endemic autotrophic and methanotrophic microbial populations (Watsuji *et al.*, 2010).

Among the previously examined Okinawa Trough hydrothermal systems, the Iheya North field hosts dominant *S. crosnieri* populations with epibiotic microbial communities that exhibit dual thiotrophic and methanotrophic productivity patterns (Watsuji *et al.*, 2010, 2012). In this study, we used *S. crosnieri* from the Iheya North field to perform microscopic observations and phylogenetic analysis of the gut microbial assemblages, as well as *in vitro* digestion experiments, thereby verifying whether the epibiotic bacterial population was ingested and digested by *S. crosnieri*. The autotrophic productivity and sulphide- and methane-oxidizing metabolisms of the dissected intestines were evaluated to determine whether sufficient chemolithotrophic activity and productivity could be achieved only by the potential gut microbial community. ^{13}C-labelled tracer experiments using living *S. crosnieri* individuals were performed to determine whether *S. crosnieri* obtains nutrition from its epibionts.

Materials and methods

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

S. crosnieri individuals were collected from the Iheya North hydrothermal field in the Okinawa Trough, Japan, during dive #1335 on 19 March 2012 (27° 47.46′N, 126° 53.81′E, depth 986 m) and dive #1619 on 29 January 2014 (27° 47.45′N, 126° 53.80′E, depth 990 m) using the JAMSTEC remotely operated vehicle (ROV) '*HyperDolphin*'. Individuals were collected from the deep-sea vent habitats using a suction sampler and stored in a confined box filled with chilled seawater in the ROV. The *S. crosnieri* individuals obtained from dive #1619 were used in sulphide and methane consumption experiments, 16S ribosomal RNA (rRNA) gene sequence analysis of the gut microbial components and epibiotic community, *in vitro* digestion experiments with epibionts and intestinal extract activity measurements. The individuals from dive #1335 were used in the other experiments described below.

Fluorescence and light microscopic examination of the intestines and setae

The intestines and setae of *S. crosnieri* were observed by light and fluorescence microscopy (BX53; Olympus, Tokyo, Japan). The full-length intestines and setae fragments were dissected from 10 and several individuals, respectively, of *S. crosnieri* stored at −80 °C. The intestines and setae fragments were placed on glass slides (76 × 26 mm; Matsunami Glass, Osaka, Japan) and gently pressed with oblong cover glasses (24 × 45 mm; Matsunami Glass). The preparations of the intestinal components and setae were examined by optical and fluorescence light microscopy (BX53; Olympus). The fluorescence was monitored at 515–550 nm with excitation at 460–490 nm.

Dye-stained epibiont tracer experiment

A dye-stained epibiont tracer experiment was conducted in the onboard laboratory. The epibionts on the setae of two living *S. crosnieri* individuals were stained with crystal violet (Wako, Tokyo, Japan), as follows. First, moisture was removed from the wet setae with paper towels. Artificial seawater

containing 0.4% (final concentration) crystal violet was infiltrated into the setae using a pipette. The artificial seawater was prepared as described previously (Watsuji *et al.*, 2012). The individuals were laid on their backs on crushed ice for 5 min. Excess artificial seawater containing the dye was removed with paper towels before two dye-labelled and two non-labelled *S. crosnieri* individuals were maintained simultaneously for 24 h at 5 °C in a 54-l tank containing 40 l of artificial seawater. The seawater in the tank was filtered continuously using a canister filter (MEGA power 9012; GEX Co., Osaka, Japan) filled with 2 kg of activated charcoal (Wako) at a flow rate of 6.6 l min^{-1} to eliminate free dye from the dye-labelled *S. crosnieri* individuals. After incubation for 24 h, the whole intestines were removed from the dye-labelled and non-labelled *S. crosnieri* individuals, and then placed on glass slides (76 × 26 mm; Matsunami Glass) before gently pressing them with oblong cover glasses (24 × 45 mm; Matsunami Glass). The preparations were observed under an optical stereo zoom microscope (YDZ-3F; Yashima Optical Co., Tokyo, Japan) on board the ship.

Preparation of intestinal extracts
Full-length intestines were removed from two individuals (carapace lengths = 57 and 62 mm), which had been preserved at −80 °C, and then homogenized in 500 µl of 40 mM Tris-HCl (pH 7.5) containing 160 mM KCl. The homogenates were centrifuged at 7000 × *g* for 10 min at 4 °C. The supernatants were used as crude extracts for the enzyme assays and *in vitro* digestion experiments with epibiotic cells. The protein contents were determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

In vitro digestion of epibionts using intestinal extracts
Portions of *S. crosnieri* setae with epibionts were removed from an individual (carapace length = 62 mm), which had been stored at −80 °C, and incubated directly for 3 days at 5 °C in microtubes containing 100 µl of the crude intestinal extract (6.4 mg protein ml^{-1}) with 0.05% (w/v) sodium azide, or 100 µl of buffer solution containing 40 mM Tris-HCl (pH 7.5), 160 mM KCl and 0.05% sodium azide. The morphological changes in the epibiotic microbial populations on the setae samples were observed before and after incubation using a light microscope (BX53; Olympus).

In addition, portions of setae of *S. crosnieri* with epibionts were removed from an individual (carapace length = 62 mm), which had been stored at −80 °C, and brushed with a paintbrush and suspended in a buffer that comprised 40 mM Tris-HCl (pH 7.5), 160 mM KCl and 0.05% (w/v) sodium azide. This epibiont cell suspension was also used in the *in vitro* digestion experiments with the crude intestinal extract as the substrate. The reaction mixture contained 900 µl of the cell suspension and 100 µl of the crude extracts (6.0 mg protein ml^{-1}). Following incubation for 3 days at 5 °C, the reaction mixture was centrifuged at 7000 × *g* for 10 min at 4 °C. Another sample was prepared in exactly the same manner, but it was centrifuged immediately without incubation. The pellets were used for DNA content quantification and the phylogenetic analysis. The DNA extraction procedure and the analysis of the bacterial 16S rRNA gene phylotype compositions of the pellets before and after incubation were performed as described in the Supplementary Information. The DNA concentrations were determined using a Quant-iT double-stranded DNA high-sensitivity assay kit and a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA).

Measurement of digestive enzyme activity levels in intestinal extracts
The activity levels of digestive enzymes in the intestinal extracts were measured as described in the Supplementary Information.

Measurement of methane and sulphide oxidation
The intestine and setae of one *S. crosnieri* individual (carapace length = 43 mm) and the intestines of two other *S. crosnieri* individuals (carapace lengths = 44 mm) were used to measure the methane-oxidizing activity in the onboard laboratory. The full-length intestines and whole setae were removed from living *S. crosnieri* individuals and homogenized using a Dounce-type homogenizer (glass–teflon) in 5 ml of ice-cold artificial seawater. Each of the intestinal homogenates from three individuals and from the whole setae of one individual was added to 95 ml of artificial seawater with dissolved methane in a 120-ml glass bottle, which was sealed with a butyl rubber stopper. The methane-containing artificial seawater was prepared by adding 1 ml of methane to the bottle and incubating at 5 °C for 2 days to dissolve the methane completely. The bottles with the methane-containing artificial seawater and the homogenate were sealed with butyl rubber stoppers and pressurized to 0.2 MPa with N_2 after adding another 1 ml of methane to the headspace gas. Although the bottles were incubated at 5 °C, 0.5 ml of the gas phase was subsampled using a syringe at specific time intervals. These subsamples were injected into a 7-ml vial (V-5A; Nichiden-Rika Glass Co., Kobe, Japan) with a butyl rubber stopper and frozen at −20 °C. The methane concentration was assayed as described previously in the onshore laboratory (Watsuji *et al.*, 2014).

Similarly, the intestine and setae from one *S. crosnieri* individual (carapace length = 36 mm) and the intestines from two *S. crosnieri* individuals (carapace length = 42 and 47 mm) were used to measure the sulphide-oxidizing activity. The full-length intestines and half of the setae assemblage

were removed from the living *S. crosnieri* individuals and homogenized as described above. Sulphide oxidation was measured using the homogenates according to the previously described batch method (Watsuji *et al.*, 2012).

Nucleic acid extraction and bacterial 16S rRNA gene clone analysis
The nucleic acid extraction procedure and the bacterial 16S rRNA gene clone analysis of the epibiotic microbial community and the cells in the *in vitro* digestion experiment and the gut microbial assemblage were conducted as described in the Supplementary Information.

^{13}C-bicarbonate tracer experiments and stable carbon isotope analysis
In the onboard laboratory, a living *S. crosnieri* individual (carapace length = 49 mm) and the full-length intestine removed from an individual (carapace length = 49 mm) were used immediately in the ^{13}C-bicarbonate tracer experiment. The living specimen and the intestine cut into six pieces were incubated in artificial seawater containing 1 mM of $NaH^{13}CO_3$ and 200 μM of sodium sulphide, as described previously (Watsuji *et al.*, 2010), and stored at −80 °C after incubation. The *S. crosnieri* individual was dissected onshore to collect the whole tissue samples of the setae, intestine and muscle. The dry weights of the whole tissue samples were determined as 141 mg for the setae, 66 mg for the intestine and 1152 mg for the muscle. The dry weight of the cut intestine used in the ^{13}C-bicarbonate tracer experiment was 61 mg. In addition, three *S. crosnieri* individuals, which were collected from the same colony as the individuals used in the tracer experiments and stored at −80 °C, were dissected onshore to analyse natural stable isotope abundances. The ^{13}C composition and the total organic carbon contents of the samples were determined using a mass spectrometer (Delta Plus XP; Thermo Finnigan, Bremen, Germany), which was coupled online via a Finnigan ConFlo III interface with an elemental analyser (FlashEA 1112; Thermo-Quest, Milan, Italy). All of the samples were analysed in triplicates.

Results

Microscopic observations of setae and intestines
The fluorescence microscopy observations showed that *S. crosnieri* setae exhibited intrinsic fluorescence at excitation wavelengths of 460–490 nm, whereas the epibiotic microbial community associated with the setae exhibited no detectable autofluorescence (Figures 1a and b). Thus, the autofluorescence of *S. crosnieri* setae was used as a bio-tracer for setae in the intestines and their components. We detected setae fragments (1–5 fragments) in the components of each *S. crosnieri* intestine (8/10 individuals examined) based on optical and fluorescence microscopy observations (Figures 1c and d). In addition, the setae found in the *S. crosnieri* intestinal components lacked evident filamentous epibiotic assemblages (Figure 1c).

Dye-stained epibiont tracer experiment
Living *S. crosnieri* individuals with dye-stained and unstained epibiotic communities were incubated together in artificial seawater, which was filtered through activated charcoal (Supplementary Figure S1). During incubation for 24 h, we found that no body parts of *S. crosnieri* were stained with crystal violet, except the dye-stained setae. After incubation, the *S. crosnieri* individuals were dissected and the dissected full-length intestines and their components were observed under an optical stereo zoom microscope. According to the microscopic observations of the *S. crosnieri* intestines and their components with the dye-stained epibiotic community, the dye pigmentation and many granules faintly coloured by crystal violet were observed (Figures 2a and c). However, no pigmentation was observed in the *S. crosnieri* intestines and their components with the unstained epibiotic community (Figures 2b and d).

In vitro digestion experiment
Portions of setae covered with epibiotic microbial populations were statically incubated in the presence or absence of the crude *S. crosnieri* intestinal extract. Microscopic observations before and after incubation showed that the abundance of epibiotic microbial populations decreased significantly in the setae incubated with the intestinal extract (Figures 3a and b), but they were apparently unchanged in the setae incubated without the intestinal extracts (Figures 3c and d).

The epibiont cell suspensions were also incubated with the crude *S. crosnieri* intestinal extract. The DNA content of the cell pellet decreased drastically during incubation with the intestinal extract (that is, 13% of the DNA content in the cell pellet before incubation) (Supplementary Table S1). However, the bacterial 16S rRNA gene phylotype composition did not differ significantly between the cell pellets before and after incubation with the intestinal extract (that is, the clonal frequencies of the *Sulfurovum*, *Thiotrichaceae* and *Methylococcales* groups were 20.9%, 59.5% and 3.5% in the cell pellets before incubation and 29.9%, 47.1% and 3.4% in the cell pellets after digestion, respectively) (Supplementary Tables S2 and S3).

Determination of digestive enzyme activity levels in intestinal extracts
The crude intestinal extracts exhibited various digestive enzyme activities, such as α-amylase,

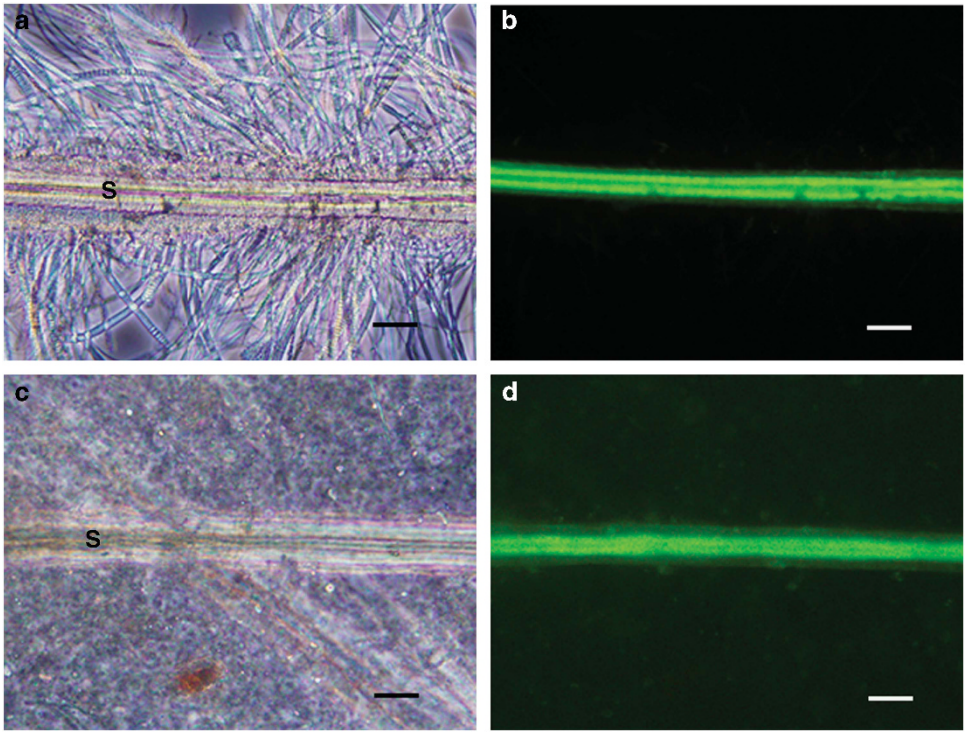

Figure 1 Optical and fluorescence microscopic observation of *S. crosnieri* setae. Optical and fluorescence microscopy of setae cut from a living *S. crosnieri* and of setae found in an *S. crosnieri* intestine is shown in the top panels (**a**, **b**) and in the bottom panels (**c**, **d**), respectively. Fluorescence microscopy shows the intrinsic fluorescence of setae (**b**, **d**). Optical microscopy shows the dense filamentous epibiotic populations and the typical morphological appearance of setae in a living individual (**a**) but the absence of epibionts on setae in the intestine (**c**). Capital S indicates a seta. Scale bars = 50 μm (**a**–**d**).

lipase and protease (Supplementary Table S4), where protease had the highest specific activity level (Supplementary Table S4). In addition, the nuclease activity was determined on the basis of agarose gel electrophoresis of genomic DNA from *Escherichia coli*, which was incubated with the crude intestinal extract (Supplementary Figure S2).

Chemolithotrophic activity of epibiotic and gut microbial communities

The rates of sulphide and methane consumption were measured using the homogenized setae and intestines (Supplementary Figure S1). The sulphide and methane concentrations drastically decreased during incubation of the homogenized setae (Figure 4). In contrast, the sulphide and methane concentrations slightly decreased during incubation in the presence of the homogenized intestines of three different individuals, although the changes in the concentrations did not differ significantly compared with those in the absence of homogenized intestines and setae (at each of the interval points, unpaired *t*-test, $P > 0.1$) (Figure 4).

Comparison of phylotype compositions and sequences in the gut and epibiotic microbial DNA assemblages

In total, 176 and 163 bacterial 16S rRNA gene clones were sequenced using the DNA extracts from the intestine and setae, respectively, of the same *S. crosnieri* individual (Supplementary Table S5). The clones that shared ⩾97% sequence identity were classified according to the same phylotype. Among the 38 gut bacterial phylotypes, eight phylotypes that represented 19% of the gut bacteria clones were very closely related to the *S. crosnieri* epibiont clones sequenced in previous studies and the present study (>99% identity) (Supplementary Table S5). The phylotypes that represented 54.5% of the gut bacterial clones and 88.9% of the epibiotic clones were affiliated with the *Sulfurovum* group within Epsilonproteobacteria, the Thiotrichaceae group and the Methylococcales group within Gammaproteobacteria (Supplementary Tables S5 and S6).

The number of phylotypes related to the Thiotrichaceae and Methylococcales groups in the epibiotic clone library was much higher than that in the gut bacterial clone library (Supplementary Table S5). Similarly, the Chao index showed that species richness levels of both the Thiotrichaceae and Methylococcales groups in the epibiotic 16S rRNA gene clone library (104 species) were much higher than those in the gut bacterial 16S rRNA gene clone library (29 species) (Supplementary Table S7). Most of the phylotypes (8/9 phylotypes) related to Thiotrichaceae and Methylococcales in the gut bacterial 16S rRNA gene clone library were found

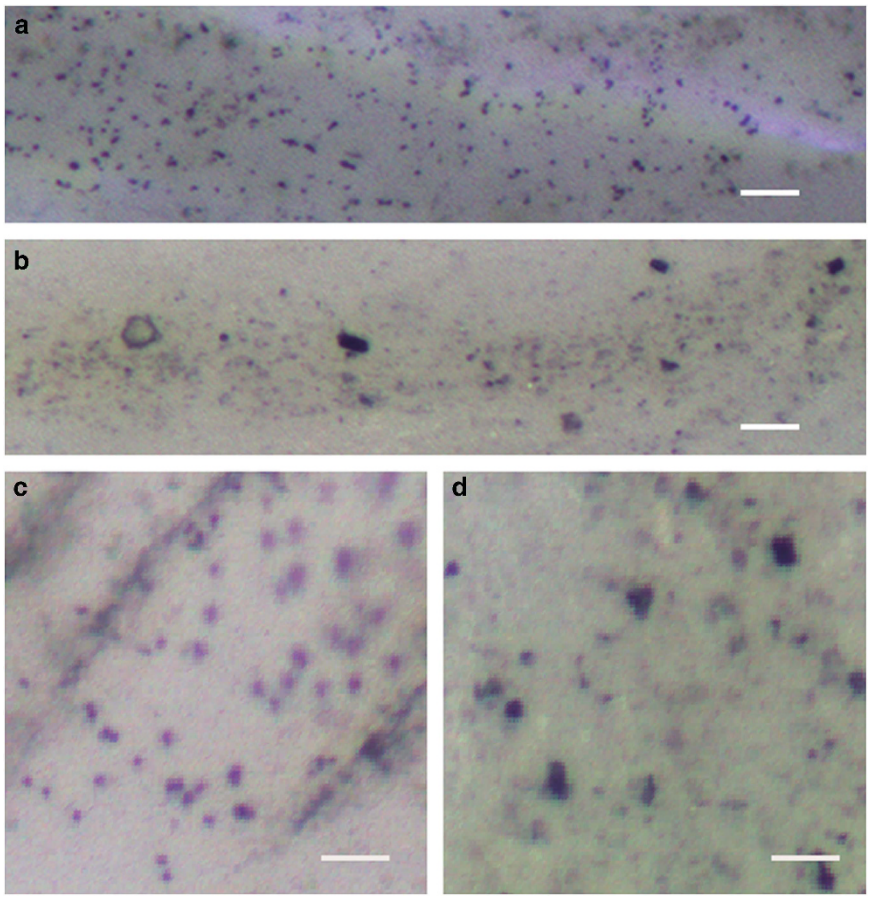

Figure 2 Microscopic observation of intestine ingredients in living *S. crosnieri* individuals with and without dye-labelled epibiont tracer experiment. Optical microscopy was performed for the intestines and their ingredients obtained from a *S. crosnieri* individual with crystal-violet-stained epibionts (**a**, **c**) and with unstained epibionts (**b**, **d**). Scale bars = 50 μm (**a**, **b**) and 20 μm (**c**, **d**).

in the epibiotic and gut bacterial clone libraries of the same individual (Supplementary Table S5). In addition, the predominant Thiotrichaceae and Methylococcales phylotypes in the epibiotic 16S rRNA gene clone library tended to be found frequently in the gut bacterial 16S rRNA gene clone library (Supplementary Table S5).

All of the epsilonproteobacterial phylotypes from the intestine and setae were phylogenetically related to members of the genus *Sulfurovum* (Supplementary Table S5). The number of *Sulfurovum*-affiliated phylotypes in the epibiotic 16S rRNA gene clone library was slightly higher than that in the gut bacterial 16S rRNA gene clone library (seven and nine phylotypes, respectively). The Chao index showed that the species richness level of the *Sulfurovum* group in the epibiotic clone library (14 species) was twice that in the gut bacterial clone library (7 species) (Supplementary Table S7). The *Sulfurovum* phylotypes in the gut bacterial clone library were not always found in the epibiotic clone library and the predominant epibiotic *Sulfurovum* phylotype (setae1_78) was not found in the gut bacterial clone library (Supplementary Table S5).

^{13}C-bicarbonate tracer experiments and stable carbon isotope analysis

^{13}C-bicarbonate tracer experiments were performed using live *S. crosnieri* and dissected intestines (Supplementary Figure S1). The ^{13}C enrichment level in setae with epibionts (3530‰) was clearly higher and the ^{13}C enrichment level in the intestine (−19.5‰) was slightly higher compared with that in the muscle (−22.8‰) (Table 1). The ^{13}C amounts assimilated in the whole setae including epibionts, the full-length intestine and the whole muscle of a living *S. crosnieri* individual for 48 h were calculated as 133 μmol, 0.22 μmol and 3.2 μmol, respectively (Table 1). The ^{13}C amount assimilated in the dissected *S. crosnieri* intestine for 48 h was 0.48 μmol (Table 1). In addition, the natural stable carbon isotope composition was determined in the tissues, including setae, intestine and muscle, of three *S. crosnieri* individuals (Table 2). The δ^{13}C value was slightly elevated in the muscles (−32.6‰) compared with that in the setae (−36.4‰) (Table 2) and these differences were significant (unpaired *t*-test, $P<0.05$). In contrast, the δ^{13}C values were similar in the muscles (−32.6‰) and intestine (−33.4‰) (Table 2), and

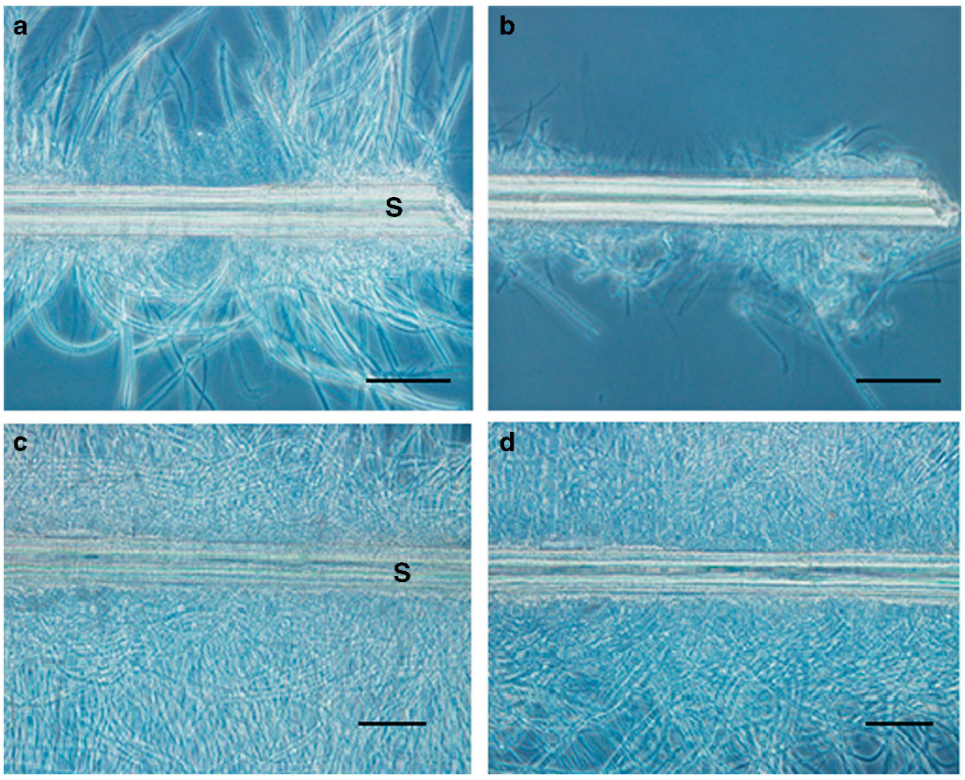

Figure 3 Microscopic observations of setae incubated with and without intestinal extract. Optical microscopy was performed to analyse setae dissected from a *S. crosnieri* individual before (**a**, **c**) and after (**b**, **d**) incubation with (**a**, **b**) and without (**c**, **d**) intestinal extract. Capital S indicates a seta. Scale bars = 50 μm (**a** and **b**).

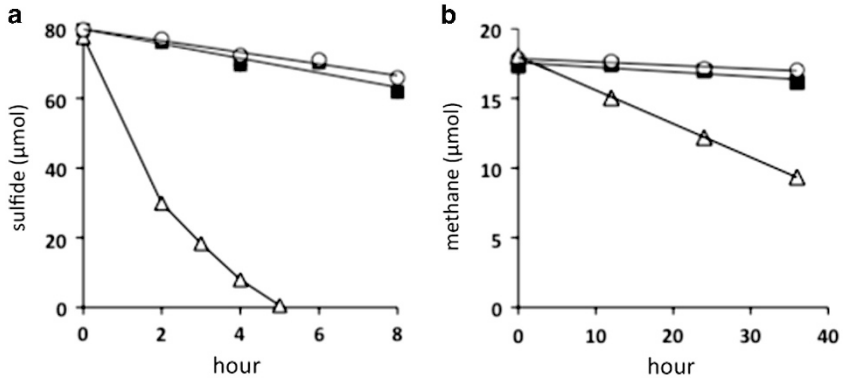

Figure 4 Time course of consumption of sulphide and methane by homogenates of *S. crosnieri* intestine and setae with epibionts. The concentrations of sulphide (**a**) and methane (**b**) were examined at the indicated intervals in the absence of the homogenate sample (■) and in the presence of homogenized setae with epibionts (Δ) and homogenized intestines (○).

there was no significant difference (unpaired *t*-test, $P > 0.05$).

Discussion

The deep-sea vent galatheid crabs, *S. crosnieri* and *K. puravida*, frequently exhibit a typical behaviour where they comb their setae, which are covered with dense epibionts, using the third maxillipeds and then bringing the maxillipeds to the mouths, although this behaviour has not been observed in *R. exoculata* (Watsuji *et al.*, 2010; Thurber *et al.*, 2011). Based on these observations, it has been hypothesized that *S. crosnieri* and *K. puravida* ingest their epibiotic microbial populations directly via this typical feeding behaviour, and digest them in their digestive organs (Watsuji *et al.*, 2010; Thurber *et al.*, 2011). In this study, we found setae fragments in almost every *S. crosnieri* intestine (Figure 1), which strongly suggests that the setae were ingested via the combing behaviour in *S. crosnieri*. In addition, it is likely that the epibiotic population on the setae, as well as the setae, are ingested during this behaviour given that *S. crosnieri* setae are always covered with dense epibiotic

Table 1 Stable carbon isotope compositions of different tissues of a living *S. crosnieri* individual and a dissected intestine before and after ^{13}C-labelled bicarbonate tracer experiments

Labelled specimen	*Tissue*	*δ^{13}C (‰)*		*Enrichment of ^{13}C/^{12}C (%)*	*Total organic carbon/whole tissue (mmol)*	*Assimilated ^{13}C/whole tissue (μmol)*
		Natural abundance[a]	*After incubation*[b]			
S. crosnieri	Setae	− 36.4	3530 ± 78	370 ± 8	3.3	133 ± 3
	Intestine	− 33.4	− 19.5 ± 0.3	1.4 ± 0.03	1.4	0.22 ± 0.005
	Muscle	− 32.6	− 22.8 ± 0.1	1.1 ± 0.01	29.1	3.2 ± 0.03
Dissected intestine	—	− 33.4	− 0.5 ± 0.6	3.4 ± 0.06	1.3	0.48 ± 0.009

[a]Values are means of three independent determinations measured in Table 2.
[b]Values were measured in triplicate and were expressed as mean ± s.d.

Table 2 Natural stable isotopic composition of different tissues of *S. crosnieri* from a natural habitat of Iheya North

Specimen no.	*δ^{13}C (‰)*		
	Setae	*Intestine*	*Muscle*
1	− 35.9 ± 0.3	− 33.3 ± 0.2	− 32.1 ± 0.1
2	− 36.0 ± 0.9	− 34.0 ± 0.7	− 32.6 ± 0.1
3	− 37.2 ± 0.5	− 33.0 ± 0.1	− 33.2 ± 0.1
Mean	− 36.4	− 33.4	− 32.6

The δ^{13}C value of a sample was measured in triplicate. They are expressed as the mean ± s.d.

microbial communities in natural deep-sea vent habitats (Figure 1a; Watsuji *et al*., 2010, 2012). However, the setae found in the *S. crosnieri* intestinal tracts were not accompanied by obvious filamentous epibiotic assemblages (Figure 1c). Typical filamentous epibiotic cells were not observed among the intestinal components of *S. crosnieri* observed via the dye-stained epibiont tracer experiment, but apparent dye pigmentation and faintly coloured granules were identified in the intestinal components (Figures 2a and c). These results suggest that the epibiotic populations on the ingested *S. crosnieri* setae and the ingested dye-stained epibionts were digested in the gut. The *in vitro* digestion experiment clearly indicated that the *S. crosnieri* epibionts, but not the setae, were digested at significant levels by the intestinal extract (Figure 3). In addition, the intestinal extract exhibited digestive enzyme activities, such as α-amylase, lipase, protease and nuclease activities (Supplementary Table S4 and Supplementary Figure S2). Therefore, these results suggest that the epibionts were ingested and digested in the gut.

The sulphide- and methane-oxidizing activities of living *S. crosnieri* individuals have previously been identified in both atmospheric and elevated hydrostatic pressure conditions (Watsuji *et al*., 2012, 2014). In addition, thioautotrophic and methanotrophic productions have been detected in the epibiotic bacterial community (Watsuji *et al*., 2010, 2012). In this study, the sulphide- and methane-oxidizing activities of the potential gut microbial community in *S. crosnieri* were found to be negligible (Figures 4a and b and Supplementary Figure S1), which indicates that the viable gut microbial community exhibited little chemolithotrophic (sulphide- and methane-oxidizing) activity and productivity to support the host's nutrition. In addition, this result suggests that the ingested epibiotic microbial populations lost their indigenous chemolithotrophic (sulphide- and methane-oxidizing) activities in the intestine because of digestion by various intestinal enzymes.

The phylogenetic diversity of gut microbial communities in deep-sea vent invertebrates that harbour epibiotic microbial communities was characterized in *R. exoculata* (Zbinden and Cambon-Bonavita, 2003; Durand *et al*., 2009). These analyses revealed that the predominant phylotypes in the *R. exoculata* gut community were related to members of *Deferribacteres*, whereas they were not identified in the *R. exoculata* epibiotic community (Durand *et al*., 2009). Based on these results, it has been speculated that a gut-specific microbial community is present in the *R. exoculata* intestine. Meanwhile, we found that many of the bacterial 16S rRNA gene sequences retrieved from the *S. crosnieri* intestine were phylogenetically related to those of the *S. crosnieri* epibionts (Supplementary Table S5). In particular, eight phylotypes that represented 19% of the gut bacterial clones were very closely related to the *S. crosnieri* epibiont clones identified in previous studies and this study (>99% identity) (Supplementary Table S5). Thus, the similar bacterial phylotypes in the gut microbial DNA assemblages and the epibiotic microbial community of *S. crosnieri* provides molecular evidence that the epibiotic microbial population is ingested and digested by *S. crosnieri* as a nutritional source for the host.

Our analysis of the phylogenetic diversity in the epibiotic community and the gut bacterial assemblage of the same individual showed that a higher diversity of Thiotrichaceae and Methylococcales phylotypes was found in the epibiotic community

compared with the gut bacterial assemblage (Supplementary Tables S5 and S7). All of the epsilonproteobacterial phylotypes from the intestine and setae were phylogenetically related to the *Sulfurovum* group and the epibiotic community exhibited a higher diversity of *Sulfurovum* phylotypes than the gut bacterial assemblage (Supplementary Tables S5 and S7). These results indicate that the phylogenetic diversity of the ingested epibionts may decline during their digestion in the intestine. Genetic microheterogeneity was also evident in the epibiotic community and the gut bacterial assemblage of the same individual, particularly in the *Sulfurovum*-affiliated phylotypes, that is, the predominant *Sulfurovum*-affiliated phylotypes in the gut bacterial assemblage were not detected in the epibiotic community (Supplementary Table S5). We cannot completely exclude the possibility that several distinctive *Sulfurovum* phylotypes from the epibiotic *Sulfurovum* phylotypes thrive specifically in the intestine, but we hypothesize that the genetic microheterogeneity in the epibiotic community and the gut bacterial assemblage may be attributable to the indigenous genetic microheterogeneity of the epibiotic community. The current analysis and previous phylogenetic analyses of the epibiotic communities in *S. crosnieri* individuals were conducted using the whole setae or different parts of setae samples, thus the phylotype compositions were represented as the average entities in an individual or several individuals (Watsuji *et al.*, 2010, 2012, 2014). However, it is likely that different parts of the setae will host similar but slightly heterogeneous epibiont phylotype compositions. The feeding-like behaviour of living *S. crosnieri* should always transfer epibionts from different parts of the setae to the gut, thus the ingested epibiotic populations in the intestine may exhibit genetic microheterogeneity. Even if the gut bacterial phylotypes were derived from the gut-endemic microbial community of *S. crosnieri*, these bacterial components would not provide a nutritional source for *S. crosnieri* because they lose their chemolithotrophic activities (Figure 4).

The proportion of *Sulfurovum*-affiliated phylotype clones was 42.6% in the gut bacterial assemblage but only 14.7% in the epibiotic bacterial community, although the proportions of Thiotrichaceae- and Methylococcales-related phylotypes in the gut bacterial assemblage decreased as compared with those in the epibiotic bacterial community (Supplementary Tables S5 and S6). These results imply that the *Sulfurovum*-affiliated epibionts tend to reside in the intestine throughout the digestive process, in contrast to the Thiotrichaceae- and Methylococcales-related epibiotic populations. However, our *in vitro* digestion experiment using an epibiont cell suspension with the intestinal extract detected no apparent phylotype-specific digestion of epibionts by the intestinal enzymes (that is, significant resistance of the *Sulfurovum* group to digestion), although the total bacterial cells and DNA levels drastically decreased during digestion (Supplementary Tables S1, S2 and S3). In many decapods, it has been reported that foods are masticated and digested in the stomach, and finer food particles are absorbed in the hepatopancreas, whereas coarser particles pass through gland filters in the intestine (Schaefer 1970; Kunze and Anderson 1979). A previous fluorescence *in situ* hybridization of *S. crosnieri* epibionts clearly demonstrated that the *Sulfurovum* epibionts had the largest cell size as well as the thickest and longest filaments in the epibiotic community (Watsuji *et al.*, 2010). Therefore, because the *Sulfurovum* epibionts have relatively larger cells and a filamentous morphology compared with other epibionts, the ingested and masticated *Sulfurovum* epibionts are more likely to be transported into the intestine via the gland filters rather than the hepatopancreas. Therefore, the *Sulfurovum* epibionts may be more likely to reside in the intestine throughout the digestive process as compared with the Thiotrichaceae- and Methylococcales-related epibiotic populations.

The phylotypes affiliated with Verrucomicrobiae and Mollicutes from the intestine were not related to the bacterial 16S rRNA gene sequences identified in the *S. crosnieri* epibionts (Supplementary Table S5). Phylotypes related to Verrucomicrobiae and Mollicutes were also detected in the gut of *R. exoculata*, where the gut-endemic microbial community would occur (Durand *et al.*, 2009). The phylotype gut_h89 belonging to Gammaproteobacteria was also not related to previously known epibiotic 16S rRNA gene sequences, although this was the predominant phylotype in the gut bacterial assemblage. Thus, these bacterial phylotypes found in the *S. crosnieri* intestine DNA assemblages may represent viable components of the gut-endemic microbial community of *S. crosnieri*.

The hydrothermal vent mussels *Bathymodiolus azoricus* and *B. puteoserpentis* are nutritionally sustained by chemolithoautotrophic and methanotrophic endosymbionts (Duperron *et al.*, 2006; Petersen *et al.*, 2011). When ^{13}C enrichment was evaluated in ^{13}C-labelled bicarbonate tracer experiments using living *B. azoricus* individuals, the ^{13}C enrichment level in the gill containing thioautotrophic endosymbionts was the highest among all tissues, whereas the ^{13}C enrichment level of tissues, including the digestive system, was slightly higher compared with that of muscles (Riou *et al.*, 2008). A similar ^{13}C tracer experiment using live *S. crosnieri* showed that the ^{13}C enrichment of setae with thioautotrophic epibionts was the highest, whereas the ^{13}C enrichment of the intestine was slightly higher compared with that of the muscle (Table 1). Thus, as found in these deep-sea vent mussels, our results may indicate that the organic carbon assimilated by the epibiotic microbial population is

supplied to the living *S. crosnieri* body via digestion in the digestive organs.

Finally, the nutritional transfer rate was estimated from the results of the ^{13}C-labelled bicarbonate tracer experiments (Supplementary Figure S1). The total amount of ^{13}C assimilated in the dissected *S. crosnieri* intestine (0.48 μmol), which was assumed to be the net ^{13}C amount assimilated by functionally active gut microbial assemblages, was much lower than that of the whole muscle in a living *S. crosnieri* individual (3.2 μmol; Table 1). Thus, it is very unlikely that ^{13}C assimilated in the intestine with gut microbial assemblages sustained the ^{13}C incorporated into the muscle and the other tissues in the body of *S. crosnieri*. In contrast, the total amount of ^{13}C assimilated in the whole setae of a living *S. crosnieri* individual (133 μmol) was much greater than that of the whole muscle (Table 1). Thus, only the ^{13}C assimilated by setae epibionts can be the source of ^{13}C supplied to muscle and other tissues in the body of *S. crosnieri*.

We determined the natural stable carbon isotope compositions of the tissues, including setae, intestine and muscle, of three *S. crosnieri* individuals (Table 2). As noted previously (Watsuji *et al.*, 2010), the highly ^{13}C-depleted values of the setae with epibionts indicated the significant contribution of the methanotrophic populations using highly ^{13}C-depleted methane in the hydrothermal fluids, in addition to the primary production of thioautotrophic epibionts. In general, a small but significant elevation in the δ^{13}C value in the animal body relative to the δ^{13}C value in the available food is observed as compared with the stable carbon isotope compositions of natural animal communities, although the δ^{13}C values of animal tissues resemble those in the animal's diet (Rau *et al.*, 1983). According to the natural carbon isotope compositions of *S. crosnieri* tissues, a small elevation in the δ^{13}C value of *S. crosnieri* muscle relative to the δ^{13}C value for the setae with epibionts was also observed, although the δ^{13}C values were almost equal in the muscles and intestines (Table 2). Thus, the natural carbon isotope compositions of different *S. crosnieri* tissues also suggest that the epibiotic microbial population on the setae is the primary diet of natural *S. crosnieri* populations in deep-sea vent habitats.

Concluding remarks

Our results indicate that the predominant deep-sea vent invertebrate *S. crosnieri* found in the Okinawa Trough hydrothermal systems is nutritionally sustained by host-associated chemosynthetic bacteria, as in the case of another deep-sea vent shrimp *R. exoculata* (Watsuji *et al.*, 2010; Ponsard *et al.*, 2012). Given that the typical feeding-like behaviour has been observed frequently in these deep-sea vent galatheid crabs in their natural habitats and under rearing conditions, it was predicted that deep-sea vent galatheid crabs such as *S. crosnieri* and *K. puravida* ingest epibiotic microbial populations and utilize them as their primary diet (Watsuji *et al.*, 2010; Thurber *et al.*, 2011). However, the nutritional transfer from the epibionts to the host has not been directly shown and its mechanism remains unclear. Therefore, in this study, our experiments and analyses demonstrated that *S. crosnieri* populations from the Okinawa Trough hydrothermal systems ingest thioautotrophic and methanotrophic epibionts via their mouth using the third maxillipeds, and digest and assimilate them as their primary nutritional source. This is a striking example, in which the manner of nutritional transfer in the ectosymbiosis between chemosynthetic bacteria and deep-sea invertebrates is clearly characterized.

Conflict of Interest

The authors declare no conflict of interest.

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