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EXPERIMENTAL ARTICLES

The Sulfate-Reducing Bacterial Community of Sulfide-Rich Water of the Ust'-Kachka Resort Spring, Perm Krai, Russia

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Abstract—Microbiological investigation of the highly mineralized, sulfide-rich cold spring of the Ust'-Kachka resort was carried out. The total number and biomass of microbial cells were 50×10^3 cell/mL and 15 μg/L, respectively. While the total microbial number was low, the sulfate reduction rate determined by the radioisotope method was relatively high (0.575 mg S L^{-1} day^{-1}). An enrichment culture was obtained on Widdel medium. According to the results of cloning the 16S rRNA gene fragments with subsequent restriction analysis, the dominant organisms were group 6 sulfate-reducing bacteria (*Desulfovibrio–Desulfomicrobium*) and the microorganism exhibiting 99% similarity to the anaerobic haloalkaliphilic bacterium *Halanaerobium hydrogeniformans*.

Keywords: sulfate reduction, sulfate-reducing bacteria, high-mineral sulfide springs

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Subterranean waters constitute a significant part of the hydrosphere, which is estimated as over one-third of the volume of the World Ocean. Development of the understanding of the mechanisms of formation, accumulation, and turnover of subterraneous waters is directly associated with advances in urban development, agriculture, and medicine. Low-mineral underground and artesian waters are among the main sources of drinking water. More saline underground waters have a curative effect and have long been used to treat a number of diseases. These aspects of the practical application of underground waters promoted microbiological investigation of the underground ecosystems as an environment harboring many physiological groups of microorganisms [1–3]. Variations in the origin, depth of occurrence, and mineral composition of the underground waters affect the structure of microbial communities and the biogeochemical processes in the water-bearing strata.

The level of mineralization of the underground waters depends on the geological structure of the site of occurrence and usually increases with depth. As a rule, highly mineralized deep waters are free from dissolved oxygen, have negative redox potential, and often contain free sulfide produced by sulfate-reducing bacterial communities [2, 3]. Investigation of formation waters of petroleum deposits provided abundant material on the activity and structure of the anaerobic microbial communities of subterranean waters. A multicomponent anaerobic microbial community (including fermenting bacteria, sulfate- and iron-reducing bacteria, and methanogenic archaea) was found to develop under these conditions [4]. The information on microbial biodiversity in the mineral waters of the wells used for medical purposes is scarcer. Investigation of such waters is usually limited to the regular chemical and sanitary microbiological analysis using the standard techniques. The mineral springs of the Ust'-Kachka resort (Perm krai, Russia) are not exceptional in this respect. Highly mineralized water from the well containing free hydrogen sulfide (~240 mg/L) is used for balneotherapeutic procedures. The water-bearing horizon (204–501 m) is associated with the early Permian deposits of fractured limestone and dolomites (Kungurian, Artinskian, and Sakmarian stages) enriched with organic matter. The water is a weakly acidic (pH 6.3–6.6) brine (65–80 g/dm^3) of sodium–chloride composition with negative E_h (–250 to –300 mV). Significant levels of sulfate (up to 6 g/L) and sulfide at low E_h values suggest active sulfate reduction in the subterranean water. Long-term stability of the mineral composition of the water (well no. 5 was drilled in 1971) indicates low water exchange with the fresh infiltration water and significant closure of the cenosis of these underground waters. However, the data on microbial biodiversity in

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the water of the cold mineral springs of the Ust'-Kachka resort are not to be found in the literature.

The goal of the present work was to investigate the activity and biodiversity of sulfate-reducing bacteria in sulfide-enriched mineral waters of this resort.

MATERIALS AND METHODS

The samples investigated were collected from the cold (10°C) mineral spring of the Ust'-Kachka resort (well no. 5) in October 2011.

The water samples were collected at the spring and either fixed with glutaraldehyde or formalin (2% final concentration) or transported intact in hermetically sealed glass vials (without air bubbles) to the laboratory of the Winogradsky Institute of Microbiology, Russian Academy of Sciences.

Total microbial numbers, biomass, and the morphological structure of the community were determined under an Olympus BX-41 fluorescence microscope (Japan) at ×1000 magnification. The samples contained a black sulfide precipitate which exhibited no fluorescence, but screened some bacteria adsorbed on the particles due to its high density. The water (30–50 mL) was filtered through black polycarbonate membranes (Osmonics, United States) with 0.22 μm pore diameter. Bacterial cells were stained with acridine orange.

The rate of sulfate reduction (SR) in the samples was determined by the radioisotope method. Immediately after sampling, the water was dispensed into 30-mL penicillin vials and sealed with rubber stoppers, avoiding air bubbles. Into each vial, 0.2 mL of sterile $Na_2{}^{35}SO_4$ solution was injected to the final activity of 20 μCi. After incubation for 1–3 days at 5–10°C, the samples were fixed with 1 mL of 2 N NaOH. The sample was then acidified with orthophosphoric acid and distilled under nitrogen flow in order to capture $H_2{}^{35}S$ by 2-phenylethylamine in the scintillation mixture. Activity of $H_2{}^{35}S$ was determined on a Packard TRI-Carb TR liquid scintillation counter (United States). The samples treated in the same way but fixed with 2 N NaOH prior to addition of $Na_2{}^{35}SO_4$ were used as the control. All measurements were carried out in two repeats in order to achieve more reliable results.

Sulfate content in the water was determined on a Staier ion chromatograph (Russia).

Enrichment cultures of sulfate-reducing bacteria (SRB) were obtained in the medium containing filter-sterilized (0.22 μm) water from the well supplemented with lactate and butyrate (1 g/L each) as carbon sources. The native spring water (1, 3, or 5 mL per 10 mL of the medium) was used as inoculum. Enrichment cultures were incubated at room temperature for 7 days and transferred to the liquid Widdel medium containing the following (g/L): NaCl, 60; $MgCl_2$, 3; $CaCl_2$, 0.5; Na_2SO_4, 3; KH_2PO_4, 0.2; KCl, 0.5. The medium was then supplemented with solutions of vitamins and trace elements [5] and organic substrates: lactate (50% solution, Merck, Germany), 1 g/L; butyrate (5% solution in distilled water), 1 g/L; and yeast extract Difco (5% solution in distilled water, 0.5 g/L. The media were prepared using the Hungate anaerobic techniques [6]. The tubes with enrichment cultures were incubated for 8 days at 18–20°C. SRB growth was indicated by an increase in sulfide concentration compared to the control. Sulfide was determined colorimetrically [7].

For PCR detection of SRB, total DNA was isolated from 15 mL of an actively growing culture using the Genomic DNA Purification Kit (Fermentas, Lithuania). PCR analysis of DNA was carried out with the oligonucleotide primers [8] specific for a site of the *dsrB* gene (β-subunit of the dissimilatory sulfite reductase, the enzyme present in all SRB and catalyzing sulfite reduction to sulfide) and with the primers [9] specific for the 16S rRNA gene sites of the six major SRB groups (table). In the latter case, the more sensitive nested PCR technique was used. For this purpose, a site of the 16S rRNA gene of *Bacteria* was amplified using the total DNA as a template (direct PCR), and the product of amplification of the bacterial 16S rRNA gene was then used as a template for PCR with the primer pairs specific for 16S rRNA gene sites of the major phylogenetic groups of SRB (nested PCR).

The PCR reaction mixture (25 μL) contained ~25 ng of the template (total DNA or the product of amplification of the *Bacteria* 16S rRNA gene in the case of nested PCR), 2.0 mM $MgCl_2$, 100 μM of each dNTP, 500 nM of the forward and reverse primers (table), and 1.25 U *Taq* DNA polymerase (Syntol, Russia). PCR was carried out in a GeneAmp PCR System 9700 amplifier (Applied Biosystems, United States) under the following conditions: 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at the annealing temperature (T_a) of the relevant primer pair, 1 min at 72°C (6 min in the case of the primers pA and pH'); and 10 min at 72°C. PCR products were analyzed by electrophoresis (110 V, 120 mA) in 1% agarose gel prepared on 1× TAE buffer and containing ethidium bromide (0.2 μg/mL) for visualization of the DNA bands under UV illumination.

Further identification of microorganisms in the enrichment culture from the Ust'-Kachka sulfide spring was carried out with the isolated DNA as the PCR template with the universal primer system 337F-907R: (5'-GAC TCC TAC GGG AGG CWG CAG-3' and 5'-CG TCA ATT CCT TTR AGT TT-3') [10]. The PCR reaction mixture (20 μL), contained 1× PCR buffer (17 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, and 2 mM $MgCl_2$), 10–50 ng of template DNA, 12 pM of each dNTP, 20 pM of each primer, and 2 U Bio*Taq* DNA polymerase (Dialat, Russia). PCR was carried out in a Gradient Mastercycler amplifier (Eppendorf, Germany) under the following conditions: 1 cycle of 3 min at 94°C, 3 min at 53°C,

Oligonucleotide primers for PCR used in the work

Primer	Specificity	Nucleotide sequence (5'–3')	Target site (16S rDNA, *E. coli* positions)	T_a, °C	Product size, bp
pA pH'	16S rDNA of *Bacteria*	AGAGTTTGATC TGGCTCAG AAGGAGGTGATCCAGCCGCA	8–28 1542–1522	37	1530
DSRp2060F DSR4R	*dsrB* gene fragment	CAACATCGTTCATACCCAGGG GTGTAGCAGTTACCGCA	″	56	350
DFM140 DFM842	Group 1 SRB (*Desulfotomaculum*)	TAGCCTGGGATAACGGCTG ATACCCGCAACTCCTAGCAC	140–158 842–823	58	700
DBB121 DBB1237	Group 2 SRB (*Desulfobulbus*)	CGCGTAGATAACCTGTCCTCATG GTAGTACGTGTGTAGCCCTGGTC	121–142 1237–1215	66	1120
DBM169 DBM1006	Group 3 SRB (*Desulfobacterium*)	CTAATACCGGATGAAGTCAG ATTCTCAAGATGTCAAGTCTG	169–183 1006–986	64	840
DSB127 DSB1273	Group 4 SRB (*Desulfobacter*)	GATAATCTGCCTTCAAGCCTGG CTTTCTGCAAAGTCGCTGCCCT	127–148 1273–1252	56	1150
DCC305 DCC1165	Group 5 SRB (*Desulfococcus–Desulfonema–Desulfosarcina*)	GATCAGCCACACTGGAACTGACA GGGGCAGTATCTTCAGAGTCC	305–327 1165–1144	56	860
DSV230 DSV838	Group 6 SRB (*Desulfovibrio–Desulfomicrobium*)	GAGCCTGCGTCTCATTAGC GTCCGACACCTAGTATCCATC	230–248 838–818	54	610

and 30 s at 72°C; 5 cycles of 30 s at 94°C, 2 min at 53°C, and 30 s at 72°C; 25 cycles of 30 s at 94°C, 30 s at 45°C, and 30 s at 72°C; and 7 min at 72°C.

PCR products were analyzed at 6 V/cm in 1.2% agarose gel stained with ethidium bromide. The BioDocII documentation system (Biometra, Germany) was used to treat the results of electrophoretic analysis. Isolation and purification of the PCR fragments was carried out using the Wizard PCR Preps Kit (Promega, United States) according to the manufacturer's recommendations.

The 16S rRNA gene fragments were cloned in the competent cells of *E. coli* DH10β using the pGEM-T Easy Vector System I Kit (Promega, United States). For each clone library, 60 colonies exhibiting the positive reaction were chosen at random. PCR with the material of the colonies was carried out using the M13 F (GTA AAA CGA CGG CCA GT, Sigma P4165) and M13R (GGA AAC AGC TAT GAC CAT G, Sigma P4290) universal plasmid primers. The presence of the insert was determined by gel electrophoresis of the plasmid DNA from the clones and of the vector plasmid without the insert.

The insert-containing recombinant clones were grouped by restriction analysis using the *Hae*III restriction endonuclease. Three clones were chosen at random from each group and sequenced on an ABI Prism 3100 automatic sequencer (Applied Biosystems, United States).

The 16S rRNA gene fragments were tested for plasmid contamination using the VecScreen program [http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen. html] and their similarity to the known microbial sequences was analyzed using the BLAST software package [http://www.ncbi.nlm.nih.gov/sutils/ genom_table.cgi].

RESULTS AND DISCUSSION

The pH (6.4), salinity (70 g/L), sulfate (5.1 g/L), and sulfide content (240 mg/L) of the sample collected in October 2011 from well no. 5 agreed with the results of the monitoring of the chemical composition of the water from this well carried out by the Russian Scientific Center of Restorative Medicine and Balneology and the Ust'-Kachka Resort in 2007–2010. At the time of sampling, the water had grayish-black color due to the presence of coarse sulfide sediment; it rapidly precipitated, and the overlying water became transparent and colorless.

Microscopy revealed microorganisms (4×10^4 cells/mL) in the supernatant water, with the biomass of 12 μg/L. The morphological diversity of microbial cells was rather low. Straight rods predominated, while the cocci were almost absent (Fig. 1a). While the number, biomass, and the morphological diversity of microbial cells in the water sample were significantly lower than in the open water bodies, including saline ones, they were similar to the average values for deep underground waters [1, 10–11]. In the preparations containing the sulfide precipitate phase, no association between the cells and the precipitate was found. However, attached bacteria (single cells and microcol-

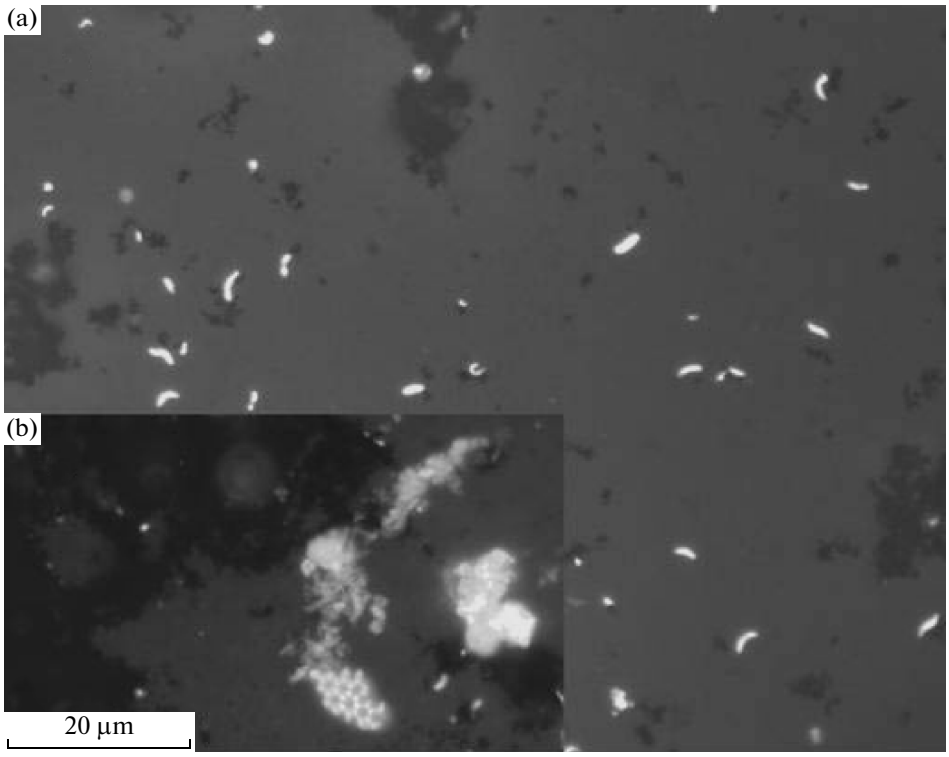

Fig. 1. Fluorescence microscopy of the water samples from well no. 5, Ust'-Kachka resort. Bacterial cells in the supernatant. Some small particles of sulfide precipitate are visible (a). Bacterial aggregates on organo-mineral particles of the carbonaceous precipitate. Some bacterial cells are not associated with the precipitate. Fuzzy glowing areas are the particles of the whitish suspension (b).

onies) were found on organo-mineral particles of the precipitate (Fig. 1b). Since such particles were not common in the precipitate, the estimated ratio of attached bacteria does not exceed 20% of bacterial number in the supernatant water.

Thus, morphologically, both attached and free-living bacteria were mostly rods with the average volume of 0.3 μm^3. The total number of microorganisms in the sample, including the precipitate, was up to 5×10^4 cells/mL, while the total biomass was up to 15 μg/L.

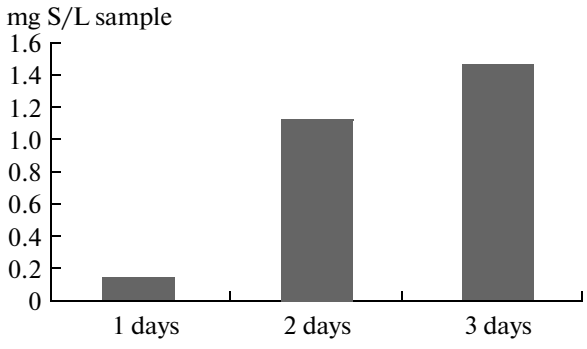

Fig. 2. Sulfate reduction rates in the water samples from well no. 5, Ust'-Kachka resort depending on the incubation time.

The rates of sulfate reduction in the native water samples are shown on Fig. 2. The SR activity depends on the incubation time, indicating active sulfate reduction in the spring water, in spite of the low microbial numbers. The highest SR rate per day (0.575 mg S L^{-1} day^{-1}) was registered on the second day of incubation. We observed similar SR rates in the water column of highly productive meromictic lakes, where organic matter formed by oxygenic and anoxygenic photosynthesis was actively transformed in the anaerobic zone. For example, SR rate below the chemocline of the saline Lake Shunet (Khakassia) was 0.5 mg S L^{-1} day^{-1} [12] and in the Lake Mogil'noe (Kil'din Island, Barents Sea), 0.43 mg S L^{-1} day^{-1} [13]. Among the subterranean ecosystems, high SR rates (up to 0.8 mg S L^{-1} day^{-1}) were observed in highly mineralized formation waters of some Azerbaijan oil deposits [4].

Enrichment cultures showed clear evidence of SRB growth after 7–8 days of incubation. Sulfide content increased almost tenfold compared to the control (uninoculated medium) and the number of microorganisms increased from 10^3 to $2.5–3.0 \times 10^6$ cells/mL. Since high concentrations of reduced sulfur compounds hindered PCR analysis in the native samples

due to the inhibition of *Taq* DNA polymerase, enrichment cultures were used for molecular detection of SRB. Application of oligonucleotide primers specific for a site of the *dsrB* gene encoding dissimilatory sulfite reductase revealed the presence of *dsrB* in the total DNA isolated from the enrichment culture.

Nested PCR revealed the presence of SRB belonging to groups 2 (*Desulfobulbus*), 5 (*Desulfococcus–Desulfonema–Desulfosarcina*), and 6 (*Desulfovibrio–Desulfomicrobium*) (Fig. 3). Thus, the SRB community of the water from well no. 5 of the Ust'-Kachka resort contained members of at least three phylogenetic groups. Importantly, both sulfate- and sulfur-reducing bacteria were possibly present, since both microbial groups contain sulfite reductase.

Cloning of the fragment of the 16S rRNA gene with subsequent restriction analysis was used for more accurate identification of microorganisms in the enrichment culture. A total of 57 clones analyzed were found to form three restriction groups containing 33, 18, and 6 clones, respectively. Sequencing of three randomly chosen clones from each group revealed the dominant microorganisms of the water samples. Analysis of the nucleotide sequences revealed that the largest group 1 (GenBank JQ829076, JQ829077) contained the microorganisms exhibiting 99% similarity to the anaerobic haloalkaliphilic (growth optimum at 7% NaCl) bacterium *Halanaerobium hydrogeniformans* [14], which was isolated from anaerobic sediments of the meromictic Soap Lake (United States) with extremely high sulfide content (up to 10 g/L). The clones of group 2 (GenBank JQ829078, JQ866620) exhibited ~90–91% homology with a broad spectrum of group 6 SRB (*Desulfovibrio–Desulfomicrobium*), with the highest similarity (92%) to *Desulfomicrobium baculatum* DSM 4028 (order *Desulfovibrionales*). This strictly anaerobic asporogenic rod-shaped sulfate-reducing bacterium was isolated from a moistened pile of manganese ore [15]. Closely related species of this genus were isolated from formation waters of a petroleum deposit [16] and from a Matsesta sulfide mineral spring [17]. The third group was represented by several clones exhibiting low similarity to the known sequences, so their precise identification was not carried out.

The results of PCR analysis showing the presence of the *dsrB* gene were different from the sequencing data. This discrepancy is understandable, since nested PCR reveals even the DNA of the organisms present in minor quantities, while sequencing of the randomly chosen 16S rRNA fragments usually reveals the dominant microbial groups. PCR detection of group 6 SRB (*Desulfovibrio–Desulfomicrobium*) was, however, confirmed by sequencing.

Thus, it may be concluded that group 6 SRB (*Desulfovibrio–Desulfomicrobium*) predominated in the enrichment cultures obtained from the samples of well no. 5 water. The 91–92% similarity of their nucleotide sequences suggests the presence of a new genus of sulfate reducers of the order *Desulfovibrionales* in Ust'-Kachka sulfide springs. The presence of organotrophic *H. hydrogeniformans* in the enrichments is not surprising, since this microorganism is able to carry out the first stage of decomposition of many organic polymers to low-molecular compounds and hydrogen, which may be further utilized by the SRB community.

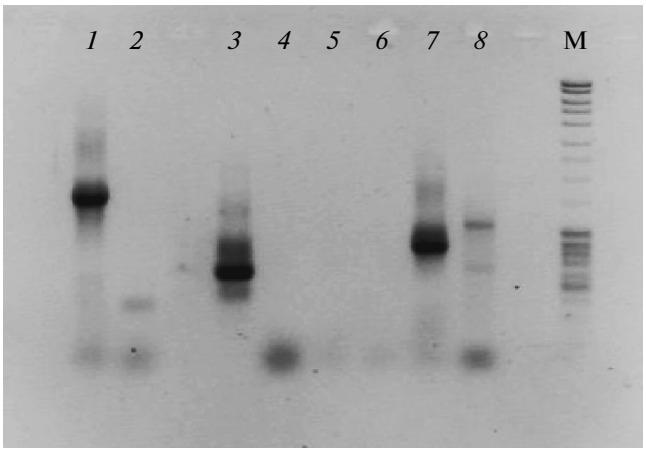

Fig. 3. Electrophoresis of PCR products in 1% agarose gel: primers pA and pH' (*1*), primers DSRp2060F and DSR4R (*2*), primers DSV230 and DSV838 (*3*), primers DBM169 and DBM1006 (*4*), primers DSB127 and DSB1273 (*5*), primers DFM140 and DFM842 (*6*), primers DCC305 and DCC1165 (*7*), and primers DBB121 and DBB1237 (*8*). M is the MassRuler DNA Ladder Mix marker (Fermentas, Lithuania).

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