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SHORT COMMUNICATION

Association of Thioautotrophic Bacteria with Deep-Sea Sponges

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Abstract We investigated microorganisms associated with a deep-sea sponge, *Characella* sp. (Pachastrellidae) collected at a hydrothermal vent site (686 m depth) in the Sumisu Caldera, Ogasawara Island chain, Japan, and with two sponges, *Pachastrella* sp. (Pachastrellidae) and an unidentified Poecilosclerida sponge, collected at an oil seep (572 m depth) in the Gulf of Mexico, using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) directed at bacterial 16S rRNA gene sequences. In the PCR-DGGE profiles, we detected a single clearly dominant band in each of the *Characella* sp. and the unidentified Poecilosclerida sponge. BLAST search of their sequences showed that they were most similar (>99% identity) to those of the gammaproteobacterial thioautotrophic symbionts of deep-sea bivalves from hydrothermal vents, *Bathymodiolus* spp. Phylogenetic analysis of the near-full length sequences of the 16S rRNA genes cloned from the unidentified Poecilosclerida sponge and *Characella* sp. confirmed that they were closely related to thioautotrophic symbionts. Although associations between sponges and methanotrophic bacteria have been reported previously, this is the first report of a possible stable association between sponges and thioautotrophic bacteria.

Keywords Deep sea · Sponge · Thioautotrophic bacteria · Associated bacteria · Symbiosis

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Introduction

Sponges are known from various marine environments (Siegl et al. 2008) and attract attention not only from biologists but also from natural product chemists because of the richness of bioactive substances (for a review, Sipkema et al. 2005). They are filter feeders, usually preying on microorganisms, but are also associated with microorganisms, which can account for as much as 57% of the biomass in the sponge (Willenz and Hartman 1989). In many cases, the associated microbes are thought to be sponge specific rather than just casual (Siegl et al. 2008; Taylor et al. 2007). Known microbial associates include autotrophic unicellular eukaryotic microalgae, archaea, cyanobacteria, and photo-

synthetic bacteria as well as a variety of heterotrophic bacteria (Taylor et al. 2007).

In deep-sea environments, such as hydrothermal vents and seeps, dense communities of thioautotrophic symbiont-containing invertebrates, e.g., vestimentiferan tubeworms and bivalves, which nutritionally depend on primary production by their symbionts, are often present (for a review, see Stewart et al. 2005). Although carnivorous sponges associated with methane-oxidizing bacteria are known from the deep sea (Vacelet et al. 1995; Vacelet et al. 1996; Vacelet and Boury-Esnault 2002), no sponge associating with thioautotrophic bacteria has yet been reported. We consider it probable that some deep-sea sponges dwelling near hydrothermal vents and/or seeps associate with thioautotrophic bacteria.

We collected a sponge in the Sumisu Caldera, Ogasawara Island chain, Japan that occurred in patches closely associated with vestimentiferan tubeworms, smelled of hydrogen sulfide, and was considered to be a prime candidate to host symbiotic thioautotrophic bacteria by one of the authors (DL). We also collected two sponges at an oil seep in the Gulf of Mexico, USA, where abundant tubeworms were also present. To examine the possibility of the presence of thioautotrophic associated bacteria in these deep-sea sponges, DNA was extracted from the sponges and analyzed using polymerase chain reaction (PCR) amplification of bacterial 16S rRNA genes in combination with denaturing gradient gel electrophoresis (DGGE). Here we provide the first evidence for an association between deep-sea sponges and thioautotrophic bacteria.

Materials and Methods

The deep-sea sponge from the Sumisu Caldera (Sumisu Caldera Sponge (SC-S)) was collected by the ROV *Hyper-Dolphin* (Dive no. 84 on 10 March 2002 at 686 m depth) at a hydrothermal vent site within the Sumisu Caldera, Ogasawara Island chain, Japan (31°28.1786′N, 140°04.2580′E) (Fig. S1A and B, Table 1). Sponge patches occurred with vestimentiferan tubeworm patches, and the collected sponges smelled strongly of hydrogen sulfide. After collection, sponges were rinsed with seawater and frozen at −80°C. For a taxonomic examination, some parts of the sponge were preserved in hexamine-saturated 10% formalin-seawater (pH 7.5; Wako Pure Chemical Industries, Osaka, Japan). By morphological examination of spicules and spicule arrangement, the sponge was found to belong to the genus *Characella* (Order, Astrophorida; Family, Pachastrellidae) according to Maldonado (2002). Molecular identification using *co1* (cytochrome oxydase subunit 1 gene: accession no. AB453834) agreed with this identification (Fig. S3A). This sponge is designated as *Characella* sp. SC-S in this study. Vestimentiferan tubeworms, *Escarpia* sp. E1, *Lamellibrachia* sp., *Alaysia* sp. A1, and *Alaysia* sp. A5, which were identified according to Kojima et al. (2003), were also collected, fixed in 70% ethanol, and stored in a freezer (−80°C) until used. Deep-sea mussels, *Bathymodiolus septemdierum* (Bsp6 and Bsp8), were also collected at the same site. Their gills were rinsed and frozen at −80°C until used.

A large white sponge [Gulf of Mexico Big White Sponge (GM-BWS); Table 1; Fig. S2A] was collected on Johnson SeaLink Dive no. 4583 on 3 September 2003 from 572 m depth at an oil seep site in the Gulf of Mexico (27°25.670′N, 93°35.421′W; Fig. S2B). A small blue sponge [Gulf of Mexico Small Blue Sponge (GM-SBS); Table 1; Fig. S2C] attached to the GM-BWS was also used for further analysis. From the GM-BWS, a slice (ca. 40 mm diameter, ca. 5 mm thickness) was cut out, and the surface layer (about 3 to 5 mm from the edge) was thereafter removed with a sterile surgical blade. The core of this slice was preserved in a DNA extraction buffer (10 mM Tris–HCl, 100 mM EDTA, pH 8.0, containing 0.5% sodium dodecyl sulfate), in which the slice was dissolved. A small portion (ca. 5 mm in diameter) of GM-SBS was preserved in the DNA extraction buffer. They were stored at room temperature for a month until used. For taxonomic identification, small portions of GM-BWS were fixed in 10% formalin-seawater and in 2.5% glutaraldehyde (TAAB, USA) in filtered seawater and were preserved in 70% ethanol until used. Morphological examination indicated that GM-BWS was attributable to the genus *Pachastrella* (Family, Pachastrellidae) according to Maldonado (2002). In the later section of this study, this sponge is designated as *Pachastrella* sp. GM-BWS. Molecular identification of this sponge was not possible because no amplicon was obtained by PCR for either the *co1* or the 18S rRNA gene with any of several primer sets. Taxonomic identification was not possible for GM-SBS because all GM-SBS samples were used for DNA extraction. However, phylogenetic analyses based on *co1* (accession no. AB453833; Fig. S3A) and 18S rRNA gene (accession no. AB453832; Fig. S3B) suggested that this sponge is in the order Poecilosclerida. In the later section of this study, this sponge is designated as Poecilosclerida sponge GM-SBS.

For PCR-DGGE analysis, DNA was extracted from the sponges, tubeworms, and mussels collected in the Sumisu Caldera and in the Gulf of Mexico (Table 1). Two frozen specimens of *Characella* sp. SC-S were cut longitudinally, and aliquots (height, width and depth $=5\times5\times2$ mm) of distal tip surface, distal tip internal core, mid-region surface, mid-region internal core, basal surface, and basal internal core were taken with a sterile spoon. DNA was extracted from small portions (ca. 2 mm diameter, 10 mm

Table 1 Sample list

Sample	Scientific name	Place of collection	Location	Depth (m)	Date of collection	Preservation for DNA analysis	Fixation for morphological taxonomy	Preservation for morophological taxonomy
Sumisu Caldera sponge	*Characella* sp. SCS (Pachastrellidae)	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	Frozen at −80°C	Hexamine-saturated 10% formalin-seawater at room temperature	70% ethanol at room temperature
Vestimentiferan tubeworm	*Escarpia* sp. E1	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	In 70% ethanol at −80°C	70% ethanol	70% ethanol at −80°C
Vestimentiferan tubeworm	*Lamellibrachia* sp.	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	In 70% ethanol at −80°C	70% ethanol	70% ethanol at −80°C
Vestimentiferan tubeworm	*Alaysia* sp. A1	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	In 70% ethanol at −80°C	70% ethanol	70% ethanol at −80°C
Vestimentiferan tubeworm	*Alaysia* sp. A5	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	In 70% ethanol at −80°C	70% ethanol	70% ethanol at −80°C
Mussel	*Bathymodiolus septemdierum* Bsp6	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	Frozen at −80°C	70% ethanol	70% ethanol at −80°C
Mussel	*Bathymodiolus septemdierum* Bsp8	Sumisu Caldera, Ogasawara, Japan	31°28.1786′N, 140°04.2580′E	686	Mar. 10, 2002	Frozen at −80°C	70% ethanol	70% ethanol at −80°C
Gulf of Mexico Big White Sponge	*Pachastrella* sp. GM-BWS (Pachastrellidae)	Gulf of Mexico	27°25.670′N, 93°35.421′W	572	Sept. 3, 2003	In DNA extraction buffer[a] at room temperature	10% formalin-seawater and 2.5% glutaraldehyde seawater	70% ethanol at room temperature
Gulf of Mexico Small Blue Sponge	Unidentified Poecilosclerida sponge GM-SBS (order Poecilosclerida)	Gulf of Mexico	27°25.670′N, 93°35.421′W	572	Sept. 3, 2003	In DNA extraction buffer[a] at room temperature	None	None

[a] DNA extraction buffer [10 mM Tris–HCl, 100 mM EDTA, pH 8.0, containing 0.5% sodium dodecyl sulfate (SDS)]

length) of the trophosomes of the vestimentiferan tube-worms and from the gills of the *Bathymodiolus* mussels. Pieces of the tissues were ground with an autoclaved mortar and pestle in 1 mL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and transferred to 2-mL sample tubes. After extraction with 1 mL TE-saturated phenol (Nippon Gene Co. Ltd., Tokyo, Japan), they were washed with 1 mL phenol/chloroform/isoamylalcohol (PCI , 25:24:1), then with 1 mL chloroform/isoamylalcohol (24:1), and the DNA was precipitated with 1 mL isopropanol.

Two hundred microliters of the dissolved sample of *Pachastrella* sp. GM-BWS in the DNA extraction buffer was dialyzed against 10 mL of TE buffer for 30 min and then with replenished 10 mL TE buffer for 24 h at 25°C by using Slide-A-Lyzer 10K dialysis cassettes (Pierce, IL, USA). The DNA was extracted with an equal volume of phenol, washed with an equivalent volume of PCI, and precipitated with an equivalent volume of isopropanol.

Extracted DNAs were purified with a GFX genomic blood DNA purification kit (GE Healthcare UK Ltd., UK) according to the manufacturer's instructions.

PCR-DGGE of 16S rRNA genes was carried out according to Muyzer et al. (1993). The fragments (V3 region, ca. 200 bp) of 16S rRNA gene sequences were amplified by PCR using the primer sets (Table 2) with AmpliTaq Gold, 10× PCR buffer and dNTP AmpliTaq Gold kit (Applied Biosystems Inc. Foster City, CA, USA). For PCR, we employed a touchdown protocol: 7 min of Taq DNA polymerase activation at 94°C; for the first 20 cycles, denaturation at 94°C for 1 min; annealing at decreasing temperature from 65°C to 56°C (1°C decrease for every 2 cycles) for 1 min; extension at 72°C for 2 min: next 15 cycles; at 94°C for 1 min; at 55°C for 1 min; at 72°C for 2 min; the final extension at 72°C for 10 min: by using GeneAmp PCR System 9600 (Applied Biosystems). For DGGE analyses, the amplicons were analyzed by using a D-code DGGE complete system (BIO RAD, CA, USA) operated at 60°C for 3.5 h at 200 V in a linear 30% to 60% denaturant agent gradient (100% denaturant agent contained 7 M urea and 40% deionized formamide) with 10% polyacrylamide gels [polyacrylamide gel, ratio of acrylamide HG (Wako Pure Chemical Industries) to bisacrylamide (Wako Pure Chemical Industries), 37.5:1]. After DGGE, the gels were soaked for 30 min in SYBR Green I nucleic acid gel stain (1:10,000 dilution; Lonza, Rockland, ME, USA) and photographed on an UV transilluminator with a CCD camera.

For sequence determination of DGGE bands, bands were excised from the gel, then DNA was extracted with 100 µL TE buffer and purified by ethanol precipitation. After checking the purity of the DNA by DGGE, the sequence of the purified DNA was analyzed with an ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the 341f and 534r primers (Table 2).

Close to full-length 16S rRNA gene fragments of the thioautotrophic bacteria were amplified from the extracted DNA by PCR using several universal primer sets (8f and 1510r for bacteria in *Characella* sp. SC-S and Poecilo-sclerida sponge GM-SBS or 8f and 1492r for bacteria in *B. septemdierum*; Table 2) and the touchdown PCR-DGGE protocol described above. Amplicons were purified by a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and cloned with a QIAGEN PCR Cloning plus Kit according to the manufacturer's instructions. After checking inserts by PCR and electrophoresis, plasmids were purified by a QIAquick Spin Purification Kit (QIAGEN). Their nucleotide sequences were analyzed with an ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems) using a BigDye Terminator v3.1 Cycle

Table 2 List of primers

Target gene/purpose	Primer	Sequence[a]	Position[b]	Reference
Bacterial 16S rDNA/ PCR-DGGE	GC-341F	5′ -CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG-3′	341–357	Muyzer et al. 1993
	534r	5′ -ATT ACC GCG GCT GCT GG-3′	534–581	
Bacterial 16S rDNA/ gene cloning	8f	5′ -AGA GTT TGA TCC TGG CTC AG-3′	8–27	Weisburg et al. 1991
	341f	5′ -CCT ACG GGA GGC AGC AG-3′	341–357	Muyzer et al. 1993
	1492r	5′ -GGT TAC CTT GTT ACG ACT T-3′	1510–1492	Hiraishi 1992
	1510r	5′ -GGC TAC CTT GTT ACG A-3′	1510–1495	Uchino et al. 1997

f forward primer, *r* reverse primer

[a] Sequence with underline, GC clamp

[b] The numbering of position is based on 16S rDNA of *Escherichia coli*

Sequencing Kit (Applied Biosystems) and the same primers as in the PCR for the sequencing reaction and specifically designed primers for sequence walking.

The most similar gene sequences to the obtained DNA sequences were searched by BLAST (Altschul et al. 1997) in GenBank. The sequences of the cloned DNAs were aligned with other sequences from the DNA databases by clustal W (Thompson et al. 1994), edited by eye with the BioEdit program (Hall 1999), and were analyzed by the neighbor-joining method with MEGA ver3.1 (Kumar et al. 2004). Reliabilities of the clusters were examined by 1,000 bootstrap re-samplings (Felsenstein 1985). Accession numbers of gene sequences of the sponges and of their associated microbes are shown in Fig. 3 and Tables S1 and S2.

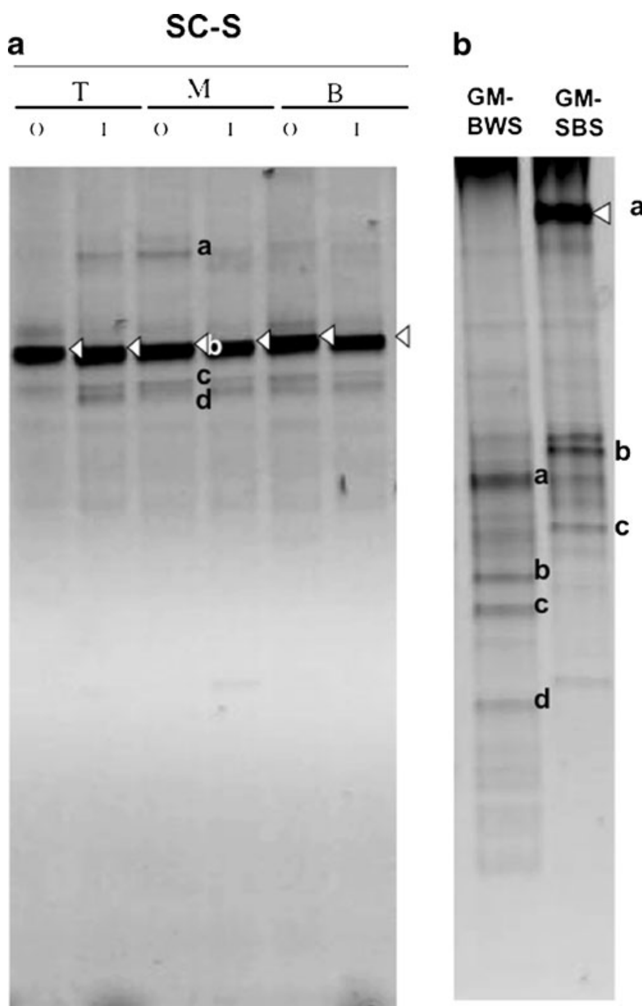

Fig. 1 DGGE profiles of the V3 region of 16S rRNA genes of the sponges from the Sumisu Caldera (**a**) and the Gulf of Mexico (**b**), which were amplified by PCR with primers, GC-341F and 534r. **a** *Characella* sp. SC-S: *O* outside surface of the sponge, *I* inner core, *T* distal tip region, *M* mid region, *B* basal region. **b** *Pachastrella* sp. GM-BWS and Poecilosclerida sponge GM-SBS. One major band was found, respectively, in the *Characella* sp. SC-S and the Poecilosclerida sponge GM-SBS (*arrowheads*). Four major bands (*a*–*d*) were detected from the *Pachastrella* sp. GM-BWS. Accession numbers of the bands are listed in Table S1

Results and Discussion

Bacteria Associated with Deep-Sea Sponges

PCR-DGGE analysis of the bacterial 16S rRNA gene sequences obtained from the *Characella* sp. SC-S showed a single major band and several minor bands (Fig. 1a). BLAST search of the DNA database (DDBJ/GenBank/EMBL) showed that the sequences of the major bands from various parts of *Characella* sp. SC-S (Fig. 1a, SC-S M-O-b and corresponding bands in other lanes) were the same and most similar to that of the thioautotrophic symbiont of *Bathymodiolus* sp. from the Juan de Fuca vents (99% nucleotide identity; Table S1; McKiness et al. 2005). The

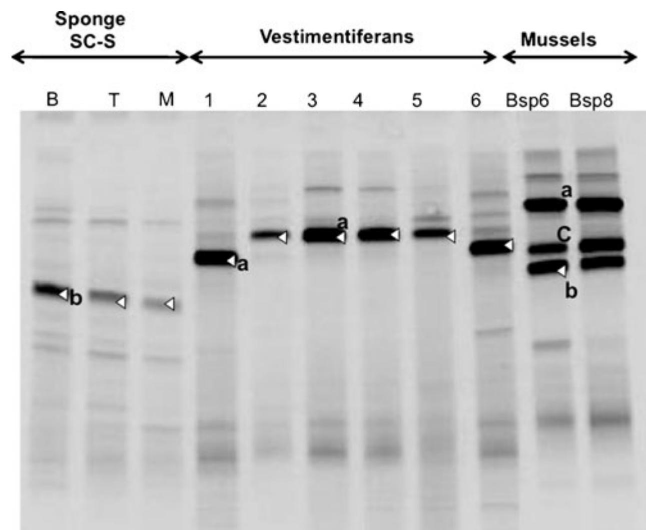

Fig. 2 DGGE profiles of V3 region of bacterial 16S rRNA gene (135–164 bp without primers) amplified by PCR with primers, GC-341F and 534r, from the *Characella* sp. SC-S, tubeworms, and mussels collected in the Sumisu Caldera. Lanes *B*, *T*, and *M*, respectively, indicate the basal (outer surface), tip (outer surface), and middle (inner core) portions of the *Characella* sp. SC-S. Mobilities of the major bands from the different parts of the *Characella* sp. SC-S (band *b* on lanes *B*, *T*, and *M*) were the same, but differed from those of the four vestimentiferan tubeworm species (*arrowheads* on *lanes 1* to *6*) and from those of two mussels (*B. septemdierum*) individuals (bands *a*, *b*, and *c* on *lanes Bsp6* and *Bsp8*). Three major bands were observed from mussels but sequence analysis showed that the middle band *c* was a mixture of sequences of *a* and *b*. A single major band was obtained from each lane of the sponge and of the vestimentiferan tubeworms [*1 Escarpia* sp., *2 Lamellibrachia* sp. (first individual), *3 Alaysia* sp. A1 (first individual), *4 Alaysia* sp. A1 (second individual), *5 Alaysia* sp. A5, *6 Lamellibrachia* sp. (second individual)]. *Arrowheads* Thioautotrophic symbiotic bacteria-like sequences. *Small letters a*, *b*, and *c* signify excised bands (see Table S1). Accession numbers are shown in Table S1

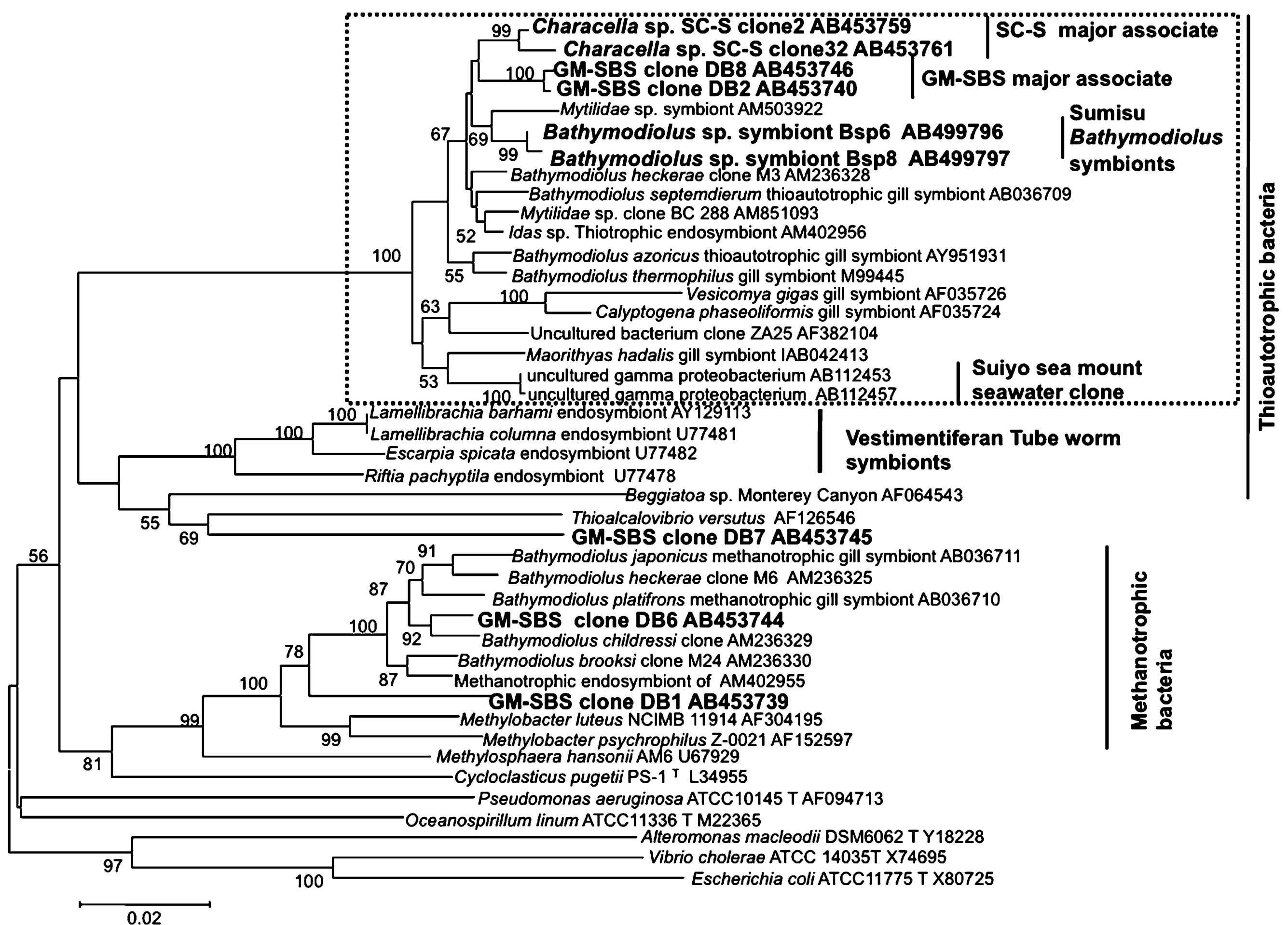

Fig. 3 Phylogenetic (neighbor-joining) tree of the nearly full length of 16S rRNA gene sequences amplified by PCR and cloned from bacteria in *Characella* sp. SC-S, Poecilosclerida sponge GM-SBS and *B. Septemdierum*. *Dotted line* A clade containing thioautotrophic symbionts of bivalves including *Bathymodiolus*, *Calyptogena*, and *Maorithyas.* Accession numbers of the corresponding sequences are shown after the names of the sequences

PCR-DGGE profile of the Poecilosclerida sponge GM-SBS also showed one major band with several minor bands (Fig. 1b). The dominant band sequence (Fig. 1b, GM-SBS-a) was most similar to that of the thioautotrophic symbiont of *B. puteoserpentis* from the Mid-Atlantic Ridge (>99% nucleotide identity; Table S1). These data suggest that these two deep-sea sponges were associated with thioautotrophic bacteria. The PCR-DGGE profile of *Pachastrella* sp. GM-BWS was quite different from that of the Poecilosclerida sponge GM-SBS even though the latter was attached to the former (Fig. 1b). The most prominent band in *Pachastrella* sp. GM-BWS (Fig. 1b, GM-BWS-a), which was not as conspicuous as that of Poecilosclerida sponge GM-SBS, was most similar to the major deltaproteobacterial sequence from the marine sponge, *Discodermia dissoluta* (Table S1; Schirmer et al. 2005), and no sequences similar to thioautotrophic gammaproteobacteria were detected (Table S1).

Sponges are filter feeders, and this raises the possibility that the thioautotroph-like bacteria detected in two of the sponges were derived from bacteria in the water column or in the sediment, including those that might be released by symbiont-containing invertebrates living near the sponges. In fact, the dominant planktonic bacterium at Suiyo sea mount has been reported to be similar to the symbiont of a *Bathymodiolus* mussel (Fig. 3, AB112453 and AB112457; Sunamura et al. 2004). To assess the possibility that the dominant bands resulted from environmental planktonic or sessile bacteria as a result of filter-feeding, DNA was extracted separately from various parts of the *Characell*a sp. SC-S. The PCR-DGGE profiles obtained from all six portions of the *Characella* sp. SC-S were very similar with the single major DNA bands of the same sequences (arrowheads in Fig. 1). This indicates that the bacterial phylotype responsible for the major band was unlikely to be a contaminant.

To further test the possibility that the dominant band in *Characella* sp. SC-S was an environmental contaminant, we compared the PCR-DGGE profile from the Sumisu Caldera sponge to those of the symbionts of various species of vestimentiferan tubeworms (*Escarpia* sp., *Alyasia* sp. and *Lamellibrachia* sp.) and a mussel species (*B. septemdierum*) collected from the Sumisu Caldera (Fig. 2). The major amplified band from the sponge had a different mobility from those of the tubeworm symbionts and from the three distinct bands from the mussel symbionts (Fig. 2, Table S1). These data indicate that the dominant band was not derived from filter-feeding on bacteria released from co-occurring thioautotrophic symbiont-containing organisms, or on free-living bacteria either near the sediment/seawater interface or in the water column. The most reasonable interpretation is that the dominant bacterial 16S rRNA gene phylotype in *Characella* sp. SC-S was an associated bacterium specific to this sponge. Two sponges from the Gulf of Mexico, the *Pachastrella* sp. GM-BWS and the Poecilosclerida sponge GM-SBS, which were collected as a single mass, had very different bacterial 16S rRNA gene DGGE profiles (Fig. 1b), even though the microbiota in their surrounding environments would almost certainly be the same. These data strongly support the hypothesis that the primary band in the PCR-DGGE of Poecilosclerida sponge GM-SBS was also not derived from an environmental bacterium, but rather from an associated bacterium specific to this sponge.

To obtain the nearly complete length of the 16S rRNA gene of the thioautotrophic associates from Poecilosclerida sponge GM-SBS, 10 clones were sequenced. In a neighbor-joining phylogenetic tree of the 16S rRNA gene sequences (Fig. 3), six of the clones out of 10 together with clones from *Characella* sp. SC-S (clones 2 and 32) formed a clade with thioautotrophic bivalve symbionts (Fig. 3, GM-SBS clones DB2 and DB8; Table S2, GM-SBS clones DB3-5 and DB10). Two clones from Poecilosclerida sponge GM-SBS formed a clade with those of free-living and symbiotic methanotrophs (Fig. 3, GM-SBS clones DB1 and 6). Of the remaining two clones, one was most similar to that of a gammaproteobacterium (Fig. 3, GM-SBS clone DB7) and the other to that of an alphaproteobacterium (Table S2, GM-SBS clone DB12).

Phylogenetic analyses of the bacterial sequences suggest that the dominant bacteria in both *Characella* sp. SC-S and Poecilosclerida sponge GM-SBS were thioautotrophic bacteria belonging to the *Gammaproteobacteria* (Fig. 3). Furthermore, these dominant bacteria in the two different sponge species, *Characella* sp. SC-S and Poecilosclerida sponge GM-SBS, which were sampled at widely separated locations in different geological settings and in different oceans, were quite closely related to each other. Taken together, the data presented here strongly suggest the occurrence of thioautotrophic-associated bacteria in sponges from both seeps and vents. Future studies demonstrating sulfur oxidation and/or inorganic carbon fixation by intact sponges, or detection of genes involved in these processes coupled with evidence of fixed carbon exchange between the putative associated bacteria and the sponge, will be necessary to demonstrate a nutritional chemoautotrophic symbiosis.

A methanotroph-like 16S rRNA gene sequence was identified in two of the ten clones from Poecilosclerida sponge GM-SBS (Fig. 3). Dual symbiosis with both methanotrophic and thioautotrophic bacteria has been reported previously in *Bathymodiolus* mussels (Distel et al. 1995; Fisher et al., 1993), and the possibility of such a dual symbiosis in this sponge deserves further study.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acid Res 25:3389–3402

Distel DL, Lee HKW, Cavanaugh CM (1995) Intracellular coexistence of methanotrophic and thiotrophic bacteria in a hydrothermal vent mussel. Proc Natl Acad Sci USA 92:9598–9602

Felsenstein J (1985) Confidence-limits on phylogenies—an approach using bootstrap. Evol 39:783–791

Fisher CR, Brooks JM, Vodenichar J, Zande J, Childress JJ, Burke RA Jr (1993) The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. Mar Ecol 14:277–289

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acid Symp Ser 41:95–98

Hiraishi A (1992) Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett Appl Microbiol 15:210–213

Kojima S, Ohta S, Yamamoto T, Yamaguchi T, Miura T, Fujiwara Y, Fujikura K, Hashimoto J (2003) Molecular taxonomy of vestimentiferans of the western Pacific, and their phylogenetic relationship to species of the eastern Pacific III. Alaysia-like vestimentiferans and relationships among families. Mar Biol 142:625–635

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Maldonado M (2002) Family Pachastrellidae Carter, 1875. In: Hooper JNA, van Soest RWM (eds) Systema porifera. A guide to the classification of sponges vol. 1. Kluwer, New York, pp 141–162

McKiness ZP, McMullin ER, Fisher CR, Cavanaugh CM (2005) A new bathymodioline mussel symbiosis at the Juan de Fuca hydrothermal vents. Mar Biol 148:109–116

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Appl Environ Microbiol 59:695–700

Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF, Hutchinson CR (2005) Metagenomic analysis reveals diverse polyketide synthetase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. Appl Environ Microbiol 71:4840–4849

Siegl A, Bayer K, Kozytska S, Hentschel U, Schmitt S (2008) Sponges and microbes—new frontiers in an ancient symbiosis. Vie et Milieu-Life Environ 58:165–174

Sipkema D, Franssen MCR, Osinga R, Tranoer J, Wijffels RH (2005) Marine sponges as pharmacy. Mar Biotechnol 7:142–162

Stewart FJ, Newton ILG, Cavanaugh CM (2005) Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces. Trends Microbiol 13:439–448

Sunamura M, Higashi Y, Miyako C, Ishibashi J, Maruyama A (2004) Two bacteria phylotypes are predominant in the Suiyo Seamount hydrothermal plume. Appl Environ Microbiol 70:1190–1198

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology and biotechnological potential. Microbiol Mol Biol Rev 71:295–347

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal-W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acid Res 22:4673–4680

Uchino Y, Yokota A, Sugiyama J (1997) Phylogenetic position of the marine subdivision of *Agrobacterium* species based on 16S rRNA sequence analysis. J Gen Appl Microbiol 43:243–247

Vacelet J, Boury-Esnault N, Filamedioni A, Fisher CR (1995) A methanotrophic carnivorous sponge. Nature 377:296–296

Vacelet J, Boury-Esnault N (2002) A new species of carnivorous deep-sea sponge (Demospongiae, Cladorhizidae) associated with methanotrophic bacteria. Cah Biol Mar 43:141–148

Vacelet J, Fialamedioni A, Fisher CR, Boury-Esnault N (1996) Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. Mar Ecol Prog Ser 145:77–85

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Willenz P, Hartman WD (1989) Micromorphology and ultrastructure of Caribbean sclerosponges. I. *Ceratoporella nicholsoni* and *Stromatospongia norae* (Ceratoporellidae Porifera). Mar Biol 103:387–402