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ORIGINAL PAPER

Isolation and diversity of planctomycetes from the sponge *Niphates* sp., seawater, and sediment of Moreton Bay, Australia

Hiroshi Izumi · Evgeny Sagulenko · Richard I. Webb · John A. Fuerst

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Abstract Planctomycetes are ubiquitous in marine environment and were reported to occur in association with multicellular eukaryotic organisms such as marine macroalgae and invertebrates. Here, we investigate planctomycetes associated with the marine sponge *Niphates* sp. from the sub-tropical Australian coast by assessing their diversity using culture-dependent and -independent approaches based on the 16S rRNA gene. The culture-dependent approach resulted in the isolation of a large collection of diverse planctomycetes including some novel lineages of *Planctomycetes* from the sponge as well as sediment and seawater of Moreton Bay where this sponge occurs. The characterization of these novel planctomycetes revealed that cells of one unique strain do not possess condensed nucleoids, a phenotype distinct from other planctomycetes. In addition, a culture-independent clone library approach identified unique planctomycete 16S rRNA gene sequences closely related to other sponge-derived sequences. The analysis of tissue of the sponge *Niphates* sp. showed that the mesohyl of the sponge is almost devoid of microbial cells, indicating this species is in the group of 'low microbial abundant' (LMA) sponges. The unique planctomycete 16S rRNA gene sequences identified in this study were phylogenetically closely related to sequences from LMA sponges in other published studies. This study has revealed new insights into the diversity of planctomycetes in the marine environment and the association of planctomycetes with marine sponges.

Keywords *Planctomycetes* · Marine sponges · 16S rRNA gene · Culture-dependent · Culture-independent · Nucleoid

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Introduction

Recently, there has been growing research interest in bacteria in the phylum *Planctomycetes* due to their unique cell biology and ecological and evolutionary significance (Fuerst and Sagulenko 2011; Jogler et al. 2012). These unique bacteria possess phenotypic characters unusual for the domain bacteria including peptidoglycan-less proteinaceous cell walls, budding reproduction, and intracellular membrane-bounded

compartmentalization within the cells (Fuerst and Sagulenko 2011) although the matter of compartmentalization and potential relation to Gram-negative walled bacteria have recently been debated (Speth et al. 2012; Santarella-Mellwig et al. 2013). Based on comparative gene sequencing analysis, the phylum *Planctomycetes* belongs to the *Planctomycetes–Verrucomicrobia–Chlamydia* (PVC) superphylum together with the phyla *Verrucomicrobia*, *Chlamydia*, and *Lentisphaera* and uncultured candidate phyla *Poribacteria* and OP3 (Wagner and Horn 2006; Gupta et al. 2012). Members of *Planctomycetes* have been proposed as transient forms between prokaryotes and eukaryotes (Reynaud and Devos 2011) due to eukaryote-like features including the presence of endocytosis-like ability for protein uptake and genes homologous to eukaryotic membrane coat proteins essential to membrane trafficking in eukaryotes (Lonhienne et al. 2010; Santarella-Mellwig et al. 2010).

Planctomycetes are ubiquitously present in natural environments including marine habitats (see review by Fuerst and Sagulenko 2011). Planctomycetes have been found in the marine water column (Delong et al. 1993; Fuchsman et al. 2012) and living in association with invertebrates (Webster et al. 2001; Pimentel-Elardo et al. 2003; Webster and Bourne 2007) and macroalgae (Bengtsson and Øvreås 2010; Lage and Bondoso 2011). A population study of prokaryotic picoplankton using the fluorescent in situ hybridization (FISH) technique revealed that the planctomycete population in surface water is less than 0.3 % of the total bacterioplankton (Schattenhofer et al. 2009). However, planctomycetes are known for their ability to attach to surfaces (Ward et al. 2006) and have been frequently found colonizing marine snow particles (Delong et al. 1993; Gade et al. 2004; Fuchsman et al. 2012). There are increasing number of studies reporting blooms of planctomycetes following diatom blooms (Morris et al. 2006; Pizzetti et al. 2011). This phenomenon was well documented in a study of the succession of *Pirellula*-like planctomycetes during a diatom bloom in Oregon coastal waters (Morris et al. 2006). Planctomycetes are believed to metabolize organic carbon released from the diatom bloom. The genome sequencing of the marine species of *Rhodopirellula baltica* revealed a high number of sulfatase genes, which are hypothesized to be involved in the initial breakdown of sulphated heteropolysaccharides produced by macroalgae (Glöckner et al. 2003). High numbers of sulfatase genes were found in all available marine planctomycete genomes including genomes of *Blastopirellula marina* and *Planctomyces maris* and on planctomycete fosmids from costal upwelling systems, suggesting the importance of marine planctomycetes for recycling of carbon from algal polymers (Woebken et al. 2007).

Planctomycetes were reported to occur in marine sponges (Webster et al. 2001; Sipkema et al. 2009; Mohamed et al. 2010). Planctomycete strains have been isolated in pure cultures from marine sponges on several occasions (Pimentel-Elardo et al. 2003; Gade et al. 2004; Sipkema et al. 2011), and the localization of planctomycetes within sponge mesohyl matrixes has been demonstrated using the FISH technique (Webster et al. 2001; Pimentel-Elardo et al. 2003). The 16S rRNA gene sequences of planctomycetes have been retrieved from marine sponges on numerous occasions using the clone library method (Webster et al. 2001; Thiel et al. 2007; Zhu et al. 2008; Sipkema et al. 2009; Mohamed et al. 2010). The phylogenetic analyses of 16S rRNA gene sequences retrieved from sponges show that some sequences of planctomycetes represent sponge-specific clusters exclusively retrieved from sponges (Simister et al. 2012). Furthermore, the identification of planctomycete 16S rRNA sequences from clone libraries prepared from sponge embryos and larvae has been reported (Enticknap et al. 2006; Schmitt et al. 2008), suggesting a possible scenario in which planctomycetes might be vertically transmitted and maintained through successive sponge generations.

In this study, the diversity of planctomycetes associated with the sponge *Niphates* sp. was examined by culture-dependent and -independent approaches. The culture-dependent approach resulted in the isolation of a large number of diverse planctomycetes including some novel lineages of *Planctomycetes* with novel phenotypic characters from *Niphates* sp. as well as sediment and water of Moreton Bay where this sponge occurs. In addition, the culture-independent study of clone libraries prepared from the sponge microbial community identified unique planctomycete sequences closely related to sequences retrieved from other sponges. The results of this study has implications for understanding the association of planctomycetes with the group of relatively poorly studied sponges called low microbial abundance (LMA) sponges, which do not harbor dense microbial

communities within their tissue unlike their well-studied counterparts, high microbial abundant (HMA) sponges.

Materials and methods

Sample collection

Samples of seawater and sediment were collected separately in sterile bottles in April 2009 from shallow water (27°29′S, 153°23′E; <1 m depth) near Moreton Bay Research Station, North Stradbroke Island, in a location where the sponge *Niphates* sp. (Queensland Museum species no. 1056) was growing. These samples were stored at 4 °C overnight before the bacterial isolation. One specimen of *Niphates* sp. was collected in October 2009. This sample was processed immediately for planctomycete isolation. The other specimen of *Niphates* sp. was collected in August 2009 for the construction of clone library. This specimen was frozen and stored at −80 °C until the molecular cloning processing. The specimen for transmission electron microscopy (TEM) experiments was collected in September 2011.

Isolation of planctomycetes

Two isolation methods were applied. The first method was based on the enrichment of inoculum prior to the plating of the enrichment culture onto the *Planctomycetes*-selective media. The inoculum of sediment was prepared by suspending 1 g of sediment in 9 mL of sterile artificial seawater (Aquasonic, NSW, Australia), while the inoculum of the sponge was prepared by homogenising 1 g of the sponge tissue in 5 mL of sterile artificial seawater using a sterile mortar and pestle after washing the sponge tissue three times with sterile artificial seawater. Seawater was used as the inoculum without addition of artificial seawater. Five different broth media with different carbon and energy sources were used for the enrichment of planctomycetes and assigned number as follows (Table 1): medium 1, M30 broth [2 g *N*-acetylglucosamine, 100 mg Na_2HPO_4, 20 mL Hutner's basal salts (Schlesner 1994), 10 mL Vitamin solution #6 (Schlesner 1994), 50 mL buffer 0.1 M Tris/HCl, pH 7.5, 920 mL artificial seawater]; 2, MChi broth [1 g chitosan (Sigma-Aldrich, MO), 100 mg Na_2HPO_4, 20 mL Hutner's basal salts, 10 mL Vitamin solution #6, 50 mL buffer 0.1 M Tris/HCl, pH 7.5, 920 mL artificial seawater]; 3, MCar broth [1 g carrageenan (Sigma-Aldrich, MO), 100 mg peptone, 20 mL Hutner's basal salts, 10 mL Vitamin solution #6, 50 mL buffer 0.1 M Tris/HCl, pH 7.5, 920 mL artificial seawater]; 4, MFuc broth [1 g fucoidan (Marinova, TAS, Australia), 100 mg peptone, 20 mL Hutner's basal salts, 10 mL Vitamin solution #6, 50 mL buffer 0.1 M Tris/HCl, pH 7.5, 920 mL artificial seawater]; 5, DPB (Staley et al. 1980) [100 mg peptone, 20 mL Hutner's basal salts, 10 mL Vitamin solution #6, 970 mL artificial seawater]. These broth media were supplemented with 200 μg/mL of ampicillin, 1,000 μg/mL of streptomycin, and 50 μg/mL of cycloheximide, except for DPB. Five milliliters of seawater or 1 mL of sediment suspension were inoculated into 75 mL of each M30, MChi, and DPB broth medium, while 0.25 mL of sponge homogenate was inoculated into each M30, MChi, MCar, MFuc, and DPB broth medium. The enrichment cultures were incubated on a rotary shaker at 28 °C for 1 month, except for the enrichment using DPB broth for which two incubation conditions were tested. One DPB enrichment culture was incubated under direct light (100 W tungsten light bulb) at room temperature without shaking, while other DPB enrichment culture was incubated in dark at 28 °C with shaking. Each week, 100 μL of each enrichment culture was plated on M13 and M30 media (Schlesner 1994) with a modification of artificial seawater level increased to full-strength. These media designated as M13+ASC and M30+ASC were made selective for planctomycetes by the incorporation of ampicillin (200 μg/mL), streptomycin (1,000 μg/mL), and cycloheximide (50 μg/mL) (Wang et al. 2002). The plates were incubated at 28 °C in the dark for 1 month, and the developed colonies were picked for single-colony purification.

The second method was based on the direct plating of the samples onto selective media. The inoculum of each sample prepared as described above was used as source of planctomycetes. Twofold dilution series of the samples up to 1/32 were prepared with artificial seawater for the seawater and sponge, while a tenfold dilution up to 10^{-4} was prepared for the sediment. M13+ASC and M30+ASC media (Table 1) were inoculated with 100 μL of each dilution. For each dilution, isolation plates were prepared in triplicate.

Table 1 List of enrichment and isolation media

	Carbon source	Selective agent	Use	Isolate designation	Reference
M30 broth	*N*-acetyl-glucosamine	ASC	Enrichment	DDM/DSM/MS33	Schlesner (1994)
MChi broth	Chitosan	ASC	Enrichment	DDC/DSC/MST	This study
MFuc broth	Fucoidan	ASC	Enrichment	MSF	This study
MCar broth	Carrageenan	ASC	Enrichment	MSC	This study
DPB-D broth	Peptone	N/A	Enrichment	DDD/DSD/MSD	Staley et al. (1980)
DPB-L broth	Peptone	Light	Enrichment	DWL/MSL	Staley et al. (1980)
M30 agar	*N*-acetyl-glucosamine	ASC	Direct plating (isolation)	DDSe30/DDSW30/ MS30	Schlesner (1994)
M13 agar	Yeast extract, peptone, glucose	ASC	Direct plating (isolation)	DDSe13/DDSW13/ MS13	Schlesner (1994)

Isolate designations in this table represent the isolates that were included in the phylogenetic tree (Fig. 1) and also isolates for which 16S rRNA sequences were deposited in GeneBank. Isolate designation with prefix 'DD' and 'DDSe' indicate isolates from sediment, while those with prefix 'DS', 'DW', 'DDSW' indicate isolates from seawater. Sponge isolates were indicated with prefix 'MS'. The letter or number following these prefix in the designation indicates isolation medium used

The plates were then incubated at 28 °C in the dark for 6 months for the seawater and sediment samples or for 1 month for the sponge sample. The developed colonies were then picked for single-colony purification using the same medium for subculturing.

Rep-PCR fingerprinting

A large number of planctomycete isolates were recovered from the sponge *Niphates* sp. using the direct plating technique. The BOX-PCR fingerprinting method was applied to determine the presence of clonal replicates among dominant isolates from the sponge displaying identical morphotypes. Isolates were selected for this fingerprinting based on the following criteria—two most numerically abundant colony types displaying (1) distinctive bright red color and (2) distinctive pink color. BOX-PCR banding patterns from genomic DNA of each isolate were produced by amplification with a primer BOXA1R (5′ CTA CGG CAA GGC GAC GCT GAC G 3′) (Versalovic et al. 1994) under the condition described by Vidgen et al. (2012) with modifications. The colony PCR technique was used for the source of DNA. A small amount of colony from an isolate pure culture was picked using a sterile pipette tip and suspended in 25 µL of the PCR reaction mix. After the gel electrophoresis, the banding patterns based on the number and position of bands were visually assessed.

16S rRNA gene sequencing of isolates

Genomic DNA was extracted from the isolates using a Wizard® Genomic DNA Purification Kit (Promega, WI) with the recommended protocol for Gram-negative bacteria. The 16S rRNA gene sequence was amplified with the 27f (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1492r (5′ TAC GGY TAC CTT GTT ACG ACT T 3′) primer set (Lane 1991). Amplified gene products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega). Nucleotide sequence analysis of the purified PCR products amplified using the primers 27f and 1492r was performed at the Australian Genome Research Facility (AGRF) using an AB3730xl DNA analyzer (Applied Biosystems, CA).

Construction of 16S rRNA clone library

In order to construct a 16S rRNA gene clone library representing the uncultured diversity of the sponge microbial community, extraction of DNA from *Niphates* sponge microbial community was performed according to the following protocol. Sponge homogenate prepared by the method described above was filtered through a 41-µm mesh filter (Millipore, MA) to remove the sponge tissue. One milliliter of the filtrate was centrifuged at 12,500 rpm for 5 min. After the removal of the supernatant, the pellet containing

sponge-associated microbial cells was washed with TE buffer. DNA was extracted from sponge-associated microbial cells using a Wizard® Genomic DNA Purification Kit (Promega) with the recommended protocol for Gram-negative bacteria. The 16S rRNA gene sequences of planctomycetes were amplified via PCR with two different primer sets in the combination of phylum *Planctomycetes*-specific forward primer and Bacteria-specific reverse primer. The amplification with the first primer set, Pla46f (5′ GGA TTA GGC ATG CAA GTC 3′) and 1390r (5′ GAC GGG CGG TGT GTA CAA 3′), were performed under the conditions described by Chouari et al. (2003), while the amplification with the second primer set, Pla40f (5′ CGG CRT GGA TTA GGC ATG 3′) and 1492r (5′ TAC GGY TAC CTT GTT ACG ACT T 3′), was performed under the conditions described by Derakshani et al. (2001). The amplified gene products were cleaned using the Wizard® SV Gel and Clean-Up System (Promega) and cloned into pGEM-T Easy Vector (Promega) following the manufacturer's instructions. Plasmid was purified using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega), and the plasmid containing the insert of correct size was screened via PCR with the primers T7 (5′ TAA TAC GAC TCA CTA TAG GG 3′) and SP6 (5′ TAT TTA GGT GAC ACT ATA G 3′). Nucleotide sequencing was performed with the primers T7 and SP6 at AGRF. The 16S rRNA gene clone sequences were deposited in the GeneBank database together with the 16S rRNA gene sequences of isolates under accession numbers JF443737–JF443806.

Phylogenetic analysis of 16S rRNA gene

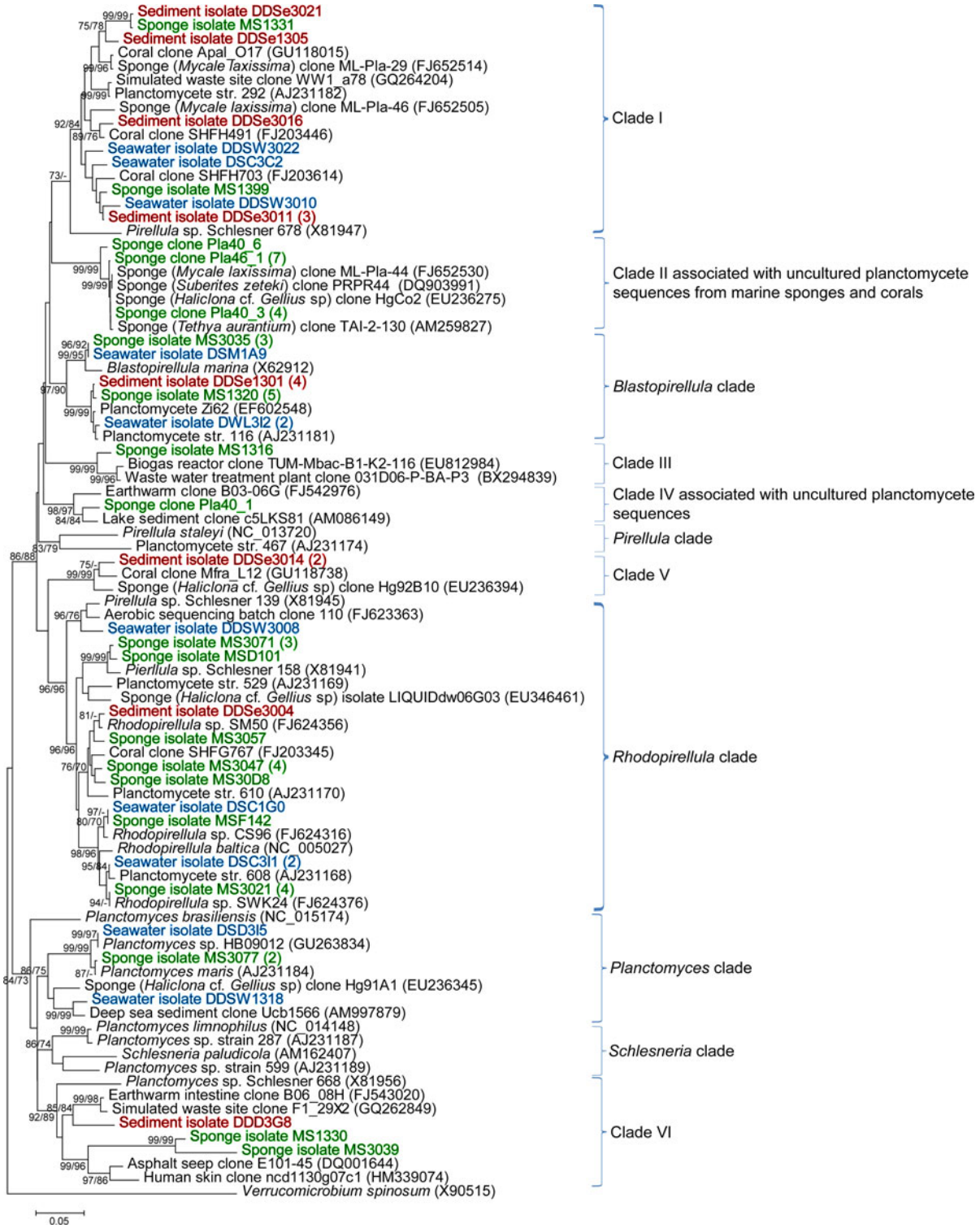
The sequences of 16S rRNA gene from isolates and from the clone library of the *Niphates* sponge microbial community were aligned against the reference sequences retrieved by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the GREENGENES program (DeSantis et al. 2006), followed by Lane masking to remove any hyper-variable region from alignments (Lane 1991). The presence of chimeric sequence was screened using BELLEROPHON program (Huber et al. 2004) available at GREENGENES website (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi). The alignment was exported into the PHYLIP program (Felsenstein 1989) for sequence similarity analysis. A similarity value below 98 % was chosen to define unique operational taxonomic units (OTUs) (Neufeld and Mohn 2006). Phylogenetic trees were constructed from the GREENGENES-generated alignment using the MEGA5.2 program (Tamura et al. 2011) by applying the neighbor-joining and maximum likelihood methods. Bootstrapping was performed using 1,000 replicates.

TEM of sponge tissue

Sponge specimens were cut into small pieces ($\sim$2 mm^3) and fixed in 3 % glutaraldehyde in filter-sterilized natural seawater. The fixed specimens were stored at 4 °C until further processing. After washing the samples in filter-sterilized natural seawater for 10 min, post-fixation was carried out in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 2 h at 4 °C. The post-fixed samples were washed in distilled water, dehydrated in a graded acetone series, and infiltrated with Epon resin. Polymerization was carried out at 60 °C for 1 day. The sponge tissue was exposed in the resin block using a razor blade and the sponge spicules were dissolved with 40 % hydrofluoric acid. Then tissue sections were cut to a 60 nm thickness using a Leica Ultracut T ultramicrotome and mounted on pioliform-coated copper grids. Sections were stained with 5 % uranyl acetate in 5 % methanol and Reynolds lead citrate and viewed using JEOL 1010 transmission electron microscope operated at 80 kV.

Ultrastructure analysis of a novel strain MS3039

The cells of the novel planctomycete strain M3039 were processed via cryosubstitution using the method described by Lonhienne et al. (2010) for TEM analysis. After the cryosubstitution process, the grids containing ultrathin sections of M3039 cells were floated on a Block solution consisting of 0.2 % w/v fish skin gelatin, 0.2 % w/v BSA, 200 mM glycine, and 1 × PBS. For detection of DNA, the grids were then floated on 8 µL of mouse anti-double strand DNA IgM monoclonal antibody (Abcam, Cambridge, UK), diluted 1:25 in blocking buffer. The grids were then washed three times in Block solution, before being incubated in 8 µL of goat anti-mouse antibody conjugated with 10 nm gold (British Biocell International, Cardiff, UK), diluted in 1:50 in Block solution. The sections were viewed using JEOL 1010 transmission electron microscope operated at 80 kV.


Sediment isolate DDSe3021
Sponge isolate MS1331
Sediment isolate DDSe1305
Coral clone Apal_O17 (GU118015)
Sponge (Mycale laxissima) clone ML-Pla-29 (FJ652514)
Simulated waste site clone WW1_a78 (GQ264204)
Planctomycete str. 292 (AJ231182)
Sponge (Mycale laxissima) clone ML-Pla-46 (FJ652505)
Sediment isolate DDSe3016
Coral clone SHFH491 (FJ203446)
Seawater isolate DDSW3022
Seawater isolate DSC3C2
Coral clone SHFH703 (FJ203614)
Sponge isolate MS1399
Seawater isolate DDSW3010
Sediment isolate DDSe3011 (3)
Pirellula sp. Schlesner 678 (X81947)
Clade I
Sponge clone Pla40_6
Sponge clone Pla46_1 (7)
Sponge (Mycale laxissima) clone ML-Pla-44 (FJ652530)
Sponge (Suberites zeteki) clone PRPR44 (DQ903991)
Sponge (Haliclona cf. Gellius sp) clone HgCo2 (EU236275)
Sponge clone Pla40_3 (4)
Sponge (Tethya aurantium) clone TAI-2-130 (AM259827)
Clade II associated with uncultured planctomycete sequences from marine sponges and corals
Sponge isolate MS3035 (3)
Seawater isolate DSM1A9
Blastopirellula marina (X62912)
Sediment isolate DDSe1301 (4)
Sponge isolate MS1320 (5)
Planctomycete Zi62 (EF602548)
Seawater isolate DWL3I2 (2)
Planctomycete str. 116 (AJ231181)
Blastopirellula clade
Sponge isolate MS1316
Biogas reactor clone TUM-Mbac-B1-K2-116 (EU812984)
Waste water treatment plant clone 031D06-P-BA-P3 (BX294839)
Clade III
Earthwarm clone B03-06G (FJ542976)
Sponge clone Pla40_1
Lake sediment clone c5LKS81 (AM086149)
Clade IV associated with uncultured planctomycete sequences
Pirellula staleyi (NC_013720)
Planctomycete str. 467 (AJ231174)
Pirellula clade
Sediment isolate DDSe3014 (2)
Coral clone Mfra_L12 (GU118738)
Sponge (Haliclona cf. Gellius sp) clone Hg92B10 (EU236394)
Clade V
Pirellula sp. Schlesner 139 (X81945)
Aerobic sequencing batch clone 110 (FJ623363)
Seawater isolate DDSW3008
Sponge isolate MS3071 (3)
Sponge isolate MSD101
Pierllula sp. Schlesner 158 (X81941)
Planctomycete str. 529 (AJ231169)
Sponge (Haliclona cf. Gellius sp) isolate LIQUIDdw06G03 (EU346461)
Sediment isolate DDSe3004
Rhodopirellula sp. SM50 (FJ624356)
Sponge isolate MS3057
Coral clone SHFG767 (FJ203345)
Sponge isolate MS3047 (4)
Sponge isolate MS30D8
Planctomycete str. 610 (AJ231170)
Seawater isolate DSC1G0
Sponge isolate MSF142
Rhodopirellula sp. CS96 (FJ624316)
Rhodopirellula baltica (NC_005027)
Seawater isolate DSC3I1 (2)
Planctomycete str. 608 (AJ231168)
Sponge isolate MS3021 (4)
Rhodopirellula sp. SWK24 (FJ624376)
Rhodopirellula clade
Planctomyces brasiliensis (NC_015174)
Seawater isolate DSD3I5
Planctomyces sp. HB09012 (GU263834)
Sponge isolate MS3077 (2)
Planctomyces maris (AJ231184)
Sponge (Haliclona cf. Gellius sp) clone Hg91A1 (EU236345)
Seawater isolate DDSW1318
Deep sea sediment clone Ucb1566 (AM997879)
Planctomyces sp. clade
Planctomyces limnophilus (NC_014148)
Planctomyces sp. strain 287 (AJ231187)
Schlesneria paludicola (AM162407)
Planctomyces sp. strain 599 (AJ231189)
Schlesneria clade
Planctomyces sp. Schlesner 668 (X81956)
Earthwarm intestine clone B06_08H (FJ543020)
Simulated waste site clone F1_29X2 (GQ262849)
Sediment isolate DDD3G8
Sponge isolate MS1330
Sponge isolate MS3039
Asphalt seep clone E101-45 (DQ001644)
Human skin clone ncd1130g07c1 (HM339074)
Clade VI
Verrucomicrobium spinosum (X90515)
0.05

◀ **Fig. 1** Phylogenetic tree of 16S rRNA gene sequences from planctomycete isolates and the sponge clone library and the related reference sequences obtained by BLAST search. The Neighbor-joining method was used to generate the phylogenetic tree. The isolates from sponges are indicated in *green* while isolates from seawater and sediment are indicated in *blue* and *red*, respectively. *Number in bracket* indicates the number of isolates or sequences within the same OTU based on the sequence similarity value of above 98 %. *Verrucomicrobium spinosum* was chosen as the outgroup and used to root the tree. Bootstrap values greater than 70 % calculated using the neighbor-joining and maximum likelihood methods are indicated at each node in order from left to right. The *scale bar* represents 0.05 nucleotide substitutions per site

Results

Isolation and phylogenetic analysis

In total 56 planctomycete isolates were cultured from the sponge *Niphates* sp. as well as sediment and seawater of Moreton Bay. Both direct plating and enrichment methods successfully yielded planctomycete strains. Based on the 16S rRNA gene sequences, the direct plating method resulted in the isolation of diverse planctomycetes and produced a large number of previously uncultured planctomycete strains. Thirty-four planctomycete isolates (61 % of the total isolates) were recovered by the direct plating method with the planctomycete-selective media. The direct plating of the environmental samples on the antibiotic-containing M30+ASC medium produced 26 isolates (46 %), while the direct plating on M13+ASC medium produced eight isolates (14 %), suggesting that M30+ASC medium, which contained *N*-acetyl-glucosamine as a sole source of carbon and nitrogen, was the preferred medium for the isolation of planctomycetes. In contrast, planctomycete strains obtained from the enrichment method were less diverse and closely related to previously cultured strains such as *Blastopirellula* strains from postlarvae of the giant tiger prawn *Penaeus monodon* (Fuerst et al. 1997) and *Rhodopirellula* strains from seawater and marine sediments (Winkelmann and Harder 2009). In terms of the diversity and novelty of isolates based on 16S rRNA gene sequences, the direct plating of the seawater and sediment samples on the planctomycete-selective M30+ASC and M13+ASC media was the most successful isolation method. In this direct plating approach, the majority of novel strains were recovered after more than 2 months of incubation. For some cases, 4 months of incubation on M30+ASC medium was required to recover slow-growing strains such as the sediment isolate DDSe3021 and the seawater isolate DDSW3022. In contrast, the direct plating of the sponge sample was proven to be difficult due to the overgrowth of fungal species within the 1 month of incubation. However, some novel planctomycete strains including the isolates MS3039 and MS1330 were recovered by this method. Despite the low number of novel strains obtained by the enrichment method, the enrichment culture containing chitosan as a sole source of carbon and nitrogen produced the seawater isolate DSC3C2, which was related to the seawater isolate DDSW3022. Moreover, the enrichment culture based on the diluted peptone broth (DPB) medium (Staley et al. 1980) produced the sediment isolate DDD3G8, which was related to the sponge isolates MS3039 and MS1330. These isolates can be considered as novel strains because of their low similarity values of 16S rRNA gene sequences to those of previously cultured strains. Among the 22 isolates (39 %) recovered by the enrichment method, 5 isolates

Table 2 Isolates within novel clades, closest database representatives, and their sequence similarity values to those representatives

Clade no.	Strain	Closest cultured representative (%)	Closest characterized representative (%)
Clade I	DDSe3021, DDSe1305, DDSe3016, DDSe3011, DDSe3012, DDSe3013, DDSW3022, DSC3C2, DDSW3010, MS1331, MS1399	Planctomycete str. 292 (<95 %)	*Blastopirellula marina* (<91 %)
Clade III	MS1316	Planctomycete str. 391 (90 %)	*Blastopirellula marina* (89 %)
Clade V	DDSe3014, DDSe3017	*Pirellula* sp. Schlesner 139 (<91 %)	*Rhodopirellula baltica* (<90 %)
Clade VI	DDD3G8, MS3039, MS1330	*Planctomyces* sp. Schlesner 668 (<89 %)	*Planctomyces brasiliensis* (<89 %)

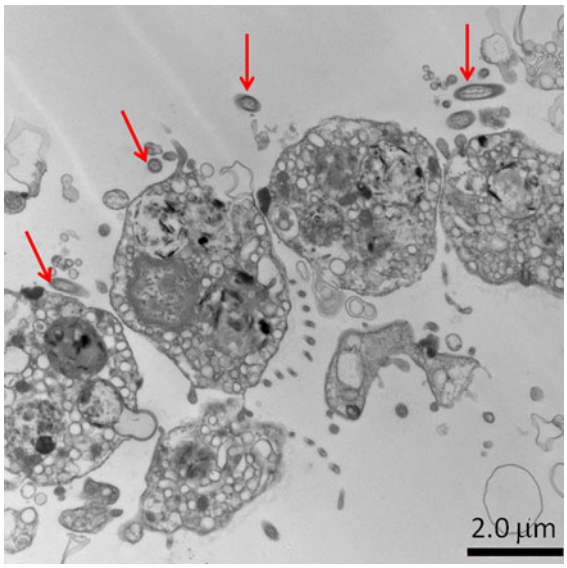


Fig. 2 Detection of bacteria in the sponge *Niphates* sp. TEM of ultrathin tissue section showing the absence of dense microbial community within the sponge tissue. *Red arrows* indicate bacteria-like cells

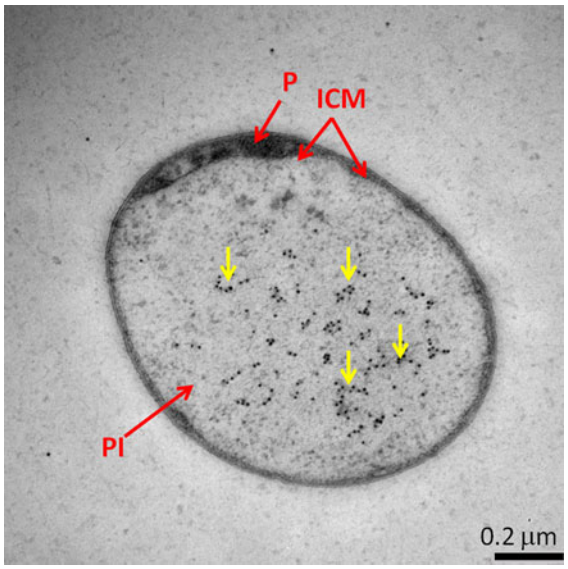


Fig. 3 TEM of high-pressure frozen cryosubstituted planctomycete strain MS3039 cell. DNA was immuno-labelled with gold particles (*yellow arrows*) and is seen as dispersed throughout the pirellulosome. Pirellulosome (Pl), paryphoplasm (P) and intracytoplasmic membrane (ICM) are indicated by *red arrows*

(9 %) were obtained from the chitosan-based enrichment culture, while 4 isolates (7 %) were obtained from the fucoidan-based enrichment culture. The carrageenan-based enrichment and *N*-acetylglucosamine-based enrichment cultures produced the lowest number of isolate with only 2 isolates (3 %) for each method.

Based on the 16S rRNA gene sequence similarity value below 98 % and the isolation sources, 33 unique OTUs were identified. Phylogenetic analysis based on the 16S rRNA gene revealed that the diversity of isolates constitutes seven genus-level clades including known marine clades of *Rhodopirellula*, *Blastopirellula*, and *Planctomyces* (Fig. 1). In addition, four novel clades, possibly comprising novel genera within the *Planctomycetes*, were identified. The formation of these clades was supported with significant bootstrap confidence, and these novel clades were designated as Clade I, III, V, and VI in Fig. 1 and Table 2. Sequence similarity values of those novel isolates to any characterized *Planctomycetes* species are below 91 %, indicating these strains might comprise novel genera (Stackebrandt and Goebel 1994). For example, Clade I consisted of 11 isolates from the seawater, sediment, and sponge in this study, one previously cultured planctomycete from Lake Fuhlensee (Griepenburg et al. 1999), and sequences of uncultured planctomycetes retrieved mostly from marine environments. Sequence similarity analysis shows that the similarity values of these isolates to the closest characterized species of *B. marina* are less than 91 %, suggesting that those strains might form a new genus associated with marine environments. In addition, two novel strains (MS3039 and MS1330) were isolated from the sponge and constitute Clade VI together with the sediment isolate DDD3G8 in the phylogenetic tree. The sponge isolates MS3039 and MS1330 have a similarity value of only 87 % to the closest 16S rRNA gene sequence retrieved from the gut of cetoniine scarab *Pachnoda ephippiata* (Egert et al. 2003).

BOX-PCR fingerprinting

The result of isolation using the direct plating method suggests that the diversity of culturable planctomycetes in the sponge was unique in its composition. The isolation plates prepared with the sponge homogenate were dominated by bright red colonies and pink colonies. Sequencing of 16S rRNA gene of the isolates

indicates that the sponge isolates were dominated by the members of the genus *Rhodopirellula*. It is estimated that sponge-derived *Rhodopirellula* with red and pink morphotypes comprised ~4,000 CFUs and 2,000 CFUs per gram of the sponge tissue, respectively. To estimate the microdiversity among the dominant isolates from the sponge, the BOX-PCR fingerprinting technique was applied. In total, 15 isolates representing the red colony morphotype and 14 isolates representing the pink colony morphotype were randomly selected and investigated using the BOX-PCR fingerprinting technique. BOX-PCR analysis of the dominant isolates with red colony morphotype showed that 73 % of the isolates had the same BOX-PCR banding pattern as *Rhodopirellula* strain MS3047, while the analysis of the second dominant group of isolates with pink colony morphotype showed that 71 % of the isolates have the same banding pattern as *Rhodopirellula* strain MS3021, indicating that these isolates represent two distinct clonal clusters or strains. The sequencing of 16S rRNA gene of representatives with the same banding pattern showed that they shared 100 % similarity in 16S rRNA gene sequence level, confirming the result of BOX-PCR.

Clone library sequences and TEM analysis of the sponge tissue

The diversity of sponge-associated planctomycetes was also assessed by a culture-independent approach. By using two sets of *Planctomycetes*-specific primers, 13 clone sequences were retrieved from the sponge microbial community. Based on the 16S rRNA gene similarity value below 98 %, these sequences represent four unique OTUs. The majority of 16S rRNA gene sequences retrieved from the clone library of the sponge *Niphates* sp. are closely related to the sequences retrieved from other marine sponges and corals (Thiel et al. 2007; Zhu et al. 2008; Sipkema et al. 2009; Mohamed et al. 2010; Sunagawa et al. 2010), forming a cluster associated with marine sponges (Fig. 1).

Those sponges from which unique planctomycete sequences were retrieved in previous studies include the known LMA sponges *Mycale laxissima* and *Haliclona* (?*gellius*) sp. (Schmitt et al. 2008; Sipkema et al. 2009). It was hypothesized that *Niphates* sp. might belong to LMA sponges based on the degree of association with microbial cells. Therefore, we investigated the association of the *Niphates* sp. with microbial cells by TEM analysis of the chemically fixed sponge tissue. The nucleoid of bacterial cells is known to appear condensed under TEM when samples are fixed chemically (Hobot et al. 1985). This artificial character was used to distinguish bacterial cells from sponge materials such as flagella in this study. TEM of the ultrathin sections of the sponge shows that sponge tissue was virtually devoid of microorganisms (Fig. 2), suggesting that the sponge *Niphates* sp. can be classified as a LMA sponge. No microbial cell was detected intracellularly within the sponge cells. A few microbial cells were found in vicinity of the sponge cells but not attached closely to the sponge cells.

Ultrastructure of a novel strain MS3039

The ultrastructure of a novel strain MS3039 was examined using a combination of cryosubstitution processing and TEM. This strain was isolated from the sponge *Niphates* sp. and has a sequence similarity value of less than 87 % to any other 16S rRNA gene sequences of planctomycetes. TEM images of sections of cryosubstituted cells of planctomycete strain MS3039 show that this strain shares the classical *Planctomycetes* character of intracellular compartmentalization. Paryphoplasm with no ribosome-like particles and an intracytoplasmic membrane defining a major pirellulosome compartment with many ribosome-like particles, features characteristic of planctomycete cell organization, were clearly evident from TEM images (Fig. 3). However, unlike other *Planctomycetes* (Fuerst 2005), the nucleoid of MS3039 was not condensed. The immunogold-labelling with anti-DNA antibody to detect the DNA shows DNA labelled with gold particles dispersed throughout the pirellulosome, indicating that the nucleoid is not condensed.

Discussion

Presence of a large number of sulfatase genes in the genomes of marine planctomycetes suggests that these marine planctomycetes are likely to play a role in the initial breakdown of sulphated heteropolysaccharides found in the marine environment (Glöckner

et al. 2003; Woebken et al. 2007). The marine planctomycetes *R. baltica* and *B. marina* are able to degrade the sulphated polysaccharide chondroitin sulphate in laboratory-grown pure cultures (Schlesner et al. 2004). Therefore, planctomycetes may play potentially key functions in nutrient recycling and the carbon and sulphur cycles in the oceans. To apply the ability of planctomycetes to utilize marine polysaccharides for the isolation of novel planctomycetes, we tested the enrichment of planctomycetes using marine polysaccharides such as fucoidan as a carbon source. Enrichment cultures including fucoidan-based and carrageenan-based enrichment media produced a large number of planctomycete colonies after plating of the enrichment culture onto *Planctomycetes*-selective media, indicating that designed broth media were supporting the enrichment of planctomycetes. Fucoidan and carrageenan are sulphated heteropolysaccharides from marine environment. Successful enrichment of marine planctomycetes using these marine sulphated polysaccharides as a carbon source suggests that the planctomycete isolates obtained by the enrichment method might be capable of utilization of these polysaccharides although this hypothesis has to be further tested by experiments with pure cultures. Despite the successful enrichment of planctomycetes, compared to the direct plating method, the enrichment method did not produce a greater diversity of isolates, and the majority of isolates recovered using the enrichment method were closely related to already cultured strains. Such isolates included mainly *Blastopirellula* strains closely related to those from postlarvae of the giant tiger prawn *Penaeus monodon* (Fuerst et al. 1997) and strains closely related to *Rhodopirellula* strains from seawater and marine sediments (Winkelmann and Harder 2009). Introduction of the enrichment step most likely reduced the diversity of isolates because readily culturable faster growing strains had overgrown the enrichment cultures. A few strains recovered using the enrichment method are considered as novel strains. In contrast, the direct plating method produced a large number of previously uncultured novel planctomycete strains. Increased incubation time for the direct plating method might have contributed to the isolation of novel planctomycete strains. These results suggest that, for cultivation of novel planctomycete strains, it might be more desirable to incorporate polysaccharides into solid media for the direct plating method instead of enrichment method because the direct plating method would eliminate the problem of overgrowing of readily culturable strains and provide direct evidence for utilization of these polysaccharides by planctomycetes.

The diversity of planctomycetes in the sponge *Niphates* sp. was assessed using culture-dependent and -independent techniques. The culture-dependent study resulted in the isolation of a large number of planctomycete strains including phylogenetically novel strains identified here for the first time. The analysis of one of those novel strains reveals that this sponge planctomycete isolate (MS3039) does not possess a condensed fibrillar nucleoid, a phenotype distinct from all other planctomycetes. All planctomycetes analysed using the cryosubstitution technique for preparation of cells for electron microscopy display condensation of the nucleoid (Lindsay et al. 2001). A condensed fibrillar nucleoid is also reported to occur in members of phylum *Verrucomicrobia*, cells of which also share a compartmented cell plan with planctomycetes (Lee et al. 2009a). Apparently, this strain is the first example of a planctomycete without such condensation of nucleoid. It is also hypothesized that the closely related strain MS1330, which constitutes the novel clade together with MS3039, might have a similar ultrastructure because clade-specific cell structure seems to be exhibited for different clades of planctomycetes; e.g. anammox planctomycetes with their anammoxosomes and *Gemmata* strains with their internal nuclear structures (Wang et al. 2002; Fuerst 2005). Lee et al. (2009b) suggested that the condensation of nucleoid plays a part in the life cycle of the planctomycete *Gemmata obscuriglobus*. Furthermore, using the electron tomography technique, Yee et al. (2012) recently showed that the nucleoid of *G. obscuriglobus* is organized in multiple nested orders, displaying the characteristics of a liquid crystalline cholesteric DNA structure. Although proteins associated with nucleoid condensation in planctomycetes are yet to be determined, genes encoding novel types of protein homologous to the bacterial nucleoid-associated protein HU were found in the genome of *G. obscuriglobus* (Yee et al. 2011). The absence of condensed fibrillar nucleoid in the sponge isolate MS3039 in this study suggests that the comparative genomics of this novel strain with other planctomycete species might provide insight into the genes and proteins associated with the condensation of nucleoid.

BOX-PCR examines the repetitive extragenic palindromic elements in bacterial genome and is a frequently used method for the strain typing of clinically important bacteria (Versalovic et al. 1994). BOX-PCR analysis of dominant isolates from the sponge indicated that these isolates represented clonal replicates of two distinct *Rhodopirellula* strains. It is hypothesized that planctomycetes that form association with sponges can replicate within the sponge environment. Therefore, the presence of large numbers of clonal replicates might suggest these planctomycete strains are actively growing within the sponge although this hypothesis needs to be tested by additional experiments using other molecular techniques.

Marine sponges can be classified into two groups based on the abundance of microorganisms that reside within their tissue. The sponges that contain dense microbial communities were termed high microbial abundance sponges (HMA), while a second group of sponges that contain only few bacteria were termed LMA sponges (Hentschel et al. 2003). The culture-independent clone library study here identified unique planctomycete sequences that are closely related 16S rRNA gene sequences derived from other sponges including known LMA sponges. TEM analysis of sponge tissue of *Niphates* sp. showed that this species can also be classified as a LMA sponge based on the low abundance of microbial cells in mesohyl of the sponge. The term 'sponge-specific' was introduced to describe the sponge-derived 16S rRNA gene sequences that form a monophyletic cluster with sequences retrieved from geographically and phylogenetically distinct sponge hosts, but not with sequences from non-sponge sources (Hentschel et al. 2002; Taylor et al. 2007). However, planctomycetes 16S rRNA gene clone sequences identified here are also related to sequences derived from corals (Sunagawa et al. 2010) and non-sponge sources such as sediments. Recent study of pyrotag-derived 16S rRNA gene sequences by Taylor et al. (2012) reported that many previously described sponge-specific sequences are widespread in non-sponge environments, albeit at low abundance. Although unique planctomycete sequences identified here do not represent true sponge-specific clusters, the possibility that these planctomycetes may establish stable associations with the host sponge cannot be excluded. The microbial community profiles of LMA sponges are reported to be different from those of HMA sponges and LMA sponges may harbour their own unique microbial communities despite low phylum-level diversity (Schmitt et al. 2011; Giles et al. 2013). The fact that related planctomycete sequences have been retrieved from several LMA sponges suggests that these planctomycetes might form stable association with LMA sponges. However, this hypothesis must be further tested by other molecular techniques. For instance, the localization of planctomycetes including cultured strains in a specific region of the sponge *Niphates* sp. is yet to be determined. Since TEM analysis of the sponge tissue showed that only a few bacteria cells are associated with mesohyl of the sponge, it is intriguing to speculate that these planctomycetes are localized in a specific region. Investigation of the spatial distribution of microorganisms in the sponge *Astrosclera willeyana* showed that planctomycetes were only found in the cortex of the sponge (Yang and Li 2012). It is also possible that planctomycetes identified in this study might be associated with the surface of the sponge rather than the sponge mesohyl. Further investigation to determine the spatial distribution of planctomycetes in *Niphates* sp. and other sponges harbouring planctomycetes is needed.

In summary, the diversity of planctomycetes associated with the sponge *Niphates* sp. was assessed using culture-dependent and -independent approaches. A diverse collection of cultured planctomycete strains including novel lineages of planctomycetes identified for the first time in this study provide new insight into the cell biology and ecological significance of planctomycetes in the marine environment. In addition, unique planctomycete clone sequences identified by a culture-independent approach revealed the association of these planctomycetes with the less known class of LMA sponges. How planctomycetes are distinguished, recruited, and maintained by the host sponges is significant to investigate because planctomycetes possess many unique features which are distinct from other bacteria. Understanding of how planctomycetes interact with sponge hosts might provide insight into the mechanism of how symbiotic bacteria establish a stable association with marine sponges. Insights into the unusual features of planctomycetes might also be obtained from study of such associations.

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