Vertical distribution of major sulfate-reducing bacteria in a eutrophic shallow meromictic lake

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Running title: SRB in a shallow meromictic lake

Abstract

The vertical distribution of sulfate-reducing bacteria was investigated in a shallow, eutrophic, meromictic lake, Lake Harutori, which is located in a residential area of Kushiro, Japan. A steep chemocline, which is characterized by gradients of oxygen, sulfide, and salinity, was found at a depth of 3.5–4.0 m. The sulfide concentration at the bottom of the lake was high (up to a concentration of 10.7 mM). Clone libraries were constructed using the *aprA* gene, which encodes adenosine-5'-phosphosulfate reductase subunit A to monitor sulfate-reducing bacteria. In the *aprA* clone libraries, the most abundant sequences were those from the *Desulfosarcina-Desulfococcus* (DSS) group. A primer set for a DSS group-specific 16S rRNA gene was used to construct another clone library, analysis of which revealed that the uncultured group of sulfate-reducing bacteria, SEEP SRB-1, accounted for nearly half of the obtained sequences. Quantification of the major bacterial groups by catalyzed reporter deposition-fluorescence *in situ* hybridization demonstrated that the DSS group accounted for 3.2–4.8% of the total bacterial community below the chemocline. Our results suggest that below the chemocline of Lake Harutori, DSS group is one of the major groups of sulfate-reducing bacteria and that these presumably metabolically versatile bacteria might play an important role in sulfur cycling in the lake.

Keywords: *Meromictic lake, sulfate-reducing bacteria, aprA, sulfide*

Introduction

The global expansion of anoxic aquatic environments has a massive influence on biological activities. For instance, in the California Current marine ecosystem, serious damage to the fish and benthic invertebrate communities is reported to have occurred due to anoxic water, and there is concern about possible further damage to the highly productive coastal environment [7]. The formation of anoxic water masses is thought to be connected to human-induced eutrophication and global warming [1], and it is speculated that the occurrence of hypoxia will increase in the coming decades [21]. Sulfide accumulation in anoxic water columns is an additional concern. In anoxic environments, sulfides are normally produced via microbial sulfate reduction. Especially in the marine environment, the sufficient supply of sulfate from seawater and of organic compounds support the growth of sulfate-reducing bacteria. Most of the sulfide is usually converted into colloidal sulfur or sulfate by bacteria, via either anaerobic or aerobic oxidation before it reaches the surface layer of the water column [21]. Therefore, the analysis of microbial sulfur cycling in oxic-anoxic interfaces is attracting increasing attention.

Meromictic lakes can provide a suitable environment to investigate the potential importance of sulfate-reducing bacteria (SRB) in anoxic water columns. In meromictic lakes, the surface layer of the lake, called the mixolimnion circulates but does not intermix with the deeper layer, called monimolimnion. Therefore, such lakes stay stratified throughout the year. At the interface between the mixolimnion and the monimolimnion, a steep chemical gradient called a chemocline is formed. A clear shift of the microbial community structures below and above the chemocline has been shown in many meromictic environments [17, 38]. Lake Harutori is located in a residential area of Kushiro, Hokkaido, Japan. This meromictic lake is consisting of water layers based on salinity. The bottom layer of Lake Harutori is known to

62 contain a very high amount of sulfide. The lake was reported to contain up to 670 mg L^{-1}
63 sulfide at one point [44], so that the toxic effects on indigenous animals and plants were a
64 matter of concern. In addition, most of the wastewater from the adjoining residential area was
65 directly discharged into the lake; therefore, the accumulation of organic compounds was an
66 environmental problem. An analysis of photosynthetic pigments revealed anaerobic
67 anoxygenic phototrophic bacteria (green sulfur bacteria) in the Lake Harutori chemocline [35].
68 Aside from that, not much is known about the microbial community in the lake.
69 In this study, we aimed to identify and quantify the main sulfate-reducing bacteria
70 beneath the Lake Harutori chemocline. To find the key players in sulfur cycling in Lake
71 Harutori, we investigated the phylogenetic diversity of sulfate-reducing and sulfur-oxidizing
72 bacteria by sequence analysis of the gene encoding adenosine 5'-phosphosulfate reductase
73 (*aprA*). Furthermore, the vertical distribution of the predominant group in
74 *Deltaproteobacteria* was revealed by using catalyzed reporter deposition-fluorescence *in situ*
75 hybridization (CARD-FISH). The study shows that *Desulfosarcina-Desulfococcus* (DSS)
76 group is one of the major groups of sulfate-reducing bacteria in the monimolimnion of Lake
77 Harutori.
78
79 **Materials and methods**
80 *Study sites and sampling*
81 Lake Harutori is a shallow meromictic lake located in Kushiro, Hokkaido, Japan (Fig. 1). The
82 lake (surface area 0.36 km^2) is a coastal lagoon in which the low salinity water is overlaying
83 water of marine origin, resulting in a permanent stratification of the water body [2]. The lake
84 is completely covered with ice in winter, usually from December to the end of March [2]. The
85 center of the lake (N42°58’20.6”, E144°24’6.6”), which has a maximum depth of 5.75 m, was

selected as the sampling point. Sampling took place in May 2012 after the ice had melted. Water samples were collected using a horizontal Van Dorn water sampler (3L, Rigosha, Tokyo, Japan) along the depths. Vertical profiles of temperature, specific conductivity and pH were measured *in situ*, with a multiparameter sensor (YSI 600XLM; YSI Inc., Yellow Springs, OH, USA). Photosynthetically active radiation (400–700 nm) was measured using a quantum meter (Model QMSS-S; Apogee instruments Inc., Logan, UT, USA). The dissolved oxygen profile was measured *in situ* with an optical dissolved oxygen meter (ProODO; YSI Inc., Yellow Springs, OH, USA).

Chemical measurements

Sulfide concentrations were measured colorimetrically with the methylene blue formation method [3]. To measure dissolved organic carbon (DOC), sulfate, and chloride, water samples were filtered through cellulose acetate syringe filter units with a 0.2 μm pore size (DISMIC-13CP; Toyo Roshi Kaisha, Tokyo). Concentrations of DOC were measured with a total organic carbon analyzer (TOC-V; Shimadzu, Kyoto, Japan). Sulfate and chloride concentrations were determined using an ion chromatograph (ICS-1500, column: IonPacAS12A, Dionex, Sunnyvale, CA, USA) with appropriate dilutions.

DNA extraction

The water samples (approximately 200 mL for each sample) were filtered through Sterivex-GV 0.22 μm pore-sized filter cartridges (Millipore, Billerica, MA, USA) immediately after the collection. The filter cartridges were frozen on dry ice and brought back into the laboratory without thawing, and stored at -20°C until DNA extraction. The filters were transferred into a 2 mL screw-capped tube with 0.5 g of sterile glass beads, 0.6 mL TE buffer

(10 mM Tris-HCl, 1 mM EDTA, pH 8), 30 μL 20% SDS and 0.6 mL phenol-chloroform-isoamyl alcohol (25:24:1; v/v/v). The tube was vigorously shaken with a beads-beater (FastPrep24; MP Biomedicals, Santa Ana, CA, USA) twice at 4.0 m s^{-1} for 30 sec. DNA in the water phase was purified using the CTAB method followed by isopropanol precipitation [43]. Extracted DNA was stored at -20°C until use.

aprA clone libraries and sequence analysis

For amplification of *aprA* genes, the primers AprA-1-FW and AprA-5-RV [25] were used. PCR conditions were as follows: an initial denaturation step at 94°C for 2 min, followed by 34 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 45 sec, with a final extension step at 72°C for 10 min. PCR products were inserted into the pCR2.1-TOPO vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA, USA) and cloned into competent *Escherichia coli* TOP10 cells according to the manufacturer's instructions. Clones that had inserts of the predicted size were screened by the direct PCR amplification with vector primers (M13F and M13R), and the PCR products were purified with isopropanol precipitation and used for sequencing reaction. The sequencing reactions were done with the ABI BigDye chemistry and analyzed with an ABI 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The *aprA* sequences were translated and the deduced amino acid sequences were aligned using ClustalW implemented in MEGA5.05 software [36]. A pairwise distance matrix was calculated based on the Poisson model. Based on the distance matrix, sequences were classified into operational taxonomic units (OTUs) using mothur software [32], with 97% sequence identity as a threshold. The AprA tree was constructed from deduced amino acid sequences using the neighbor-joining method with bootstrap analysis with 1,000 replications.

DSS-specific 16S rRNA gene clone library

The primers DCC305 and DCC1165 [4] were used to amplify DSS-specific 16S rRNA genes. The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 1 min, with a final extension step at 72°C for 7 min. The PCR products were cloned and sequenced as described above. The sequences were aligned using ClustalW implemented in MEGA5.05 software and classified using mothur software into OTUs based on 98% sequence identity. Final phylogenetic analyses of the 16S rRNA genes were performed with the ARB software package [23] using the ARB reference database SILVA SSU release 108. All sequences were automatically aligned with the SINA web aligner (http://www.arb-silva.de) [28] and the alignments were subsequently optimized manually. The phylogenetic tree was constructed by neighbor-joining and maximum-likelihood analysis with different sets of filters. For construction of the reference tree, only nearly full-length sequences (>1300 bp) were considered. Partial sequences obtained in this study were inserted into the tree by parsimony criteria without allowing changes in the overall tree topology.

Nucleotide sequence accession numbers

Sequence data reported in this study are available in the DDBJ, GenBank and EMBL databases under the following accession numbers: 16S rRNA genes (AB894629-AB894655), and *aprA* genes (AB894656-AB894821).

Probe design

157 An oligonucleotide probe SEEP1d-468 was designed using the probe design tool in the ARB
158 software package [23]. The probe specifically targets the 16S rRNA sequences of SEEP SRB-
159 1d group obtained in Lake Harutori and several environmental clones (Fig. 4). The probe was
160 tested for coverage (target group hits) and specificity (outgroup hits) in silico with the ARB
161 probe match tool [23]. Probe specificity was checked on 618,442 prokaryotic sequences of the
162 SILVA SSU Ref dataset Release 108 [29]. Specific CARD-FISH conditions were determined
163 by hybridizing the probe to the lake water sample at formamide concentrations of 40%, 50%,
164 60%, and 70%, as explained in the following section. A deltaproteobacterial strain PL12 [8]
165 was used as a control having a single mismatch.
166
167 *Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)*
168 Water samples were fixed with paraformaldehyde solution (final concentration 0.9%
169 [v/v]) at 4°C. Afterwards, 200 µL aliquots were filtered onto polycarbonate membrane filters
170 (Cyclopore Track Etched Membrane; pore size, 0.22 µm; 25-mm diameter; Whatman, NJ,
171 USA) within 40 hours and stored at -20°C until further processing. Inactivation of endogenous
172 peroxidases was done by incubating the filters in 0.01 M HCl with 0.3% H_2O_2 for 10 min at
173 room temperature. Cells were permeabilized by incubating the filters in 15 µg mL^{-1} proteinase
174 K (dissolved in 0.1 M Tris–HCl, 0.05 M EDTA [pH 8.0]) for 3 min at 37°C [37]. CARD-
175 FISH and subsequent staining with 4′,6-diamidino-2-phenylindole (DAPI) followed a
176 previously published protocol [27] with the following modification: hybridization was
177 performed for 2 h, and tyramide signal amplification was performed for 15 min, both at 46°C.
178 The washing temperature was 48°C. For the probes SEEP1d-1420 and SEEP1d-468,
179 hybridization and washing temperatures were 35°C, and tyramide signal amplification was
180 performed for 30 min at room temperature. Oligonucleotide probes labeled with horseradish

peroxidase were purchased from Biomers (Ulm, Germany). Probe sequences, probe concentrations and formamide concentrations required for specific hybridization are given in Table S1. Hybridized samples were examined with an epifluorescence microscope (Axioplan2; Zeiss, Germany). The given CARD-FISH counts are the means calculated from 40–70 randomly chosen microscopic fields, corresponding to 2,000 DAPI-stained cells.

Results

Physico-chemical properties of Lake Harutori

In Lake Harutori, a steep chemocline was found between 3.5 and 4.0 m below the surface. Physicochemical parameters are summarized in Figure 2. The thermocline almost coincided with the chemocline. The temperature of the lake bottom was 9°C. Photosynthetically active radiation decreased with depth in the mixolimnion and, in the chemocline, declined to 0% of the surface intensity. Oxygen and sulfide formed opposite concentration gradients. The sulfide concentration increased below the chemocline, reaching a concentration of 10.7 mM at the bottom of the lake. The chloride concentration in the middle of the monimolimnion was about half of the concentration of that in seawater, meaning that the water was brackish. The sulfate concentration was much lower than that of seawater and the maximal concentration was 1.4–2.1 mM in the chemocline. The concentration of DOC increased below the chemocline.

AprA diversity in around the chemocline

Clone libraries for *aprA* were generated from water taken from three different depths (3.0, 3.5, and 4.0 m) and contained 62, 42, and 62 clones, respectively. Based on a 97% identity cut-off of the deduced amino acid sequences, 51 distinct OTUs were identified (Table 1). Representative sequences of each OTU were selected to be shown in the phylogenetic tree in

Figure 3. The phylogenetic analysis of the deduced AprA amino acid sequences revealed a clear difference in the dominant sequences in each library. In the 3.0 m library, from water taken from above the chemocline, OTU A2 was the most dominant OTU (Table 1). The closest cultured species to OTU A2 was *Desulfatitalea tepidiphila* (94–97% sequence identity) [9]. In the upper part of the chemocline at 3.5 m, OTU A1, A3, and A7 were the dominant OTUs (Table 1). Sequences from OTU A3 were closely related to *Desulfocapsa thiozymogenes* (98–100%), which is known to disproportionate inorganic sulfur compounds [10]. Sequences of OTU A7 were related to *Desulfobacterium vacuolatum* (96–97%), which can oxidize a wide variety of organic acids completely [20]. OTU A1 was also the most abundant OTU in the lower part of the chemocline (4.0 m). The closest, but still distantly related, cultured organism was *Desulfofaba gelida* (88–92%), which is a psychrophilic incomplete oxidizer [16]. All the above-mentioned species and OTUs belong to the family *Desulfobacteraceae*. A few sequences related to spore-forming sulfate reducers in the genus *Desulfotomaculum* were found, however, they were all distantly related to cultured species (Table 1). Sequences related to those of known sulfur oxidizers were found at a depth of 3.0–3.5 m, which is above and in the upper part of the chemocline. Of these sulfur-oxidizing bacteria, OTU A11 was related to "*Candidatus* Pelagibacter ubique" (85% identity; EAS84493; Fig. S3), which is one of the most abundant and common species in the pelagic zone of the ocean [30]. Some OTUs were related to *Sulfuricella denitrificans* [18] (Fig. S3), and OTU A5 was related to *Thiobacillus denitrificans* [12] (Fig. S3). OTUs A13 and A20 formed a distinct clade with other environmental clones; however, their function is uncertain (Fig. 3a).

Abundance of SEEP SRB-1d in DSS-specific 16S rRNA gene clone library

The most abundant sequences in the *aprA* clone libraries were those related to the DSS group (Fig. 3b; OTU A1). Therefore, a 16S rRNA gene clone library using a DSS-specific primer set was constructed from a water sample taken at 4.5 m. This clone library contained a total of 27 sequences of ca. 820 bp in size. The sequences are grouped into 10 different OTUs based on 98% identity (Fig. 4). All but one of the sequences (OTU7) are in the DSS group. Almost half of the sequences (13 out of 27 sequences) were grouped into OTU1, which is distantly related to *Desulfofaba fastidiosa* (94.2–94.7% identity). An environmental clone obtained in the Guaymas Basin has high sequence identity (98.5–99.6%) with the sequences in OTU1. OTU1 sequences were grouped into the SEEP SRB-1d cluster in the phylogenetic tree shown in Figure 4, and had no mismatch with the probe DSS658 (Fig. S1) used for the CARD-FISH experiments.

Design and evaluation of new probe SEEP1d-468

An oligonucleotide probe SEEP1d-468 was designed to specifically detect OTU1 in SEEP SRB-1d group, which comprised nearly half of the sequences obtained from the above mentioned DSS group-specific 16S rRNA gene clone library (Fig. 4). The new probe SEEP1d-468 has coverage of nearly half of the SEEP SRB-1d group and two outgroup hits in OTU4 (Fig. 4). When the probe tested with 20% formamide concentration with hybridization temperature at 46°C, only spotty signals were observed. Bright signals were observed at 40% formamide concentration with the hybridization and washing temperature at 35°C. No signal was observed from the reference strain PL12, which has a single mismatch to probe SEEP1d-468, under the same hybridization condition. A previously published probe SEEP1d-1420 [33] showed bright signals in the lake water sample with the same hybridization condition as the probe SEEP1d-468. Since the sequence information about the probe-binding site of

253 SEEP1d-1420 was not available from the DSS specific clone library, the newly designed
254 probe SEEP1d-468 was mainly used for the further analyses.
255
256 *Abundance of DSS bacteria in the monimolimnion*
257 Total cell numbers (DAPI) ranged from 1.3×10^7 to 1.5×10^7 cells mL^{-1} in the mixolimnion,
258 and decreased somewhat in a depth of 3.0 to 3.5 m (Fig. 5). Total cell numbers increased
259 below 3.5 m, reaching up to 3.5×10^7 cells mL^{-1} in the bottom part of the water column (4.8
260 m depth). Total bacterial cell numbers (probe EUB I-III) also increased below the chemocline,
261 reaching up to 1.9×10^7 cells mL^{-1} at 4.8 m. Deltaproteobacteria comprised 7.2–22.1% of
262 total bacteria below 3.0 m (Fig. 5). Among deltaproteobacteria, cells from the DSS group
263 were predominant (probe DSS658). At 4.5 m, in the middle of the monimolimnion, the ratio
264 of DSS was 67% of the deltaproteobacteria (Fig. 6). The cell numbers of SEEP SRB-1d group
265 (probe SEEP1d-468) ranged from $2.3 \times 10^5 – 6.3 \times 10^5$ cells mL^{-1} below the chemocline, and
266 comprised 43.5–79.0% of DSS group (Fig. 6). Another probe SEEP1d-1420 also showed the
267 similar cell numbers, which ranged from $2.6 \times 10^5 – 5.7 \times 10^5$ cells mL^{-1} below the
268 chemocline. The typical morphology of the hybridized cells with probes DSS658 and
269 SEEP1d-468 were coccoid (2–3 μm), as shown in Figure S1. Cells were dispersed or formed
270 small aggregates.
271
272 **Discussion**
273 *Unique characteristics of Lake Harutori*
274 The monimolimnion of Lake Harutori contained a very high concentration of sulfide—up to
275 10.7 mM. Below the chemocline, the seawater seems to be diluted because the chloride
276 concentration is nearly half of the seawater. If the water were diluted homogeneously, the

277 sulfate concentration should remain at half of that in seawater, around 14 mM. However, only
278 0.4–2 mM of sulfate was detected *in situ* (Fig. 2). The sum of the sulfate and sulfide
279 concentrations comes close to 13 mM, which possibly means that most of the sulfate in the
280 monimolimnion had been converted into sulfide. Active sulfate reduction by bacteria would
281 explain the presence of such a high sulfide concentration in the monimolimnion.
282
283 *Diversity of sulfate-reducing and sulfur-oxidizing bacteria in Lake Harutori*
284 Analysis of the *aprA* clone libraries revealed a high diversity of sulfate-reducing and sulfur-
285 oxidizing bacteria. The dominant groups of those bacteria differ around the three different
286 depths of the chemocline. It appears that the availability of electron acceptors and salinity
287 affected the composition of the bacterial community in the lake. Sulfur-oxidizing bacteria
288 were found only where oxygen was available. Among those, some sequences obtained from
289 3.0 m were related to *Sulfuricella denitrificans* [18] and *Thiobacillus denitrificans* [12], which
290 have been isolated from freshwater. Both of them are facultatively anaerobic
291 chemolithoautotrophs. It has been shown that not all of sulfur oxidizers have *aprA* gene [24];
292 therefore we cannot exclude other sulfur oxidizers, such as *Epsilonproteobacteria*, are also
293 present.
294 In the deeper layer of the chemocline, the sulfate-reducing bacterial community is
295 dominated by a single OTU, OTU A1 (Table 1). OTU A1 is in the family *Desulfobacteraceae*
296 and is distantly related to species in the DSS group.
297
298 *Abundance of the DSS group in sulfate-reducing bacteria*
299 In the high diversity of sulfate-reducing and sulfur-oxidizing bacteria observed in the *aprA*
300 clone library, the most dominant sulfate-reducing bacterial OTU was distantly related to

301 species in the DSS group (OTU A1, Table 1). Nearly half of the sequences obtained from the
302 DSS-specific 16S rRNA gene clone library were related to an uncultured group of
303 *Deltaproteobacteria* (OTU1, Fig. 4), previously called as SEEP SRB-1d [33]. Both OTUs 1
304 and A1 are members of the *Desulfobacteraceae*, and the relative abundance of the sequences
305 was very high in the libraries from the anoxic monimolimnion. The OTUs might correspond
306 to each other, but require additional evidence to demonstrate genomic linkage.
307 Members of the DSS group are known as nutritionally versatile SRB [42]. Most of them
308 oxidize organic compounds completely to CO_2, and several species can grow autotrophically
309 with H_2 and sulfate. The DSS group of bacteria is abundant in the upper layer of sediments in
310 many aquatic environments, such as permanently cold marine sediment [31], sediments above
311 gas hydrate [15], temperate tidal flat sediment [26], and a meromictic Lake Cadagno sediment
312 [34]. However, in anoxic water columns, only a few reports about the abundance of the DSS
313 group are available. For example in a permanently anoxic water column in the Black Sea,
314 SRB detected with the SRB385 probe are abundant below 100 m, where sulfide appears, and
315 comprise up to 2–8% of total DAPI counts [22]. The abundance of DSS almost coincides with
316 the cell counts obtained with the SRB385 probe [22, 40]; therefore, DSS is the most dominant
317 group of SRB in the anoxic layer of the Black Sea water column. Some sequences from the
318 DSS group are found in an anoxic brine pool of the Gulf of Mexico [11], below the
319 chemocline of the stratified lagoon in Clipperton atoll [6], and below the chemocline of the
320 meromictic Lake Suigetsu [19]. In contrast, very few sequences from the DSS group have
321 been detected in seasonal anoxic water masses such as those in Saanich Inlet [41], and the
322 Arabian Sea [5], which indicates that the DSS group might prefer permanently anoxic
323 environments in which reduced sulfur compounds can accumulate.

Many SEEP SRB-1d cells were observed in the anoxic layer of the water column (Fig. 6). It has been suggested that SEEP SRB-1 subgroups interact with ANME-2 [15, 33] or use hydrocarbons including short-chain alkanes [13,14]. However, in Lake Harutori, we could not detect ANME-2 cells by CARD-FISH with the specific probe ANME-2 538 [39] (data not shown). In addition, the cells detected with the probe SEEP1d-468 and DSS658 were often dispersed as single cells or formed chain-like small aggregates by themselves. Hence, the specific association of the SEEP SRB-1d with ANME-2 cells is unlikely in this lake. There is a possibility that SEEP SRB-1 have a preference for the availability of hydrocarbons. We detected methane (approximately 1.5 mM) in the monimolimnion of Lake Harutori (data not shown), but no data is available for other hydrocarbons. Further study will be required to confirm this hypothesis.

The DSS group in the chemocline of Lake Harutori can probably use various organic compounds including persistent substances and might prefer a permanently anoxic environment, and are therefore able to be one of the major SRB in the bottom of Lake Harutori. Further cultivation and incubation studies will be required to estimate the contribution of these SRB to sulfide production and carbon remineralization in anoxic water columns, which may expand in the near future.

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496

Figure captions

Fig. 1 Map of Lake Harutori in Kushiro, Hokkaido, Japan.

Fig. 2 Depth profiles of temperature, pH, specific conductivity, concentrations of chloride, dissolved oxygen, sulfate, sulfide, and dissolved organic carbon in the water column of Lake Harutori in May 2012.

Fig. 3 Phylogenetic tree based on deduced AprA amino acid sequences retrieved from Lake Harutori water column. The tree was constructed based on a distance matrix (119 amino acid positions) by using the neighbor-joining method. Bootstrap value estimation was based on 1000 replicates (only values above 50% are shown). Numbers of sequences obtained in each clone library were indicated in parentheses; 3.0 m, 3.5 m, and 4.0 m in that order. a) Overview tree. b) Magnified tree of *Desulfobacteraceae* and *Desulfobulbaceae*. The bar represents 5% estimated sequence divergence.

Fig. 4 Phylogenetic tree showing the affiliation of Lake Harutori 16S rRNA sequences to selected reference sequences of *Desulfobacteraceae* of *Deltaproteobacteria*. Representative sequences from each OTU are in boldface type. The name of the representative clone and the number of the grouped sequences are in parentheses. Probe specificity is indicated by the vertical lines. The bar represents 10% estimated sequence divergence.

Fig. 5 Abundance of microbial cells in Lake Harutori water column determined by CARD-FISH. DAPI (closed circle), EUBI-III (closed triangle), Delta495a (closed square), and DSS658 (open circle).

519 Fig. 6 Abundance of *Deltaproteobacteria* (probe Delta495a), DSS group (probe DSS658)
520 and SEEP SRB-1d cells (probes SEEP1d-1420 and SEEP1d-468) in the anoxic
521 layer of Lake Harutori, determined by CARD-FISH.

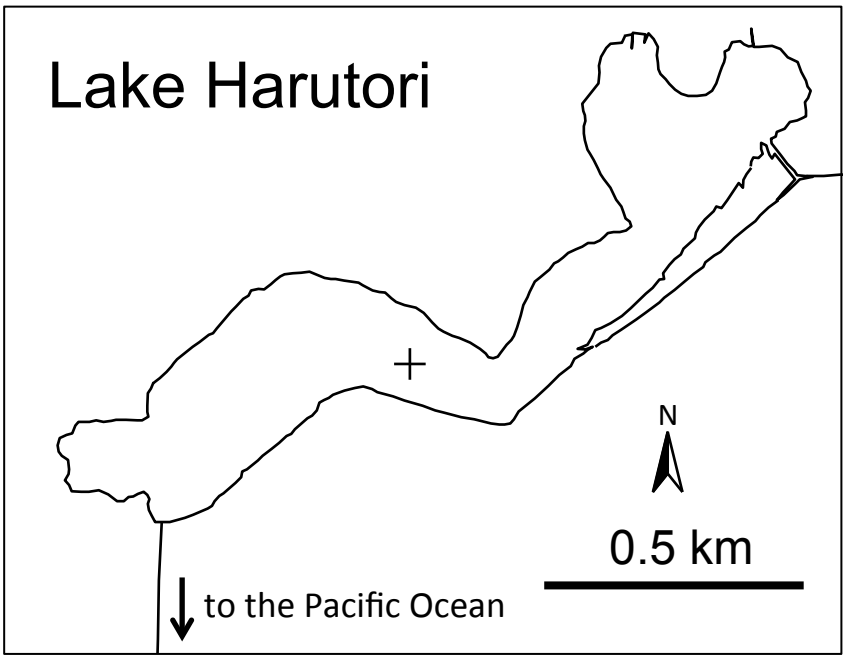

Fig. 1

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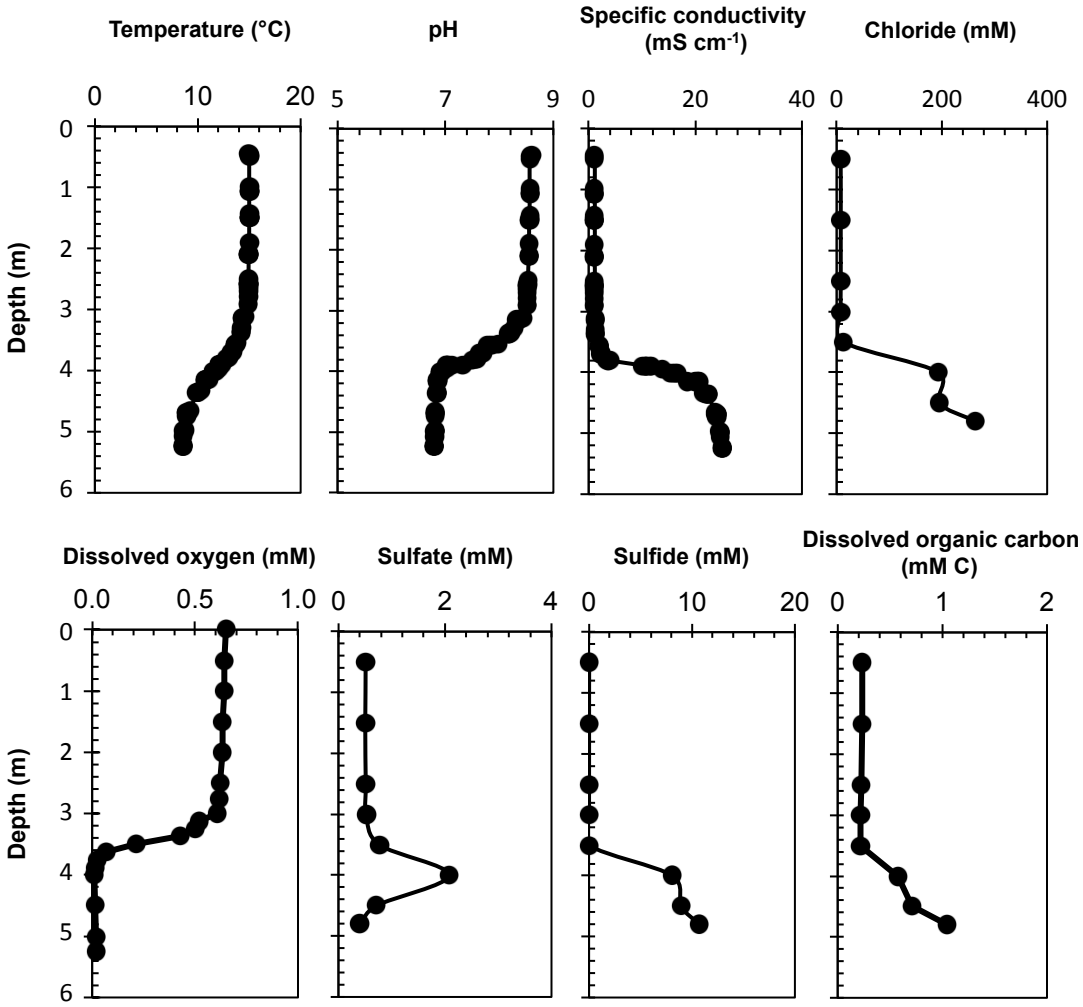

Fig. 2

Kubo et al.

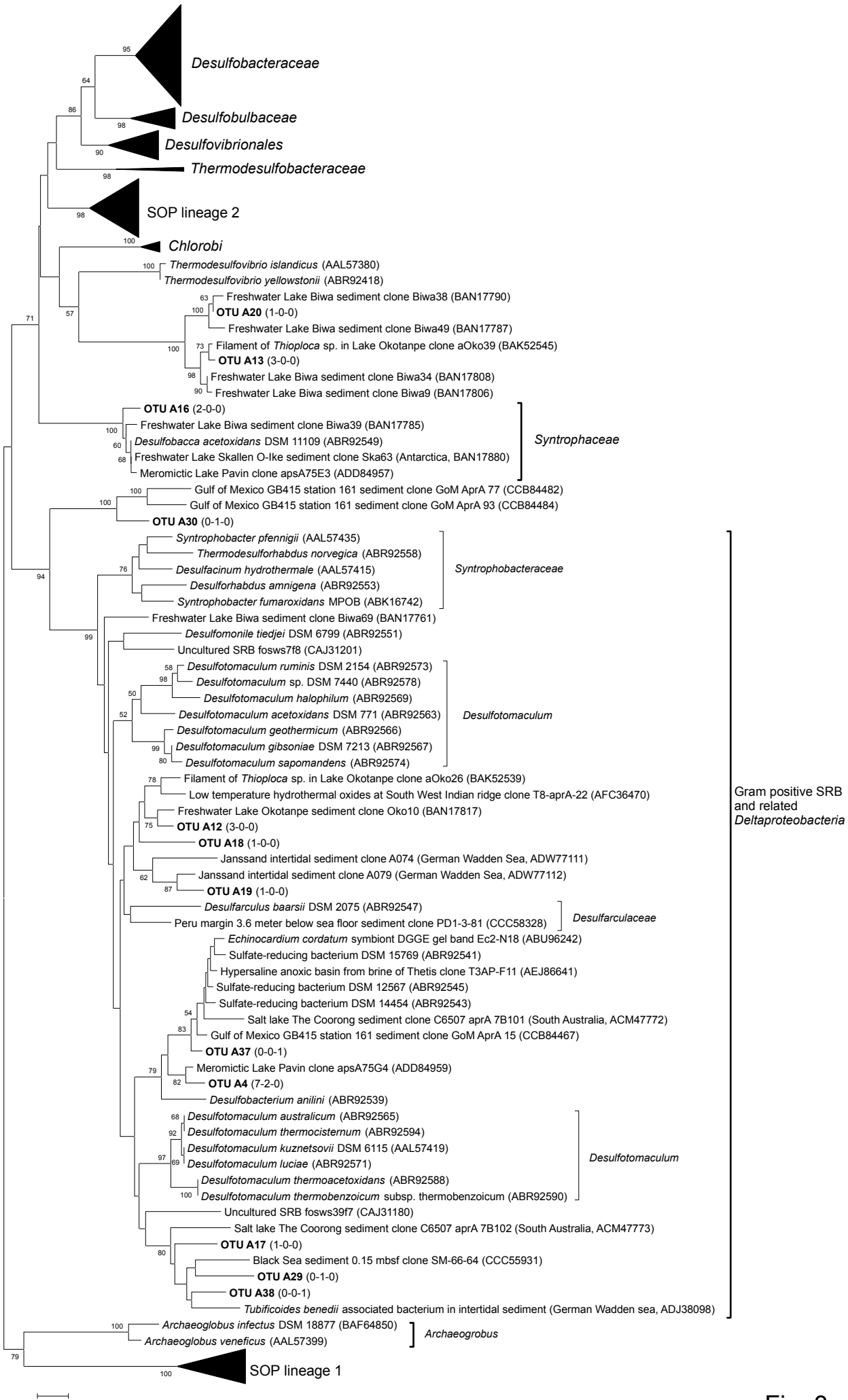

Fig. 3a

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Fig. 3b

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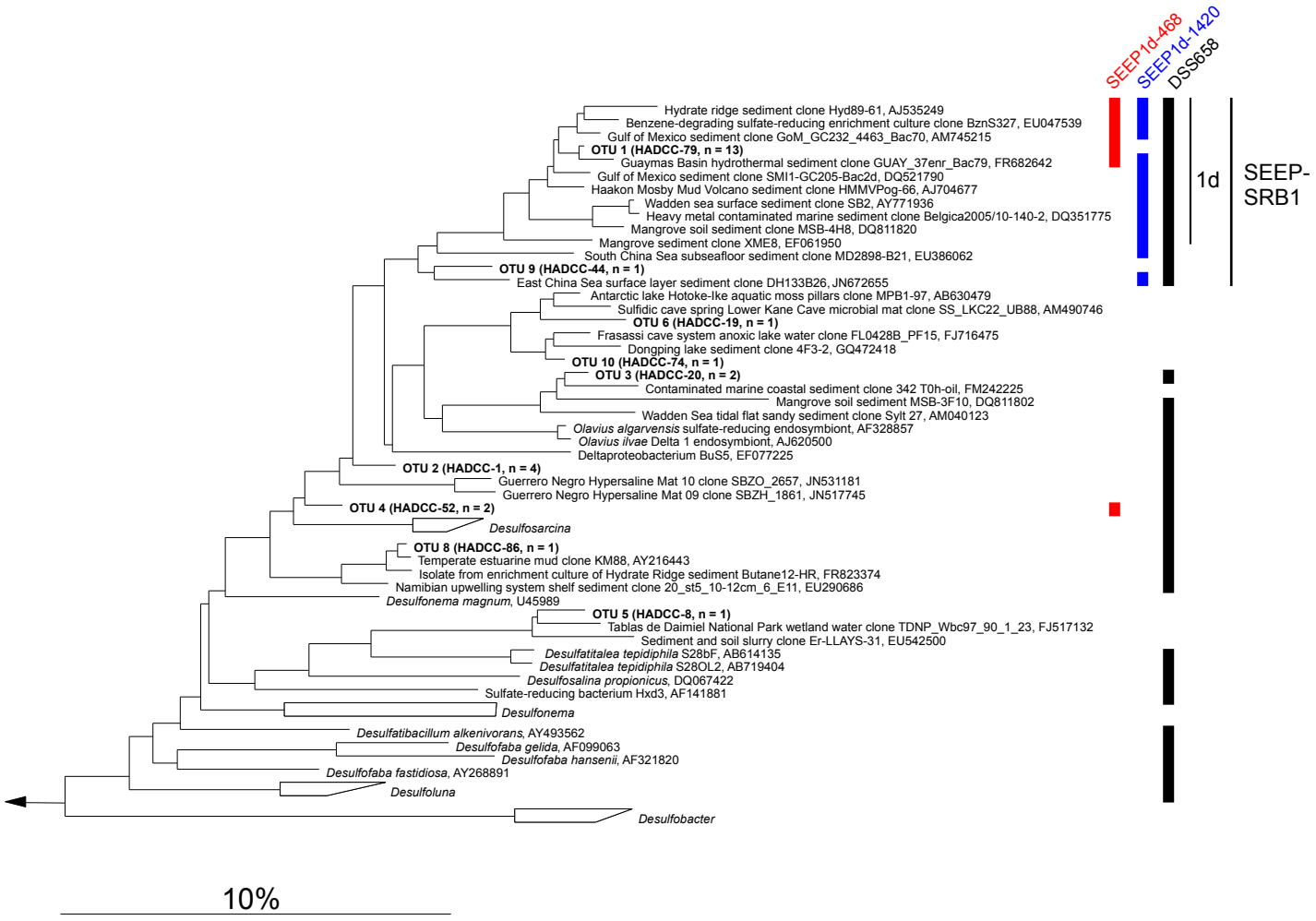

Fig. 4

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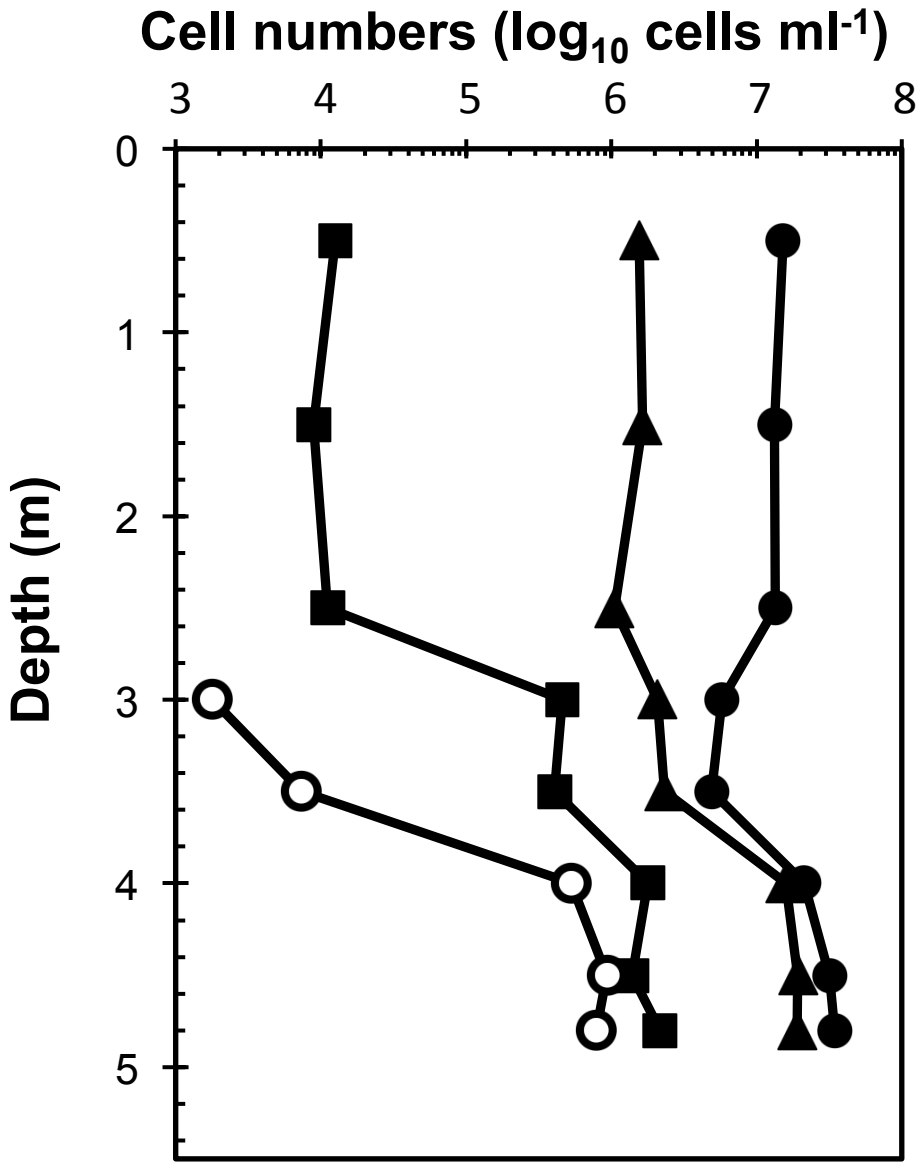

Fig. 5
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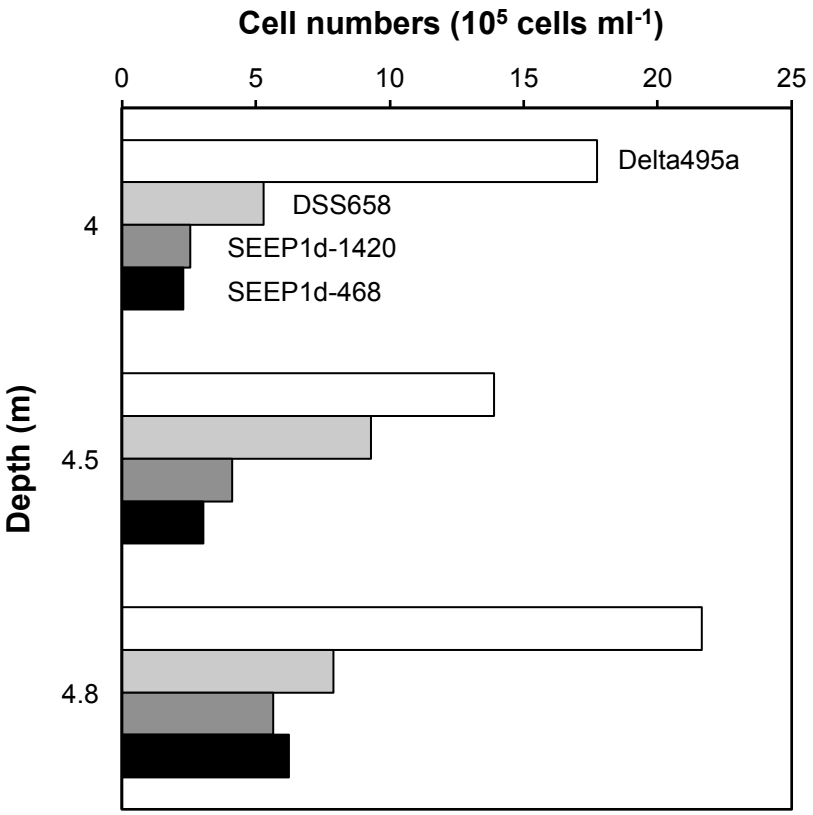

Fig. 6
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Table 1. Phylogenetic affiliation of classified OTUs of aprA clone sequences and the number of clones in each clone libraries.

Putative function	Phylogenetic affiliation	OTU	Closest cultured strain[a]	Identity (%)	Number of clones		
					3.0 m	3.5 m	4.0 m
Sulfate reduction	*Desulfobacteraceae*	OTU A1	*Desulfofaba gelida* PSv29 (AAL57385)	88-92	0	8	41
		OTU A2	*Desulfatitalea tepidiphila* S28OL2 (BAM15569)	94-97	10	0	0
		OTU A6	*Desulfatitalea tepidiphila* S28OL2 (BAM15569)	92-93	0	0	8
		OTU A7	*Desulfobacterium vacuolatum* IbRM (AAL57391)	96-97	0	7	0
		OTU A8	*Desulfofaba gelida* PSv29 (AAL57385)	88-89	5	0	0
		OTU A9	*Desulfofaba gelida* PSv29 (AAL57385)	94-96	1	4	0
		OTU A14	*Desulfobacterium indolicum* InO4 (AAL57390)	96	0	0	3
		OTU A15	*Desulfobacterium indolicum* InO4 (AAL57390)	96	0	1	1
		OTU A24	*Desulfobacterium autotrophicum* HRM2 (AAL57375)	97	0	1	0
		OTU A25	*Desulfobacterium autotrophicum* HRM2 (AAL57375)	95	0	1	0
		OTU A26	*Desulfofaba gelida* PSv29 (AAL57385)	92	0	1	0
		OTU A27	*Desulfatitalea tepidiphila* S28bF (BAM15567)	97	0	1	0
		OTU A28	*Desulfobacterium indolicum* InO4 (AAL57390)	94	0	1	0
		OTU A32	*Desulfobacterium indolicum* InO4 (AAL57390)	94	0	0	1
		OTU A33	*Desulfobacterium indolicum* InO4 (AAL57390)	93	0	0	1
		OTU A34	*Desulfatitalea tepidiphila* S28OL1 (BAM15568)	92	0	0	1
		OTU A35	*Desulfatitalea tepidiphila* S28OL1 (BAM15568)	92	0	0	1
		OTU A36	*Desulfatitalea tepidiphila* S28OL1 (BAM15568)	92	0	0	1
		OTU A47	*Desulfofaba gelida* PSv29 (AAL57385)	93	1	0	0
		OTU A48	*Desulfonema magnum* Montpellier (AAL57389)	94	1	0	0
		OTU A49	*Desulfonema magnum* Montpellier (AAL57389)	92	1	0	0
		OTU A50	*Desulfobacterium autotrophicum* HRM2 (AAL57375)	94	1	0	0
		OTU A51	*Desulfatitalea tepidiphila* S28OL2 (BAM15569)	91	1	0	0
	Desulfobulbaceae	OTU A3	*Desulfocapsa thiozymogenes* Bra2 (AAL57433)	98-100	0	8	1
		OTU A31	*Desulforhopalus vacuolatus* Itk 10 (ABR92535)	99	0	0	1
	Syntrophaceae	OTU A16	*Desulfobacca acetoxidans* ASRB2 (ABR92549)	96	2	0	0
	Gram positive SRB and related *Deltaproteobacteria*	OTU A4	*Desulfobacterium anilini* Ani1 (AAL57425)	86-88	7	2	0
		OTU A12	*Desulfotomaculum kuznetsovii* 17 (AAL57419)	86	3	0	0
		OTU A17	*Desulfotomaculum kuznetsovii* 17 (AAL57419)	83	1	0	0
		OTU A18	*Desulfotomaculum geothermicum* BSD (AAL57382)	83	1	0	0
		OTU A19	*Desulfotomaculum kuznetsovii* 17 (AAL57419)	79	1	0	0
		OTU A29	*Desulfotomaculum thermoacetoxidans* CAMZ (ABR92588)	78	0	1	0
		OTU A37	*Desulfobacterium anilini* Ani1 (AAL57425)	88	0	0	1
		OTU A38	*Desulfotomaculum kuznetsovii* 17 (AAL57419)	80	0	0	1
Sulfur oxidation	Lineage 1[b]	OTU A11	*Thiocapsa rosea* 6611 (ABV80104)	79	4	0	0
		OTU A21	*Thiocapsa rosea* 6611 (ABV80104)	92	1	0	0
		OTU A22	*Thiocapsa rosea* 6611 (ABV80104)	90	1	0	0
		OTU A23	*Lamprocystis purpurea* ThSch 12 (ABV80005)	89	0	1	0
	Lineage 2[b]	OTU A5	*Thiobacillus denitrificans* GP3 (ABV80033)	98-100	4	4	0
		OTU A10	*Sulfuricella denitrificans* skB26 (BAI66427)	97-98	4	0	0
		OTU A39	*Sulfuricella denitrificans* skB26 (BAI66427)	97	1	0	0
		OTU A40	*Sulfuricella denitrificans* skB26 (BAI66427)	97	1	0	0
		OTU A41	*Sulfuricella denitrificans* skB26 (BAI66427)	91	1	0	0
		OTU A42	*Sulfuricella denitrificans* skB26 (BAI66427)	92	1	0	0
		OTU A43	*Sulfuricella denitrificans* skB26 (BAI66427)	83	1	0	0
		OTU A44	*Sulfuricella denitrificans* skB26 (BAI66427)	85	1	0	0
		OTU A45	*Sulfuricella denitrificans* skB26 (BAI66427)	87	1	0	0
		OTU A46	*Thiothrix* sp. CT3 (ABV80023)	100	1	0	0
Uncertain	Unclassified[c]	OTU A13	N.A.	N.A.	3	0	0
		OTU A20	N.A.	N.A.	1	0	0
		OTU A30	N.A.	N.A.	0	1	0
				Total number of clones	62	41	62

[a] Closest cultured strains were determined according to BLASTP search result. GenBank accession number of AprA protein is in parentheses.
[b] Classification of the lineages of sulfur-oxidizing bacteria is according to Meyer and Kuever [24].
[c] Sequences have less than 70% sequence identity to any known sequences were grouped as Unclassified.
N.A., Not available