Effects of Hydrogen Sulfide on Bacterial Communities on the Surface of Galatheid Crab, *Shinkaia crosnieri*, and in a Bacterial Mat Cultured in Rearing Tanks

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To investigate the effects of H2S on the bacterial consortia on the galatheid crab, *Shinkaia crosnieri*, crabs of this species were cultivated in the laboratory under two different conditions, with and without hydrogen sulfide feeding. We developed a novel rearing tank system equipped with a feedback controller using a semiconductor sensor for hydrogen sulfide feeding. H2S aqueous concentration was successfully maintained between 5 to 40 µM for 80 d with the exception of brief periods of mechanical issues. According to real-time PCR analysis, the numbers of copies of partial 16S rRNA gene of an epibiont of the crabs with H2S feeding was three orders of magnitude larger than that without feeding. By phylogenetic analysis of partial 16S rRNA gene, we detected several clones related to symbionts of deep sea organisms in Alphaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, and Flavobacteria, from a crab with H2S feeding. The symbiont-related clones were grouped into four different groups: Gammaproteobacteria in marine epibiont group I, Sulfurovum-affiliated Epsilonproteobacteria, Ose dx mucocfloris endosymbiont-affiliated Epsilonproteobacteria, and Flavobacteria closely related to CBG group bacterial epibiont of Rimicaris exoculata. The other phyotypes were related to Roseobacter, and some Flavobacteria, seemed to be free-living psychrophiles. Furthermore, white biofilm occurred on the surface of the rearing tank with H2S feeding. The biofilms contained various phyotypes of Gammaproteobacteria, Epsilonproteobacteria, and Flavobacteria, as determined by phylogenetic analysis. Interestingly, major clones were related to symbionts of Alviniconcha sp. type 2 and to endosymbionts of Ose dx mucocfloris, in Epsilonproteobacteria.

Key words: hydrogen sulfide, epibiont, symbiont, tank, feedback control

The chemosautotrophic ecosystem is supported by chemooautotrophs, which are organisms that are capable of fixing carbon using chemical energy obtained from the oxidation of reduced compounds blowing up from hydrothermal vents, such as sulfuric compounds, methane, and hydrogen (18). Several benthic invertebrates are hosts of chemosautotrophic bacteria in cells of specialized tissues and on the surface of their bodies, and they obtain nutrition from the bacteria (2).

*Shinkaia crosnieri*, a galatheid crab, is one of the most predominant animal species that massively aggregate close to hydrothermal vents on the subseafloor in the deep sea. The crab is observed only in areas near active vents in the Okinawa Trough, western Pacific (25, 26). The crab has not only strong, stout, sparse setae on the dorsal surface of its carapace, chelipeds, and walking legs, but also long, soft, dense plumose setae on the ventral surface of its body (1). According to previous studies (27, 30), epibiotic filamentous symbionts inhabit the surface of the plumose. Phylogenetic analyses of 16S rRNA sequences in those studies showed no significant difference in the epibiotic phylotype composition of the individuals collected from different sites. The major subdivisions of the phyotypes were Epsilonproteobacteria and Gammaproteobacteria. In particular, specific phylogenetic clusters related to the genus *Sulfurovum* have been found in epibiotic 16S rRNA gene clone libraries. A free-living *Sulfurovum* sp. NBC37-1 isolated from a vent field is one of the most well-characterized strains (17, 31). According to whole genome sequencing (17), NBC37-1 is a mesophilic hydrogen- and sulfur-oxidizing chemolithoautotroph and has key genes associated with sulfur reduction (psr families) and oxidation (sox families). Further culture-dependent experiments showed that the strain can grow utilizing two different types of sulfur related energy metabolism, namely, hydrogen-oxidizing sulfur respiration and thiosulfate-oxidizing nitrate/oxygen respiration in the deep sea (17). Such experiments also provide evidence for the operation of the hydrogenase-coupled polysulfide reductase pathway as well as the Sox system; therefore, the strain and its relatives may inhabit the mixing zones between oxidative and reductive areas where the physical and chemical conditions are optimal for sulfur-oxidizing nitrate/oxygen and hydrogen-oxidizing respiration (31).

On the other hand, Saito (21) reported that *Shinkaia crosnieri* obtains many of its lipids from *Bathymodiolus* mussels and chemosynthetic symbionts on the basis of the characteristics of fatty acid composition of the crab. Miyake

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et al. (15) have reported that the crabs graze on epibiotic bacteria using their mouth-parts in a tank rearing system with an artificial hydrothermal vent system; therefore, we consider that the crab is able to obtain many nutrients from epibionts on its surface. However, there has been no information on how environmental, physical and chemical conditions affect the epibiont consortium on the surface of the crab, since it is difficult to control experimental conditions, particularly H$_2$S concentration, in the laboratory. The continuous supply of hydrogen sulfide, which is a highly poisonous and reactive gas, at low levels has also been difficult.

In the applied microbiology research field, there have been reported high cell density cultivation techniques with feedback control of volatile and toxic materials such as alcohols at a low level in a jar fermenter by detecting evaporated compounds in the exhaust gas by a semiconductor sensor (6, 9). We applied technology for the H$_2$S control system of the rearing tank for deep-sea invertebrates, which is also capable of controlling temperature and dissolved oxygen. Using the newly developed rearing tank system, we first examined the effect of low-concentration H$_2$S on epibiontic bacterial communities in an artificial environment. The aim of this experiment was to examine the effects in clear cause-effect relationships between H$_2$S and bacterial communities by novel controlled growth conditions in the rearing tanks. Moreover, a white bacterial mat was obtained from the surface of the rearing tank. On the basis of the results of phylogenetic analysis, we also describe here the bacterial consortium structure. Finally, we discuss the effects of hydrogen sulfide on deep-sea bacteria and the potential use of the rearing tank experimental system.

Materials and Methods

Collection of S. crosnieri from deep-sea vent

Individuals of S. crosnieri were collected from a deep-sea hydrothermal field: Iheya North field (27°47.46’N, 126°53.80’E, depth = 981 m) in the Okinawa Trough, Japan, by a remotely operated vehicle (ROV), ‘HyperDolphin’ of Japan Agency for Marine-Earth Science and Technology (JAMSTEC). The individuals collected, which were still alive after the rapid pressure and temperature change during recovery, were immediately moved to shipboard tanks containing surface seawater at 4°C.

Rearing tanks for S. crosnieri.

Fig. 1A shows the rearing tank system including the H$_2$S feeding system (gas sensor, feedback control system, and gas-providing apparatuses), temperature control system (a thermostat controller and a cooler), and dissolved oxygen (DO) control system (DO sensor, mixing pump, and degassing system). The main tank of 100 and 200 L and their lids (1 in Fig. 1A) were made of acrylic plastic plates from Iwaki Pumps (Tokyo, Japan). The tin dioxide semiconductor sensor unit for H$_2$S (2 in Fig. 1A, TGS-825; Figaro Eng., Osaka, Japan) was equipped with an electric circuit unit placed inside the lid of the rearing tank. The sensor detected H$_2$S in the head space of the tank as voltage signals. The signals are approximately proportional to the H$_2$S concentration in tank water (Fig. 1B). The sensor was connected to a detection relay (3 in Fig. 1A, 2411D-1-04-H0-T5-A; Tsuruga Electric, Osaka, Japan) and a data logger (4 in Fig. 1A, LR5042; Hioki, Nagano, Japan). An electromagnetic valve and a pump were controlled by on-off signals by the detect relay. Hydrogen sulfide gas was provided from the cylinder of liquefied H$_2$S via the valve to a gas exchange unit (8 in Fig. 1A, STERAPORE degassing module, MHF0504MBFT; Mitsubishi Rayon, Tokyo, Japan). The gas was dissolved in water that passed through the unit. The water was degassed by a gas trap (9 in Fig. 1A) and introduced into a porous tetrafluoroethylene (PTFE) tube (10 in Fig. 1A, POREFLON tube, TB-0302; Sumitomo Electric Fine Polymer, Tokyo, Japan), and circulated to the gas-exchange unit. The dissolved H$_2$S was diffused from the circulating water to the tank water via the PTFE tube and sea sand bed (11 in Fig. 1A). The H$_2$S concentration in the tank water was controlled to between 20 and 50 µmol L$^{-1}$. The concentration was set to avoid chemical generation of white matter in the tank water by H$_2$S in preparatory experiments, because the optimal concentration for the crabs was unknown. The concentration of H$_2$S in the Iheya North field has been reported to be over a wide range from 2.6 mmol kg$^{-1}$ to not detected (7). A digital optical sensor (Oxymax W COS61-D; Endress+Hauser, Switzerland) was used for detecting DO. The signals were analyzed by a transmitter (Liquisis M COM223; Endress+Hauser). The aeration pump (14 in Fig. 1A) and degassing system (16, 17, and 18 in Fig. 1A) were controlled by the transmitter. DO was controlled by the system at between 1.0 and 1.5 mg L$^{-1}$, which corresponds to between 8.12 and 12.2% of saturation at 5.2°C. pH was continuously measured by a pH sensor (MicroPH; Aquabase, Kanagawa, Japan). Temperature was controlled at 5.25±2.5°C by a
thermostat controller (TC-100; Iwaki Pumps) and a cooling unit (AZ-151X; Iwaki Pumps). Artificial seawater (ROHTOMARINE II; Iwaki Pumps) was used as the tank water. For a comparative experiment without H$_2$S feeding, a tank without an H$_2$S feeding system (2 to 11 in Fig. 1A) was also prepared. Sixty crabs were transferred to 200-L tanks with an H$_2$S feeding system, and thirty crabs to a 100-L tank without a feeding system.

**Determination of H$_2$S concentration in tank water**

A colorimetric method for the quantification of H$_2$S concentration in tank water was carried out by a previously reported method (32) with modification. N,N-dimethyl-p-phenylene diammonium dichloride (14.3 mM) and FeCl$_3$ (18.5 mM), solutions were prepared in 0.6 M HCl as test reagents. One milliliter of tank water was gently mixed with 0.1 mL of a test reagent. The samples were incubated at room temperature for 5 min. The absorbance of the samples at 670 nm was measured by a spectrometer (UV-1000; Hitachi, Tokyo, Japan).

**Microscopy**

Microscopy was carried out using microscope systems (Eclipse E600, Nikon, and Olympus BX51 systems) equipped with a digital camera. Then, 4,6-diamidine-2-phenylindole dihydrochloride (DAPI)-stained samples were prepared as follows. Samples of the setae of *S. crosnieri* crabs and biofilms were collected in deionized water and centrifuged at 10,000×g for 1 min. The supernatant was removed and the samples were washed in 1 µg mL$^{-1}$ DAPI in methanol solution and covered for 15 min at room temperature with DAPI solution. DAPI solution was removed by centrifugation, and the setae were then gently washed with methanol immediately. The washed samples were dispersed in phosphate-buffered saline (pH 6.8) and used for microscopy.

**DNA extraction**

Samples of the setae of *S. crosnieri* (approximately 20 mg wet weight) were collected immediately after washing the crabs with filtered artificial sea water, and centrifuged at 9,100×g for 1 min. The supernatant was removed carefully. Wet weight of a portion of setae samples was measured. Genomic DNA of samples were extracted from setae immediately by a DNA extraction kit for soil (ISOL for Beads Beating; Nippon Gene, Tokyo, Japan). Extraction was carried out following the manufacturer’s instructions.

**Real-time PCR analysis**

To estimate the proportion of bacteria on the setae of the crabs, which were cultivated with and without feeding with H$_2$S, real-time PCR was performed. A SYBR Premix Ex Taq II kit (Takara, Shiga, Japan) was used for real-time PCR analysis. Partial 16S rRNA gene sequences as target genes were amplified by PCR using bacterial 16S rRNA-universal oligonucleotide primers Bac27F and Univ1492R (11). Thermal cycling was performed using a GeneAmp PCR System 2700 (Applied Biosystems), with preliminary denaturation at 94°C for 4 min, followed by 27 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 2 min, and a final elongation at 72°C for 2 min. Amplified 16S rRNA gene products were cloned using the above TA cloning kit. The TA clones were used for sequencing. Single phylogenetic clone types (phylotypes) were obtained from the sequence analyses. The phylotypes were compared with those of related sequences obtained from DNA Data Bank, Japan (DDBJ) and GenBank of the National Center for Biotechnology Information via the BLAST search program and aligned using Clustal X ver. 2.0 software by the neighbor-joining method (12). The phylogenetic tree was visualized using TreeView software version 1.6.6.

**Phylogenetetic analysis**

To analyze epibiotic clones on the surface of the crabs, the obtained real-time PCR products were used directly as follows. The products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). Purified products were cloned using a TA cloning kit (QIAGEN PCR Cloning$^\text{\textregistered}$ kit; QIAGEN, Germany) in accordance with the manufacturer’s instructions. The recombinant colonies were transferred to LB medium with 50 µg mL$^{-1}$ ampicillin and incubated at 37°C overnight. Recombinant plasmids were extracted using the QIAprep Miniprep system (QIAGEN) and sequenced with an ABI PRISM 3100 DNA Analyzer and an BigDye Terminator Cycle Sequencing kit, version 3.1 (PE Biosystems, Foster City, CA, USA). In the analysis of bacterial biofilms, 16S rRNA gene sequences were amplified with the bacterial 16S rRNA-universal oligonucleotide primers Bac27F and Univ492R (11). Thermal cycling was performed using a GeneAmp PCR System 2700 (Applied Biosystems), with preliminary denaturation at 94°C for 4 min, followed by 27 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 2 min, and a final elongation at 72°C for 2 min. The amplified 16S rRNA gene products were cloned using the above TA cloning kit. The TA clones were used for sequencing and phylogenetic analysis. Single phylogenetic clone types (phylotypes) were obtained from the sequence analyses. The phylotypes were compared with those of related sequences obtained from DNA Data Bank, Japan (DDBJ) and GenBank of the National Center for Biotechnology Information via the BLAST search program and aligned using Clustal X ver. 2.0 software by the neighbor-joining method (12). The phylogenetic tree was visualized using TreeView software version 1.6.6.

**Nucleotide sequence accession numbers**

The sequence data have been submitted to EMBL/GenBank/ DDBJ databases under accession numbers AB697010 to AB697038.

**Results**

**Cultivation of S. crosnieri with epibionts**

To investigate the effects of H$_2$S feeding that are separate from the other effects, including hydrostatic pressure, temperature, and the other chemicals as far as possible, *S. crosnieri* crabs were cultivated in rearing tanks with and without H$_2$S feeding. The temperature and DO of the rearing tank without H$_2$S feeding were maintained at 5°C and 1 mg L$^{-1}$ as set points during the cultivation period. pH was kept at approximately 8.0 during cultivation periods (data not shown). Fig. 2 indicates the parameters measured during the cultivation with H$_2$S feeding. Four crabs died 1 d after starting cultivation when H$_2$S was not fed into the tank; stress during shipping probably affected these dead crabs. In the early phase of cultivation (first week), the effects of H$_2$S on the activities of the crabs were examined. H$_2$S was initially introduced in pulses until an H$_2$S concentration of approximately 100 µmol L$^{-1}$ was reached in the tank water (Fig. 2A). The concentration of this compound increased rapidly during feeding and decreased markedly after stopping the feeding. The rapid decrease in H$_2$S concentrations may have been mainly due to the spontaneous oxidization of H$_2$S. The initial pulse introduction of the compound did not have harmful effects on the survival of the crabs (Fig. 2C). Then, H$_2$S was continuously introduced into the tank, and its concentration was maintained between 5 and 60 µmol L$^{-1}$ by the feedback control system. Feeding was successfully maintained before the cooling unit malfunctioned 36 d
after starting the cultivation. During the period before the malfunction, the temperature of the tank water was well controlled at 5.2±0.2°C, and DO concentration initially decreased to approximately 1 mg L\(^{-1}\) in the initial 7 d (Fig. 2B). The decrease in DO concentration may have been caused by not only the degassing-DO control system, but also the respiration of crabs and aerobic bacterium. pH gradually decreased from 7.95 to 7.45 during the same period (Fig. 2B). Unfortunately, the malfunction of the cooling unit markedly increased the tank temperature to 12.6°C for several hours, which killed four crabs. We immediately transferred the active crabs into the back-up rearing tank system; therefore, DO concentration primarily increased to approximately saturation. The increased DO concentration gradually decreased to approximately 1 mg L\(^{-1}\) in the following 5 d. pH also increased to 8.2 in the new artificial sea water, and gradually decreased. Three additional crabs died during the period between the 43rd day and the 55th day. It was possible that the death of the crabs was caused by the high temperature following the malfunction of the cooling unit. Finally, 44 of the initial 60 crabs remained (survival of crabs was 73.3%) after 84 d of cultivation when the crabs were cultivated with H\(_2\)S feeding. On the other hand, 27 of the initial 30 crabs survived (survival of crabs was 90.0%) when the crabs were cultivated without H\(_2\)S feeding (data not shown). The survival rate of crabs with H\(_2\)S feeding seemed to be lower than that of crabs without H\(_2\)S feeding. Active busy setae were observed a few days after continuous feeding of H\(_2\)S, and their color changed from brown to cream. Furthermore, white bacterial films (biofilm) formed and increased on the surface of the tank wall (Fig. 3A) and sea sand in the feeding unit (Fig. 3B) (11 in Fig. 1A).

**Estimation of amount of epibionts on surface of setae**

To estimate the amount of epibiotic bacteria on the surface of setae of crabs with and without H\(_2\)S feeding after

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**Fig. 2.** Time courses of H\(_2\)S concentration, pH, DO concentration, temperatures, and survival rate. Time courses of H\(_2\)S concentration (A), pH, DO, and temperature (B), and survival rate of the crab as a percentage (C). Symbols: pH (▲), DO (●), and temperatures (○).

**Fig. 3.** Images of biofilms. Image of biofilm occurring on the surface of the tank wall (A), image of biofilm occurring on the surface of the sea sand bed (B, 11 in Fig. 1A), phase difference microscopy image of a portion of biofilm (C), and epifluorescence microscopy image of a portion of DAPI-stained biofilm (D).

**Fig. 4.** Microphotographs of setae of crabs with and without H\(_2\)S feeding. Images of setae of crabs with feeding (A and B) and without feeding (C and D) are shown. Phase difference microscopic images (A and C) and DAPI-stained images (B and D) are shown.
cultivation for 84 d, microscopy observations and real-time PCR analysis were performed. Microscopy images of representatives of DAPI-stained setae are shown in Fig. 3. The numbers of epibionts on setae after cultivation with H$_2$S feeding (Fig. 4A and B) were obviously larger than those without H$_2$S feeding (Fig. 4C and D). Interestingly, in the images of setae of crabs with feeding, spherical and cocoid epibionts were interestingly found along the filaments. The majority of epibionts on the setae of the crabs collected from the environment are filaments, as shown by previous studies (27, 30). The amount of epibionts estimated by real-time PCR analysis is shown in Fig. 5 as the copy number of target DNA (partial 16S rRNA gene) per wet weight of a sample. Three crabs from each cultivating condition were used for the analysis. The amount of epibionts of crabs with feeding reached the target $1.8 \times 10^9$, $2.7 \times 10^9$, and $8.2 \times 10^9$ copies per gram of wet weight of setae, whereas those of crabs without H$_2$S feeding reached $2.7 \times 10^8$, $7.1 \times 10^8$, and $1.9 \times 10^9$ copies g$^{-1}$. Although differences in the target DNA copy numbers of individual samples were observed under the same conditions, the number of copies of target DNA of epibionts of crabs with feeding was three orders of magnitude larger than that of crabs without feeding.

**Phylogenetic analysis of epibionts**

To determine the phylogenetic affiliation of epibionts on the surface of the setae, phylogenetic analysis was performed on the basis of the partial 16S rDNA sequences. As shown in Fig. 6, thirteen phyotypes of epibionts of the crabs cultivated with H$_2$S feeding were placed in four different phyla, including Alphaproteobacteria, Gammaproteobacteria, and Epsilonproteobacteria and Flavobacteria.

One of the signature epipsymbiotic bacteria of *S. crosnieri*, epibiotic phyotype 5, was related to the Sulfofervum-affiliated bacteria group in Epsilonproteobacteria, which was also often detected in different *S. crosnieri* (27, 30). It was also related to ectosymbiotic clones isolated from the deep-sea limpet, *Symmetromphalus* aff. *hageni* (4), a gill endosymbiont of the hydrothermal vent gastropod, *Alviniconcha* aff. *hessleri* (22), ectosymbiont of the hydrothermal shrimp, *Symmetromphalus* (19), a gill endosymbiont (29). An *Osedax* sp. of the most closely related isolate is a cold-adapted mesophilic and heterotrophic bacteria (28).

**Rimicaris exsulcata** (5), and a deep-sea snail of a *Lepetodrilus* associated *Episoloproteobacterium* (4).

In this study, other phyotypes were also detected in *Episoloproteobacteria*, as a bone-eating worm *Oxedas mucofloris* endosymbiotic-related phyotypes, namely, *Oseax scomberoides* endosymbiotic-affiliated bacteria (29). There was no description of the endosymbiont in *Epsilonproteobacteria* affiliated to *Oxedas mucofloris* in a recent paper (29). An *Arcobacter* sp. of the most closely related isolate is a cold-adapted mesophilic and heterotrophic bacteria (28). Species in the genera *Arcobacter* are often detected from deep sea sulfur mats near and on seeps (16). The phyotypes are thought to be deep sea sulfur oxidizers. Other signature epipsymbiotic bacteria of *S. crosnieri* are well known to cluster, namely, marine epibiont group I (30), belonging to the phylum of Gammaproteobacteria. Epibiotic phyotypes 8 and 9 are located in marine epibiont group I, although the most closely related sequence is that of *Cocilemonas flava* (GenBank Accession AB49521) isolated from the sand snail *Umbonium costatum*, which lives by burrowing into shallow sand or mud (23). Various sequences related to the symbionts of deep-sea invertebrates close to the phyotypes were located as the *Thyasira* sp. symbiont (19), *Marithyas haddal* gill thioautotrophic symbionts (3), *Alviniconcha* sp. type I endosymbiont (22), *Siboglinum fiordicum* endosymbionts (24), *Oligobrachia hauknomosbiensis* endosymbiont (13), *Kiwa* sp. ectosymbiont (4), *Symmetromphalus* aff. *hageni* ectosymbiont (4), *Spirobachiria tripeira* symbiont (20), and *Rimicaris* sp.
electosymbiont (5), which seem to be putative sulfur-oxidizing bacteria.

Epibiotic phyletype 14 was detected in the phylum of flavobacteria. The most related sequence was that of an epibiotic of R. exoculata, namely, R. epibiont-associated flavobacteria (5). Furthermore, the other phyletypes were located near those of free-living Alphaproteobacteria and Flavobacteria. Epibiotic phyletypes 7 and 11 were related to Formosa sp. (14), which were detected by denaturing gradient gel electrophoresis analysis of a commercial shallow raceway marine recirculation system, and related to Lacinatrix sp. strain 5H-3-7-4 (8), which is a polysaccharide-degrading strain isolated from the subseafloor sediments. All of the phyletypes belonging to Alphaproteobacteria were related to the species of rhodobacterales, which are often isolated from marine environments as determined on the basis of GenBank information. The putative free-living bacteria were observed as spherical and cocoid by microscopy.

Phylogenetic analysis of biofilms

During the cultivation experiment, white bacterial mats and films accumulated on the surfaces of the bed of sea sand in the H$_2$S feeding device and tank wall (Fig. 3A and B). The DAPI-stained films were then observed by microscopy (Fig. 3C and D). The biofilms seemed to consist of bacterial consortia; therefore, phylogenetic analysis was carried out to examine the profile of the growth-stimulated bacteria in biofilm, which were collected from the tank wall 70 d after the start of cultivation. The phylogenetic tree of consortia in the biofilms is shown in Fig. 7. Fourteen biofilm phyletypes were located in Gammaproteobacteria and Epsilonproteobacteria and Flavobacteria; however, no phyletype belonging to Alphaproteobacteria was detected.

Phyletypes were categorized into the following groups:
- R. epibiont-associated Flavobacteria, biofilm phyletype 14;
- Sulfurovum-affiliated bacteria, biofilm phyletype 5;
- and Osedax mucifloris endosymbiont-affiliated bacteria, biofilm phyletypes 1, 7, 11, and 13. Interestingly, seven phyletypes were closely related to the sequences of endosymbionts of Alvinicroncha sp. type 2 (22), and hydrothermal vent eubacterium PVB_OTU_6, with sequence identities of more than 96%. Phyletypes that clustered with endosymbiont sequences were detected from snails collected from the Vienna Woods site, Manus Basin, 3°9.8'S/150°16.7'E, 2510 m. The clade was named group B in a previous study (22).

Biofilm phyletype 3 of Gammaproteobacteria was closely related to free-living Thiomicrosira species with 96% sequence identity; therefore, the phyletype was derived from free-living Thiomicrosira sp and was different from marine epibiont group I.

Discussion

To investigate the effects of hydrogen sulfide feeding on epibiotic communities on the surface of the deep-sea crab, S. crosnieri, a rearing tank with an H$_2$S-supply mechanism was developed in this study for the first time. The crabs and epibionts were cultivated for 84 d using the tank system, and the structures of epibionts and bacterial mats obtained secondarily were analyzed by microscopy, real-time PCR analysis, and phylogenetic analyses.

Kawagucci et al. (7) reported that H$_2$S was not detected near the Bathymodius colony in the Iheya North field; however, the quantification of H$_2$S in natural environments, especially in the deep sea, is difficult in terms of accuracy because as a reducing agent, H$_2$S spontaneously oxidizes during a long sampling period; therefore, there is possibility that the estimation was affected by spontaneous oxidation. This is one reason why there are few detailed reports on the effects of H$_2$S on the invertebrates and microorganisms in the deep sea. We developed a simple semiconductor gas sensor and on-off control system attached to the rearing tank, and controlled H$_2$S at a specific concentration, between 5 and 40 µmol L$^{-1}$ during two distinguishable periods excluding the period when the cooling unit malfunctioned. From our cultivation experiments (Fig. 2A), H$_2$S at concentrations lower than 40 µmol L$^{-1}$ was continuously provided under a microaerobic condition of DO concentrations between 1.0 and 1.5 mg L$^{-1}$, which corresponds to saturation between 8.12 and 12.2% at 5.2°C. These conditions stimulated the growth of deep-sea chemosynthetic bacteria related to sibomibionts in this study. Based on the findings, therefore, the conditions may be representative conditions near hydrothermal vents in the deep sea, where chemoautotrophic ecosystems are located. In other words, H$_2$S concentrations near the hydrothermal vents may be higher than those estimated by sampling analyses. The physical and chemical conditions for their growth, obtained from this experiment, will also be significant information for the isolation of deep-sea chemosynthetic bacteria.

From the microscopy findings (Fig. 4) and real-time PCR analysis (Fig. 5) of the surfaces of setae, the growth of epibiotic bacteria appeared to be stimulated by a low concentration of H$_2$S, as determined by the comparison between the two different cultivations with and without H$_2$S feeding. Furthermore, phylogenetic analysis of epibionts (Fig. 6) showed that the rearing tank system increased not only the number of epibionts such as Sulfurovum-affiliated bacteria and marine epibiont group I but also R. epibiont- and Osedax-affiliated sibomibiont groups and several free-living heterotrophic bacteria. According to the results, putative sulfur-oxidizing bacteria belonging to Sulfurovum-affiliated bacteria and marine epibiont group I seem to operate via sulfur-oxidizing respiration by using H$_2$S and its spontaneously oxidized compounds including sulfur, thiosulfate, and sulfite, in the artificial deep sea environment at a low temperature and a low DO concentration with H$_2$S provided continuously. A deep-sea isolate Sulfurovum sp. NBC37-1, which is related to an epibiont of S. crosnieri, was reported to be able to use elemental sulfur as both an electron acceptor and donor via its hydrogen-sulfur respiration and thiosulfate-oxidizing nitrate/oxygen respiration pathway under “mixing zone” conditions, where the redox condition shifted from reductive to oxidative, near a hydrothermal vent corresponding to the present artificial condition (17). R. epibiont-affiliated bacteria belonging to Flavobacteria and Osedax-affiliated sibomibiont groups belonging to Epsilonproteobacteria seemed to be cold-adapted mesophilic and heterotrophic bacteria. These bacteria may secondarily grow using metabolites from chemosynthetic bacteria and the crabs.

Phylogenetic analysis of biofilms (Fig. 7) showed that...
the phylotypes belong to *Rimicaris* epibiont-associated *Flavobacteria*, *Sulfurovum*-affiliated bacteria, and *Osedax* *mucifer* endosymbiont-affiliated bacteria, which correspond to the above results of the analysis of epibions (Fig. 6). Growth of these bacteria would depend on chemical and physiological conditions other than gravitational pressure, and host-symbiont linkages might not necessarily be a significant factor. On the other hand, marine epibiont group I belonging to *Gammaproteobacteria*, which have been detected in epibiont phylotypes, was not detected. This finding implies that marine epibiont group I tightly bind to their host by a physiological linkage, not only because of environmental factors. Interestingly, one of the major clades of phylotypes was closely related to an endosymbiont of the snail, *Alviniconcha* sp. type II, which was collected from the deep sea near the Hawaiian Islands. Significant growth factors for these bacteria are the chemical and physical conditions.

There are a lot of reports about the host-symbiont linkage of invertebrates through field work approaches (2–5, 13, 17, 19, 20, 22, 24, 27, 29, and 30). Such approaches pose difficulty in clarifying the cause-effect relationship between growth factors and bacterial growth. In the present study, we were able to examine preliminarily the effects of H$_2$S on the deep-sea crab *S. crosnier* and its epibionts in the laboratory, using our own novel rearing tank. Our rearing tank system will be a powerful tool for understanding the host-symbiont linkages of invertebrates inhabiting areas near deep-sea vents, particularly the bivalves, *Calypogena* spp., which are considered to possess a strong host-symbiont linkage because of the reduced genome of the symbiont (10). Further developments of cultivation experiments are important for studies of deep-sea invertebrates and microorganisms. Further results of cultivating organisms will be described elsewhere in the future.

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