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EXPERIMENTAL ARTICLES

Activity and Structure of the Sulfate-Reducing Bacterial Community in the Sediments of the Southern Part of Lake Baikal

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Abstract—The rates of sulfate reduction (SR) and the diversity of sulfate-reducing bacteria (SRB) were studied in the sediments of the Posol'skaya Banka elevation in the southern part of Lake Baikal. SR rates varied from 1.2 to 1641 nmol/(dm^3 day), with high rates (>600 nmol/(dm^3 day)) observed at both deep-water stations and in subsurface silts. Integral SR rates calculated for the uppermost 50 cm of the sediments were higher for gas-saturated and gas hydrate-bearing sediments than in those with low methane content. Enrichment cultures were obtained in Widdel medium for freshwater SRB. Analysis of the 16S rRNA gene fragments from clone libraries obtained from the enrichments revealed the presence of SRB belonged to the genus *Desulfosporosinus*, with *D. lacus* as the most closely related member (capable of sulfate, sulfite, and thiosulfate reduction), as well as members of the order *Clostridiales*.

Keywords: sulfate reduction, sulfate-reducing bacteria, gas-saturated and gas hydrate-bearing sediments, Lake Baikal

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Sulfate-reducing bacteria (SRB) are a group of anaerobic prokaryotes using sulfate as the terminal electron acceptor. Various organic compounds (mostly of low molecular mass) and hydrogen, which are produced at the first stage of organic matter (OM) decomposition by a community of aerobic and anaerobic microorganisms possessing a set of hydrolytic enzymes, may act as electron donors for dissimilatory sulfate reduction. Capacity of some SRB for anaerobic respiration with electron acceptors other than sulfate (elemental sulfur, thiosulfate, nitrate, Mn(IV), Fe(III), etc.) [1, 2], as well as their efficient systems of antioxidant protection [3, 4], provide for their occurrence in diverse natural and anthropogenic habitats. In reduced sediments of seas and sulfate-rich lakes (>1 mM) SRB are known to play the major role in OM mineralization [5, 6]. Resistance to low concentrations of dissolved oxygen makes it possible for SRB to grow actively at the oxic–anoxic interfaces in the water of meromictic lakes [7–9] and in the sediments [10], where SRB numbers may reach 10^5–10^8 cells per mL water or cm^3 sediment. In anaerobic horizons of freshwater basins, the biogeochemical activity of SRB is limited by low concentrations of sulfate, which is almost completely reduced by these microorganisms in the uppermost sediment horizons [11]. However, since K_m for sulfate consumption varies from 5 to 30 μM, SRB are well adapted to low sulfate concentrations and remain active in low-mineral environments.

Lake Baikal, the deepest freshwater lake on Earth, belongs to the group of low-mineral lakes with the total salt concentration of ~100 mg/L. According to numerous measurements, the average sulfate concentration in the water of various Lake Baikal sites is 5.5 mg/L [12]. Even at high depths, the near-bottom water horizons contain high levels of dissolved oxygen (87–100% of air saturation) [13]. Active development of SRB communities in the deep-water zone is therefore possible only in subsurface sediment horizons below the oxidized sediments; the latter usually form 3–15 cm layers, depending on the site.

Application of specific growth media made it possible to reveal viable SRB cells in Lake Baikal sediments over 35 years ago [14]. Later, Namsaraev et al. [15] used radioisotope techniques to reliably demonstrate detectable sulfate reduction (SR) rates from 0.3 to 1200 nmol/(dm^3 day) in the upper 15–20 cm of the

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Characteristics of the sampling stations in the southern Lake Baikal

Station no./depth	Sampling device	Coordinates	Brief characterization of the sediment
3/51	BT, GC	52°04.7965′ N 105°54.2216′ E	0–2 cm, oxidized brownish-yellow clayey aleurite; deeper, dark-gray and gray aleuro-pelitic silt with dark layers
4/508	GR, GC	52°03.2460′ N 105°54.2681′ E	0–1 cm, oxidized brownish-gray clayey aleurite; below to 50 cm, dark gray aleuro-pelitic silt with dark layers; below 50 cm, striated soft gray silt with dark layers
5/500	BT, GC	52°02.1705′ N 105°50.6025′ E	0–1 cm, oxidized brownish-gray clayey aleurite; below, gray aleuro-pelitic silt with dark layers; below 20 cm, gas-bearing silt with gas caverns; below 75 cm, GH
9/111	BT	52°07.3393′ N 105°58.9617′ E	0–1 cm, oxidized brown aleuro-pelitic silt; 1–50 cm, dark gray aleuro-pelitic silt; below 50 cm, light gray soft clays
10/850	BT, GC	52°10.4786′ N 105°48.5499′ E	0–1 cm, oxidized brownish-yellow aleuro-pelitic silt; 1–90 cm, dark gray and gray aleuro-pelitic silt with indication of gas saturation below 40–50 cm; below 90 cm, gray clayey silt with numerous lamellar GH layers

* BT, benthic tube, GC, gravity corer, GR, grapple.

sediments. The highest rates of this process were reported at the area where the wastewater of the Baikal'sk pulp-and-paper mill was discharged. The highest SR rates detected in deep-water sediments did not exceed 7 nmol/(dm^3 day). The SRB number in the sediments of various Lake Baikal areas determined on Postgate medium varied from 0.5×10^3 to 2.1×10^5 cells/cm^3 wet silt. Immunofluorescence analysis revealed the presence of *Desulfovibrio desulfuricans* and *Desulfotomaculum guttoideum* in the sediments (up to 3.3×10^4 cells/cm^3). Analysis of C_{org} consumption for sulfate reduction and CH_4 formation indicated that methanogenic archaea usually played the major role in the terminal phase of OM decomposition in Lake Baikal sediments, while SRB were actively involved in the degradation processes only at certain sites [15].

Significant SO_4^{2-} concentrations (up to 15 mM) were revealed in the pore water in the sediments of the Malen'kii mud volcano in the southern part of the lake at the depth of 1370–1393 m [16, 17]. In the sediments of this volcano, SR rates varied from 0.001 to 0.7 nmol/(cm^3 day) and were significantly higher than the SR rates measured previously in Lake Baikal deep-water sediments.

Investigation of the structure of the microbial community of the St. Petersburg methane seep by 16S rRNA gene pyrosequencing and analysis of the functional genes revealed predominance of methanogenic archaea of the orders *Methanomicrobiales* and *Methanosarcinales* in the upper sediment layers [18]. Importantly, neither SRB, nor the known uncultured anaerobic methanotrophic archaea were revealed in the upper sediments and in the gas hydrate (GH) zone at the depth of 80–85 cm. Sulfate distribution profile in the pore water exhibited, however, a pronounced maximum (~0.17 mM) [18], indicating sulfate reduction in the upper sediments, similar to other geochemically active areas of Lake Baikal [17]. Since below 20 cm sulfate content in pore waters decreased almost to zero, the absence of anaerobic methanotrophic archaea, which, in a consortium with SRB, are responsible for anaerobic methane oxidation [19–21], was understandable. Thus, while the data on SR rates in Lake Baikal sediments are available in the literature, almost no data exist concerning the structure of microbial communities responsible for sulfate reduction in these sediments.

The goal of the present work was to investigate the rates of sulfate reduction and to reveal the structure of the SRB communities in gas-saturated and hydrate-bearing sediments of the southern part of Lake Baikal.

MATERIALS AND METHODS

Sampling and measurement of sulfate reduction rates. Bottom sediments were sampled in June 2012 from R/V *G.Yu. Vereshchagin* at the Posol'skaya Banka elevation. The map of the polygon showing the sampling site locations is presented on Fig. 1. Coordinates of the sampling stations, their depths, and sampling equipment used are listed in the table.

Sulfate reduction rates were determined by the radioisotope method with ^{35}S-labeled sulfate. Immediately after heaving on board, the sediments (3 mL) from the relevant horizons were collected into cut-off 5-mL plastic syringes and sealed with butyl rubber stoppers. The label (0.2 mL ^{35}S–SO_4^{2-}, 10 μCi) was injected through the stopper. After incubation in a refrigerator for 1–2 days, the samples were fixed with 1 mL 2N KOH prior to transportation to the stationary

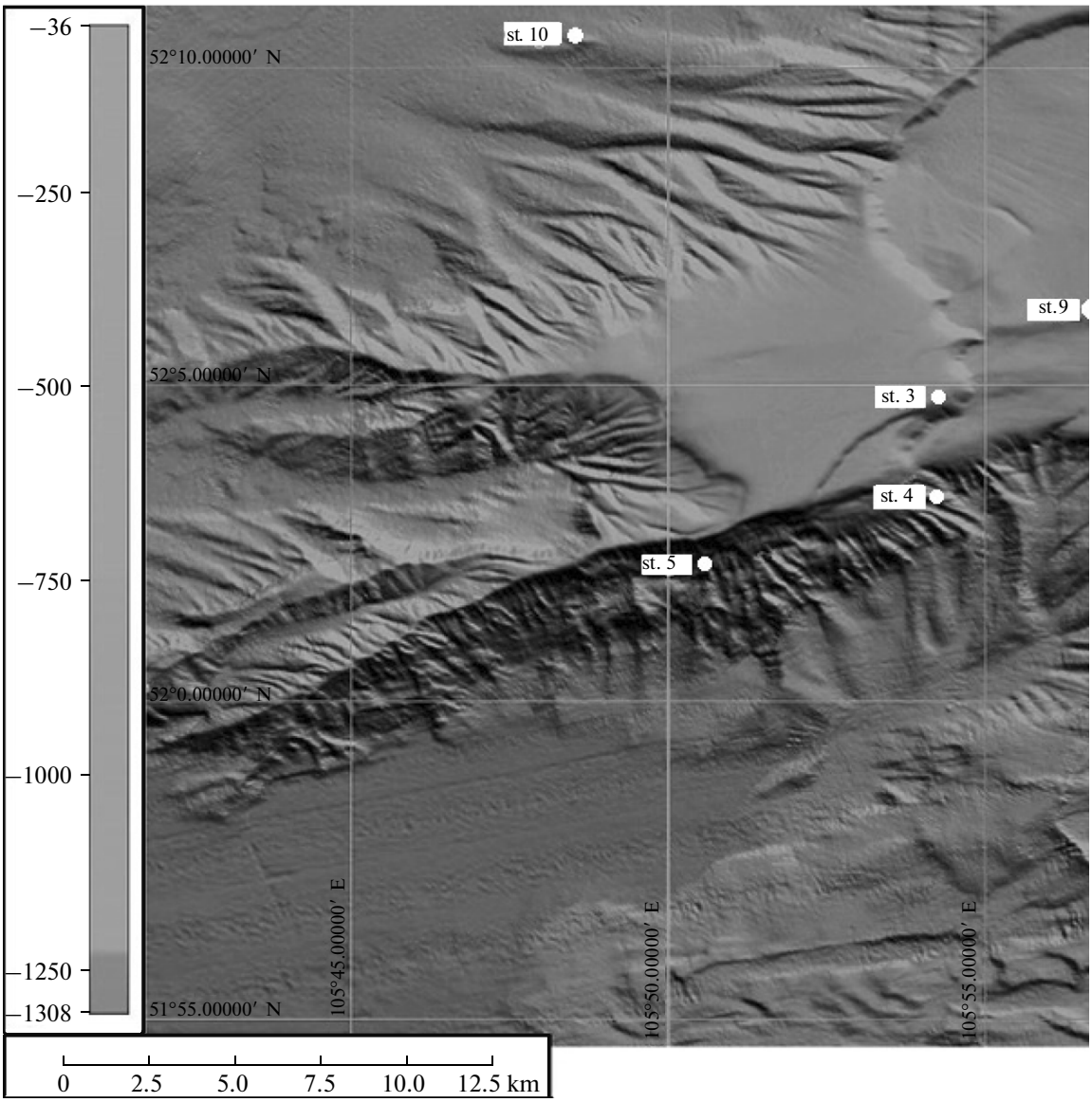

Fig. 1. Multibeam bathymetric map of the Posol'skaya Banka polygon, with sampling stations marked in white (the mapping was supported by the program of the Presidium of the Russian Academy of Sciences, project no. 23.7 and Fonds Wetenschappelijk Onderzoek, project no. 1.5.198.09).

laboratory. The samples were then treated as described previously [22].

Pore water was obtained by centrifugation of the sediments for 10 min at 8000 *g* in a TsUM-1 centrifuge (Russia). Sulfate concentration in pore water was measured on a Stayer ion chromatograph (Russia).

Methane content in the sediments was measured by headspace analysis [23] on an EKhO-PID chromatograph (Russia) equipped with a flame ionization detector (2 m packed column, 2 mm inner diameter, Porapak as the sorbent, isothermal mode at 100°C).

Enrichment cultures. Enrichment cultures of sulfate-reducing bacteria were obtained in liquid Widdel medium for freshwater SRB [24] supplemented with vitamins and yeast extract (0.5 g/L).

The media were prepared using the Hungate anaerobic technique [25]. Elevated sulfide concentration compared to the control was an indicator of SRB growth. Sulfide was determined colorimetrically with *N*,*N*-dimethyl-*p*-phenylenediamine according to Trüper and Schlegel [26] at 670 nm.

The first series of enrichments, which did not contain selenite ($Na_2SeO_3 \cdot 5H_2O$) or tungstate ($Na_2WO_4 \cdot 2H_2O$), were incubated for 30 days at 18–20°C. These trace elements were added in two subsequent reinoculations, since without them poor or no growth occurred. Growth of enrichment cultures with selenite and tungstate was maintained for 14 days.

Four enrichments (st. 4, 0–1 cm; st. 5, 1–3 and 45–50 cm; and st. 9, 1–3 cm) resulted in significant sulfide production. These cultures were used for analysis of the 16S rRNA genes.

Molecular identification techniques. DNA (30–50 μg/mL) was isolated from enrichment cultures according to [27]. In order to obtain the most representative sampling for subsequent cloning, PCR amplification of the 16S rRNA gene fragments was carried out using the Univ11F–Univ1492R universal primer system [28]. The PCR reaction mixture (50 μL) contained the following: 1× BioTaq DNA polymerase buffer solution (17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 2 mM $MgCl_2$); 12.5 nmol of each dNTP; 50 ng template DNA; 5 pmol of each primer; and 3 U BioTaq DNA polymerase (Dialat, Russia). The PCR profile was as follows—one cycle: 9 min at 94°C, 1 min at 55°C, and 2 min at 72°C; 30 cycles: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and one cycle for 7 min at 72°C. PCR products were analyzed by electrophoresis in 2% agarose gel at 6 V/cm. PCR products were isolated and purified using the Wizard PCR Preps kit (Promega, United States) according to the manufacturer's recommendations.

PCR fragments were cloned in the competent cells of *E. coli* DH5α. Ligation was carried out using the pGEM-T and pGEM-T Easy Vector Systems kits (Promega, United States) according to the manufacturer's recommendations. Transformation of the competent cells was carried out according to the standard procedure [29]. The clones with the PCR fragment inserts (20 to 24 clones per each of the four libraries) were selected using X-gal/IPTG. Plasmid DNA was isolated from 12-h cultures of *E. coli* DH5α grown in liquid LB medium with ampicillin (50 U/mL) using the Wizard Minipreps kit (Promega, United States) according to the manufacturer's recommendations. The plasmid DNA samples were stored at –18°C.

The PCR fragments of the 16S rRNA genes were sequenced according to Sanger on an ABI PRISM 3730 automatic sequencer (Applied Biosystems, United States) using the BigDye Terminator v. 3.1 kit (Applied Biosystems, United States) according to the manufacturer's recommendations; the 11F primer was used for sequencing [28]. Initial analysis of the similarity of the 16S rRNA gene sequences to the known GenBank sequences (http://www.ncbi.nlm.nih.gov/genbank/) was carried out using the BLAST software package and the RDP search engine (http://rdp.cme.msu.edu) with reliability threshold of at least 95%. The operational taxonomic units (OTUs) comprising the sequences with at least 98% similarity were accepted as the phylotypes representing the members of the community at the species level. These OTUs were used for further analysis. The phylogenetic tree was constructed using the neighbor-joining algorithm implemented in the TREECONW software package (http://bioinformatics.psb.ugent.be/psb/Userman/treeconw.html), and using the reference sequences from the RDP and GenBank databases.

The nucleotide sequences of the 16S rRNA genes were deposited to GenBank under accession nos. KF220589–KF220610.

RESULTS AND DISCUSSION

Lithology of the sediments collected at the Posol'skaya Banka polygon did not show considerable variation (table). At all stations, the upper oxidized layer of the sediments did not exceed 2 cm. Reduced gray aleuro-pelitic silts with dark layers of hydrotroilite (amorphous iron sulfide) lay below. At the shallow stations 3 and 9 (51 and 111 m, respectively), the upper 15–20 cm of the sediments had low methane content of up to 45 μmol/dm^3 (Fig. 2). In the sediments of the deep-water stations (500, 508, and 850 m for stations 4, 5, and 10, respectively), where GH were revealed in the sediment column, methane concentration exceeded 90 μmol/dm^3 even in the upper layers (Fig. 2).

Sulfate content in pore water of the stations studied varied from 9 to 130 μM with the highest sulfate concentrations in the uppermost 25 cm of the sediment. Below 45 cm, sulfate content in pore waters did not exceed 20 μM (Fig. 3).

The rates of SR are presented on Fig. 4. At shallow stations 3 and 9, considerable activity of the SRB community was observed in the upper 20 cm of the sediments. Below 20 cm, SR decreased sharply and did not exceed 32 nmol/(dm^3 day). In deep-water, gas-saturated and GH-bearing silts of stations 4, 5, and 10 SR rates also peaked in the upper 5–15 cm of the sediment. However, unlike the shallow stations, a considerable activity of the SRB community was observed at the depths of 40–50 cm (Fig. 3), where high methane content was detected in the sediments. SR rates measured at the Posol'skaya Banka stations varied from 1.2 to 1641 nmol/(dm^3 day), with the highest rates (over 600 nmol/(dm^3 day) were revealed at both shallow and deep-water stations in subsurface (5–15 cm) grayish silts with hydrotroilite layers (Fig. 4). These results agree with the data on SR rates in the gas-saturated and GH-bearing sediments of the Malenky mud volcano [17]. In the latter case, SR rates varied within the range of 1 to 800 nmol/(dm^3 day), with the highest rates of this process also occurring in the interface between oxidized and reduced sediments at the sediment depth of 15–20 cm.

Since in the recent studies of deep-water sediments of the St. Petersburg methane seep the presence of SRB was not revealed by pyrosequencing of the 16S rRNA genes and analysis of the functional genes [18], conventional enrichment culture techniques were used in the present work. Widdel medium for freshwater SRB was inoculated with the material from subsurface sediment horizons (the zone of transition from

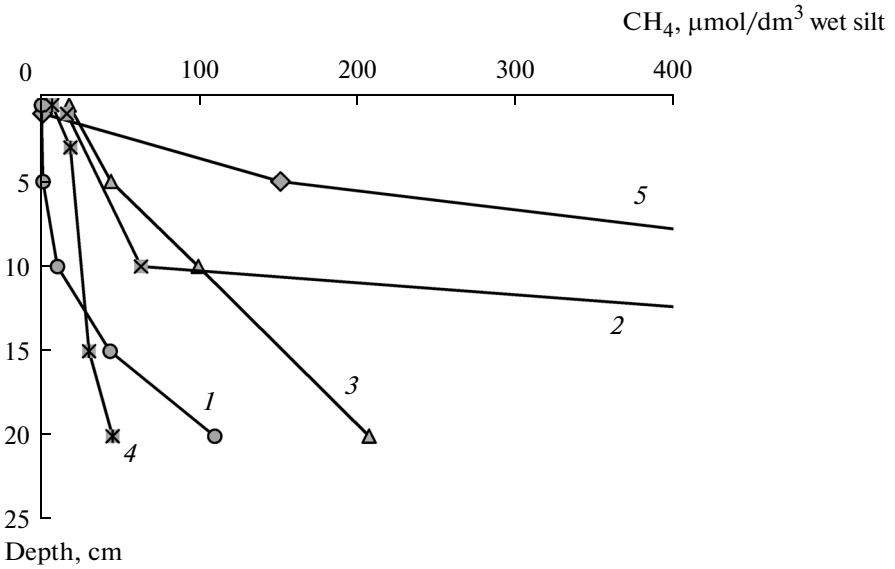

Fig. 2. Methane content in the upper sediment horizons of some stations of the Posol'skaya Banka area, Lake Baikal: st. 3 (*1*), st. 4 (*2*), st. 5 (*3*), st. 9 (*4*), and st. 10 (*5*).

the oxidized to reduced silts, where sulfate reduction is usually activated) and from the gas-bearing horizon of station 5 (45–50 cm, above the GH-containing layer). Since many SRB are known to retain activity in oxidized sediments [3, 4], material from the upper oxidized layer of station 4 was also used for inoculation.

For analysis of the clone library obtained from the enrichment from the upper (0–1 cm) sediment layer (st. 4), 20 clones with the fragments of the 16S rRNA gene sequences were selected. The dominant phylotype of this library, 1-0-otu1 (11 clones) was classified as a *Desulfosporosinus* species with 96% similarity with the type strain of the most closely related species, *Desulfosporosinus auripigmenti* (formerly *Desulfotomaculum auripigmentum*). The next most abundant phylotype, 1-0-otu2 (5 clones) was most closely related to the type strain of *Tissierella creatinini* (96% similarity), while the remaining minor phylotypes (1 clone each) were related to *Clostridium* and *Acetivibrio* species (95–99% similarity). The sulfate reducer *D. auripigmenti* [30] was originally isolated from freshwater lake sediments in the United States. Abundance of anaerobic proteolytic bacteria *Tissierella* and *Clostridium* may be due to the relatively high OM content in Lake Baikal sediments. Moreover, the species *C. thiosulfatireducens*, which is closely related to one of the clones, is known to reduce thiosulfate and sulfur [31].

According to analysis of the 16S rRNA gene fragments, the library (19 clones) of the enrichment culture from the subsurface sediment layer (1–3 cm) of station 5 exhibited the highest diversity. The dominant phylotype 1-3-5-otu1 (6 clones) was related to the phylotype 1-0-otu2 from the upper layer and to *Tissierella creatinini* (95%). The remaining phylotypes represented the minor components (1–2 clones); with a single exception, they belonged to the order *Clostridiales*. Only six phylotypes, however, were identified as members of the genera *Clostridium, Anaerosporobacter, Sedimentibacter*, and *Desulfosporosinus* (93–98% similarity). Thus, unlike the uppermost layer library, in the subsurface sediment layer sulfate reducers of the genus *Desulfosporosinus* were a minor component; the most closely related species, *D. lacus* (98% similarity), was, like *D. auripigmenti*, isolated from freshwater lake sediments [32]. Other minor phylotypes of the order *Clostridiales*

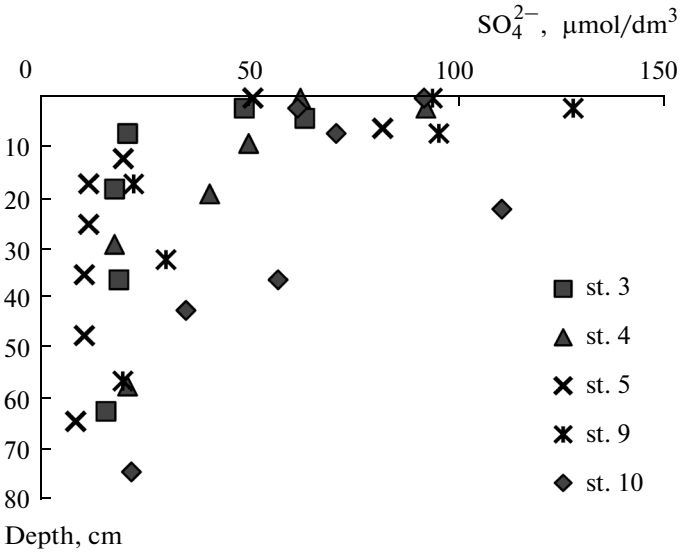

Fig. 3. Concentrations of sulfate ion in pore waters of the Posol'skaya Banka sediments, Lake Baikal.

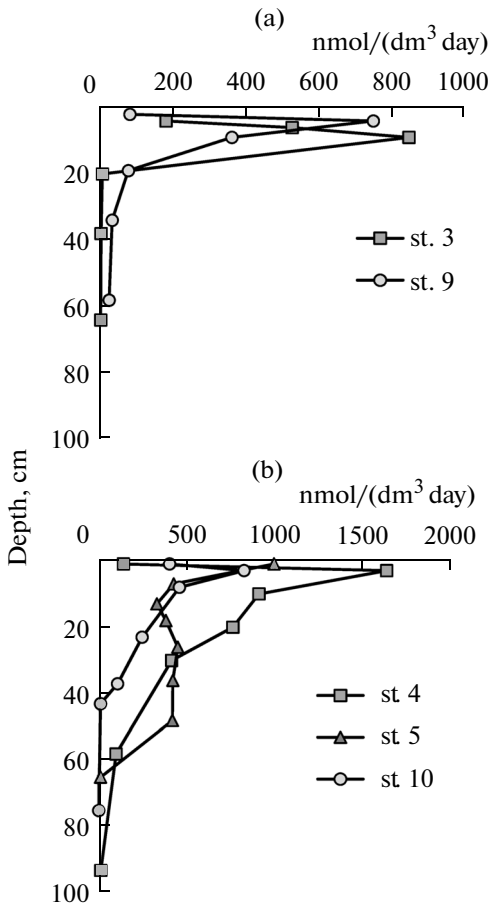

Fig. 4. Sulfate reduction rates in the Posol'skaya Banka sediments: shallow sediments (51 and 111 m) without gas hydrate layers (a) and deep-water sediments (500, 508 and 850 m) with gas hydrate layers (b).

exhibited no relation to the known species and genera of this order, but had high similarity (up to 99%) to uncultured microorganisms from various sources (Fig. 5). The remaining minor phylotype from this library belonged to the order *Natranaerobiales*; as a member of this order it was closely related to an unidentified microorganism from an iron-reducing enrichment culture (Fig. 5).

The library (24 clones) from the subsurface layer (1–3 cm) of the shallow station 9 exhibited lower diversity. The dominant phylotype 1-3-9-otu1 (23 clones) was related (98% similarity) to the species *C. crotonatovorans*, while the only minor phylotype (1 clone) belonged to a sulfate reducer of the genus *Desulfobulbus*, order *Desulfobacterales* of the *Deltaproteobacteria* (98% similarity to *Desulfobulbus propionicus* DSM 2032 isolated from freshwater silt in Germany). Since recent publications report the isolation of new clostridia capable of dissimilatory sulfate reduction [33], predominance of *Clostridiales* in the enrichments may also indicate their involvement in sulfidogenesis.

The library from the enrichment inoculated with deep sediment samples (45–50 cm) of station 5 contained 24 clones. The dominant phylotype 45-50-otu1 (14 clones) belonged to the order *Selenomonadales* (99% similarity with the type strain *Sporotalea propionica*). The next abundant phylotype (7 clones) was most closely related to *Desulfosporosinus* (98% similarity to *D. lacus* STP12^T), while the only minor phylotype (1 clone) was almost identical (99.8%) to one of the minor phylotypes of the 1-3-5 library and belonged to the genus *Clostridium* (97% similarity to the type strain of *Clostridium bowmanii*).

Thus, sulfate-reducing bacteria of the genus *Desulfosporosinus* were found in both surface (0–1 cm) and deep (45–50 cm) layers of the Posol'skaya Banka sediments. The most closely related member of this species, *D. lacus*, is able to reduce sulfate, sulfite, and thiosulfate.

Isolation of pure cultures of the Lake Baikal sulfate reducers by transferring the colonies formed on anaerobic freshwater Widdel agar medium into liquid media is presently under way. After isolation of pure SRB cultures, their molecular identification will be carried out.

Integral SR rates calculated for the upper 50 cm of the sediments were higher in gas-bearing and GH-bearing sediments than in shallow sediments with low methane content (Fig. 4). Higher SR rates in the silts of stations 4, 5, and 10 may be associated with the development of methane-oxidizing anaerobic communities. A consortium of methanotrophic archaea and sulfate-reducing bacteria is known to be responsible for anaerobic methane oxidation (AOM) in marine sediments, with sulfide production associated with anaerobic methane oxidation responsible for 40% and more of the SR rate in methane-rich reduced sediments [21, 34]. Sulfate content in reduced silts of Lake Baikal is, however, too low for the geochemically significant sulfate-dependent methane oxidation. While AOM was revealed in Lake Baikal silts by radioisotope techniques, with the maximum usually close to the GH layers [17], molecular techniques did not reveal methane-oxidizing archaea ANME-1, 2, or 3, which are responsible for AOM in marine sediments. In methane-enriched Lake Baikal sediments the archaea producing methane may be responsible for AOM [35]. Alternatively, other microorganisms than sulfate reducers (e.g., Fe^{3+} or Mn^{4+}-reducers) may act as electron acceptors in the AOM consortium. It may be also possible that denitrifying bacteria, rather than archaea, may be responsible for AOM in Lake Baikal sediments according to the recently described intracellular oxygenic mechanism [36]. Higher SR rates in methane-enriched sediments probably also result from enhanced content of organic matter in these silts due to activity of methanotrophic microorganisms.

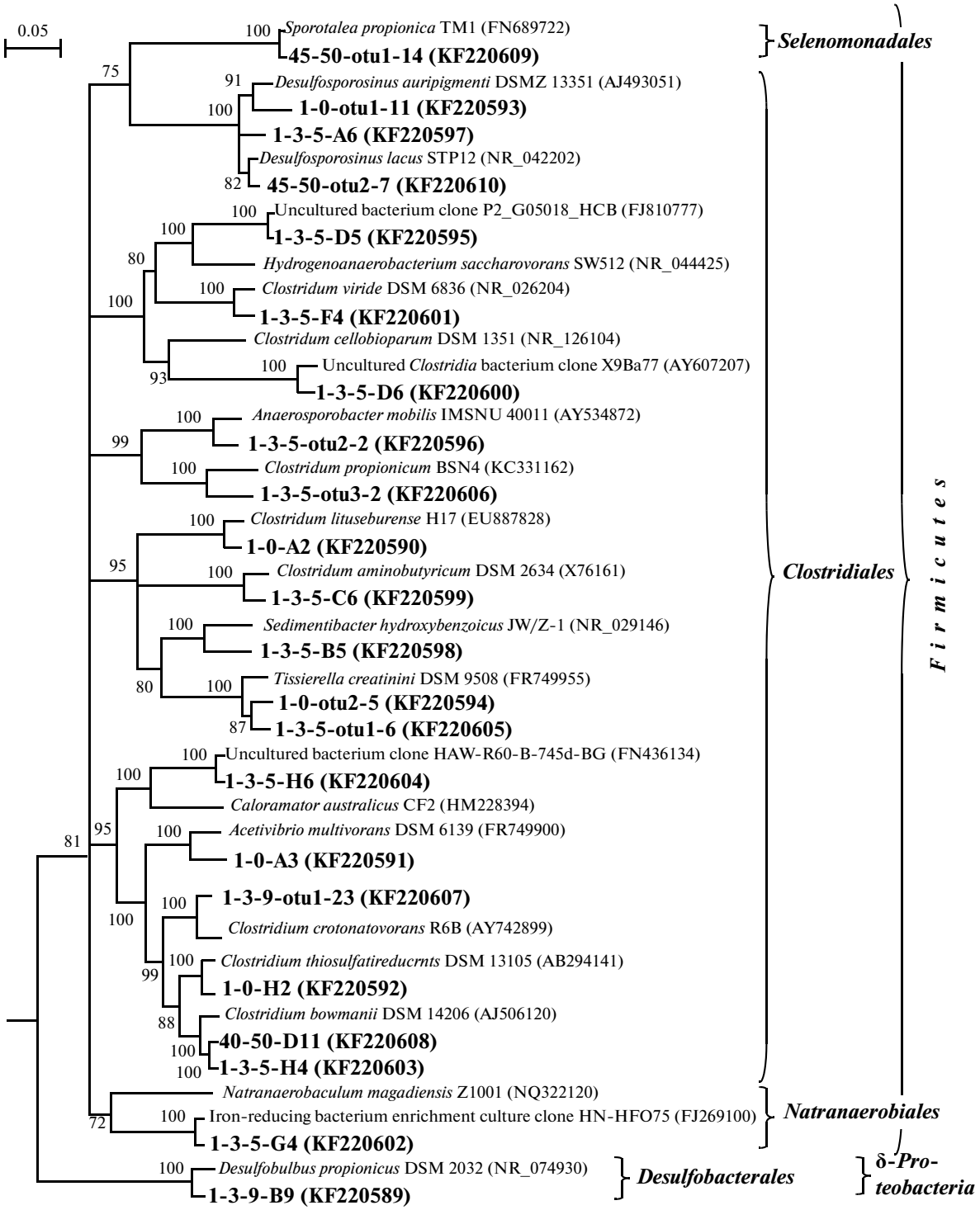

Fig. 5. Phylogenetic tree of the 16S rRNA genes revealed in enrichment cultures of sulfate-reducing bacteria from the southern Lake Baikal sediments. The sequences obtained in the present work are in boldface. Scale bar indicates the evolutionary distance corresponding to 5 nucleotide replacements per 100 nucleotides. The numerals indicate the branching order determined by bootstrap analysis of 1000 alternative trees (values above 70 were considered significant).

In all studied sediments, independent of sampling location, considerable SR rates were detected in subsurface horizons (1–60 cm). Importantly, SR activity in the surface sediments was accompanied by elevated sulfate levels, which were 1.5–2 times higher than in the near-bottom water. Sulfate accumulation in Lake Baikal upper sediments may probably result from rapid turnover of reduced and oxidized sulfur com-

pounds in the sediments, which involves sulfate-reducing and sulfur-oxidizing bacteria. Intense SR results in a rapid decrease in sulfate concentration and in formation of a diffusion flow of this ion from the water column into the sediments. Sulfide and other reduced sulfur compounds (thiosulfate and elemental sulfur) produced in the course of sulfate reduction are utilized by sulfur-oxidizing filamentous bacteria morphologically resembling *Thioploca*, which were revealed in all upper sediments of the polygon. These bacteria may prevent the release of reduced sulfur compounds into the water column and provide for sulfur turnover in the sediments.

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