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EXPERIMENTAL ARTICLES

Sulfate Reduction in Underground Horizons of a Flooded Coal Mine in Kuzbass

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Abstract—Although dissimilatory sulfate reduction (DSR) is an important microbial process in subterranean aquifers, its geochemical consequences in this ecosystem remain insufficiently studied. The absence of data on the process rate under in situ conditions prevents quantitative estimation of the sulfur reservoir. This research is aimed at investigation of microbial sulfate reduction in subterranean aquifers associated with the Severnaya coal mine in Kuzbass. Water samples were collected from an artesian borehole broaching the underground horizons of the flooded mine. During over 10 years of sampling the water temperature fluctuated within a narrow range (10–13°C); the water was anoxic (–112 to –174 mV) and contained up to 6 mg/L sulfide. Analysis by high-throughput sequencing of the 16S rRNA genes showed that sulfur-oxidizing bacteria *Sulfurovum, Sulfuricurvum, Sulfurospirillum*, and *Thiothrix* predominated in the community. No phylotypes with known ability to carry out DSR were detected. Measurement of sulfate reduction rates with $Na_2^{35}SO_4$, showed the process to be relatively active, resulting in up to 178 g of reduced sulfur per year at the borehole discharge. Two organisms representing minor components of the community, a psychrophilic and acidophilic *Desulfomicrobium* sp. DI and a moderately thermophilic *Desulfotomaculum* LL1, were isolated in pure culture by varying the cultivation condition in a bioreactor. These members of the "rare biosphere" may be responsible for production of reduced sulfur species, which are used by a diverse and numerous sulfur-oxidizing community.

Keywords: subterranean biosphere, rare biosphere, coal beds, sulfate reduction, *Desulfomicrobium*, *Desulfotomaculum*

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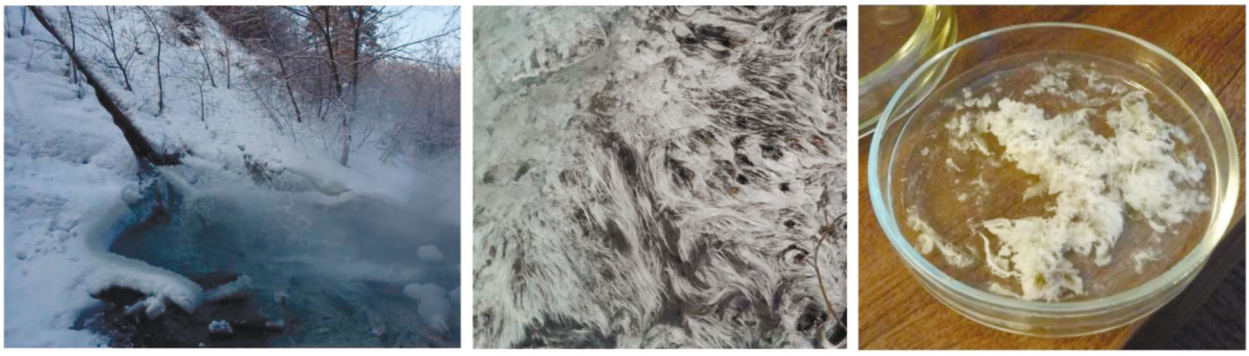
Dissimilatory sulfate reduction is a key microbial process in subterranean aquifers (Orcutt et al., 2013; Momper et al., 2017; Bell et al., 2018). Although sulfate-reducing prokaryotes of the subterranean biosphere have been a subject of extensive research (Moser et al., 2005; Chivian et al., 2008; Magnabosco et al., 2014; Karnachuk et al., 2019), their geochemical activity still remains insufficiently understood. While some studies discussed biochemistry of sulfate reduction based on molecular data (Anantharaman et al., 2016), in situ rates of these processes have not been determined. Identification of functional markers of dissimilatory sulfate reduction and even evaluation of their transcriptional activity is insufficient to obtain quantitative estimates of sulfur species reservoirs. To date, global sulfate reduction rates have been evaluated in marine ecosystems (Bowles et al., 2014), but it has been impossible to reproduce this research for deep terrestrial aquifers.

In most cases, subterranean ecosystems are characterized by oligotrophic conditions, which specifies the low growth rates of prokaryotes inhabiting them. However, aquifers associated with sediment rocks (oil or coal beds) may contain sufficient amounts of organic substances to sustain active heterotrophic growth. Along with microorganisms that consume hydrocarbons, lignite, or unmetamorphosed plant remains, these microbial communities may contain a significant portion of those that can utilize low-molecular-weight organic acids. Formation of acetate, formate, and oxalate in the course of lignite metamorphism was confirmed experimentally in a study that searched for carbon sources in the subterranean biosphere (Glombitza et al., 2009).

Our research concerns microbial sulfate reduction in subsurface aquifers associated with a coal mine in the Kuznetsk basin (Kuzbass). The mine Severnaya is located in the city of Kemerovo, where the Kuzbass



Parameter	July 13, 2006	July 24, 2015	Jan. 15, 2017 **	July 12, 2017	Nov. 8, 2018	Oct. 17, 2019	Feb. 2, 2020
T, °C	13.80	12.50	8.70	12.10	11.60	9.80	11.70
pH	7.58	7.58	8.54	7.87	7.60	7.65	7.60
Eh, mV	ND*	ND	–40.00	–174.00	–170.00	–112.00	ND
SO_4^{2-}, mg/L	21.80	ND	25.10	ND	5.50	ND	5.50

* ND, no data.

** Samples collected downstream from the discharge site.

Fig. 1. Borehole Ku-5 and microbial fouling. The table lists the dates of sample collection and the physicochemical parameters of water.

coal deposits were discovered in the 18th century; it was established in 1934. In 1998, the mine was shut down using the conventional "wet" technique involving flooding with interruption of drainage. To control the groundwater level and prevent flooding of residential buildings near the mine field, a borehole was drilled; according to different sources, its depth is 260 to 400 m. The borehole is located in a natural topographic depression on the bank of the Tom river, where groundwater discharge. The artesian borehole has a debit of 166–170 m^3/h. The discharge site features considerable overgrowth with *Thiothrix* mats, which utilize hydrogen sulfide present in the groundwater (Kadnikov et al., 2019). The composition of the microbial community of groundwater was analyzed by high-throughput sequencing of 16S rRNA genes, which showed that it was dominated by sulfur-oxidizing bacteria *Thiovirga*, *Thiothrix*, *Sulfurovum*, and *Sulfuricurvum* (Kadnikov et al., 2019). However, no phylotypes known to be capable of dissimilatory sulfate reduction were found, and the origin of hydrogen sulfide sustaining the diverse microbial community of groundwater and the discharge site remained an open question.

In the present work, to identify the origin of H_2S in the borehole waters, sulfate reduction rate was measured using radioactively labeled sulfate, and sulfate reducers representing minor components of the community were enriched in bioreactor.

MATERIALS AND METHODS

Sample collection and physicochemical characteristics of water. Samples of water from the borehole Ku-5 and microbial mats were collected during the period of 2006–2020. The dates of sample collection are indicated in Fig. 1. Water was collected at the discharge of the borehole, except for the samples from January 2015, when this site was inaccessible because of the snow sheet, and water was collected from the stream ~10 m downstream from the borehole. Samples of microbial mats were collected from the metal borehole casing at the site of discharge. Microbial mat samples for sulfidogen cultivation were collected on July 24, 2015. The composition of the microbial community was studied using the samples collected on January 15, 2017 and February 12, 2020. Sulfate reduction rates were measured in the samples collected on February 12, 2020. Microbial mat samples designated for cultivation were placed in sterile plastic tubes and stored refrigerated until inoculation in the bioreactor.

Physicochemical characteristics of the water (pH, temperature, and redox potential) were determined using an HI18314F pH-meter (Hanna Instruments, Germany). Sulfide content was determined after fixation with 10% zinc acetate by spectrophotometry with paraphenylenediamine (Cline, 1969). The element composition of water was determined by mass spectrometry with inductively coupled plasma as described previously (Karnachuk et al., 2015).

Measurement of sulfate reduction rate. To determine sulfate reduction rates under in situ-like conditions, water samples were dispensed into 30-mL penicillin vials and sealed with air-tight rubber plugs. Aliquots (200 μL) of $Na_2{}^{35}SO_4$ (4 μCi, Perkin Elmer, United States) were injected into the vials with a syringe through the rubber plug. All measurements of sulfate reduction rates were performed in three replicates. The samples were incubated at 12°C for 24, 48, and 72 h and then fixed with 1 mL of 1 N KOH. In the laboratory, reduced sulfur forms were separated by acidic distillation (acid-volatile sulfides, AVS) and by reduction with $CrCl_2$ (chromium-reducible sulfur, CRS, which includes elemental sulfur, pyrite, and organic sulfur) as described previously (Karnachuk et al., 2006).

Isolating pure cultures of sulfidogens and analysis of their physiology. Samples of microbial mats were inoculated into a 5-L Biostat B plus desktop bioreactor (Sartorius Stedim Biotech GmbH, Göttingen), with agitation at 100 rpm and with pH and temperature control. The Widdel and Bak (WB) medium (Widdel and Bak, 1992) had the following composition (g/L): Na_2SO_4, 4; KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1; $MgCl_2 \cdot 6H_2O$, 0.4; KCl, 0.5; $CaCl_2$, 0.113; 2 mL vitamin solution, and 1 mL trace element solution, as well as Na_2SeO_3 and Na_2WO_4 solution (1 mL). Lactate (18 mM) was used as an electron donor and $Na_2S \cdot 9H_2O$ served as a reducing agent. Ultrapure nitrogen (99.9%) was sparged at the rate of 25 mL min^{-1}. The temperature in the bioreactor varied from 17 to 50°C. The pH level was maintained at 7.5 during the entire experiment. At intervals, samples were taken from the bioreactor to determine the concentrations of protein and H_2S, as well as to assess cell morphology by phase contrast microscopy. Since the bioreactor was sparged with nitrogen, H_2S concentrations were considered only as a marker of culture response to temperature variations.

DNA isolation, sequencing, and phylogenetic analysis. A 20-L water sample was filtered through 0.22-μm filters. The filters were homogenized by grinding in liquid nitrogen, and metagenomic DNA was isolated using a Power Soil DNA Isolation Kit (MO BIO Laboratories, United States). The same reagents were used to isolate DNA from the microbial mat.

The composition of the prokaryotic communities was analyzed based on the sequences of variable region V3–V4 of the 16S rRNA gene amplified by PCR with the primers PRK341F (5'-CCTACGGGRBGCASCAG-3') and PRK806R (5'-GGACTACYVGGGTATCTAAT-3'). PCR fragment libraries were prepared for sequencing using the Nextera XT DNA Library Prep Kit (Illumina, United States) according to the protocol proposed by the manufacturer. The obtained libraries were sequenced on a MiSeq (Illumina) using the MiSeq Reagent Kit V3 (in the form of paired reads, 2 × 300 nt), and overlapping reads were merged using FLASH v 1.2.11 software (Magoč and Salzberg, 2011). As a result, 17186 sequences of 16S rRNA gene fragments were obtained for the water sample, and 18259 sequences were obtained for the microbial mat sample. Sequences of either set were clustered into operational taxonomic units (OTUs) based on the 97% identity level, and chimeras were removed with the Usearch software (Edgar, 2010). Taxonomic identification of OTUs was performed with the SINA classifier using the SILVA database of rRNA gene sequences with default settings (Pruesse et al., 2012).

To determine the phylogenetic position of the isolates, 16S rRNA gene sequences were amplified with the primers 27F–1492R. The procedures of DNA isolation and amplification were as described previously (Frank et al., 2016). The obtained 16S rRNA gene sequences were deposited into the GenBank NCBI under accession numbers MT500787.

RESULTS

Physicochemical characteristics of water of borehole Ku-5 and sulfate reduction rate. The physicochemical characteristics of water discharging from borehole Ku-5 have been monitored since 2006. During the period of monitoring, the temperature of the discharging water varied in the narrow range of 9.8–13.8°C and did not depend on the season (Fig. 1). The medium reaction was neutral and remained nearly constant during the entire period of observations. The outlying values obtained in January 2017 (pH 8.7 and T = 8.7°C) are explained by the fact that these samples were collected not at the discharge site but downstream, where the waters from the residential sector located upstream are drained. During the entire period of observations, water was anoxic, with a redox potential varying from −112 to −174 mV, and had a pronounced smell of hydrogen sulfide. The concentration of H_2S measured in July 2017 was 5.4 mg/L. The discharge site and the stream bed are covered with pronounced microbial mats in the form of white filaments (Fig. 1).

The average sulfate reduction rate in the water samples collected in February 2020 was 88.8 ng S_{red}/L/day. Only 25% of all reduced sulfur was in the form of hydrogen sulfide (AVS). Most part of the label was detected in the form of CRS, which may include pyrite (FeS_2), elemental and organic sulfur. Experiments with radioactively labeled sulfate were performed to investigate the effect of duration of sample incubation under near-in situ conditions. Comparison of the samples incubated for 1, 2, and 3 days showed that the highest rate of sulfate reduction (119.7 ng S_{red}/L/day) was observed after 3 days of incubation. Presumably, this value represents the best estimate of the actual rate of this process in the subsurface aquifer,

Table 1. Composition of the microbial communities of water of borehole Ku-5 and microbial mat developing at the discharge site determined in samples collected in February 2020.

Phylogenetic group	Share of 16S rRNA gene sequences, %	
	water	microbial mat
Euryarchaeota (*Methanomassiliicoccales*)	2.65	–
Woesearchaeota	10.08	–
Bacteroidetes	–	12.69
Chloroflexi	4.32	1.34
Cyanobacteria	–	10.50
Epsilonproteobacteria (*Sulfurovum*)	40.12	23.39
Epsilonproteobacteria (*Sulfuricurvum*)	16.89	3.22
Epsilonproteobacteria (*Sulfurospirillum*)	–	1.00
Patescibacteria	1.37	3.66
Gammaproteobacteria (*Halothiobacillaceae*)	14.37	3.67
Gammaproteobacteria (*Crenothrix*)	0.56	6.04
Gammaproteobacteria (*Thiothrix*)	6.75	14.18
Other	2.88	20.31

since the microbial activity assessed over shorter incubation periods might have been decreased because of changes in physicochemical conditions.

Composition of microbial communities was determined along with evaluation of sulfate reduction rates. Both the water community and the microbial mats were dominated by bacteria (Table 1). In the microbial mat, archaea were absent. The bacterial community was principally composed of sulfur-oxidizing *Sulfurovum, Sulfuricurvum, Sulfurospirillum*, and *Thiothrix* species. The microbial mat contained a significant portion of cyanobacteria (10.5%). Deltaproteobacteria of the orders *Desulfobacterales* and *Desulfovibrionales* were the prokaryotes with known ability to reduce sulfate detected in the water samples; they constituted 0.12 and 0.01% of the community, respectively. The same two groups were detected in the microbial mat, where they constituted only 0.02% taken together. It should be noted that most part of the detected representatives of *Deltaproteobacteria* belonged to the classes *Myxococcales* and *Bdellovibrionales*, for which the capability of sulfate reduction has not been described to date.

Pure cultures of sulfate-reducing bacteria and their physiology. Pure cultures of sulfate-reducing bacteria (SRB) were isolated using enrichment cultivation in a bioreactor. Under these conditions, it is possible to modulate different culture parameters, such as temperature or electron donor supply, and create optimal conditions for development of sulfidogens representing minor components of the community. The concentration of sulfide in the medium was considered as a marker of sulfate reduction. Initially, 18 mM lactate was used as the sole electron donor. In borehole Ku-5, the water temperature slightly fluctuated around 12.5°C. To simulate near-in situ conditions, we maintained the temperature of 17°C, which is the lowest temperature that could be achieved by circulation of tap water in the jacket of the bioreactor vessel. Figure 2 shows the dynamics of biomass and sulfide concentration in the bioreactor during the first 500 h of cultivation. Evaluation of morphotypes in samples of the bioreactor culture revealed predominance of motile rods, which coincided with an increase in H_2S concentration in the medium after 185 h of cultivation. Samples collected at this point were used to obtain a pure sulfidogen isolate represented by motile rods 1.5–2 μm long and ~1 μm in diameter; it was designated strain DI (Fig. 3). The culture was further purified using serial dilutions, and colonies were obtained on solid WB medium with lactate as the sole electron donor.

Phylogenetic analysis of the 16S rRNA gene sequence of the strain DI showed that it belonged to the genus *Desulfomicrobium* (Fig. 4). Its closest relatives are several species: *D. norvegicum* with an identity of 16S rRNA gene sequences 99.79%, *D. baculatum* (99.65%), *D. apsheronum* (99.65%), and *D. macestii* (99.58%). The strain was moderately psychrophilic, growing at temperatures from 4 to 28°C with an optimum at 15°C, and stable active growth was maintained at 4°C. Incubation at 37°C resulted in a loss of motility by DI cells and their lysis within 12 h. At the same time, strain DI was found to be moderately acidophilic

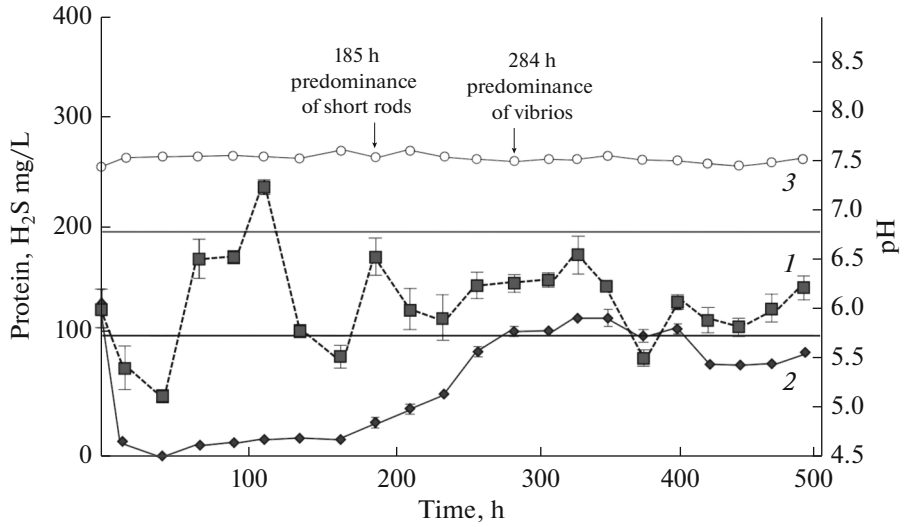


Fig. 2. Dynamics of biomass (*1*), sulfide concentration (*2*), and pH (*3*) in the digester during the first 500 h of cultivation. Vertical bars show standard deviations calculated for three replicates.

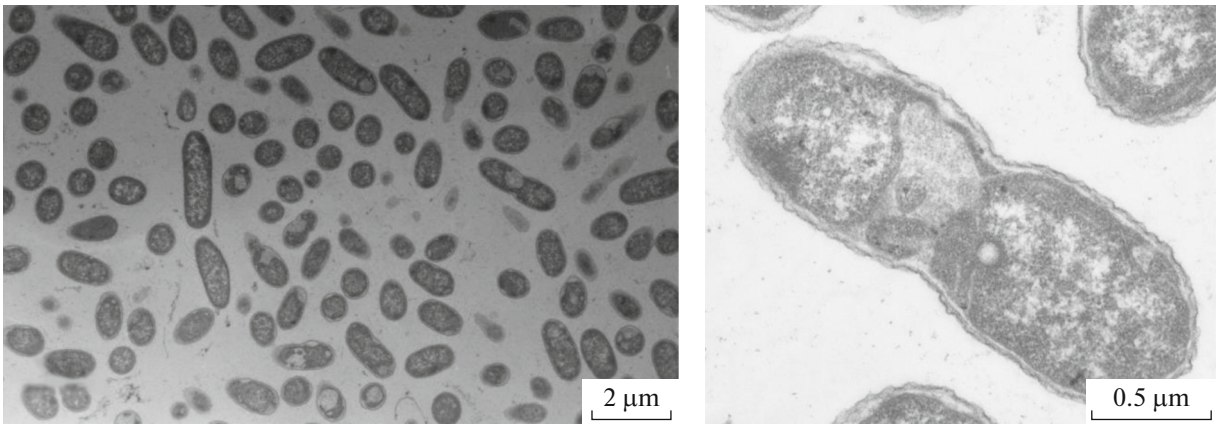


Fig. 3. Microphotographs of ultrathin sections of *Desulfomicrobium* sp. DI cells.

and grew in the pH range of 4–6.5 with an optimum at pH 5. The highest NaCl content still compatible with cell growth was 1.2%; no growth was observed at 1.5% NaCl. Optimum NaCl concentrations in the medium were 0–1%. The number of compounds that could be utilized by *Desulfomicrobium* sp. DI as electron donors for sulfate reduction included lactate, succinate, glycerol, ethanol, fructose, glucose, mannose, and sucrose. The activity of sulfate reduction was highest in the cultures growing on lactate, glycerol, and fructose. No growth was observed on citrate, butyrate, propionate, or formate. The strain did not utilize glycine, alanine, or cysteine.

After 1456 h, the temperature in the bioreactor was gradually increased to reach 50°C at 2030 h after the beginning of cultivation. At 2109 h, the culture was dominated by spore-forming rods, and the sulfide concentration reached 400 mg/L. From this sample, a pure culture of a spore-forming sulfidogen was isolated and designated strain LL1. The culture was further purified by heating at 90°C for 30 min with subsequent serial dilutions. Phylogenetic analysis of the 16S rRNA gene sequence showed that this strain belonged to the genus *Desulfotomaculum* (Fig. 4). Its closest relative is the recently described *Desulfotomaculum ferrireducens* (Yang et al., 2016) with an identity of 16S rRNA gene sequences of 98.93%. The strain is moderately thermophilic and grows in the temperature range of 28–55°C with an optimum at 50°C. Considering that the threshold level of 16S rRNA gene sequence identity for species differentiation is 98.7% (Chun et al., 2018), LL1 may represent a novel strain of *D. ferrireducens*.

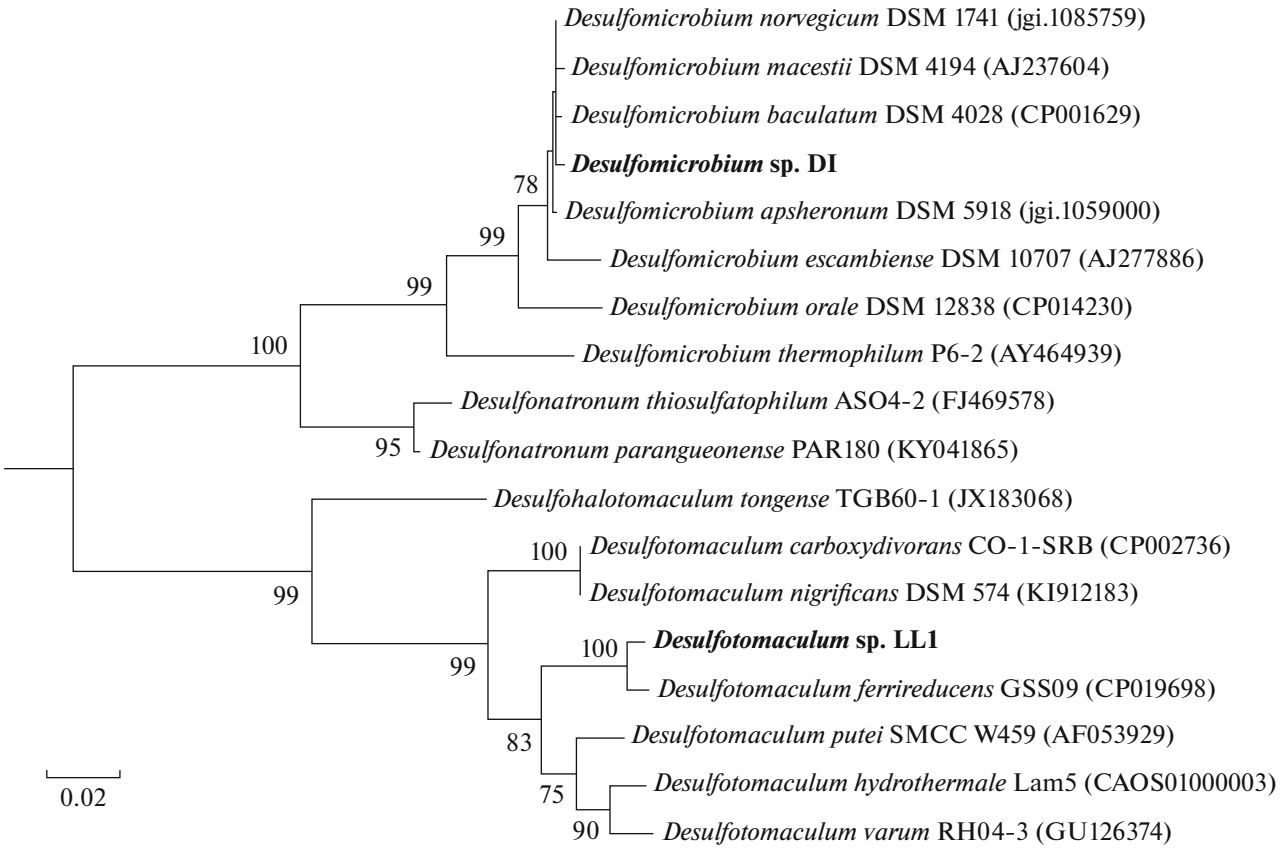


Fig. 4. Phylogenetic tree showing the position of isolates DI and LL1 based on the analysis of 16S rRNA gene sequences using the Maximum Likelihood approach. Bootstrap support values were calculated for 500 replicates.

DISCUSSION

The observed sulfate reduction rate in the water of the borehole Ku-5 was 119.7 ng S_{red}/L per day, which is relatively low in comparison to the known rates determined in the water column of surface water bodies with an anoxic zone, such as meromictic lakes. However, considering the borehole debit of 166–170 m^3/h, the amount of reduced sulfur delivered with the discharging water constitutes 475–488 mg S_{red} per day. Thus, the borehole discharge generates a reduced sulfur flux (calculated per hydrogen sulfide) that may be as high as 173–178 g S per year. This figure exceeds the rate of sulfate reduction determined in subterranean horizons associated with lignite beds in Germany, which amounts to 8.4 mM (768 mg) sulfate per year (Detmers et al., 2001). The maximal sulfide concentration observed in this work reached 1.34 mM (129 mg/L), which significantly exceeds the level observed for borehole Ku-5. Probably, the low sulfate concentration (5.5 mg/L) observed during the measurements of sulfate reduction rate in Ku-5 is related to active sulfate consumption by bacteria.

It should be noted that, during short-term sample incubation in our experiments evaluating sulfate reduction rate, reduced sulfur was predominantly present in the form of the CRS fraction, which includes elementary and organic sulfur, as well as pyrite, and not as hydrogen sulfide (AVS). The concentrations of iron determined in the course of monitoring were low, ranging from 0.27 to 0.67 mg/L. Therefore, it seems unlikely that considerable amounts of pyrite were formed in the water. We suppose that the dominant community of sulfur-oxidizing prokaryotes rapidly oxidizes hydrogen sulfide to sulfur and probably further to sulfate. This is an evidence of intensive sulfur turnover in the water and the subterranean aquifer. Apparently, sulfur-oxidizing organisms that develop abundant colonies at the discharge site, primarily *Thiothrix*, act as a sort of filter.

It remains unclear what electron acceptor is utilized by sulfur-oxidizing microorganisms. It is possible that oxygen penetrating the water–rock system may give rise to oxic microzones, similarly to known anoxic microzones in oxic habitats. Some *Thiothrix* species can also utilize nitrate as an alternative acceptor. In January 2017, the observed nitrate concentration was 6.7 mg/L. However, taking into account that this particular sample was collected in the stream and not at the borehole discharge site, it is possible that nitrates originated from household wastewaters. It cannot be ruled out that household wastewaters are

Table 2. Levels of genomic sequence identity of *Desulfomicrobium* species closely related to *Desulfomicrobium* sp. DI.

ANI			
	D. norvegicum	*D. apsheronum*	*D. baculatum*
D. norvegicum	100	93.90	86.85
D. apsheronum	93.90	100	86.44
D. baculatum	86.85	86.44	100
AAI			
	D. norvegicum	*D. apsheronum*	*D. baculatum*
D. norvegicum	100	94.50	88.33
D. apsheronum	94.51	100	87.71
D. baculatum	88.33	87.71	100

filtered into the subterranean aquifer of the flooded mine.

Using enrichment cultures grown in a bioreactor under changing conditions, we obtained two SRB isolates: the moderately psychrophilic and acidophilic *Desulfomicrobium* sp. DI and the moderately thermophilic *Desulfotomaculum* sp. LL1. The presence of psychrophilic forms was not an unexpected finding, since the annual variation of temperatures of the discharging water lies in the range of 11.7–13.8°C. The fact that strain DI preferred moderately acidic conditions may be related to oxidation of pyrite present in coal. This process generates zones with elevated proton concentrations, similarly to the phenomenon of acidic mine drainage waters described for deposits of metal sulfides (Kaksonen et al., 2008). *Desulfomicrobium* sp. may play an important role in biotopes associated with coal beds: it was shown that they constituted a major component (10.9%) of a lignin-degrading bacterial community (Wang et al., 2013).

The presence of spore-forming thermophilic *Desulfotomaculum* can be explained by the fact that mine flooding results in formation of an aquifer complex of hydraulically linked subterranean horizons. Therefore, microorganisms from deeper horizons with elevated temperatures can reach the waters of the borehole. This can be compared to the discovery of spore-forming thermophilic *Desulfotomaculum* in marine sediments; supposedly, they originate from hydrothermal vents and hydrocarbon seeps (O'Sullivan et al., 2015). The authors proposed a hypothesis that hydrothermal vents on the ocean bottom represent a sort of seed bank from which microbial spores can be carried to remote regions and give rise to vegetative cells under appropriate conditions.

Apparently, both sulfate-reducing prokaryotes isolated by enrichment cultivation represent rare biosphere of the biotope concerned, since neither phylotype could be detected by high-throughput sequencing of the 16S rRNA gene. In spite of the high identity level of marker gene sequences, the isolated strain *Desulfomicrobium* sp. DI may represent a new species, because it differs significantly from closely related *Desulfomicrobium* species in its physiological parameters. To date, no psychrophilic member of the genus has been described; moreover, the ability to utilize sugars is also unknown in members of *Desulfomicrobium*. Genomic sequences are currently available for some close relatives of the strain DI: *D. baculatum*, *D. norvegicum*, and *D. apsheronum*. Our analysis of genomic levels of average nucleotide identity (ANI) and average amino acid identity (AAI) confirmed that these organisms belong to different species, and ANI did not exceed the conventional species-level threshold of 95–96% (Chun et al., 2018) (Table 2). Obviously, species of the genus *Desulfomicrobium* cannot be discriminated based on their 16S gene sequences.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals or human participants performed by any of the authors.

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