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EXPERIMENTAL ARTICLES

Phylogenetic Diversity of Bacteria in Soda Lake Stratified Sediments

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Abstract—Various previously developed techniques for DNA extraction from samples with complex physicochemical structure (soils, silts, and sediments) and modifications of these techniques developed in the present work were tested. Their usability for DNA extraction from the sediments of the Kulunda Steppe hypersaline soda lakes was assessed, and the most efficient procedure for indirect (two-stage) DNA extraction was proposed. Almost complete separation of the cell fraction was shown, as well as the inefficiency of nested PCR for analysis of the clone libraries obtained from washed sediments by amplification of the 16S rRNA gene fragments. Analysis of the clone library obtained from the cell fractions of stratified sediments (upper, medium, and lower layers) revealed that in the sediments of Bitter Lake 3 most eubacterial phylotypes belonged to the class *Clostridia*, phylum *Firmicutes*. They were probably specific for this habitat and formed a new, presently unknown high-rank taxon. The data obtained revealed no pronounced stratification of the species diversity of the eubacterial component of the microbial community inhabiting the sediments (0–20 cm) in the inshore zone of Bitter Lake 3.

Keywords: saline and soda lakes, DNA extraction, 16S rRNA genes

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Soda lakes are unique ecosystems with doubly extreme conditions: high carbonate alkalinity in the solution, which provides for a stable, extremely high pH of about 10, and high salinity [1]. Such conditions favor the dominance of prokaryotic components in haloalkalophilic microbial communities. Investigation of biodiversity of such habitats is of importance, since unique autochthonous microorganisms often found abounding under extreme conditions are not found in other ecosystems. Moreover, the study of the functional structure of microbial communities of soda lakes gives new information about the limits of stability of microbial communities under extreme conditions.

Many papers dealt with the investigation of the structure of haloalkaliphilic microbial communities from different soda lakes. These studies revealed that a complete system functions in these lakes, performing all the basic elemental cycles and represented by major phylogenetic branches of prokaryotes [1–5]. However, since brackish or moderately saline lakes were studied in most of these works, the traits of these processes under extremely haloalkaliphilic conditions and characteristics of the microorganisms that carry them out have been insufficiently investigated and may differ significantly from those already studied.

Apart from traditional cultivation techniques, the diversity of prokaryotes in saline and soda lakes was also studied using molecular biological methods based primarily on PCR amplification and construction of the 16S rRNA clone libraries. This approach was, for example, used in the study of soda lakes of Wadi An Natrun in Egypt [6], Inner Mongolia in China [7] and soda Mono Lake in the United States. [8] Communities of soda lakes of Ethiopia were recently studied using high-performance pyrosequencing [9].

Bacterial communities of the sediments of several soda lakes of the Kulunda Steppe (Altai, Russia) were studied in a single work [10], which applied amplification and denaturing gradient gel electrophoresis to PCR fragments of the 16S rRNA genes to reveal dependence of bacterial phylogenetic diversity in soda lakes on the salinity of the lakes.

The distribution of different physiological groups of microorganisms in natural habitats is uneven and is determined by environmental physicochemical parameters—illumination, content of oxygen and other compounds, temperature, etc. However, few works have been devoted to the study of stratification of microbial communities in soda lakes. In particular, it follows from the data on the vertical distribution of microorganisms in the various layers of the Mono Lake water column that the composition of microbial

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Table 1. Characteristics of soda lakes and sediment samples used for testing the methods of DNA extraction and analysis of the 16S rRNA gene clone libraries

Lake	Designation	pH	Salinity, g/L	Description		Phytobiomass
				brine	sediment	
Cock Lake	1Kl	9.80	200	Green	Black sand	Many degrading *Cladophora* and dying *Artemia*
Bitter Lake-1	2Kl	10.23	400	Oily-yellow	Black silt	Very thin cyanobacterial biofilms under the salt crust
Bitter Lake-3	3KL	9.90	200	Green	Pinkish clay	Many dying *Cladophora*
Tanatar-6	4KL	9.80	250	Green	Gray clay	Many eukaryotic algae, mainly *Dunaliella*
Tanatar-3	5KL	9.85	100	Transparent	Black sand	None; many *Artemia*
Tanatar—trona, crystallizing water body	6KL	9.60	380	Brown	Black sand	None; the color is probably due to the presence of natronophilic archaea

Gray indicates the sample used for the analysis of clone libraries of the 16S rRNA genes.

communities of the mixolimnion and oxycline differed insignificantly and were much poorer than in the anaerobic and more saline monilimnion and chemocline, with none of the inhabitants of the monimolimnion detected in the oxycline or higher [8].

The goal of the present work was to study the species diversity of the bacterial community of stratified sediments of Bitter Lake 3 in the Kulunda Steppe using the 16S rRNA gene as a molecular marker.

MATERIALS AND METHODS

Characterization of the samples. Samples of sediments from different hypersaline alkaline (soda) lakes of the Kulunda Steppe (Altai, Russia) were collected in July 2012. General characteristics of the lakes and the samples are presented in Table 1. Sediment samples with near-bottom brines were collected from undisturbed columns obtained with a bottom sampler. Typically, samples were taken from three layers: the upper surface aerobic layer with bottom water (lc), the medium 5–10 cm layer (2c), and the lower layer of 10–20 cm (3c). In the laboratory, sediment samples (10 cm^3) were resuspended in 50 mL of 1 M NaCl, and after homogenization coarse fractions were removed in several steps using low-speed centrifugation. The colloidal fraction was concentrated, washed again with brine, and the resulting duplicate sample of about 0.5 cm^3 was stored frozen at –80°C prior to DNA extraction.

Isolation of DNA. The method of DNA extraction (MoBio) used previously [11, 12] for similar samples was found to be efficient only for a part of the newly acquired samples, probably due to the extreme increase in salinity of the lakes in 2012. Experiments were therefore performed with different sets of buffers and reagents to optimize the method of DNA extraction from the newly acquired samples. The efficiency of the variants of DNA isolation was assessed by PCR with the universal primers for the 16S rRNA genes. The following methods were used:

(1) alkaline lysis with sodium dodecyl sulfate and purification using the Wizard Plus Minipreps DNA Purification System (Promega);

(2) a method for DNA isolation from soil samples using the MoBio Power Soil DNA Isolation reagent kit (MoBio Laboratories, United States);

(3) indirect method—cell separation using a buffer with polyvinylpyrrolidone (PVP);

(4) dissolution of silicates with alkali and purification using the MoBio Power Soil DNA Isolation reagent kit according to [13];

(5) lysis with proteinase K and SDS in a buffer containing activated charcoal, PVP, cetyltrimethylammonium bromide (CTAB), and precipitation with polyethylene glycol according to [14];

(6) grinding in liquid nitrogen with glass dust (buffer with chelating agents and nonionic detergent), lysis with proteinase and SDS, CTAB, and cleaning;

(7) pretreatment with hexane/diethyl ether (1/1); DNA isolation according to method 5, followed with desalting using Sephadex G-50 gel filtration;

(8) lysis with proteinase K and SDS, sonication, DNA sorption on MagneSil® Paramagnetic Particles (Promega), washing with guanidine thiocyanate and ethanol, treatment with chloroform, followed with desalting using Sephadex G-50 gel filtration;

(9) lysis with proteinase K and SDS, sorption on the sorbent Wizard MaxiPreps DNA Purification Resin (Promega, United States), washing with GuHSCN and ethanol on a minicolumn, further purification of the eluate with chloroform after the minicol-

umn and subsequent desalting using Sephadex G-50 gel filtration;

(10) new integrated two-stage method, which combines direct and indirect methods for the isolation of DNA. The new method included:

Stage 1—Separation of the cell fraction. A weighed portion of the sample was suspended in a buffer of the following composition (in proportion of 400 μL of buffer per 100 mg of the sample): 1.5 M NaCl, 0.1 M Na phosphate buffer (pH 8.0), 1% PVP, 1% Triton X100, and 50mM EDTA. The sample was incubated for 50 min on a shaker (250 rpm), the silt was precipitated, and the supernatant was collected and placed in a refrigerator. The buffer was added to the residue in a ratio of 200 μL per 100 mg, and the mixture was incubated on a shaker for 15 min. The silt was precipitated, the supernatant was added to the previous one, and the procedure was repeated. The combined supernatants were subjected to differential centrifugation: soil particles were precipitated at 5000 *g*, and cells were then collected at 18000 *g*. DNA was isolated from the cells by the standard method (lysis with 1% SDS in 0.2 M NaCl at 65°C with RNase, SDS sedimentation with potassium acetate and sorption on the Wizard Maxipreps DNA Purification Resin (Promega), washing with Wash Solution and further purification of DNA on a minicolumn).

Stage 2—DNA isolation from the remaining precipitate. The precipitate was suspended in a buffer containing 0.1 M Tris-HCl (pH 8.0), 0.1 M EDTA, and 1% PVP, followed by fractional ultrasonication with three low-power pulses. SDS was added to 2% and guanidine thiocyanate was added to 5 M, and the mixture was incubated for 25 min at 65°C. Activated carbon (1/2 of the precipitate mass) was added, the mixture was incubated for another 15 min at 65°C, and again treated with 3 pulses of ultrasound. SDS was precipitated with potassium acetate; the supernatant was washed with phenol-chloroform (1: 1). DNA was precipitated with isopropanol and then dissolved in 50 μL of mQ and further purified on a Sephadex G50 column according to the Wizard technology.

Amplification of the 16S ribosomal RNA gene fragments. The isolated DNA was verified for PCR suitability. The 5'-fragment of the gene of bacterial 16S ribosomal RNA with a length of about 510 bp was amplified in all cases, and for some samples a 5'-fragment of approximately 1100 bp length was amplified using universal bacterial primers system [15].

To verify the isolated DNA for PCR amplification suitability, test amplification was performed in 25 μL of reaction mixture of the following composition: DNA polymerase buffer (2 mM $MgCl_2$; 17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8), 15 nmol of each deoxyribonucleotide triphosphate, 5 pmol of the forward Univ11F and reverse Univ519R primers, 1 U of BioTaq DNA polymerase, and 2 μL of extracted DNA. For amplification of the fragments for cloning, 12.5 nmol of deoxyribonucleotide triphosphates and 2.5 pmol of each primer were taken. The following temperature–time profile was used: the first two cycles at 94°C for 3 min, 53°C for 1 min and 72°C for 2 min; further 49 cycles at 94°C for 12 s, 55°C for 12 s, and 72°C for 30 s; and final elongation for 7 min at 72°C.

DNA of *Escherichia coli* was used as a positive control.

To obtain PCR fragments with DNA isolated from the washed sediments of Bitter Lake 3, nested PCR was performed. Primary PCR was performed with the primers Univ11F–Univ1110R and with the same concentrations of reagents as test PCR. For primary amplification the following time-temperature profile was used: the first cycle at 94°C for 3 min, 53°C for 1 min, and 72°C for 2 min; the following 29 cycles at 94°C for 10 s, 55°C for 10 s and 72°C for 10 s; and final elongation for 5 min at 72°C.

Part of the resulting PCR fragment was purified by precipitation. For this purpose, 1.5 μL of 3 M of sodium acetate and 15 μL of isopropanol were added to the mixture obtained after PCR (15 μL), incubated for 1 h at –20°C, and centrifuged for 20 min at 20800 *g* at 4°C. The resulting precipitate was washed with 500 μL of 70% ethanol, centrifuged for 5 min at 20800 *g* and 4°C. The precipitate was dried at 37°C and dissolved in 15 μL of deionized water.

Secondary (nested) PCR was performed with the same concentrations of reagents as test PCR. The template was 2 μL of the mixture obtained after primary PCR diluted 200-fold with deionized water, undiluted primary PCR fragment purified by precipitation, or the primary PCR fragment purified by precipitation and diluted 100-fold. With each type of template two PCR were made, one with the primer pair of Univ11F and Univ519R, and the second with a pair of primers Univ341F and Univ907R. The secondary PCR was performed at the same temperature–time profile as the primary.

Analysis of PCR products was performed by electrophoresis in 0.8% agar gel in 1× TAE-buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 μg/mL) at 6 V/cm^2. Electrophoresis results were documented using a BioDoc II gel documentation system (Biometra). Commercial DNA molecular markers GeneRuler 1 kb Plus and GeneRuler 100 bp Plus (ThermoScientific) were used as fragment size standards.

Cloning and sequencing of the 16S rRNA gene fragments. Competent cells of *E. coli* strain DH5[α] were used for the cloning of PCR fragments. The ligation reaction was carried out using the pGEM-T and pGEM-T Easy Vector Systems reagent kit (Promega, United States) according to the kit manual. Transformation of competent cells was performed by the standard procedure. The clones containing inserts of PCR fragments (20–24 clones were obtained for each of the four libraries) were selected using blue-white selection

with X-gal/IPTG. Plasmid DNA was isolated from 12-h cultures of *E. coli* DH5[α] grown in liquid LB medium with ampicillin (50 U/mL, using the Wizard Minipreps reagent kit (Promega, United States) according to the kit manual. The resulting plasmid DNA preparations were stored at –18°C.

Statistical analysis of the clone libraries. Representativeness of the libraries of the 16S rRNA genes, i.e., adequacy of the sample size of clones for reliable analysis of the community structure, was assessed by calculating the Good's coverage of the libraries according to the formula: $(1 - n/N) \times 100$, in which n is the number of single clones and N is the total number of clones in the library [16]. Shannon's diversity index and the estimated number of phylotypes Chao 1 were calculated using the EstimateS program (Version 8, R.K. Colwell, http://purl.oclc.org/estimates). The dominance index was calculated as the ratio of clone number of the dominant phylotypes to the total number of clones.

Phylogenetic analysis of the sequences. Preliminary analysis of the nucleotide sequences of the 16S rRNA genes was carried out using the BLAST program in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and classifiers of the databases RDP (http://rdp.cme.msu.edu) and SILVA (http://www.arb-silva/aligner/de). The sequences were processed and aligned using BioEdit (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) with built-in CLUSTALW. To eliminate chimera, the sequences were checked using the CHECK_CHIMERA online system from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu). Operational taxonomic units (OTUs) were accepted as the phylotypes representing members of the community at the species level which combine sequences with the similarity level of 98% and above. Identified OTUs were used for further analysis. Phylogenetic trees were constructed using the neighbor-joining algorithm implemented in the TREECONW software package (http://bioinformatics.psb.ugent.be/psb/Userman/treeconw.html) using the reference sequences from GenBank database.

Deposition of the nucleotide sequences. The resulting nucleotide sequences of the 16S rRNA gene fragments were deposited in the GenBank database under the accession numbers KJ704708–KJ704772.

RESULTS AND DISCUSSION

Optimization of methods for DNA isolation from hypersaline soda lake sediments. The first stage of the work was to test the applicability of different methods of DNA extraction—both those described in the literature and in our modification—to the samples of sediments from hypersaline soda lakes. The criterion of quality of the obtained DNA preparations was their suitability as a template for PCR, which was performed with universal primers for the 16S rRNA gene fragment (~510 bp). None of the nine tested methods of DNA extraction was found to be effective and universal for all the studied sediment samples. Therefore, based on the results of testing of these methods (data not shown) and the literature data, a two-step method for isolating DNA was developed comprising separation of the cells with a buffer, DNA extraction according to the Wizard technology, and isolation of residual DNA from the washed precipitate by sonication or chemical lysis with SDS as methods of cell disruption, as well as multi-step purification by treatment with a nonpolar solvent with the addition of guanidine thiocyanate and activated charcoal followed by phenol–chloroform treatment and gel filtration.

This method allowed us to isolate PCR-suitable DNA from eight different samples (Fig. 1). DNA extraction from the cells was successful for all samples except the silt with trona from the hypersaline lake Tanatar 1, presumably due to enrichment of the sample with attached forms of bacteria poorly separable form silt particles. At the same time, DNA from the washed precipitate was obtained in all cases, including sediment from Tanatar 1, which further confirms the inadequacy of using only the indirect method and the comparative efficiency of the two-stage method.

Comparative study of clonal libraries based on 16S rRNA genes derived from one sediment sample using the developed two-stage DNA isolation method. To test the universality of this method of DNA extraction and further phylogenetic analysis of the bacterial community in soda lake anaerobic sediments, a sample from the upper sediment layer (1c, 0–5 cm) of Bitter Lake 3 was taken, which was not used for development of the procedure. During DNA isolation according to the developed technique, we succeeded in obtaining the PCR product from the DNA of the cell fraction (Stage 1); we, however, failed to obtain a PCR product of the DNA sample obtained from the washed precipitate (Stage 2). Nevertheless, an attempt was made to obtain a PCR fragment from the washed precipitate, using the method of nested PCR, which resulted in a PCR fragment with a length of about 500 bp. Both types of PCR fragments derived from the DNA of the cells and DNA from the precipitate were cloned, and thus, two libraries were obtained of 75 and 95 clones for each of the stages of DNA extraction. All clones from the libraries made from DNA of the cells (hereinafter referred to as "cell library"), and DNA from the washed precipitate (hereinafter "the precipitate library") were sequenced. The sequences obtained were divided into operational taxonomic units (OTUs) according the level of similarity—sequences with similarity of 98% or higher were assigned to one OTU (intraspecific level)—and then the search for related sequences in the GenBank database was carried out using the BLAST NCBI, followed by OTU classification.

BLAST analysis of the sequences of the 16S rRNA gene fragments from the cell library showed that they

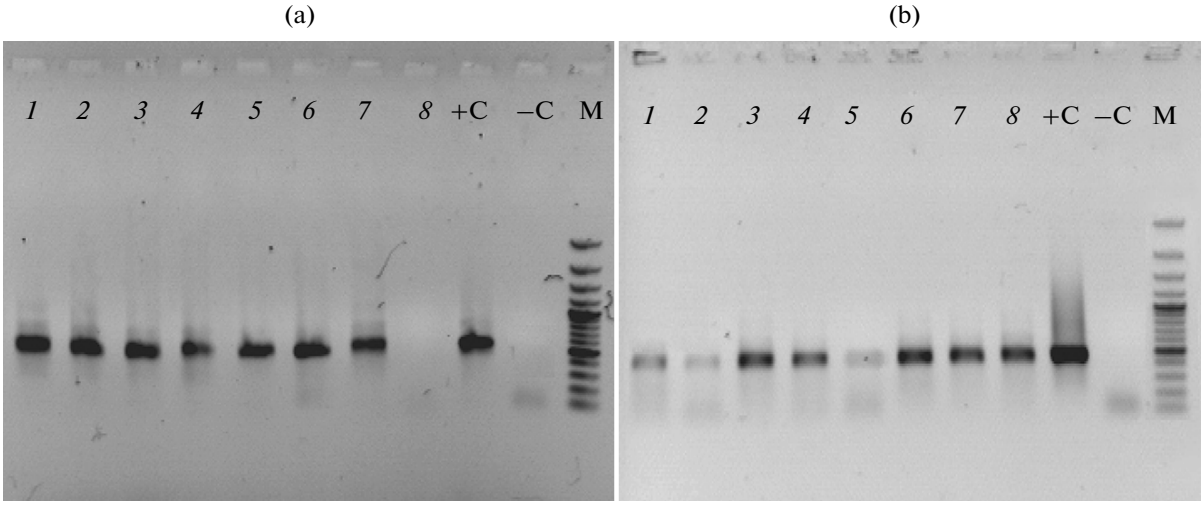


Fig. 1. Electrophoretic separation of PCR products with DNA extracted using the two-stage method: from the separated cells (a) and from washed sediments (b). Lanes *1–8* correspond to samples: 1KL-1c, 1KL-2c, 2KL-1c, 2KL-2c, 2KL-3c, 4KL, 5KL, 6KL. Lanes +C (a and b), positive control, lanes –C (a and b), control of the reagents. Lanes M (a and b), DNA marker 100bp plus (from 100 to 3000 bp), DNA at 80 ng is in brighter bands.

aligned at a sufficiently high percentage of nucleotide identity only with similar sequences of uncultured organisms (Table 2). Most taxonomic units were only classified to the level of family, order, or even class.

The greatest number of sequences (both in the number of OTUs and in the total number of clones included in them) belonged to the class *Clostridia*. The presence of clostridia with different types of metabolism in anaerobic sediments of soda lakes was also shown by cultural methods [17]. While it is usually impossible to determine the type of metabolism of a microorganism from its phylogeny, most of clostridia represented in the libraries could be primary or secondary hydrolytic anaerobes. The sample also contained, although in minor amounts, representatives of the genus *Thioalkalivibrio*, which, according to various sources, are obligate inhabitants of soda lakes, although not always detected by clonal PCR analysis of the 16S rRNA gene fragments. Thus, the observed diversity of bacteria in the cell fraction of sediments may be considered autochthonous to soda lakes.

Unlike OTUs of the cell library, most OTUs obtained from the washed precipitate were determined to the genus or even species (Table 2). However, these results are questionable because they do not correspond to known data on the microbiota of soda lakes. The library of precipitates included a large number of soil phylotypes (*Bradyrhizobium japonicum*) and freshwater (*Caulobacter* sp.) and phytopathogenic (*Sphingomonas melonis*) species, as well as representatives of the skin microbiota and microbiota of the respiratory tract of terrestrial animals (*Propionibacterium acnes*, *Streptococcus* spp.). This is probably due to contamination of lake sediments with the microbiota of surrounding soil and surrounding fauna occurring during floods, rain, or wind; the introduced cells then either remain in an inactive state or die. Destructive processes in the sediments of hypersaline lakes are slow, and the remains of the introduced biomass and DNA are preserved and remain attached to the silt particles, but the total concentration of them is so small that they are not identified by primary PCR after almost complete extraction of the cell fraction. This does not exclude the possibility of distortion of the quantitative proportion of phylotypes in the resulting library of precipitates due to the known artifact (bias) of nested PCR, used in this case for the amplification of 16S rRNA gene fragments. Thus, it was shown that the application of this method is not a reliable method of detection of the autochthonous microflora in natural samples. At the same time, detection of the phylotypes of cyanobacteria and some other species that may be autochthonous to the microbiota of soda lakes (for example, *Methylocystis* sp.) in only the DNA from the washed sediment may indicate that the indirect method of DNA isolation has limitations because not all members of the microbiota may be present in the cell fraction.

Statistical and phylogenetic analysis of cell libraries of stratified sediments. To analyze stratification of the microbial community of soda lake sediments from Bitter Lake 3, additional DNA extraction was conducted using only the indirect method (the cell fraction) from two deeper layers: the medium layer at 5–10 cm (2c) and the lower layer 10–20 cm (3c). As a result, together with the previously obtained library from the upper layer of 0–5 cm (1c) on the template of the isolated DNA, three libraries were obtained and analyzed, consisting of a total of 226 clones. Table 3 represents results of statistical analysis of the 16S rRNA gene sequences of these three cell libraries. Good's coverage was relatively low and approximately

Table 2. The distribution of clones in the libraries of the 16S rRNA genes obtained by two-stage extraction of DNA from the upper layer of the sediment from soda Bitter Lake

Cell fraction					Washed sediment				
No. OTU	Number of clones	Closest relative	Identity, %	Accession	No. OTU	Number of clones	Closest relative	Identity, %	Accession
1	30	Uncultured Firmicutes bacterium clone x215	99	GQ848219	1	26	*Streptococcus mitis* KCOM 1379	99	GU045389
2	17	Uncultured bacterium clone 23J36	98	EU645214	2	20	*Bradyrhizobium japonicum* NA110	99	JN392462
3	8	Uncultured bacterium clone 1-1B-19	91	JF417910	3	9	*Sphingomonas melonis*	100	AB334774
4	3	Uncultured Firmicutes bacterium clone CSS2	96	JX240586	4	8	Bacterium HTCC8036	99	EF616603
5	3	Uncultured bacterium clone x55	99	GU083671	5	6	*Arthrobacter davidanieli*	100	AF099202
6	3	Uncultured bacterium clone MiJ16	95	EU645127	6	5	*Caulobacter segnis* ATCC 21756	95	DQ493433
7	2	*Roseinatronobacter monicus* ROS 35	99	NR_043914	7	5	*Sphingomonas rhizogenes* BW59UT1570	100	JF276901
8	2	Uncultured bacterium clone SSS47	94	EU592468	8	4	*Methylocystis heyeri* H2	97	NR_042531
c1-F09	1	Uncultured bacterium clone MoJ28	96	EU645251	9	3	*Gordonia sputi* DSM44019	100	X80627
c1-F06	1	Uncultured bacterium clone SA_57	91	JQ738944	10	2	*Propionibacterium acnes* HL096PA1	99	CP003293
c1-F07	1	Uncultured Firmicutes bacterium clone x23	94	GQ848204	11	2	*Methylobacterium radiotolerans* A219	97	HF585373
c1-F12	1	Uncultured low G+C gram-positive bacterium clone WN-FSB-22	98	DQ432093	s1-G05	1	*Brevundimonas vesicularis* G1-1-80	99	KC494327
c1-H09	1	Uncultured low G+C gram-positive bacterium clone WN-HSB-258	98	DQ432330	s1-G01	1	*Cyanobacterium stanieri* PCC 7202	95	CP003940
c1-G02	1	Uncultured bacterium clone MHSed-75	94	JF780883	s1-C12	1	*Corynebacterium pseudogenitalium* CIP106714	99	AJ439348
c1-A02	1	Uncultured low G+C gram-positive bacterium clone WN-HWB-61	99	DQ432363	s1-A11	1	*Aeromonas hydrophila* subsp. *anaerogenes* CIP106714	99	AJ439348
c1-H02	1	*Thioalkalivibrio nitratireducens* DSM 14787	99	NR_102486	s1-B12	1	*Stenotrophomonas maltophilia* YB-6	99	AB513656
Total	77				Total	95			

Table 3. Data of statistical processing of cell libraries prepared from three layers of Bitter Lake soda lake sediment

Sample	Number of clones in the library	Number of phylotypes (OTUs > 98%)	Good's Coverage (%)	Calculated number of phylotypes Chao 1	Shannon-Weaver index of species diversity (H)	Evenness (H/H_{max})	Index of dominance
Upper layer (1c)	75	15	90	22 (min 16, max 51)	1.92	0.71	0.39
Medium layer (2c)	71	22	83	46 (min 28, max 123)	2.58	0.84	0.21
Lower layer (3c)	80	28	82	61 (min 36, max 169)	2.92	0.88	0.20

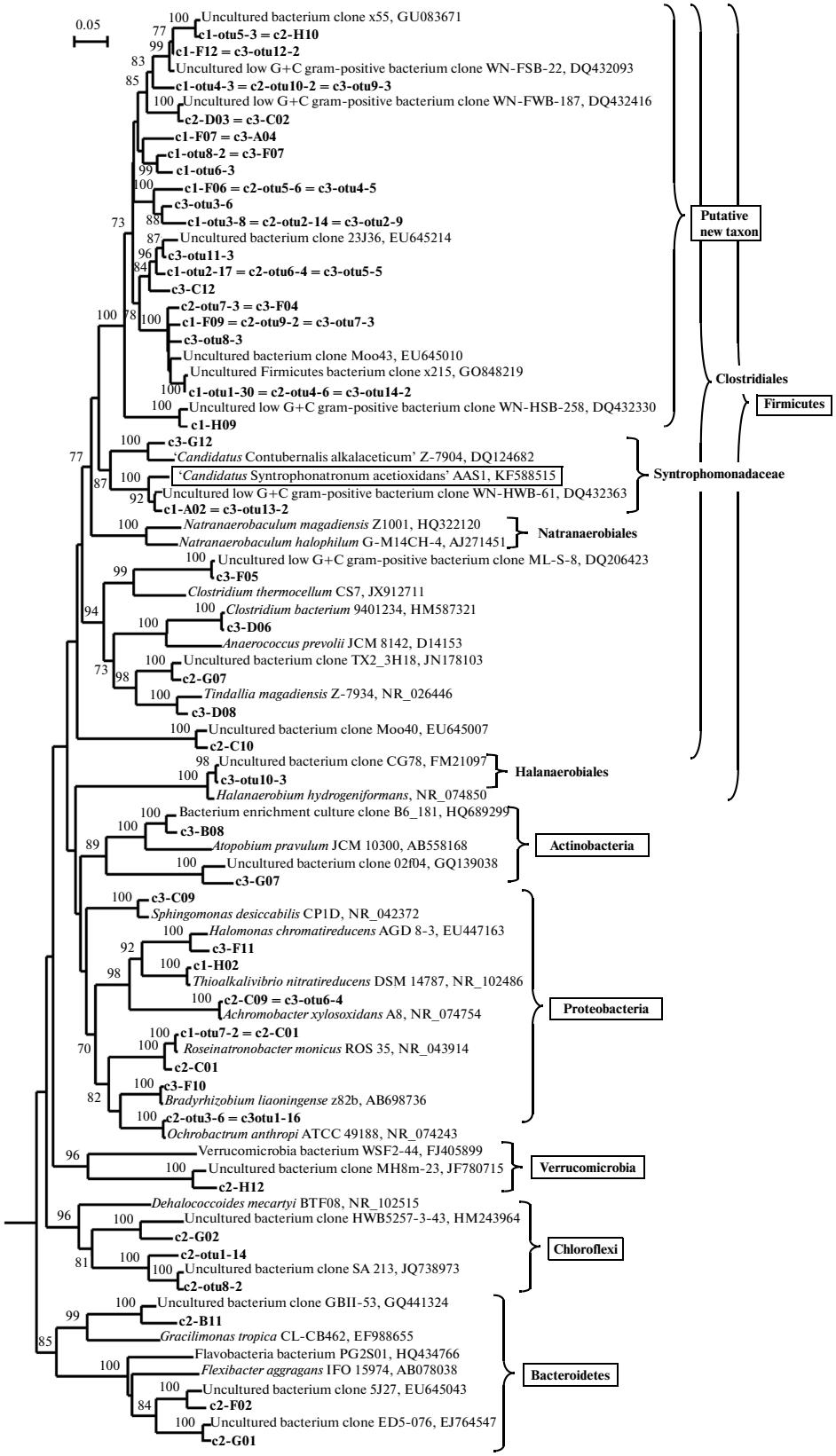
the same for all libraries, comprising 82–90%, while the estimated number of phylotypes Chao 1 was also significantly higher than that obtained in the experiment. Thus, it is necessary to take into account that the investigated libraries did not fully reflect the composition of the studied natural communities.

The lowest diversity was observed in the upper layer; the number of represented groups was significantly higher in the medium and lower layers. The calculated indices of diversity and uniformity increased in the third layer. Small number of phylotypes in the upper layer was probably due to stronger dominance of the most represented phylotypes (index of dominance in the upper layer was two times higher than in the medium and lower ones, while uniformity, on the contrary, increased in the third layer).

This marked dominance may be due to the fact that in the upper layer the conditions are most favorable for one of the discovered fermenting anaerobes, possibly caused by the greatest amount of polymers and other organic compounds available for fermentation by this dominant microorganism. Less energetically favorable processes in the lower layers of the sediments can be carried by a smaller total number of microorganisms, whereby the degree of uniformity in the lower layers increases, as well as the diversity represented in the library.

Distribution of the phylotypes identified in the studied libraries is shown on Figs. 2 and 3. According to preliminary analysis using the online classifiers RDP and SILVA, most phylotypes identified in all three layers were classified as belonging to the family *Synthrophomonadaceae* of the order *Clostridiales*, the phylum *Firmicutes*. However, high levels of sequence similarity of the identified phylotypes was detected only for uncultured organisms from various sources, including soda lakes. In particular, the dominant phylotype from the upper layer had 99% similarity with an uncultured and unidentified representative of *Firmicutes* from Xiarinur soda lake in China (GQ848219), and the next dominating phylotype had 98% similarity with an uncultured and unidentified representative of *Firmicutes* from soda Soap Lake, Washington State, United States (EU645214). Several phylotypes were also close to a variety of uncultured representatives of *Firmicutes* from soda lakes of Wadi An Natrun [6]. This was indicative of the autochthonous bacteria represented by these phylotypes in the sediments of soda lakes. However, the level of sequence similarity of the 16S rRNA genes of most detected phylotypes of the *Firmicutes* with described representatives of this phylum (including members of the family *Synthrophomonadaceae*) did not exceed 85%. At the same time, according to the phylogenetic tree (Fig. 2), the majority of the detected phylotypes of *Firmicutes* (36 out of 65 unique phylotypes and 69% of the total number of clones in all libraries) formed a separate large cluster within this phylum. Thus, by the level of divergence of the 16S rRNA gene sequences, and according to its particular position on the phylogenetic tree, the identified cluster of *Firmicutes* (*Clostridiales*) corresponded at least to the status of a family; assignment of its members to the family *Synthrophomonadaceae* is therefore doubtful, and its taxonomic status remains unknown. At the same, uncultured members of this cluster not only dominated in the diversity of the community in general, but many unique phylotypes were revealed in more than one layer. So far, no representatives of this unknown taxon have been isolated into pure culture, which emphasizes the need to continue the microbiological research in this direction.

Fig. 2. Phylogenetic tree of the 16S rRNA gene sequences of cell libraries (shown in bold) and the closest sequences of cultured and uncultured bacteria (the designations 1c, 2c, and 3c at the beginning of the names of taxonomic units indicate these taxonomic units as belonging to the libraries of three layers, respectively; OTU numbers are presented in descending order of the number of clones in them; single clones are designated by letters and numbers). The 16S rRNA gene sequence of a syntrophic clostridium AAS1 is framed. The tree was constructed using a neighbor-joining algorithm. The scale indicates the evolutionary distance corresponding to 5 substitutions per 100 nucleotides. The numerals show the accuracy of the branching order determined by bootstrap analysis of 1000 alternative (values greater than 70 are shown).


0.05
Uncultured bacterium clone x55, GU083671
c1-otu5-3 = c2-H10
c1-F12 = c3-otu12-2
Uncultured low G+C gram-positive bacterium clone WN-FSB-22, DQ432093
c1-otu4-3 = c2-otu10-2 = c3-otu9-3
Uncultured low G+C gram-positive bacterium clone WN-FWB-187, DQ432416
c2-D03 = c3-C02
c1-F07 = c3-A04
c1-otu8-2 = c3-F07
c1-otu6-3
c1-F06 = c2-otu5-6 = c3-otu4-5
c3-otu3-6
c1-otu3-8 = c2-otu2-14 = c3-otu2-9
Uncultured bacterium clone 23J36, EU645214
c3-otu11-3
c1-otu2-17 = c2-otu6-4 = c3-otu5-5
c3-C12
c2-otu7-3 = c3-F04
c1-F09 = c2-otu9-2 = c3-otu7-3
c3-otu8-3
Uncultured bacterium clone Moo43, EU645010
Uncultured Firmicutes bacterium clone x215, GO848219
c1-otu1-30 = c2-otu4-6 = c3-otu14-2
Uncultured low G+C gram-positive bacterium clone WN-HSB-258, DQ432330
c1-H09
Putative new taxon
c3-G12
'Candidatus Contubernalis alkalaceticum' Z-7904, DQ124682
'Candidatus Syntrophonatronum acetioxidans' AAS1, KF588515
Uncultured low G+C gram-positive bacterium clone WN-HWB-61, DQ432363
c1-A02 = c3-otu13-2
Syntrophomonadaceae
Natranaerobaculum magadiensis Z1001, HQ322120
Natranaerobaculum halophilum G-M14CH-4, AJ271451
Natranaerobiales
Uncultured low G+C gram-positive bacterium clone ML-S-8, DQ206423
c3-F05
Clostridium thermocellum CS7, JX912711
Clostridium bacterium 9401234, HM587321
c3-D06
Anaerococcus prevolii JCM 8142, D14153
Uncultured bacterium clone TX2_3H18, JN178103
c2-G07
Tindallia magadiensis Z-7934, NR_026446
c3-D08
Uncultured bacterium clone Moo40, EU645007
c2-C10
Clostridiales
Firmicutes
Uncultured bacterium clone CG78, FM21097
c3-otu10-3
Halanaerobium hydrogeniformans, NR_074850
Halanaerobiales
Bacterium enrichment culture clone B6_181, HQ689299
c3-B08
Atopobium pravulum JCM 10300, AB558168
Uncultured bacterium clone 02f04, GQ139038
c3-G07
Actinobacteria
c3-C09
Sphingomonas desiccabilis CP1D, NR_042372
Halomonas chromatireducens AGD 8-3, EU447163
c3-F11
c1-H02
Thioalkalivibrio nitratireducens DSM 14787, NR_102486
c2-C09 = c3-otu6-4
Achromobacter xylosoxidans A8, NR_074754
c1-otu7-2 = c2-C01
Roseinatronobacter monicus ROS 35, NR_043914
c2-C01
c3-F10
Bradyrhizobium liaoningense z82b, AB698736
c2-otu3-6 = c3otu1-16
Ochrobactrum anthropi ATCC 49188, NR_074243
Proteobacteria
Verrucomicrobia bacterium WSF2-44, FJ405899
Uncultured bacterium clone MH8m-23, JF780715
c2-H12
Verrucomicrobia
Dehalococcoides mecartyi BTF08, NR_102515
Uncultured bacterium clone HWB5257-3-43, HM243964
c2-G02
c2-otu1-14
Uncultured bacterium clone SA 213, JQ738973
c2-otu8-2
Chloroflexi
Uncultured bacterium clone GBII-53, GQ441324
c2-B11
Gracilimonas tropica CL-CB462, EF988655
Flavobacteria bacterium PG2S01, HQ434766
Flexibacter aggragans IFO 15974, AB078038
Uncultured bacterium clone 5J27, EU645043
c2-F02
Uncultured bacterium clone ED5-076, EJ764547
c2-G01
Bacteroidetes

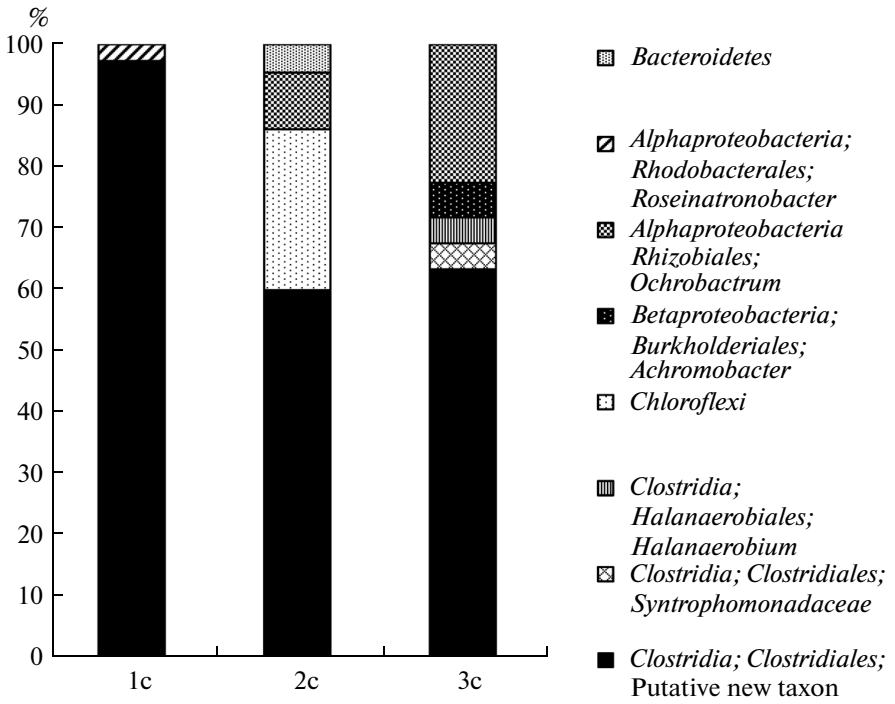


Fig. 3. Diagram of distribution of different groups of bacteria detected in the cell libraries in the layers. The figure shows only the OTUs, individual clones are not shown.

However, in the libraries of the upper and lower layers among the minor components of the *Firmicutes*, several phylotypes were found which really belonged to the family *Synthrophomonadaceae*. One of them was close (94.9% sequence similarity) to the syntrophic bacterium *Candidatus* 'Contubernalis alkalaceticum' [18], and 2 other phylotypes close to *Synthrophomonadaceae* exhibited a high level of similarity (96.0%) with the 16S rRNA gene of an acetate-fermenting clostridium AAS1, a part of the syntrophic association with an extremely natronophilic sulfate-reducing bacterium of the genus *Desulfonatronospira*. This association was isolated from the same natural habitats and was able to oxidize acetate sulfate as an electron acceptor in saturated soda brines. A new clostridium of this association was recently described as a new genus and species (*Candidatus* 'Syntrophonatronum acetioxidans') [19]. It can be noted that a similar uncultured bacterium (98.8% sequence similarity with the phylotypes detected in this study) was found in soda lakes of Wadi An Natrun [6]. Exactly how the oxidation of acetate occurs in hypersaline soda lakes is still unclear, but a high degree of similarity of the cultivated member of the syntrophic association and of uncultured clostridia from soda lakes samples confirms that the process occurs in the studied natural conditions.

Unlike the phylotypes of a proposed new taxon within *Clostridiales*, other phylogenetic divisions of *Firmicutes* were mainly represented by minor components of the community and were different for different layers of the sediment. In particular, among the *Clostridiales* and *Halanaerobiales* of the lowest layer of the sediments, phylotypes close to the known bacteria of hypersaline habitats, *Tindallia* and *Halanaerobium*, were found. It can be noted that a large number of representatives of *Firmicutes* was also detected in a previous work [10].

A considerable part of the clones of the library from the medium layer were representatives of the phylum *Chloroflexi* (total of 15 clones, 3 phylotypes). Most likely, these phylotypes represented nonphtotrophic members of the phylum, but the level of sequence similarity of the 16S rRNA genes was low, both with uncultured clones (not exceeding 93%) and with the closest described species of this phylum *Dehalococcoides mccartyi* (84%). What role they play in the studied community remains unclear. Furthermore, among the minor components of the libraries, the phylotypes of such autochthonous inhabitants of soda lakes as members of the genera *Thioalkalivibrio, Halomonas* and *Roseinatronobacter* were found in different layers. However, the libraries from the medium and lower layers were enriched with the representatives of supposedly introduced microflora, in particular, soil or anthropogenic *Ochrobactrum* sp. (9 and 21% of the total number of clones in each library, respectively).

Fig. 4 represents a diagram showing the number of phylotypes (OTUs), general and non-overlapping (individual), for different layers (in parentheses the percentage of individual clones or clones belonging to the group of overlap of the total number of clones in all three layers is shown). Over 50% of all the clones

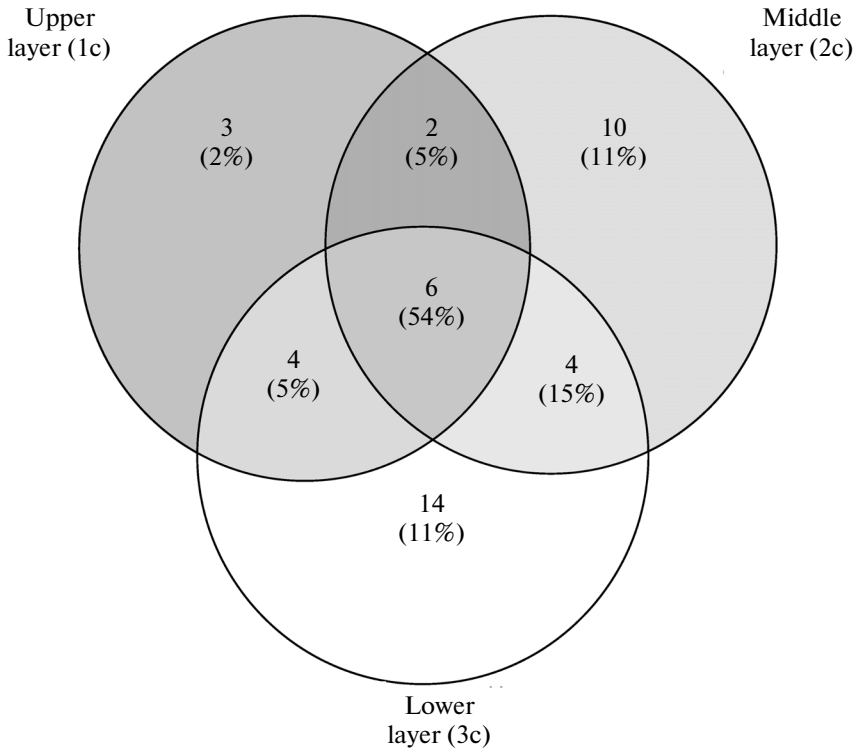


Fig. 4. A diagram illustrating the number of common and unique phylotypes in the cell libraries of different layers. In parentheses the percentage of clones belonging to the overlap group or individual clones of the total number of clones in all three layers are given.

belonged to six phylotypes common to all three layers, and up to 25% of the clones belonged to the overlap group for two different layers. Non-overlapping (individual) phylotypes were in most cases minor groups represented by only one clone, so it is impossible to say with confidence that the OTUs were really unique to a particular layer. As mentioned above, the statistics indicate that the number of investigated clones is insufficient for complete characterization of the community.

Our results demonstrate the absence of pronounced stratification of bacteria and are consistent with previously obtained results [10], which also found no significant stratification over the depth of sediments in other investigated Kulunda Steppe lakes differing in the degree of salinity. This may be due both to natural causes and to the features of the used methods of laboratory analysis. Stratification of the microbial community in the sediments of soda lakes can be weakly expressed because of the rather stable conditions in the bulk sediment, as opposed to the sediment surface and the water column. Insufficiency of the sample may also be an important factor: since some processes can be carried out by a relatively small number of cells, only minor groups are probably stratified, i.e., the ones we have not detected in the taken number of clones. For example, such active anaerobic functionaries as acetogens, sulfate reducers, and methanogens are typically present in the anaerobic sediments in minor amounts and are reliably detected only by specific functional primers.

Although considerable experience has been accumulated in molecular ecological studies of prokaryotic diversity in extreme environments, such as soda lakes, our work revealed the fact that the most difficult circumstance for these studies may be the stage of DNA isolation from environmental samples. The effectiveness of this phase, the selectivity of the results for different groups of prokaryotes constituting a natural community, can significantly affect the assessment of biodiversity of prokaryotes present in the studied habitats. This fact has already been noted earlier, especially for soil habitats, for which the methods of DNA extraction were discussed in specialized works [20, 21].

Testing a significant number of previously developed methods for the isolation of DNA from samples with a complex physicochemical structure (soil, silt, and sediment) and the modifications of these methods proposed in this paper allowed us to assess their suitability for isolation of DNA from the sediments of hypersaline soda lakes and to develop the most efficient modification of the indirect method of DNA extraction.

The ambiguity of interpretation of the data of analysis of the 16S rRNA gene libraries, obtained by nested PCR on template DNA from the washed sediments,

indicates the need for further research on continued improvement of the methods of DNA extraction in order to increase their degree of universality.

At the same time, the results of phylogenetic analysis of the communities of bacteria from the cell fractions of studied sediments revealed unexpected features of this composition, which may be important for carrying out further experiments to isolate new, unknown bacteria from the studied extreme ecosystem.

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