Community Structure of Bacteria Associated with Sheaths of Freshwater and Brackish *Thioploca* species

Running title: Bacterial communities on freshwater/brackish *Thioploca* sheaths

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Abstract

Bacterial communities associated with sheaths of *Thioploca* spp. from two freshwater lakes (Lake Biwa, Japan, and Lake Constance, Germany) and one brackish lake (Lake Ogawara, Japan) were analyzed with denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments. The comparison between the DGGE band patterns of bulk sediment and *Thioploca* filaments of Lake Biwa suggested the presence of specific bacterial communities associated with *Thioploca* sheaths. As members of sheath-associated communities, bacteria belonging to *Bacteroidetes* were detected from the samples of both freshwater lakes. A DGGE band from *Thioploca* of Lake Biwa, belonging to candidate division OP8, was quite closely related to another DGGE band detected from that of Lake Constance. In contrast to the case of freshwater lakes, no bacterium of *Bacteroidetes* or OP8 was detected from *Thioploca* of Lake Ogawara. However, two DGGE bands from Lake Ogawara, belonging to *Chloroflexi*, were quite closely related to a DGGE band from Lake Constance. Two DGGE bands obtained from Lake Biwa were closely related to phylogenetically distant dissimilatory Fe(III)-reducing bacteria. Cloning analyses for a dissimilatory sulfite reductase (DSR) gene were performed on the same samples used for DGGE analysis. The results of the analyses suggest that sheaths of freshwater/brackish *Thioploca* have little ecological significance for the majority of sulfate reducers.

INTRODUCTION

Bacteria encompass a phylogenetically expansive range of organisms, and their vast diversity is illustrated by their various cell sizes ranging over several orders of magnitude in volume (29), as well as numerous variations in physiological properties. Nitrate-storing sulfur oxidizers are known to be a group of bacteria which have the largest cells, and their cell sizes are inextricably linked to their ecological strategies. The volume of their large cells is mostly occupied by vacuoles, which serve as nitrate reservoirs. The accumulated nitrate enables them to oxidize reduced sulfur compounds even in the absence of external electron acceptors (7, 21, 28).

All known nitrate-storing sulfur oxidizers belong to a particular phylogenetic cluster (1, 14, 28, 31). At the present time, they are classified into three genera based on their morphological traits. The bacterium with the largest cell volume belongs to the genus *Thiomargarita*, and its appearance can be described as a chain of spherical cells (28). Bacteria of the other two genera, *Beggiatoa* and *Thioploca* are filamentous. Discrimination between these two genera is dependent on the presence or absence of a common sheath. *Thioploca* species have sheaths that surround bundles of trichomes, whereas *Beggiatoa* species are present as individual trichomes (12).

The common sheaths, which are the genus marker of *Thioploca*, also reflect their unique behavior. The sheaths function as a pathway for vertical gliding movements

in sediment. With the ability to store nitrate intracellularly and vertical migration, they overcome spatial dissociation between electron donor and acceptor (10). This strategy is especially effective in the situations where stable spatial dissociation is formed between sulfide and nitrate. The marine sediments of upwelling areas characterized with intensive sulfate reduction and high nitrate concentration in overlaying water are typical habitats of *Thioploca* species, where they can monopolize substrate. In the sediments of shelf along coast of Chile and Peru, *Thioploca* species exist as dominant organisms in terms of biomass (7, 10).

Hence, the common sheath is quite an important structure for members of the genus *Thioploca*, but it can also have ecological significance for other organisms. The organic material composing the sheaths theoretically could provide substrates for fermentation or other microbial activities. In addition, the microenvironment around the sheaths is thought to be affected by activities of *Thioploca* trichomes. Therefore, the sheaths are likely to provide specific habitats for some kinds of bacteria. For instance, it has been demonstrated that sheaths of marine *Thioploca* are densely covered with filamentous sulfate reducers (*Desulfonema* sp.), and they are thought to provide sulfide to *Thioploca* (8). However, there are only a few studies on sheath-associated bacteria of marine *Thioploca* species, and knowledge on freshwater or brackish species is still lacking. With their substantial biomass and the characteristic of linking the sulfur, nitrogen and carbon cycles, *Thioploca* populations are thought to have great influence on

substance cycling in their habitats (34). Interactions between *Thioploca* species and sheath-associated bacteria might be a key to understanding their ecology and their impact on cycling of organic and inorganic compounds. In the present study, the community structures of the bacterial flora associated with sheaths of freshwater and brackish *Thioploca* species were analyzed with denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes. In addition, cloning analysis of dissimilatory sulfite reductase (DSR) gene was performed in order to detect sulfate reducers that might provide sulfide to *Thioploca*.

MATERIALS AND METHODS

Sampling sites and collection of *Thioploca* specimens In Lake Biwa, *Thioploca* and sediment samples were collected from two sites: station A (35° 23.4 N, 136° 7.7E, 90 m water depth) and station B (35°11.9 N, 135° 59.2 E, 70 m water depth). Station A corresponds to the site of previous studies (16, 17, 18). *Thioploca* filaments (sheaths with trichomes) were collected by dredge net (18, 24), and the sediment samples were obtained with an Ekman-Birge grab sampler or a core sampler. Accumulated clumps of filaments on the dredge net were immediately transferred into a container with sediment and water from the collection site. All samples were kept at 4°C in the dark until processing in the laboratory.

The sediment samples from Lake Constance were obtained with an Ekman-Birge grab sampler from a shoreline site with 1-6 m water depth. The sediment samples were immediately sieved to condense *Thioploca* filaments. The following filaments washing procedure and genomic DNA extraction from them were performed on the same day at the University of Constance laboratory.

Lake Ogawara is a brackish lake located at northern end of the main island of Japan, Honshu. The sampling was performed at a site of 16 m water depth (40° 49.3 N, 141° 20.1E). The *Thioploca* filaments were collected from the sediment by sieving. The collected filaments were kept in water from the collection site at 4°C until processing in the laboratory.

DNA extraction For the analyses of sheath-associated microorganisms, DNA was extracted from washed filaments of *Thioploca*. For the washing, filter-sterilized water from each of the respective lake was prepared. The filaments were rinsed in the filtered water of the respective lake to wash out sediment particles. Subsequently, residual contaminating materials were removed with forceps. The filaments were further washed with at least 3 changes of lake water, and then kept at -20°C until DNA extraction. From the washed whole filaments, including trichomes and sheaths, genomic DNA was extracted by the method described previously (18). DNA extraction from bulk sediment of Lake Biwa was carried out as described previously (16).

Sequencing of 16S rRNA gene for *Thioploca* sp. from Lake Ogawara For *Thioploca* sp. from Lake Ogawara, sequence analysis on 16S rRNA gene was performed as described previously (18). With the specific primers, three overlapping regions of 16S rDNA were amplified from the genomic DNA extracted from the washed filaments. The three fragments were sequenced, and the resulting sequences were combined based on their consensus in overlaps, to obtain nearly complete 16S rDNA sequence (18).

PCR-DGGE analyses of bacterial communities The bacterial flora of filaments and bulk sediment were analyzed with PCR-DGGE. From the extracted genomic DNA samples, fragments of 16S rRNA genes were amplified with the combination of eubacterial primer 341F-GC and universal primer 907R (22). For the PCR amplification of 16S rDNA, two kinds of thermal cycles were tested for each sample. The touch-down cycle with annealing temperature of 65 to 55°C was performed as previously described (5). However, this cycle might cause bias originating from differences in the affinity of the primers to 16S rRNA gene sequences (11). Therefore, another cycle with a lower constant annealing temperature at 45°C was also tested (16). In addition to the analysis using universal primer pair described above, analysis with the selective primer pair was also performed. The primer combination SRB385 (with GC-clamp) and 907R has been used for the DGGE analysis targeting δ-Proteobacteria,

although it also amplifies 16S rRNA gene of other organisms (13, 27, 30). In the PCR amplification with this primer pair, each thermal cycle consisted of 2 min of denaturation at 94°C, 1.5 min of annealing at 56°C and 2 min of elongation at 72°C. The total cycle number was 30. DGGE was performed as previously described (16). From the resulting DGGE gels, several bands were excised, reamplified, and then sequenced.

Cloning analyses for DSR gene The community structures of sulfate reducers associated with the sheaths of *Thioploca* were investigated by cloning analysis of the gene for dissimilatory sulfite reductase (DSR). From the same DNA samples analyzed with DGGE, fragments of DSR gene were amplified with the primer pair DSR1Fdeg and DSR4Rdeg (15) and PCR conditions previously described (33). For Lake Biwa, the sample from the site A was analyzed as representative. The amplification products obtained were electrophoresed in 1.1% (w/v) agarose gel and bands of expected size were excised from the gel. The DNA fragments were extracted from the excised pieces of gel using QIAquick Gel Extraction kit (Qiagen), and then ligated into a pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA, USA). The vectors were transformed into competent TOP10 cells (Invitrogen) to establish clone libraries. From four resulting clone libraries, (three from filaments and one from sediment), 15 colonies each were randomly picked for analyses. With the provided primer pair M13F (-20) and M13R, regions including the cloned inserts were amplified by PCR directly from the cells. The resulting PCR products were purified and then partial nucleotide sequences (ca. 650 bases) of

gene for α-subunit of DSR were determined.

Phylogenetic analyses The phylogenetic analysis for the DGGE bands of 16S rDNA fragment was performed with the software package ARB (http://www.mikro.biologie.tu-muenchen.de). The sequences were aligned with the provided dataset using the alignment tool of the ARB package. The aligned sequences were inserted into the provided phylogenetic tree with the maximum-parsimony algorithm.

From the obtained partial nucleotide sequence of DSR gene, amino acid sequences were deduced based on the standard genetic code table. The deduced sequences were aligned using the program ClustalX (32), and genetic distances were calculated with the program MEGA2 (19). The clones which share more than 194 amino acid in 202 available sites were considered to be member of the same operational taxonomic unit (OTU). Phylogenetic relationship of the OTUs and DSR sequences in the DDBJ/EMBL/Genbank database were also deduced with ClustalX and MEGA2.

Nucleotide sequence accession numbers The sequences of the DGGE bands HY1, HY2, and HY4 have DDBJ/EMBL/Genbank accession numbers AF452893-AF452895. The other sequences determined in this study have been assigned the numbers

xxxxxx-yyyyyyy.

RESULTS

Characteristic of *Thioploca* sp. from Lake Ogawara *Thioploca* sp. from Lake Ogawara was morphologically similar to that from Lakes Biwa and Constance, with the diameter of the trichomes ranging from 3.0 to 4.0 μm. It has been reported that freshwater *Thioploca* species have constricted zones on their sheaths (18); however, no constriction was observed on the sheaths of *Thioploca* sp. from Lake Ogawara. The obtained 16S rRNA gene sequence of *Thioploca* sp. from Lake Ogawara showed quite a high similarity to that of *Thioploca* sp. from Lakes Biwa and Constance. In a pairwise comparison of 1374 nucleotide positions, there were only four mismatches each between the sequence of *Thioploca* sp. from Lake Ogawara and that of the species from Lakes Biwa and Constance.

DGGE analysis of bacterial communities associated with *Thioploca*. In the DGGE analyses of the bulk sediment of Lake Biwa, the same band patterns were obtained from sites A and B, and there was no significant difference between the results of the two PCR cycles (results not shown). The band patterns of *Thioploca* filaments from these two sampling sites were also quite similar to each other; however, they were

entirely different from those of the bulk sediment (Fig. 1). No differences originated from the annealing temperature used in PCR. The sequences were determined for eight DGGE bands. Bands HY1 and HY5 belonged to *Bacteroidetes* (Fig. 2). Band HY2 was identified as a member of OP8, a candidate division without cultured representatives. The sequence of band HY4 was closely related to a dissimilatory Fe(III)-reducing bacterium *Geothrix fermentans* (3). HY8, another band, was also closely related to an Fe(III)-reducing bacterium; however, the bacterium *Rhodoferax ferrireducens* belongs to β-Proteobacteria (6) and is phylogenetically distant from *G. fermentans*. All other bands were related to β-Proteobacteria (Fig. 2), and *Thioploca* itself could not be detected as a DGGE band.

In the case of Lake Constance, the DGGE band patterns changed drastically depending on the PCR cycle (Fig. 3A). When fragments of 16S rDNA were amplified in a PCR cycle with annealing at 45℃, a band of remarkable intensity was observed in DGGE. However, this band was not observed in the amplificate obtained from the touch-down cycle. This conspicuous band was sequenced and was shown to have originated from *Thioploca* itself. Another band found only in the PCR product of the cycle with annealing at 45°C was labeled BO11. Its sequence was related to that of the non-vacuolar *Beggiatoa* species. Among the DGGE bands from the touch-down cycle, five bands were sequenced. Two of these bands—BO6 and BO7—belonged to *Bacteroidetes* (Fig. 2). Band BO8 was closely related to band HY2 from Lake Biwa.

Band BO4 was grouped within the cluster of diatom chloroplast, and BO9 was related to uncultured bacteria of *Chloroflexi*.

Using the template of genomic DNA extracted from the *Thioploca* filaments of Lake Ogawara, two PCR cycles resulted in similar DGGE band patterns; however, the relative intensity of each band was slightly altered (Fig. 3B). The band that had originated from *Thioploca* itself was observed to be the major band in both cases. Three sequenced bands—OG3, OG4, and OG7—were grouped within the *Chloroflexi*, and two of these were quite closely related to band BO9 from Lake Constance (Fig. 2). Band OG1 was clustered with unidentified clones from a benzoate-degrading methanogenic consortium (according to the description provided in the DDBJ/EMBL/Genbank database). Band OG5 was also related to these clones, although only partial sequences could be determined for this band. Band OG6 was also sequenced partially; however, phylogenetic analysis based on the limited sequence length indicated that band OG6 was grouped with δ-Proteobacteria (Fig. 2)

In the DGGE analysis with the primer pair SRB385 and 907R, several closely related sequences were obtained from *Thioploca* samples of different lakes (Fig. 2, Fig. 4). The *Thioploca* samples from two sites in Lake Biwa—sites A and B—exhibited an identical band pattern that featured two major bands (Fig. 4). The sequences of these bands—HY-s1 and HY-s3—were identical to those of the DGGE bands HY2 and HY4, obtained with the universal primer pair. Another sequenced band HY-s2 showed a high

similarity to band BO9 from Lake Constance and OG3 from Lake Ogawara (Fig. 2). The other two sequenced bands were related to environmental clones belonging to the *Holophaga-Geothrix* lineage (Fig. 2). In the case of Lake Constance, two major bands were identical to the bands that appeared in the analysis with the universal primer pair. Bands BO-s1 and BO-s2 corresponded to BO8 and BO9, respectively. Band BO-s3 is closely related to band HY4 from Lake Biwa (Fig. 2). The other sequenced band BO-s4 was related to sulfate-reducing bacteria of genus *Desulfomonile* (Fig. 2). Although one of the bands from Lake Ogawara had a migration position similar to that of bands of OP8 from freshwater lakes, band OG-s4 did not show a close phylogenetic relationship to OP8. The other bands from Lake Ogawara also did not have any close cultured relatives, and none of these bands was identified as δ-Proteobacteria.

Cloning analyses of the DSR gene DSR gene sequences belonging to wide-ranging lineages were obtained from the clone libraries. Based on sequence similarity, 60 clones from four libraries were sorted into 32 OTUs, and their phylogenetic relationship was analyzed (Fig. 5).

The clones from the bulk sediment of Lake Biwa belonged to eight OTUs. Among these OTUs, three (dsr 12, dsr 18, and dsr 20) occurred with the highest frequency; however, two of them were also detected in the other clone libraries. Four OTUs were specific to the sediment of Lake Biwa, and seven clones belonged to these

OTUs.

With regard to the DSR clones obtained from the *Thioploca* filaments of Lake Biwa, 9 of 15 clones belonged to OTUs unique to this sample. Two OTUs were inferred to be members of the group of complete oxidizers. OTU dsr7 was related to the DSR genes of the *Desulfobacter* species, and dsr3 was related to the *Desulfonema/Desulfosarcina/Desulfococcus* assemblage, which is known to be a versatile group. Two OTUs, comprising three clones each, were related to incomplete oxidizers, i.e., dsr9 was related to *Desulfomonile tiedjei* and dsr23 was related to *Desulforhopalus*. The remaining five OTUs were scattered in the phylogenetic tree of DSR that includes all known lineages of sulfate-reducing prokaryotes (SRP).

In the case of Lake Constance, 13 clones belonged to 9 OTUs that were unique to Lake Constance. Two OTUs (dsr2 and dsr5) were related to complete oxidizers, and OTU dsr21 was grouped within the cluster of the *Desulfobulbus* species. All other seven OTUs belonged to the phylogenetic group consistent with clones from environmental samples, including clones obtained in this study.

The DSR gene clones from the *Thioploca* filaments of Lake Ogawara were also phylogenetically diverse, and most clones belonged to Lake Ogawara-specific OTUs. Two OTUs (dsr1 and dsr4) were grouped within the *Desulfonema/Desulfosarcina/Desulfococcus* assemblage, and dsr6 was related to the *Desulfobacter* species. Three clones of OTU dsr22 were members of the cluster formed

by *Desulforhopalus* and *Desulfobulbus* spp. One dsr24 clone was related to *Thermodesulforhabdus norvegica* and *Desulfacinum infernum*. The other OTUs were clustered with clones obtained in this study, without any cultured close relatives.

DISCUSSION

DGGE analyses of 16S rRNA genes The PCR-DGGE band profiles of several replicated samples of *Thioploca* filaments from sites A and B in Lake Biwa showed an almost identical band pattern. This uniformity indicates that the DGGE bands of washed *Thioploca* filaments originate from specific bacteria that are closely associated with *Thioploca* and do not originate from accidental contaminants. No band appeared in the DGGE analyses of both the bulk sediment and washed filaments. These findings indicated that members of the bacterial community could be differentiated from the bulk sediment community by the method employed in this study.

In some cases, a fragment of the *Thioploca* rRNA gene was not detected as a DGGE band, although the analyzed *Thioploca* filaments contained trichomes and therefore *Thioploca* genomic DNA. This may result from the low proportion of *Thioploca* DNA to general bacterial DNA. *Thioploca* cells have a much larger cell volume than other bacteria, resulting in a lower DNA content per biovolume. The presence of *Thioploca* DNA in the DNA extracted from complete filaments was verified

by PCR with *Thioploca*-specific primers (data not shown). Another reason might be the affinity of primer 907R to rDNA sequences. The 16S rDNA sequences of *Thioploca* spp. from Lakes Biwa, Constance, and Ogawara have one mismatch to 907R used here. They would therefore produce fewer PCR products than perfectly matched bacteria, particularly at the higher annealing temperature. The lower annealing temperature increases the efficiency of amplification of templates with mismatches (11), and this effect was demonstrated clearly in the case of Lake Constance (Fig. 3A).

Primer SRB385 was originally designed as a probe for FISH to detect SRP of δ-Proteobacteria (2). This primer has been used in several studies for DGGE analysis targeting δ-Proteobacteria (13, 27, 30); however, it was also known that this primer detects several bacteria other than the targeted group. In this study, among 14 sequenced bands, only one band was identified as belonging to members of δ-Proteobacteria. However, analysis with this selective primer emphasized the similarity of the sheath-associated community structure among freshwater lakes. The sequences of the DGGE bands HY2 and BO8 perfectly matched with those of primer SRB 385. As expected, these sequences were detected as bands HY-s1 and BO-s1 in DGGE with the selective primer. Although band HY4 has several mismatches with SRB 385, its sequence was also detected in the analysis with SRB 385. A sequence that was closely related to this band was detected from Lake Constance; this was the DGGE band BO-s3. Similarly, despite mismatches, the sequence of band BO9 was detected in the analysis

with SRB 385 as band BO-s2. Close relatives of this band were also detected from Lake Biwa (HY-s2).

The washing procedure was required for discriminating sheath-associated bacteria from other sediment dwelling bacteria; however, it might also cause loss of bacteria that have close interactions with *Thioploca*. Although some important bacteria may have been missed in this manner or may remain undetected due to other reasons, the bacteria that were detected from *Thioploca* sheaths probably interact with *Thioploca* in some manner.

Phylogenetic affiliations of sheath-associated bacteria Sequences that were grouped into *Bacteroidetes* were detected (HY1, HY5, BO6, and BO7) from all three sites in the two freshwater lakes. In the previous analysis of the bulk sediment of Lake Biwa, no sequence of this lineage was detected as a major DGGE band (16). The isolated members of *Bacteroidetes* catabolize various polymeric substances, and several culture-independent studies have demonstrated the association of *Bacteroidetes* with the degradation of complex organic materials (4, 25, 26). The bacteria detected here may be utilizing sheath material as a substrate.

Band HY2 from Lake Biwa was a member of the candidate division OP8, a phylum-level group of uncultured bacteria (9). Band BO8 obtained from the sheaths of Lake Constance was very closely related to HY2. The physiology of OP8 bacteria is

unknown, although habitat characteristics suggest that their metabolism is anaerobic (9, 20).

In contrast to the similarity between the results obtained from the two freshwater lakes described above, no bacterium of *Bacteroidetes* or OP8 was detected as the major DGGE band from the *Thioploca* filaments of Lake Ogawara. This distinction appears to reflect differences in the environmental condition between freshwater and brackish lakes, although the presence of *Bacteroidetes* or OP8 on the sheaths of *Thioploca* sp. from Lake Ogawara has not yet been excluded. In spite of the apparent differences described above, two DGGE bands detected from Lake Ogawara—OG3 and OG4—were closely related to the DGGE band BO9 from Lake Constance. These bands belonged to *Chloroflexi*, and another band OG7 was also a member of this cluster.

Band HY4 from Lake Biwa was closely related to an Fe(III)-reducing bacterium—*Geothrix fermentans*—that was isolated from a hydrocarbon-contaminated aquifer (3). The sequence of the other DGGE band obtained from the same sample was closely related to that of another Fe(III)-reducing bacterium *Rhodoferax ferrireducens* (6). These data suggest the presence of an unknown specific interaction between *Thioploca* and Fe(III)-reducing bacteria.

The DGGE band OG6, which was related to sulfate-reducing bacteria, was obtained from the *Thioploca* filaments of Lake Ogawara. This organism may provide sulfide to *Thioploca* trichomes and accept sulfate from them.

Band BO4 from the *Thioploca* filaments of Lake Constance was closely related to the plastid of diatoms; however, it is unlikely that the diatom was actually associated with the *Thioploca* filaments. The detected diatom might be condensed and attached to the *Thioploca* filaments during sieving. Band BO11 was related to the filamentous *Beggiatoa* species, and filamentous bacteria might also be condensed by sieving.

In previous studies, application of fluorescence *in situ* hybridization (FISH) on the sheaths of marine *Thioploca* indicated the presence of filamentous sulfate-reducing bacteria (8) and α-Proteobacteria (23). In contrast, the sequence of these organisms was not detected in the present study. This distinction might reflect a difference between marine and freshwater/brackish *Thioploca* species in terms of sheath-associated communities. However, whether specific bacteria can be detected as a DGGE band or not is dependent on their relative abundance to other bacteria. Another point that should be taken into account is that different techniques have different biases, which are exposed in their results.

Cloning analyses of the DSR gene In general, the sulfate concentration and sulfate reduction activities in freshwater environments are significantly lower than those in marine environments. Therefore, a possible interaction between freshwater *Thioploca* species and sulfate reducers would have more critical significance for both organisms than in the case of marine sediment. However, in the analysis of the 16S rRNA gene, no

DGGE band related to known SRP was detected from the sheaths of organisms from either of the freshwater lakes. For a more specific and detailed investigation on SRP, cloning analysis of the DSR gene was performed. In the analysis, diverse sequences were obtained from the *Thioploca* sheaths of freshwater lakes, and there was no obvious domination by a specific lineage. These results do not conflict with the results of the 16S rRNA gene-based analyses because high diversity may correspond to low abundances of the respective SRP.

In contrast to the freshwater lakes, a 16S rRNA gene sequence related to SRP was obtained in the DGGE analysis performed on the *Thioploca* filaments of Lake Ogawara. The DGGE band OG6 was related to *Thermodesulforhabdus norvegica* and *Desulfacinum infernum*, and accordingly, the DSR clone related to these bacteria was obtained from the *Thioploca* filaments of Lake Ogawara (Fig. 2, Fig. 5, dsr24). However, the overall result of the cloning analysis of the DSR gene was similar to that of freshwater lakes, i.e., it was characterized by clones of diverse phylogenetic lineages and showed no obvious domination. Among 15 clones from Lake Ogawara, only one belonged to the OTU shared with other freshwater lakes. It is likely that this result reflects the differences in the environmental condition of freshwater and brackish lakes.

In the comparison of SRP community structures between *Thioploca* sheaths and bulk sediment of Lake Biwa, some lineages, including plural clones, were observed only in the sediment or in the sheaths. On the other hand, four OTUs were detected from both

the *Thioploca* sheaths and sediment, and these OTUs occupied a substantial portion of both libraries (6/15 in sheath and 8/15 in sediment). This is in contrast to the clear differentiation between the bacterial community structures of *Thioploca* filaments and bulk sediment demonstrated by 16S rDNA-based DGGE analysis. This incomplete differentiation and low relative abundance of SRP on the sheath suggest that the *Thioploca* sheaths have little ecological significance for the majority of SRP. This unexpected result suggests that the ecology of freshwater *Thioploca* is rather different from that of marine species.

Although the sheaths have no significance for the majority of SRP, the possibility of the presence of SRP that are specialized to inhabit *Thioploca* sheaths has not been excluded. Three clones each from the *Thioploca* filaments of Lakes Biwa and Ogawara and two clones from Lake Constance were grouped within the cluster of *Desulforhopalus*/*Desulfobulbus* species. In addition, no clone of this lineage was obtained from the bulk sediment of Lake Biwa. Sulfate-reducing bacteria of this lineage may have specific ecological relationships with freshwater/brackish *Thioploca* species through the exchange of sulfide and sulfate. To reveal the significance of adhesion to *Thioploca* sheaths, further quantitative study on these bacteria would be required.

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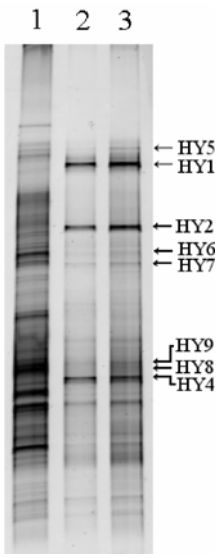

Figure 1. DGGE band profile of sediment and *Thioploca* filaments from Lake Biwa. Lane 1, sediment of site A; lane 2, *Thioploca* filaments from site A; lane 3, *Thioploca* filaments from site B. The bands indicated with an arrow were sequenced.

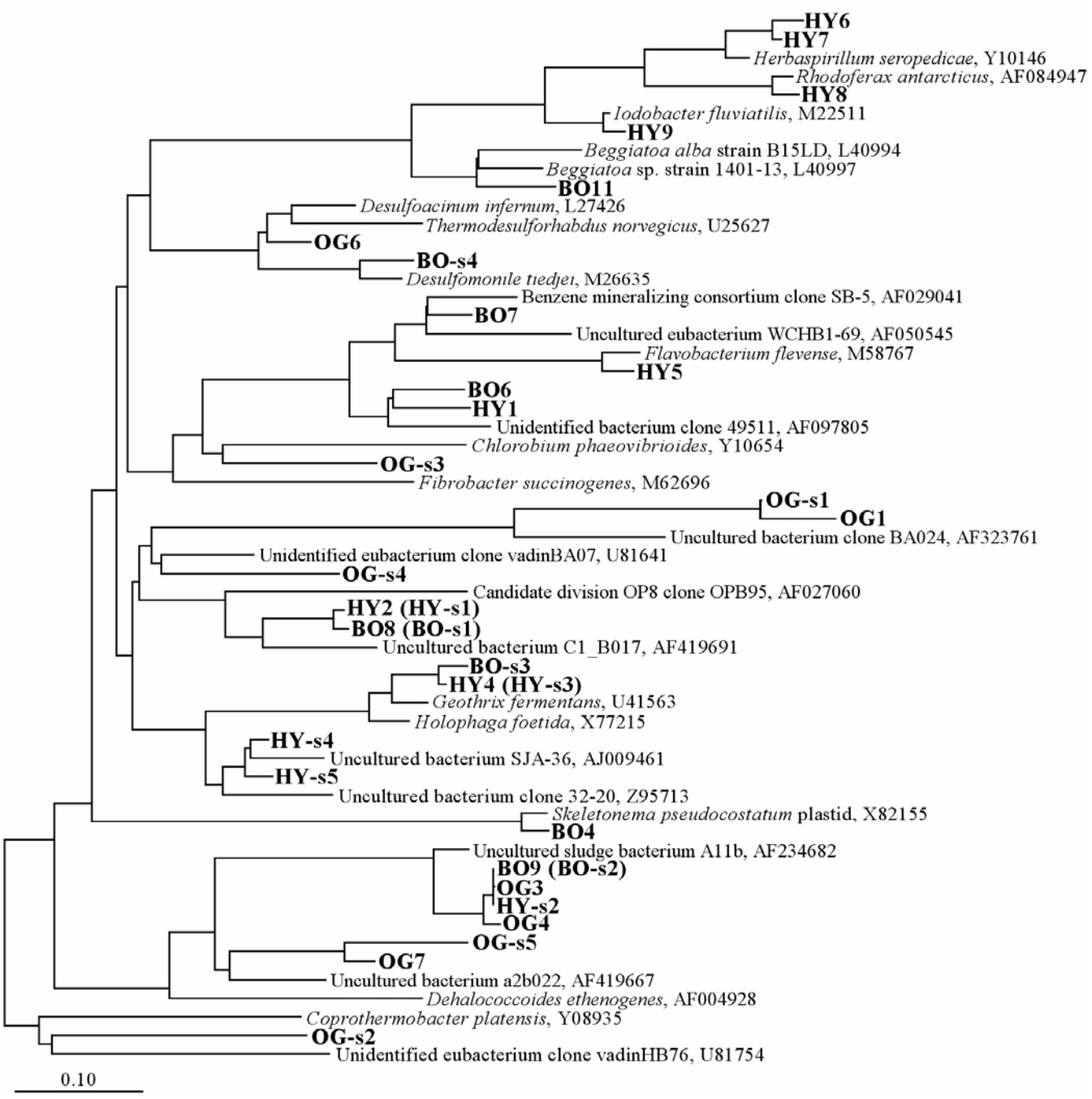

Figure 2. Phylogenetic affiliation of DGGE bands obtained in this study. The tree is based on the results of a maximum-parsimony analysis of the dataset in the ARB software package. The labels of DGGE bands are defined in Fig. 1, Fig. 3, and Fig .4. The band OG5 was excluded from the tree because the determined sequence was too short to obtain a reliable result. The scale bar represents 10% estimated divergence.

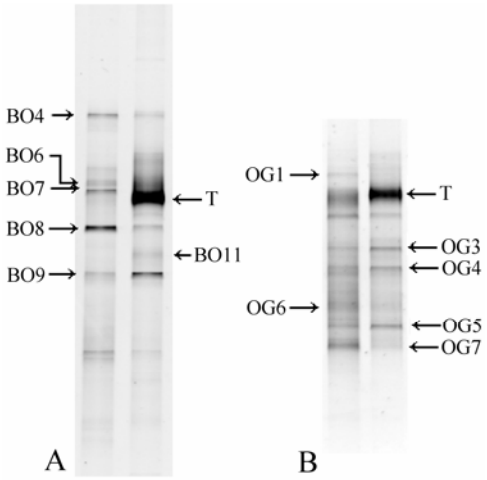

Figure 3. Different DGGE band patterns obtained from the same primer pair and same template DNA extracted from *Thioploca* filaments from Lake Constance (A) and Lake Ogawara (B), as results of two different PCR cycles. Left lane, product of the touch-down cycle; right lane, product of the cycle with lower annealing temperature. The bands indicated with an arrow were sequenced. The bands indicated with “T” were originated from *Thioploca* itself.

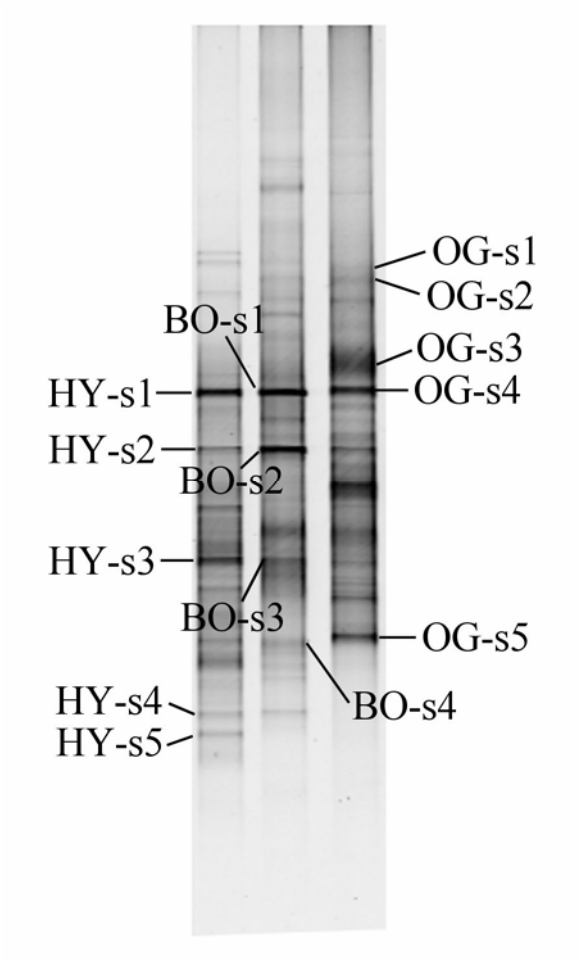

Figure 4. DGGE band profiles of 16S rRNA gene fragments amplified with the primer pair GC-SRB385 and 907R, from *Thioploca* sheaths. Lane 1, site A in Lake Biwa; lane 2, Lake Concetance; lane 3, Lake Ogawara.

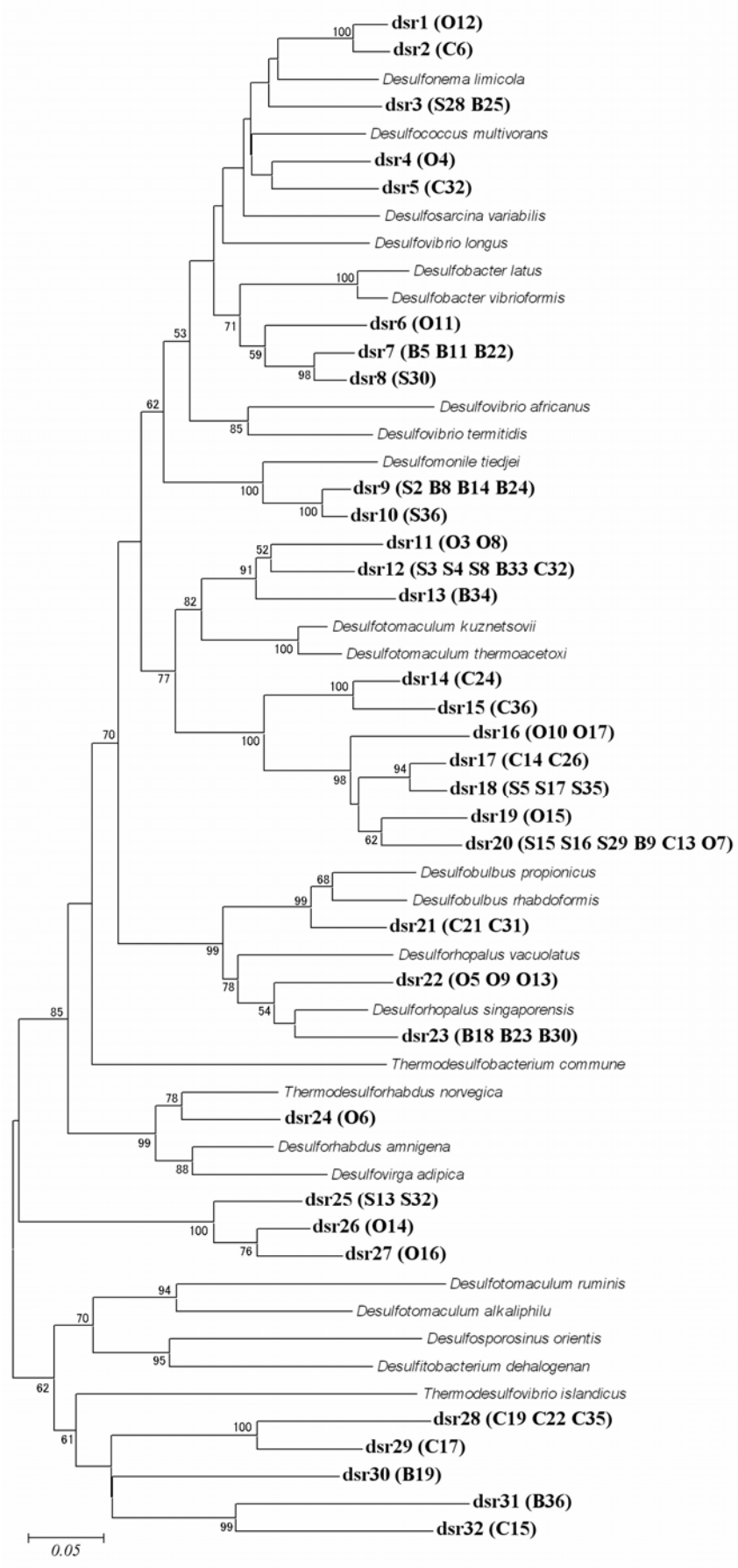
dsr1 (O12)
dsr2 (C6)
Desulfonema limicola
dsr3 (S28 B25)
Desulfococcus multivorans
dsr4 (O4)
dsr5 (C32)
Desulfosarcina variabilis
Desulfovibrio longus
Desulfobacter latus
Desulfobacter vibrioformis
dsr6 (O11)
dsr7 (B5 B11 B22)
dsr8 (S30)
Desulfovibrio africanus
Desulfovibrio termitidis
Desulfomonile tiedjei
dsr9 (S2 B8 B14 B24)
dsr10 (S36)
dsr11 (O3 O8)
dsr12 (S3 S4 S8 B33 C32)
dsr13 (B34)
Desulfotomaculum kuznetsovii
Desulfotomaculum thermoacetoxi
dsr14 (C24)
dsr15 (C36)
dsr16 (O10 O17)
dsr17 (C14 C26)
dsr18 (S5 S17 S35)
dsr19 (O15)
dsr20 (S15 S16 S29 B9 C13 O7)
Desulfobulbus propionicus
Desulfobulbus rhabdoformis
dsr21 (C21 C31)
Desulforhopalus vacuolatus
dsr22 (O5 O9 O13)
Desulforhopalus singaporensis
dsr23 (B18 B23 B30)
Thermodesulfobacterium commune
Thermodesulforhabdus norvegica
dsr24 (O6)
Desulforhabdus amnigena
Desulfovirga adipica
dsr25 (S13 S32)
dsr26 (O14)
dsr27 (O16)
Desulfotomaculum ruminis
Desulfotomaculum alkaliphilu
Desulfosporosinus orientis
Desulfitobacterium dehalogenan
Thermodesulfovibrio islandicus
dsr28 (C19 C22 C35)
dsr29 (C17)
dsr30 (B19)
dsr31 (B36)
dsr32 (C15)
0.05

Figure 5. Phylogenetic relationships of DSR clones obtained in this study. The tree was constructed with minimum evolution method, based on deduced amino acid sequences of representative clones of each OTU. Phylogenetic distances were calculated with Poisson correction. Numbers on nodes are percentage values of 500 bootstrap resampling (values larger than 50 are shown). The names of clones belonging to respective OTU are represented in parentheses. Name of clone prefixed “S”, “B,” “C,” and “O” indicate the clones from the sediments of Lake Biwa, *Thioploca* sheaths of Lake Biwa, sheaths of Lake Constance, and sheaths of Lake Ogawara, respectively.