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EXPERIMENTAL ARTICLES

Molecular Identification of Filterable Bacteria and Archaea in the Water of Acidic Lakes of Northern Russia

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Abstract—Wetland ecosystems are the natural centers of freshwater formation in northern Russia lowland landscapes. The humic acidic waters formed in bogs feed the numerous lakes of the northern regions. One milliliter of the water in these lakes contains up to 10^4 ultrasmall microbial cells that pass through "bacterial" filters with a pore size of 0.22 μm. The vast majority of these cells do not grow on nutrient media and cannot be identified by routine cultivation-based approaches. Their identification was performed by analysis of clone libraries obtained by PCR amplification of archaeal and bacterial 16S rRNA genes from the fraction of cells collected from water filtrates of acidic lakes. Most of the obtained bacterial 16S rRNA gene sequences represented the class *Betaproteobacteria* and exhibited the highest homology of (94–99%) with 16S rRNA genes of representatives of the genera *Herbaspirillum, Herminiimonas, Curvibacter*, and *Burkholderia*. The archaeal 16S rRNA gene clone library comprised genes of *Euryarchaeota* representatives. One-third of these genes exhibited 97–99% homology to the 16S rRNA genes of taxonomically described organisms of the orders *Methanobacteriales* and *Methanosarcinales*. The rest of the cloned archaeal 16S rRNA genes were only distantly related (71–74% homology) to those in all earlier characterized archaea.

Keywords: filterable microbial forms, acidic lakes, 16S rRNA gene clone libraries.

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The freshwater sufficiency and quality are among the major challenges that the humankind faces in the 21st century. Wetland ecosystems are the most important natural centers of freshwater formation in the lowland landscapes typical of the boreal zone of Russia and other countries of the northern hemisphere. The humic acidic waters formed in wetlands feed the numerous lakes and rivers of the northern regions. Analysis of the waters of small acidic lakes located in swampy catchments of upper Volga showed that their microflora is primarily represented by small cells that are difficult to identify by fluorescent in situ hybridization and standard set of group-specific probes [1]. Preliminary studies showed that a considerable fraction of these cells passed through 0.22-μm-pore-size "bacterial" filters, which are commonly used for removal of microbial cells from water, nutrient media, serums, etc.

Despite the long-standing interest in filterable microbial forms, they remain poorly studied. The term *ultramicrobacteria* was first used in 1981 to describe marine heterotrophic bacteria with a cell diameter of less than 0.3 μm that showed slow growth on standard nutrient media and did not increase in size upon prolonged cultivation in the laboratory [2]. Later, the interpretation of this term was revised because it turned out that many of these small objects are starvation cell forms and acquire "normal" size when the substrate is available [3, 4]. Currently, the term ultramicrobacteria is used to denote cells with a volume of less than 0.1 μm^3 [4, 5].

Many ultramicrobacteria can pass through 0.22-μm-pore-size filters. The acknowledgment of this fact by microbiologists held back for a long time by the fact that filterable cell forms either do not grow on standard nutrient media or form microcolonies invisible to the unaided eye [2, 3, 6]. The problem of unculturability of filterable forms was in part solved by using strongly diluted nutrient media and prolonged incubation. Application of this approach for the analysis of water samples from a number of rivers, lakes, and ponds resulted in isolation of filterable bacterial forms which included representatives of *Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, Actinobacteria*, and *Spirochaetes* [6]. A wide phylogenetic diversity of isolates was also yielded by cultivation of filterable bacterial forms from samples of 120 thousand years old Greenland ice [7].

However, none of cultivation-based methods embrace the entire diversity of the microorganisms present in a sample. A good alternative is the use of molecular methods, although few studies have been performed on molecular identification of filterable microbial forms. Their list includes studies of filtered Mediterranean Sea water [8], uranium mine groundwater [9], and waters of an acidic *Sphagnum* peat bog

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Limnological and hydrochemical characteristics of the studied lakes[a]

Lakes	Area, km^2	Depth, m	pH	Water color index, Pt-Co	Conductivity, μS/cm	DOM, C/L	Total N, mg/L	Total P, mg/L	Trophic status	Bacterioplankton number, million cells/mL	Filterable cells, %
Lake Motykino	0.02	2.5	4.5–4.8	11–55	10	5.9	0.34	0.03	Acidic oligotrophic	1.03 ± 0.1	3.07
Lake Dubrovskoe	0.19	1.1	4.4–4.6	111–291	20	34.1	0.64	0.04	Acidic dystrophic	3.89 ± 0.4	0.43

Note: [a] [1, 12].

[10]. Identification of the major populations of filterable bacteria in water sampled from several rivers and lakes of Switzerland showed that one milliliter of these waters contained about 10^4 microbial cells that passed through 0.22-μm-pore-size filters, which constituted from 0.03 to 3.6% of the total bacterioplankton numbers [11]. No similar investigations have so far been performed for the water bodies of Russia. Therefore, the aim of the present study was enumeration and molecular identification of filterable microorganisms in the waters of acidic lakes in the catchment area of upper Volga.

MATERIALS AND METHODS

Study sites. The acidic dystrophic Lake Dubrovskoe and the acidic oligotrophic Lake Motykino belong to a group of numerous small boreal lakes located in the swampy Mologa–Sheksna catchment area of upper Volga, within the territory of the Darwin State Nature Reserve (Vologda oblast, 58°10′ N, 37°33′ E). The structure of the catchment system and the mode of water arrival determine the considerable differences between the studied lakes in the degree of humification, pH value, and chemical composition of water. The oligotrophic Lake Motykino (pH 4.7) is located near the top of a peat bog complex and is to a considerable extent (40%) fed ombrotrophically. The dark-water dystrophic Lake Dubrovskoe (pH 4.4) is located lower along the catchment area slope and is primarily fed by surface waters of an ombrotrophic bog (up to 90% of the annual inflow) [12]. The main characteristics of the studied water bodies are presented in the table. Water samples were taken from the surface of the lakes (depth of 0.2–0.5 m).

Enumeration of filterable cells by microscopy. To determine the total cell number of bacterioplankton, lakewater samples (1 to 2 mL) were filtered through 0.22-μm-pore-size Millipore membrane filters with the use of a vacuum pump. The filters with trapped cells were cut into four sectors, stained with a 0.5 μM solution of the DNA-specific fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) for 10 min, washed with distilled water, and dried. The number of trapped cells was determined using a Zeiss Axioplan 2 microscope (Jena, Germany) with a Zeiss 02 light filter specific for DAPI staining. Cells were counted in 100 microscope fields with subsequent calculation of their number in 1 mL of water. The numbers of cells in water samples passed through a 0.22-μm-pore-size filters were determined after trapping the cells from 5 mL of filtered water on 0.09-μm-pore-size filters with subsequent cell count on these filters by the above-described procedure.

Enumeration and phylogenetic identification of filterable cells by means of cultivation. The feasibility of enumeration of filterable cells by plating onto nutrient media was estimated using a Lake Dubrovskoe water sample as an example. The water filtered through 0.22-μm-pore-size filters was plated onto two nutrient media of different compositions:

(1) SB2 medium, containing (mg/L) KNO_3, 50; NH_4Cl, 50; $MgSO_4 \cdot 7H_2O$, 60; $CaCl_2 \cdot 2H_2O$, 20; yeast extract, 50; sodium pyruvate, 300; and Difco agar, 18 g/L. The medium pH was adjusted to 5.2 with alginic acid (20 mg per liter of medium).

(2) Standard R2A agar medium (Difco) that was diluted 100-fold.

To prevent fungal growth, the media were supplemented with 30 mg/L of cycloheximide. The number of colony forming units (CFU) was counted 2 months after inoculation. Representative colonies were transferred to the same media to obtain pure cultures. Identification of the isolates was performed by determining partial sequences (about 500 bp) of 16S rRNA genes.

Molecular identification of filterable prokaryotic forms. The fraction of ultrasmall cells was collected by passing 100 mL of water through 0.22-μm-pore-size nitrocellulose filters (Millipore) and subsequent trapping of filterable forms on filters with a pore size of 0.09 μm (Millipore). For each sample, three filters with cells trapped on them were used to isolate DNA with the use of FastDNA SPIN kit for soil (Biol 101, United States) according to the manufacturer's recommendations. The obtained total DNA was used as a template in polymerase chain reaction (PCR). PCR amplification of bacterial 16S rRNA gene fragments

(about 1490 bp long) was performed with the use of the 9f and 1492r bacterial primers [13]. The amplification of archaeal 16S rRNA gene fragments (about 800 bp long) was performed with the use of the 109f and 915r primers [14]. PCR was run in a PE GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer Applied Biosystems, United States). PCR products were checked by electrophoresis in a 1.2% agarose gel with subsequent staining with ethidium bromide and visualization in a UV transilluminator. The amplicons were cloned with the use of a pGem-T Easy Vector SystemII kit (Promega). Recombinant clones were selected by amplification of the cloned fragments with the vector-specific primers T7 and SP6. The clones were sorted by restriction analysis using MspI and RsaI endonucleases for bacterial amplicons and HhI and BsuRI (HaeIII) endonucleases for archaeal amplicons. Electrophoresis of the restriction products was performed in a 2.5% agarose gel.

Isolation and purification of plasmid DNA was carried out using Wizard® Plus Minipreps DNA Purification System (Promega). Nucleotide sequences were determined on an ABI 377A sequencer (Perkin-Elmer Applied Biosystems, United States) and edited using the SeqMan (Laser Gene 7.0; DNA Star Package) software. The comparison of sequences with those available in the GenBank database was performed using the Blast2 software (http://www.ebi.ac.uk/Tools/blast2/). The clonal libraries were checked for the presence of chimeras with the use of the Bellerophon 3.0 software (http://compbio.anu.edu.au/bellerophon/bellerophon.pl). Phylogenetic dendrograms were constructed using ARB software package (http://www.arb-home.de). The statistical significance of the dendrograms was estimated using Phylip software package by constructing 1000 alternative trees and bootstrap analysis.

The newly determined nucleotide sequences of the 16S rRNA genes of filterable prokaryotic forms have been deposited in GenBank under accession numbers JN825208–JN825301.

RESULTS

Filterable cell numbers. The total bacterioplankton cell counts in the water of Lakes Dubrovskoe and Motykino were $(3.89 \pm 0.40) \times 10^6$ and $(1.03 \pm 0.10) \times 10^6$ cells/mL, respectively. After passing the water samples through 0.22-μm-pore-size filters, the cell numbers in filtered water were determined to be $(1.69 \pm 0.53) \times 10^4$ and $(3.16 \pm 0.43) \times 10^4$ cells/mL, or 0.4 and 3.1% of the total microbial cell numbers in the water of Lakes Dubrovskoe and Motykino, respectively (table).

Cultivation. The microbial cell numbers determined by plating unfiltered water of Lake Dubrovskoe on R2A (1 : 100) and SB2 media were 1.68×10^5 and 1.25×10^5 CFU/mL, respectively. The CFU number determined on the same media after their inoculation with filtered water was three orders of magnitude lower, comprising 0.93×10^2 and 2.00×10^2 CFU/mL. Thus, only 0.5–1.2% of the cells present in filtered water were able to produce colonies upon plating on solid media.

Reinoculation of the colonies grown after plating of filtered water allowed 11 bacterial strains to be isolated as pure cultures. Determination of partial sequences of 16S rRNA genes of the isolates showed their high similarity (97–99%) to 16S rRNA genes of representatives of known species of the genera *Mesorhizobium, Bradyrhizobium, Pseudomonas, Sphingomonas*, and *Agrobacterium*. The cells of all isolates were rods with a diameter of 0.3–0.5 μm; apparently, these isolates did not represent the numerically dominant populations of filterable cells. Only the 16S rRNA genes of *Pseudomonas* isolates, strains DSB-4 and DSB-11, were identical to the 16S rRNA genes revealed in filtered water by molecular analysis (see below).

Molecular identification of filterable bacteria. The compiled library of the 16S rRNA genes of filterable bacteria included 86 clones, of which 25 were obtained from Lake Dubrovskoe, and 61 from Lake Motykino. 74 of the cloned 16S rRNA genes belonged to representatives of the class *Betaproteobacteria*, four and two represented *Alphaproteobacteria* and *Gammaproteobacteria*, respectively, and six represented the phylum *Actinobacteria* (Fig. 1).

The filterable forms of betaproteobacteria revealed in the water of acidic lakes belonged to the families *Oxalobacteraceae, Comamonadaceae*, and *Burkholderiaceae*. The cloned 16S rRNA gene fragments exhibited highest homology (94–99%) to the 16S rRNA genes of representatives of the genera *Herbaspirillum, Herminiimonas, Curvibacter*, and *Burkholderia*.

The nucleotide sequences of clones DB27 and DB28, representing the class *Gammaproteobacteria*, were highly similar (99%) to the 16S rRNA genes of *Pseudomonas* representatives isolated from a melt water stream of an Arctic glacier [15] and identical to the 16S rRNA genes of strains DSB-4 and DSB-11, isolated from filtered lakewater by means of plating.

The four clones that formed a common cluster with representatives of the class *Alphaproteobacteria* exhibited only remote relatedness (about 90% 16S rRNA gene identity) with taxonomically described organisms and were most close (95–97%) to the 16S rRNA gene sequence of the uncharacterized bacterium Ellin362 isolated from Australian soil [16].

The six clones representing the phylum *Actinobacteria* were most close (95%) to the 16S rRNA gene of a typical representative of freshwater ultramicro bacterioplankton, '*Candidatus* Planktophila limnetica' [17].

Molecular identification of filterable archaea. The library of cloned archaeal 16S rRNA genes obtained from filtered water of acidic lakes included 120 genes of *Euryarchaeota* representatives, of which 48 sequences

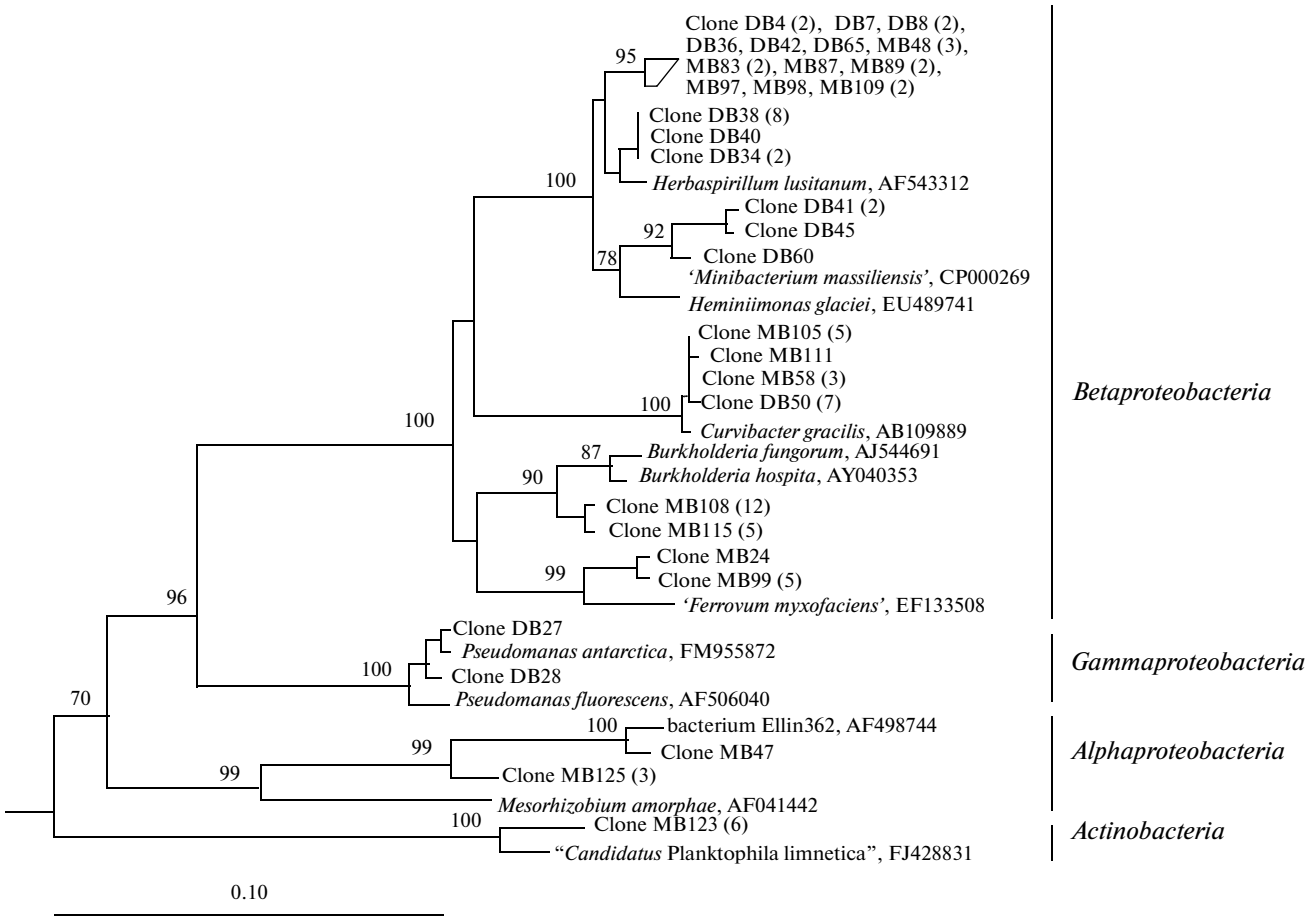

Fig. 1. Phylogenetic dendrogram constructed based on comparative analysis of the bacterial 16S rRNA gene sequences retrieved from filtered water and the 16S rRNA genes of some representatives of the *Proteobacteria* and *Actinobacteria*. The clones obtained from the water of Lake Dubrovskoe and Lake Motykino are designated by letters DB and MB, respectively. As outgroups, the 16S rRNA genes of *Gemmata obscuriglobus* (X54522), *Isophaera pallida* (AJ231195), *Schlesneria paludicola* (AM162407), and *Planctomyces limnophilus* (X62911) were used. Bar, 0.1 substitutions per nucleotide position.

were retrieved from Lake Dubrovskoe and 72, from Lake Motykino (Fig. 2).

Only one-third of these clones exhibited high homology (97–99%) to the 16S rRNA genes of taxonomically described organisms, which represented the orders *Methanobacteriales* and *Methanosarcinales*. 34 clones formed common clusters with the 16S rRNA genes of methanogens from the genus *Methanobacterium* and with clones earlier obtained from Finland and US wetlands [18, 19] and from permafrost soil in Russia [20]. Two groups of three and four clones obtained from the filtered lakewater exhibited high homology to the 16S rRNA genes of methanogens from the genera *Methanobrevibacter* and *Methanosarcina*, respectively.

Other cloned archaeal 16S rRNA gene sequences were only distantly related (71–74%) to those in taxonomically described archaea. However, they displayed higher similarity (85–99%) and formed a common cluster with a number of clones earlier obtained in the course of molecular studies of various, primarily northern, ecosystems, such as meromictic lakes [21], Arctic shelf and northern rivers [22–25]. At present, this archaeal cluster does not contain cultured representatives; therefore, the biology and physiology of its members are vague.

DISCUSSION

So far, only in one work by researchers from Switzerland was a qualitative approach used to study filterable microbial cells in natural freshwaters [11]. The present study was the first to address the cell number and phylogenetic composition of filterable microbial forms in fresh water bodies of the north of Russia. The number of cells that passed through 0.22-μm-pore-size filters and their fraction in the microbial pool of the two lakes that we investigated was in the same range as the values determined for lakes and rivers of Switzerland [11]. The highest cell number of filterable forms was found in the water of Lake Motykino, which is more oligotrophic than Lake Dubrovskoe (table).

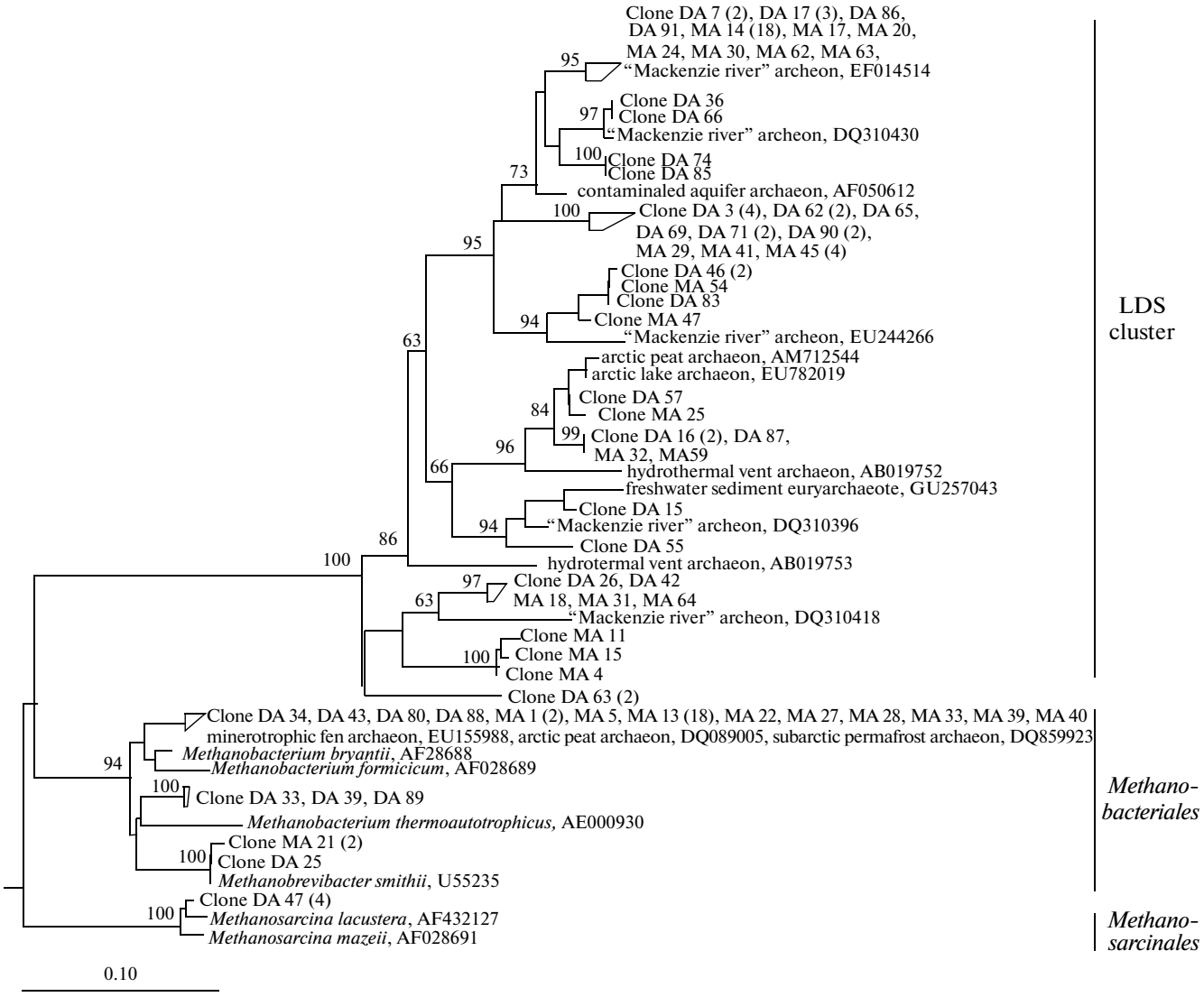

Fig. 2. Phylogenetic dendrogram constructed based on comparative analysis of the archaeal 16S rRNA gene sequences retrieved from filtered water, the 16S rRNA genes of some representatives of the orders *Methanosarcinales* and *Methanobacteriales*, and a number of 16S rRNA gene clones obtained in molecular studies of various ecosystems. The clones obtained from the water of Lake Dubrovskoe and Lake Motykino are designated by letters DA and MA, respectively. As outgroups, the 16S rRNA genes of *Archaeoglobus fulgidus* (AE000965), *Archaeoglobus profundus* (AF297529), *Nanoarchaeum equitants* (AJ318041), and *Sulfolobus solfataricus* (X03235) were used. Bar, 0.1 substitutions per nucleotide position.

Cultivation on solid media allowed only a minor fraction (0.5–1.2%) of cells present in filtered water to be revealed. The spectrum of bacteria grown on nutrient media had little in common with the spectrum of microorganisms detected by molecular analysis. The only exceptions were *Pseudomonas* representatives, which were revealed by both cultivation and analysis of 16S RNA gene clone libraries. It cannot be excluded that the presence of a low number of *Mesorhizobium, Bradyrhizobium, Sphingomonas*, and *Agrobacterium* cells in the filtrates was accounted for by occurrence of some defects in the filters (pore superimposition). Archaea failed to be revealed by cultivation. Therefore, the cultivation-based approach appears to be inappropriate for identification of filterable cells; the molecular approach provided more comprehensive knowledge of their diversity.

Most of the filterable bacterial forms revealed in the waters of Lakes Dubrovskoe and Motykino belonged to the class *Betaproteobacteria*. Representatives of this class are usually dominant in the bacterioplankton of acidic lakes [1]; among them, there are many organisms with ultrasmall cells [26–28]. Thus, several clones obtained in the present work exhibited highest homology to the 16S rRNA genes of *Herminiimonas glaciei*, an ultramicrobacterium with an average cell

volume of 0.043 μm^3, isolated from Greenland ice [27]. One more example of a closely related organism is the taxonomically uncharacterized ultramicrobacterium *'Minibacterium massiliensis'*, found in ultrapure filtered water used in hospitals for hemodialysis [26]. The groups comprising largest numbers of clones formed common clusters with bacteria of the genera *Herbaspirillum* and *Curvibacter*, whose cells are thin curved rods with a diameter of 0.3–0.5 μm [29, 30]. It is possible, however, that under conditions of substrate limitation, the sizes of these bacteria are smaller than those recorded after growth on laboratory media. A considerable number of 16S rRNA genes revealed in filtered water were close to those of *Burkholderia* representatives, among which there are many conditionally pathogenic forms.

Detection of pseudomonads in filtered water has repeatedly been reported [3, 8, 31]. Apparently, in natural environments, the size of some cells of these bacteria is within the ultramicro range.

Ultrasmall forms of actinobacteria, which are typical components of the bacterioplankton of oligotrophic and oligomesotrophic lakes, have been studied and characterized in sufficient detail [17, 32, 33]. Conversely, virtually nothing is known about the alphaproteobacterial subgroup to which the clones obtained in the present work belong. The only cultured representative of this subgroup, strain Ellin362, was isolated after prolonged incubation of soil inocula on a strongly diluted nutrient medium [16]; however, the characteristics of this strain have not been described.

Only a small fraction of filterable archaeal forms revealed in the water of Lakes Dubrovskoe and Motykino were close to taxonomically characterized archaea, namely to those of the families *Methanobacteriales* and *Methanosarcinales*. Among them, most clones represented methanogens of the genus *Methanobacterium*, which was little surprise given that the latter have long but flexible and very thin cells.

Two-thirds of cloned 16S rRNA genes of filterable archaea fell into a broad taxonomically uncharacterized phylogenetic cluster within the *Euryarchaeota*. The first sequences belonging to this cluster were obtained during the analysis of the microbial communities of aquifer waters (AF050612) [34] and deep-sea hydrothermal vents (AB019752) [35]. Subsequently, a large group of similar 16S rRNA genes was revealed during the study of the sediments of the meromictic Lake Dagow in northern Germany [21]. The authors of the latter paper designated the new cluster LDS (Lake Dagow Sediment). However, this abbreviation is not commonly accepted, and some authors address this cluster as a group of "uncharacterized *Euryarchaeota*". The 16S rRNA genes of representatives of this group have been found in various ecosystems, primarily northern ones, such as rivers, lakes, and wetlands of Arctic and boreal zones, permafrost, etc. [20, 22–25]. The low homology of nucleotide sequences of this group to the 16S rRNA genes of earlier characterized groups of *Archaea* suggests its class-level status within the *Euryarchaeota*. So far, there were no data on the biology of representatives of this new group. Our study allows us to assume that many members of this group are represented by ultramicroforms that are able to pass through 0.22-μm-pore-size filters. The question as to whether such ultrasmall archaea are widespread in freshwaters of northern ecosystems needs further experimental studies.

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