Ecology of microorganisms from springs of national park "Alkhanai" (Transbaikalia, Russia)

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Abstract. The composition and diversity of microbial communities from the cold ultra-fresh oligotrophic seeps and River Sukhoe Ubzhogoe of the National Park "Alkhanai" were determined via culture-dependent vs. culture-independent approaches. The microbial community of six groundwater seeps determined by 16S rDNA cloning, and sequencing consisted of four dominant genera belonging to the classes Betaproteobacteria and Gammaproteobacteria; they were Duganella sp., Janthinobacterium sp., and Pseudomonas sp., Serratia sp., respectively. Uncultured bacteria were presented by the phylum Bacteroidetes, classes Flavobacteriia (Flavobacterium sp.), Cytophagia (Flexibacter sp.), and Chitinophagia (Sediminibacterium sp.); the phylum Proteobacteria, classes Betaproteobacteria (Comamonadaceae) and Gammaproteobacteria (Acinetobacter sp.); and the phylum Actinobacteria, class Actinomycetia (Rhodococcus sp.). The same chemoorganoheterotrophic bacteria were cultivated as dominant species from the waters of the investigated seeps. Still, differences in the microbial communities were found in a proper combination of the four dominant forms of bacteria in the appropriate ecosystems. One of the peculiarities of the cultivable bacteria was the production of colored pigments violacein and prodigiosin, known for their therapeutic properties. The subject is promising for fundamental and applied research. Curative properties of cold seeps were discussed, and it was proposed that the water quality could assume medicinal qualities due to the specific composition of microorganisms.

1. Introduction

The ability to adapt to their environment is one of the essential features of living creatures, most clearly represented by bacteria. Diversity, abundance, and the distribution of microorganisms in extreme environments are determined by the efficiency of their adaptive mechanisms [1, 2]. Natural underground waters are extreme ecosystems for life and have fundamental differences from other surface water ecosystems. The main one deals with the total or partial isolation from the inflow of organic matter and energy from the surface [3]. To survive in the conditions of high or low temperature, low accessibility of inorganic and/or organic compounds, and in the complete or temporary absence of oxygen and light, microorganisms have to contain a full set of subsistence, including biochemical mechanisms for energy support, nitrogen fixation, and organic compound synthesis [3].

Recently, studies of microbial communities of these ecosystems (deep, groundwater, and subsurface of the Earth) receive much attention due to their relict origin, isolation from the biosphere, and attempts...
to determine the extreme limits of life our planet [4, 5]. The study of these relict organisms in laboratory conditions is complex. However, modern techniques now allow for the exploration of these specific communities [6, 7]. National Park "Alkhanai" is a unique natural site. On a relatively small area, cold ultra-fresh oligotrophic groundwaters have been found in the form of small springs and seeps (arshans). Our study aimed to investigate the ecology of microbial communities, such as structure, phylogenetic and functional diversity, in the cold ultra-fresh oligotrophic seeps of the National Park "Alkhanai".

2. Material and methods

2.1. Geology and geographic characteristics of the studied area
National Park "Alkhanai" is located in the Duldurginskij region of Transbaikalia, Russia. The area is geologically confined to the juncture of Khentey Dauria and Aga zones that border the Onon-Turinskij deep fault. Its activation in the Jurassic period (145–200 million years ago) has been noted, accompanied by the appearance of sagging and paleovolcanoes. The main paleovolcanoe is Mount Alkhanai, with a height of 1662 m.

Cold ultra-fresh oligotrophic groundwater seeps are the main feature of the area overflowed as small springs (arshans) and are considered curative. By the conditions of deposition, nature of water circulation, and connection with certain lithological and stratigraphic units, the territory belongs to the aquifer system of the Jurassic volcanic rocks. Water-bearing rocks are fractured zones of weathered volcanic rocks of the alkhanai series. The thickness of the zone hydrogeological effective fracture consists of 60‒100 m and depth to groundwater up to 1.5–3.0 m. Aquitard for the complex is long-term and seasonal frost. The abundance of water in the complex is irregular and slightly higher compared to other water-bearing complexes. Within Alkhanayskij massif in the most mountainous part, on the area of 21.5 km², 14 springs with a total flow rate of 89.3 L / s have been found, whereas, at the foot of the Mount Alkhanai, individual springs flow rate consists of 10–15 L / s. These springs are sodium, bicarbonate-calcium, mixed according to water chemistry. Waters are ultra-fresh with a total mineralization of 50.42 mg / L and pH of 5.9–6.6 [8].

2.2. Sampling and on-site sample treatment
Water samples were collected from the six seeps (Seeps 1 and 2, and Eye, Cardiac, Gastric, and Kidney Arshans) and River Sukhoe Uzbghoeg at the exit point to the surface in August 2009 and July 2010 (table 1). For microorganism cultivation, water was collected in sterile Falcon tubes, stored, and transported at in situ temperature. For microscopic analysis in the field, aliquots of 10 ml of water were filtered through a nitrocellulose filter with a pore diameter of 0.22 microns. For molecular genetic studies in the field, at least 1.5 liters of water sample were concentrate on the bacterial filter (filter diameter 47 mm, pore size 0.22 mkm) and fixed with 70% ethanol. Physico-chemical characteristics of water (pH, salinity, dissolved oxygen, and temperature) were determined using a field laboratory Multi-340i (WTW, Germany).

2.3. Cultivation of chemoorganoheterotrophic microorganisms
In the laboratory, as soon as possible after collection (up to eight hours), 1 ml of the sample was inoculated onto agar nutrient medium NSY [9] for the enrichment cultures cultivation. Each sample was plated in triplicate. Incubation was carried out at in situ temperature in a refrigerator to avoid light. Bacterial growth was monitored visually and by light microscopy. The viability of microorganisms was judged by colony-forming units (CFU / mL). Isolation of pure cultures was carried out on a modified medium "3+" [10], and purity was determined by microscopic analysis. According to the standard procedure, microscopic analysis of water samples was performed using a microscope Micmed P-13/2 (× 1350) [11].
2.4. Molecular identification of microorganisms
DNA from cell cultures and bacterial material concentrated on the filters was isolated using a commercial kit Bacterial genomic DNA isolation kit (Axogen, USA) by the procedure adopted. The polymerase chain reaction was performed on conservative bacterial primers [12]. Ligation was done with selected amplicons with GeneJET™ PCR Cloning Kit (Fermentas) and CaCl₂-competent cells of *Escherichia coli* (strain XL-1) [13]. Strain and clone sequencing was performed on an automatic sequencer ABI310A (ABI PRISM 310 Genetic Analyzer) in the CPC "Genomika" SB RAS (Novosibirsk). Comparative analysis of the obtained sequences was done with the software package FASTA (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). Verification of the sequences for the presence of chimeric structures was confirmed with Pintail (http://www.bioinformatics-toolkit.org/Pintail/index.html).

3. Results and discussion

3.1. Microbiological characteristics
Microscopic examination revealed the complexity of the microbial communities of the studied ecosystems. Different origins of the groundwater formation and the special conditions of release of groundwater to the surface have affected the structure of microbial communities (table 1).

Table 1. Physico-chemical and microbiological characteristics of cold ultra-fresh oligotrophic seeps and River S. Uzbzhogoe.

| Physico-chemical and microbiological characteristics | Seep 1 | Seep 2 | Springhead of the River Sukhoe Uzbzhogoe | Upstream of the River Sukhoe Uzbzhogoe | River Sukhoe Uzbzhogoe Eye Arshan | River Sukhoe Uzbzhogoe Cardiac Arshan | River Sukhoe Uzbzhogoe Gastric Arshan | River Sukhoe Uzbzhogoe Kidney Arshan |
|-----------------------------------------------------|--------|--------|------------------------------------------|--------------------------------------|---------------------------------|---------------------------------------|-------------------------------------|-------------------------------------|
| Name of sampling site                                | Al-1   | Al-2   | Al-3                                     | Al-4                                 | Al-4.1                          | Al-4.2                                | Al-4.3                              | Al-4.4                              |
| Temperature, °C                                      | 0.6    | 1.5    | 1.3                                      | 2.7                                  | 2.7                             | 2.7                                   | 2.9                                  | 2.8                                  |
| pH                                                   | 6.5    | 6.4    | 6.3                                      | 6.3                                  | 6.7                             | 6.5                                   | 6.7                                  | 6.7                                  |
| Mineralization, mS/m                                 | 28     | 50     | 36                                       | 37                                   | 31                              | 52                                    | 54                                   | 53                                   |
| Oxygen, mg/L / % of saturation                       | 7.60/66.6 | 7.51/90.4 | 7.85/92.8                     | 7.40/64.3                           | 7.40/64.3                      | 7.40/64.3                            | 7.40/64.3                           | 7.40/64.3                           |
| Total bacteria number (TBN), ×10⁵ cells/mL           | 0.06   | 0.15   | 3.73                                     | 2.33                                 | 1.98                            | 3.01                                  | 0.78                                 | 3.45                                 |
| Rod size (h=d), mkm                                  | 2.0–4.0 | 2.0–4.0 | 1.5–3.0                                 | 2.0–4.0                             | 0.8–6.0                        | 0.8–6.0                               | 0.8–6.0                             | 0.8–6.0                             |
| Cocci size (h=d), mkm                                | 0.8–1.0 | 1.0–1.5 | 1.0–1.5                                 | 1.0–1.5                             | 0.4–1.6                         | 0.4–1.6                               | 0.4–1.6                             | 0.4–1.6                             |
| Biomass, mg/L                                       | 0.36   | 0.45   | 14.29                                    | 11.69                                | 8.69                            | 7.80                                  | 0.90                                 | 8.33                                 |
| Number of cultivated microorganisms (NCM), ×10⁶ CFU/mL | 0.70   | 0.07   | 0.22                                     | 1.50                                 | 1.53                            | 3.50                                  | 0.91                                 | 4.00                                 |
| Number of morphotypes of cultivated microorganisms   | 3      | 4      | 3                                        | 4                                    | 4                               | 4                                     | 4                                    | 4                                    |
| Fraction of NCM from TBN, %                          | 1.16   | 0.04   | 0.005                                    | 0.06                                 | 0.07                            | 0.12                                  | 0.12                                 | 0.11                                 |

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Thus, the water of Seeps 1 and 2, located at an altitude of 1492–1946 m above sea level (masl), was characterized by low microbiological indicators, which varied slightly: the total bacteria number (TBN) from 0.06 to 0.15×10^6 cells / mL, biomass 0.36–0.45 mg / L, the number of chemoorganoheterotrophic bacteria 0.07-0.70×10^3 CFU / mL. Analysis of the structural parameters of the microbial community of the seeps located nearby in the riverbed of River S. Ubzhogoe (≈1390 masl) showed more significant variation. For example, the microbial community of the Eye Arshan with the TBN corresponded to mesotrophic level has been characterized by high biomass of 8.69 mg/L compared with other investigated seeps. However, TBN of water from the Gastric Arshan was 2 to 4 times lower than in other seeps and has not exceeded 0.78×10^6 cells / mL. According to the light microscopic investigation, the microbial community has corresponded to the low-temperature water of ultra-fresh and oligotrophic origin and presented monotonous morphometric composition of bacteria: sticks of various shapes (short straight, slightly curved, with rounded ends, with inclusions), rod-shaped cells with a round or tapered end, small cocci, single cells, or, in some cases, short chains.

Cultivation has shown that chemoorganoheterotrophic bacteria represented a relatively small fraction (0.005–0.06%) of the TBN in the water of the River S. Ubzhogoe. In the water samples from Seep 1, their content reached 1.16%, whereas, in the Eye Arshan, it did not exceed 0.07%. Low values of the number of cultivated microorganisms (NCM, table 1) were observed in the water of Seeps 1 and 2, Gastric Arshan (0.70, 0.07, and 0.91×10^3 CFU / mL, respectively), and in the water of the River S. Ubzhogoe (1.50–1.53×10^3 CFU / mL); they corresponded to the oligotrophic level and microorganism distribution in the freshwater river ecosystems [14]. The maximum values of the indicator were found in the Cardiac and Kidney Arshans of 3.5 and 4.0×10^4 CFU / mL, respectively.

The results of the quantitative enumeration of pigmented chemoorganoheterotrophic microorganisms in the studied waters have established an unusually high amount varied from 0.1 to 90% of the microbial community. The cultivation of microorganisms from the arshans located along the River S. Ubzhogoe has allowed us to distinguish four morphotypes of bacteria (table 1); three were pigmented and had black-purple, light-purple, and yellow-colored colonies, and one non-pigmented small, semitransparent colony. Different quantitative combinations of these bacteria have been determined in the water of the selected arshans. From the waters of Seep 1, reliable growth was obtained for one morphotype of pigmented bacteria with a red-colored colony and two morphotypes of non-pigmented colonies — small semitransparent and large opaque ones. Non-pigmented bacteria with a large white semitransparent colony and small shiny ones were cultured from the waters of the river S. Ubzhogoe (table 1).

3.2. Molecular investigation of microbial communities

Comparative analysis of 16S rRNA gene fragments of pure cultures and native samples showed a low diversity of bacteria and revealed three major phyla of Eubacteria: Proteobacteria, Bacteroidetes, and Actinobacteria. Cultured chemoorganoheterotrophic bacteria were identified as representatives of the phylum Proteobacteria, species Duganella sp., Janthinobacterium sp. (class Betaproteobacteria), Pseudomonas sp., and Serratia sp. (class Gammaproteobacteria). Representatives of the phylum Bacteroidetes belonged to different classes: Flavobacteria (Flavobacterium sp.), Cytophaga (Flexibacter sp.), and Chitinophagia (Sediminibacterium sp.), as well as widespread representatives of the phylum Proteobacteria, classes Betaproteobacteria (fam. Comamonadaceae) and Gammaproteobacteria (Acinetobacter sp.), and phylum Actinobacteria, class Actinomycetia (Rhodococcus sp.) have been identified by molecular cloning. It should be noted that all representatives of different classes of the phylum Bacteroidetes have shown high homology to both cultivated and uncultivable microorganisms from high mountain lakes in Germany, Spain, and Canada [15, 16].

The study showed that the microbial diversity of the seep and river waters had been represented mainly by Gram-negative chemoorganoheterotrophic asporogenous bacteria. The main ones in the community structure are aerobic bacteria with a respiratory type of metabolism, which used oxygen as the terminal electron acceptor. In some cases, nitrate can act as an alternative electron acceptor (Pseudomonas sp., Serratia sp.). Organic substances (acetate, lactate, formiate, ethanol, glucose, mixture of amino acids) can serve as electron donors and carbon sources for the vast majority of bacteria.
Some bacterial species, like *Janthinobacterium* sp., can utilize citrate and ammonia as the only source of carbon and nitrogen. The presence of flagella and the capability of sliding movement or twitching motility have been found for most of the isolated bacteria. These functional properties of microorganisms might be an essential adaptation to the extreme conditions of the groundwater and the capacity for rapid acclimatization at the exit to the surface [3].

Phylogenetic analysis revealed that the red-pigmented bacteria were identified as *Serratia* spp., purple-pigmented as *Duganella* sp. and *Janthinobacterium* sp., and yellow-pigmented as *Pseudomonas* spp. According to known data, the purple pigment violacein is produced by a limited number of Gram-negative bacterial species, including *Duganella* spp. [17] and *Janthinobacterium* spp. [18]. Red pigment prodigiosin produced by Gram-negative and Gram-positive bacteria, including *Serratia* spp., Actinomycetes (*Streptomyces coelicolor* A3), various marine bacteria such as *Hahella chejuensis* KCTC 2396 and *Pseudoalteromonas denitrificans* [19]. Pigments of yellow color like phenazine-1-carboxylic acid (and some other phenazine derivatives) are produced by bacteria of genus *Pseudomonas* [20, 21].

All pigments are biologically active secondary metabolites, and in some studies, it has been proved that violacein has antiprotozoal, antibacterial, and antifungal activities [22, 23, 24]. Investigation of red-coloured pigments attracts increasing interest nowadays because of their immunosuppressive and anticancer properties, as well as antifungal, antibacterial, antimalarial, and antiprotozoal activities [19]. A broad spectrum of chemical agents such as pyocyanin, chlororaphine, oxychlororaphine, phenazine-1-carboxylic acid, and aeruginosine with antibiotic properties has been isolated from different strains of *Pseudomonas* spp. It was shown that they exhibited antibiotic activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi. Besides antibiotics, the colored substances synthesized by pseudomonads include vitamins, riboflavin, folic acid, and pterins.

The role of these pigments in bacterial life and metabolism is currently not fully understood. However, studies demonstrate the possible role of the pigment violacein in ecological competition among the microorganisms [24]. It is suggested that bacteria can synthesize violacein in response to stress conditions and long-term growth and survival in extreme environments. Investigation of the molecular mechanisms indicates that the prodigiosin biosynthesis affects numerous environmental and physiological signals, including temperature, oxygen, pH, light, and the changes of the carbon and nitrogen source, presence of inorganic phosphate, and various cations and anions [19]. Thus, research on extreme natural environments may be promising for elucidating the role of these pigments in bacterial life.

4. Conclusions
The investigation of composition and diversity of microbial communities from the cold ultra-fresh oligotrophic seeps and River Sukhoe Ubzhogoe of the National Park "Alkhanai" via culture-dependent vs. culture-independent approaches revealed the representatives of three dominant phyla of Eubacteria Proteobacteria, Bacteroidetes, and Actinobacteria. The main representatives have shown high homology to both cultivated and uncultivable microorganisms from high mountain lakes. Dominant species of the cultivable microorganisms were pigmented chemoorganoheterotrophic bacteria. One of the peculiarities of the cultivable bacteria was the production of colored pigments violacein and prodigiosin, known for their therapeutic properties. One of the adaptation strategies of microorganisms from underground coldwater seeps of Mount Alkhanai might be pigment production in response to changes in environmental conditions (light, oxygen, temperature, etc.).

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