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EXPERIMENTAL ARTICLES

Production of Gaseous Hydrocarbons by Microbial Communities of Lake Baikal Bottom Sediments

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Abstract—Production of gaseous hydrocarbons by the microbial community of the Posolsky Bank methane seep bottom sediments (southern Baikal) was studied at 4°C. Formation of both methane and a heavier gaseous hydrocarbon, ethane, was detected in enrichment cultures. The highest methane concentrations (6.15 and 4.51 mmol L^{-1}) were revealed in enrichments from the sediments from 55-cm depth incubated with sodium acetate and H_2/CO_2 gas mixture, respectively. A decrease in activity of aceticlastic methanogens and a decrease in methane concentration produced by hydrogenotrophic archaea occurred with depth.The highest concentration of ethane was revealed in enrichments from the microbial community of the layer close to gas hydrates (75 cm) incubated with CO_2 as a substrate. According to analysis of the 16S rRNA gene fragments from the clone library, these enrichments were found to contain members of the phylum *Crenarchaeota* forming a separate cluster with members of the class *Thermoprotei*. The phylum *Euryarchaeota* was represented by nucleotide sequences of the organisms homologous to members of the orders *Methanococcales*, *Methanosarcinales*, and *Thermoplasmatales*.

Keywords: microbial community, methane and ethane formation, gas-bearing and gas hydrate bottom sediments, Lake Baikal

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Methane formation in marine and freshwater sediments is due either to microbial organic matter (OM) transformation or to thermocatalytic processes [1, 2]. Microbial methane is characterized by low levels of homologues (ethane, propane, and butane), which is usually three levels of magnitude lower than the concentration of methane (C_1/C_{2+} >1000). In thermogenic gas, the content of methane homologues is considerably higher, reaching 10–15 vol % (C_1/C_{2+} < 100) [3]. Apart from methane, ethane, and propane, unsaturated hydrocarbons C_2–C_4 (ethylene, propylene, and butylene) are also detected in the sediments. These compounds are typically not components of the thermogenic gas, but may be formed as intermediate products of OM decomposition [4].

Gaseous hydrocarbons of different genesis differ in the carbon isotopic composition. Thus, ethane of microbial origin contains more isotopically light ^{12}C ($\delta^{13}C$–C_2 < –40‰), while thermogenic ethane has higher $\delta^{13}C$ values exceeding –40‰ [5]. Capacity of microorganisms for production of gaseous hydrocarbons other than methane was first reported in 1948 [6]. It is presently known that ethanogenesis is carried out by methanogenic archaea under the conditions required for methanogenesis (anoxic conditions, low Eh, etc.) [7–10]. The mechanisms of biogenic ethane formation and the biochemistry of the microorganisms involved in this process remain, however, unknown. Since no ethane-producing strain was isolated in pure culture, the mechanisms responsible for microbial C_2H_6 formation are deduced from the results of experiments with microbial communities supplemented with certain substrates [6–10].

In the sediments of Lake Baikal, similar to marine sediments, both microbial and thermogenic methane are present [11, 12]. Microbial methane is found in the sediments all over Lake Baikal, while biogenic ethane ($\delta^{13}C$–C_2 from –62.7 to –60.0‰) was revealed at a single site, the Peschanka cold seep (southern Baikal) [13]. A number of structures at the bottom of the lake are responsible for the discharge of gas containing admixtures of ethane (up to 3–5 vol % in some cases);

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according to its $\delta^{13}C-C_2$ values (from −27.6 to −23.3‰), this gas is of thermogenic origin. In some areas of the lake, high ethane concentrations dissolved in pore water result in formation of gas hydrates (GH), not only of the KS-1 structure (monolithic hydrate), but also of the KS-2 structure (granulated hydrate). They differ in the size of cavities in the crystalline structure and in the ethane content in the hydrate gas [12, 14].

Investigation of the processes resulting in biogenic ethane formation in Lake Baikal bottom sediments, which are probably responsible for its detection in some areas, has not been carried out previously. Thus, investigation of the processes of microbial formation of methane and its homologues, as well as detection and taxonomic description of the microorganisms and microbial consortia responsible for this process, are important tasks.

The goal of the present work was to carry out laboratory experiments for investigation of the processes of formation of gaseous hydrocarbons by the microbial community of the Posolsky Bank station, where GH were detected, as well as high methane and ethane concentrations, and to determine the phylogenetic diversity of microorganisms in enrichment cultures.

MATERIALS AND METHODS

Sampling and determination of hydrocarbon gas content and pore water composition. Samples of gas hydrate-bearing bottom sediments collected at the Posolsky Bank methane seep (southern Baikal, St. 5, GC-4) were investigated. The station coordinates, depth, and the sampler type were described previously [15].

Gaseous hydrocarbons in the sediments and in experimental vials were determined by head-space technique [16]. Methane content in the gas phase was determined on an ECHO-PID chromatograph equipped with a flame ionization detector and a 2 m × 2 mm Porapak-filled packed column under isothermic conditions at 100°C. The gas volume for analysis was 0.03–0.05 mL (volume of the needle of a chromatographic syringe).

Pore water composition was determined as described previously [17].

Enrichment cultures. The experiments were carried out in 15-mL vials with Pfennig bicarbonate medium containing the following (g/L): NaCl, 0.3; NH_4Cl, 0.33; KH_2PO_4, 0.33; $MgCl_2 \cdot 6H_2O$, 0.33; $CaCl_2 \cdot 2H2O$, 0.33; $NaHCO_3$, 1; resazurin, 0.001; vitamin solution, 1 mL; and trace element solution [18], 1 mL. The vials were inoculated (1 mL) with the sediments from the core at 0, 25, 55, 75 (sediment above GH), 80, 100, and 145 cm (GH-bearing layers). In order to maintain strictly anoxic conditions, the media were boiled, supplemented with a reducing agent ($Na_2S \cdot 9H_2O$, 0.5 g/L), and incubated under nitrogen.

Ability of the microbial community to form gaseous hydrocarbons via the acetate pathway was studied in the medium supplemented with sodium acetate (2 g/L). Enrichments for hydrogenotrophic methanogens were incubated under the H_2/CO_2 (80 : 20) gas mixture. The vials were incubated for three moths at 4°C.

Methanogenic enrichment cultures were obtained in liquid Pfennig medium using the Hungate anaerobic technique [19] and serial dilutions [18].

Molecular identification techniques. DNA was extracted from enrichment cultures according to a modified procedure for enzymatic lysis with subsequent phenol–chloroform extraction [20]. The 16S rRNA genes were amplified using the archaeal primers Arch-20F (5'-TCCCGGTTGATCCYGCCRG)/Arch-915R (5'-GTGCTCCCCCGCCAATTCCT).

Cloning and transformation of the 16S rRNA gene fragments were carried out using the pGEM-T Easy Vector Systems kit (Promega, United States) according to the manufacturer's recommendations.

Sanger reaction using the BigDye Terminator Kit v. 3.1 (Applied Biosystems) and analysis of its products on an ABI 3130xl analyzer were carried in the Genomika collective use center, Siberian Branch, Russian Academy of Sciences (Novosibirsk).

The sequences were edited using the BioEdit v. 7.1.9 software package [21]. Homological sequences in the GenBank database were determined using BLAST (www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis was carried out with the MEGA 5.2 software package [22] using the neighbor-joining and the Kimura's two parameters algorithm. The branching order was determined by bootstrap analysis of 100 alternative trees. The 16S rRNA gene sequences obtained in the present work were deposited to GenBank under accession nos. KJ736828–KJ736834.

Microbial morpology was studied by fluorescence and electron microscopy. For transmission electron microscopy, the samples were resuspended in sterile water, applied to formvar-coated grids, dried, and examined under a Leo 906E transmission electron microscope (ZEISS, Germany) at the Ul'tramikroanaliz baze.

For epifluorescence microscopy, the sample was dehydrated in a series of ethanol solutions, stained with 4,6-diamino-2-phenylindole (DAPI, 0.5 μg/mL), incubated for 3 min, and examined under an Axio Imager M1 microscope (Zeiss, Germany).

RESULTS

Lithological characterization of the bottom sediments. The sediments contained reduced moist aleuro-pelite with aleurite admixture and dark spots or layers of amorphous iron sulfide (hydrotroilite). At 30–80 cm, "clotted" aleuro-pelite with numerous degassing cracks was found. Humidity decreased with

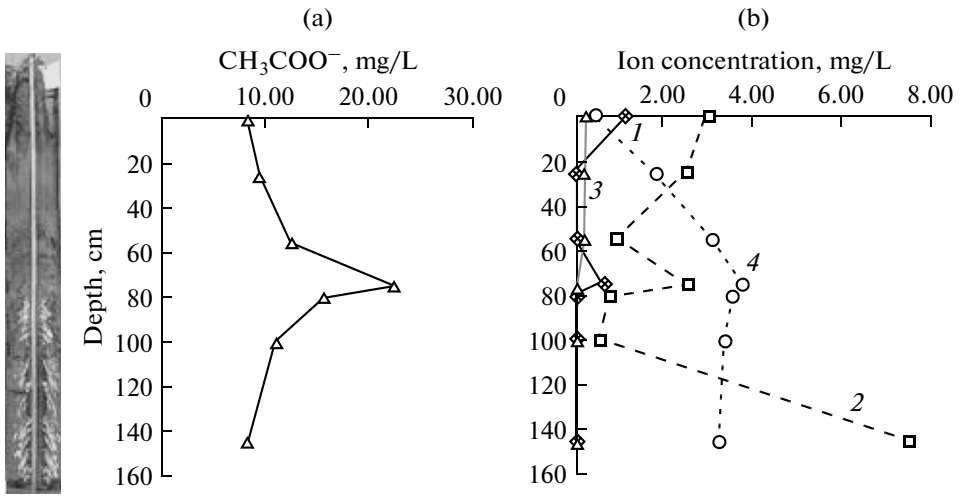

Fig. 1. Distribution of acetate (a) and sulfate (*1*), iron (*2*), manganese (*3*), and ammonium (*4*) ions (b) in pore water of St. 5, GC-4.

depth, while "clottedness" increased. In the 80–150 cm horizons, GH were present as separate layers or as masses occupying significant spaces almost devoid of rock (97% hydrate content by volume). The gas hydrate array was oblique (Fig. 1).

The sediments from the core of St. 5, GC-4 had high gas content, with up to 112 mL CH_4 per dm^3 at 50-cm depth. The main methane homologue in the gas was ethane. Thus, at 60 cm ethane concentration was 2.2% (vol/vol), while propane and butane were present in trace amounts. The $\delta^{13}C–C_1$ value was –44.0‰, while $\delta^{13}C–C_2$ was –27.4‰, indicating its thermogenic origin.

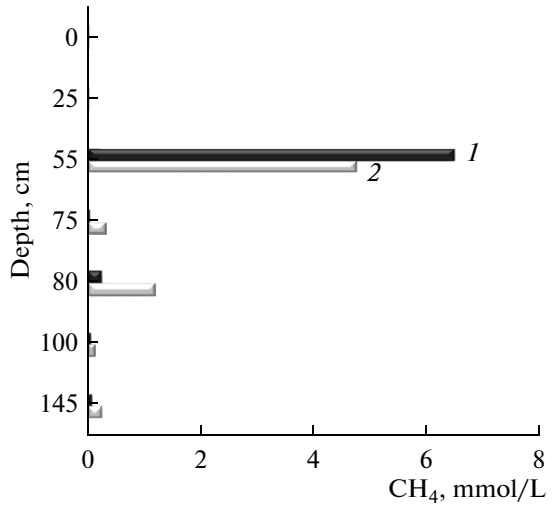

Fig. 2. Methane concentration in enrichment cultures inoculated with St. 5 GC-4 bottom sediments and incubated with acetate (*1*) and CO_2 (*2*).

Pore water in all sediment layers was of the hydrocarbonate–calcium–sodium type with salinity comparable to the values for pore water from background sites (100–120 mg/L). Acetate concentration varied from 8.27 mg/L in the uppermost sediment layers to 22.35 mg/L in the layer at the border with GH (75 cm). Very low concentrations of sulfate were found in these horizons (1.08 and 0.6 mg/L, respectively). Pore water of the GH-bearing sediments contained considerable amounts of acetate, chloride, and iron ions and less significant amounts of ammonium (Fig. 1).

Enrichment cultures. After three months of anaerobic incubation at 4°C, methane and ethane were detected in experimental vials.

Methane concentrations for enrichment cultures from the upper sediment horizons grown in media with H_2/CO_2 and with sodium acetate were 0.001 and 0.002 mmol/L, respectively. For the sediment samples from 25-cm depth, the values were ≤0.018 and ≤0.021 mmol/L, respectively (Fig. 2).

The highest methane concentrations were revealed in enrichments from microbial communities collected at 55-cm depth (6.15 and 4.51 mmol/L for cultivation with acetate and CO_2, respectively).

Initial concentrations of acetate in the sediments had no effect on the amount of methane produced. Thus, while pore water of the sediments adjacent to the GH layers (75 and 80 cm) were found to contain elevated acetate concentrations (Fig. 1), methane concentrations for enrichments with acetate and H_2/CO_2 were 0.03–0.23 and 0.3–1.13 mmol/L, respectively. This tendency did not change with depth: in the enrichments inoculated with communities from 100 and 145 cm, 0.06–0.07 and 0.133–0.24 mmol/L methane was produced via the aceticlastic and hydrogenotrophic pathway, respectively.

Apart from methane, all enrichments were found to produce ethane (Fig. 3). Enrichments inoculated with

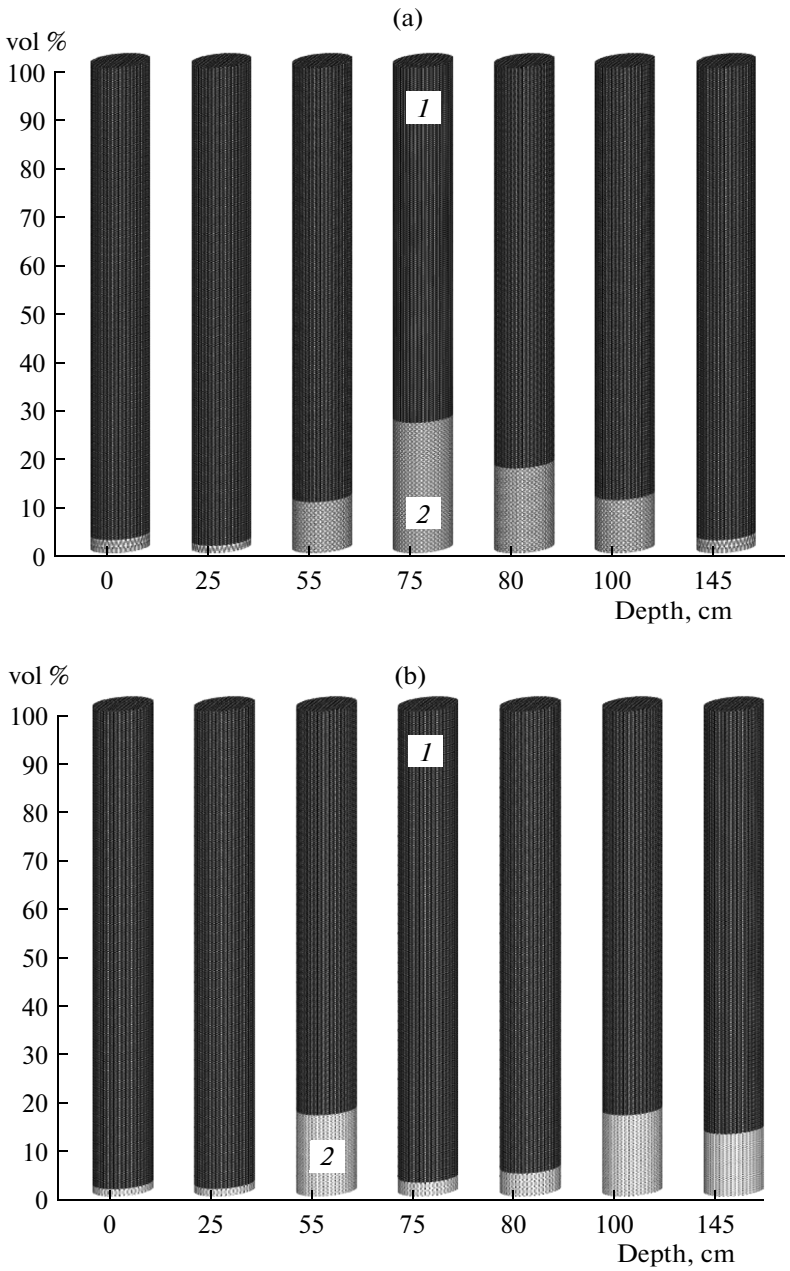

Fig. 3. Ratio of gaseous hydrocarbons in hydrogenotrophic (a) and aceticlastic (b) enrichment cultures inoculated with St. 5 GC-4 bottom sediments: methane (*1*) and ethane (*2*).

the samples of reduced sediments yielded considerably more methane than those with the surface sediments. The highest ethane concentration among gaseous hydrocarbons (26.8 vol %) was revealed in the samples with CO_2 as a carbon source and inoculated with the microbial community from the horizon adjacent to the GH layer (75 cm). On the medium with acetate, the highest C_2H_6 concentration (16.7 vol %) was found in the cultures inoculated with sediments from 55 and 100 cm.

Phylogenetic structure of a microbial community from an enrichment culture. An enrichment culture of anaerobic microorganisms obtained by serial dilutions was used for investigation of the composition of the microbial community in the deep sediment layer where ethane formation was detected. For analysis of the clonal library, 24 clones with the 16S rRNA gene fragments were selected. Phylogenetic analysis revealed archaea related to the phyla *Euryarchaeota* and *Crenarchaeota* (Fig. 4). Most nucleotide sequences (14) belonged to the phylum *Crenarchaeota* and formed a separate cluster together with an uncultured archaeon of the class *Thermoprotei*. This class comprises thermophilic and extremely thermophilic

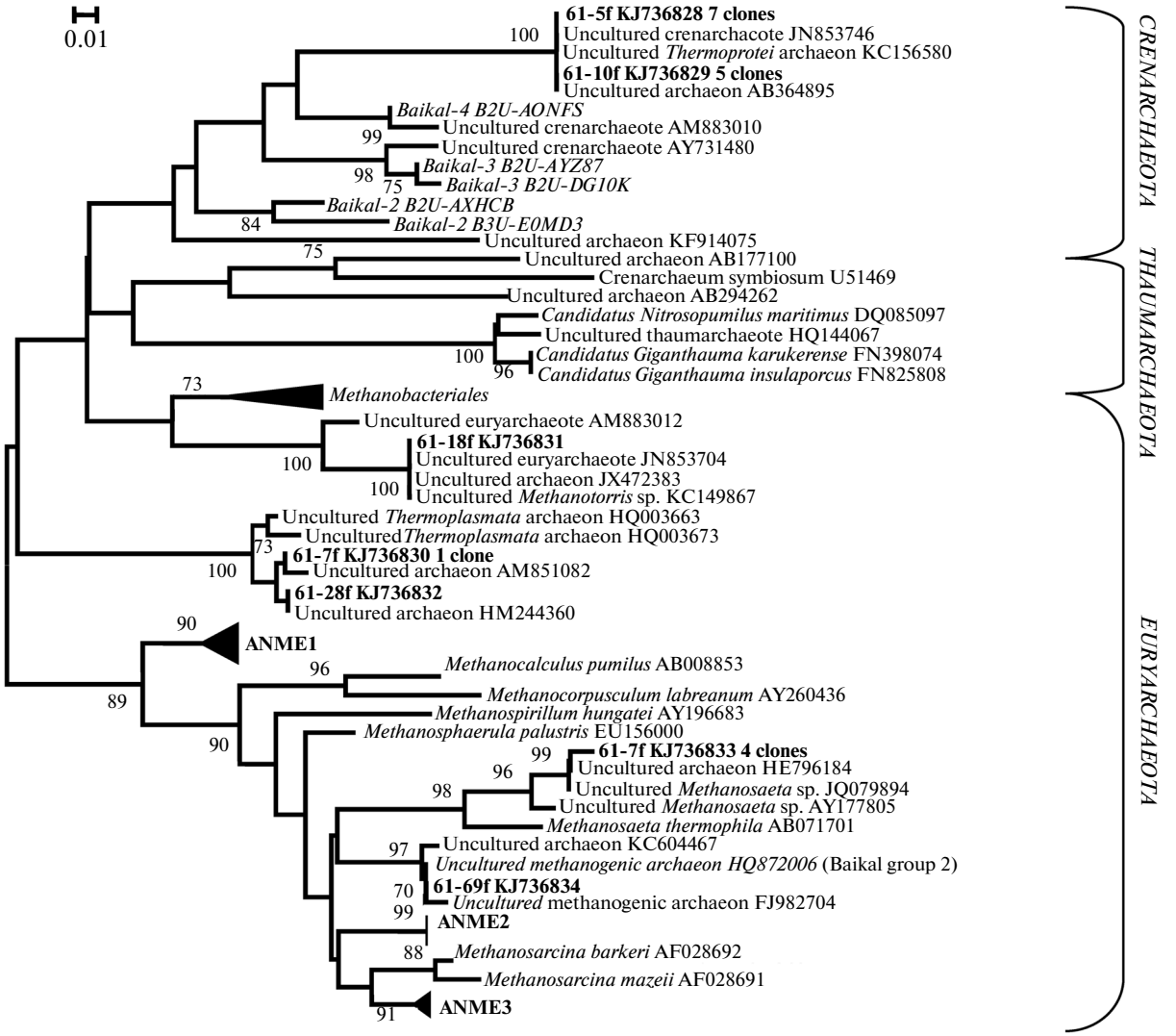

Fig. 4. Phylogenetic tree of the 16S rRNA genes from the archaeal enrichment from the bottom sediments (100 cm) of the Posolsky Bank methane seep, southern Baikal. The sequences obtained in the present work are in boldface. The sequences of the Baikal group's archaea from the Sankt Petersburg methane seep are in italics. Scale bar, 1 nucleotide replacement per 100 nucleotides. The numerals indicate the branching order determined by bootstrap analysis of 100 alternative trees (values exceeding 70 were considered significant).

organisms, which have been found in thermal springs, volcanic calderas and soils of volcanic fields, and black smokers. Some members of this class were found in activated sludge of anaerobic sewage treatment facilities [23]. Two phylotypes of this phylum (61-5f and 61-10f) exhibited 98–99% homology to the sequences of uncultured *Crenarchaeota* from the sediments of Central African Lake Kivu (Acc. no. JN853746) and from Arctic waterlogged areas (AB364895). None of the lineages of *Crenarchaeota* obtained by pyrosequencing of DNA from the Sankt Petersburg methane seep (central Baikal) were revealed in the enrichment culture (Fig. 4).

The phylum *Euryarchaeota* was represented by 10 clones related to methanogenic archaea of the orders *Methanococcales* and *Methanosarcinales*. Three clones (11f, 47f, 28f) were identified as members of the order *Thermoplasmatales*. This order comprises archaea carrying out oxidation of organic substrates coupled to sulfur reduction under oxic or anoxic conditions [24]. The closest relatives of the Baikal clones include the sequences from anaerobic sediments of a shallow lake in Spain (HQ003663, HQ003673), from the upper sediment horizons of Lake Honghu, China (HM244360), and from the sediments of an Alpine lake (AM851082). According to the results of phylogenetic analysis, one sequence (61-18f) was identified as belonging to the order *Methanococcales* (99% probability), while six sequences belonged to the order *Methanosarcinales* (98–99% homology). One sequence belonging to this order formed a cluster together with Baikal group 2 sequences from the

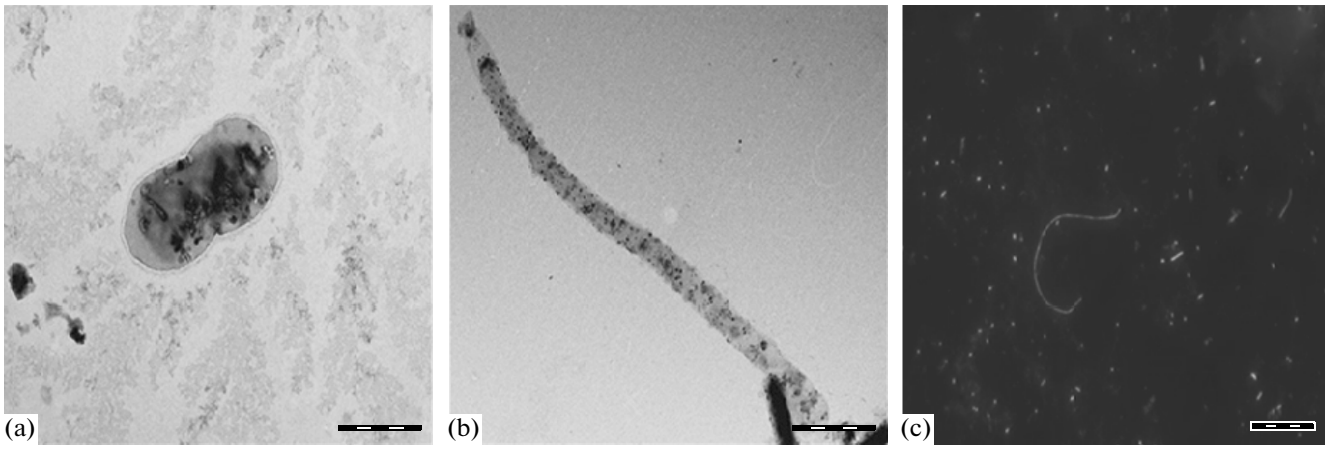

Fig. 5. Morphology of the cells from the enrichment culture inoculated with Posolsky Bank deep sediment (100 cm): transmission electron microscopy (a, b) and epifluorescence microscopy (c).

St. Petersburg methane seep obtained by pyrosequencing [25].

Investigation by epifluorescence microscopy and transmission electron microscopy revealed rods 4–8 μm long, single or in chains and surrounded by sheaths, as well as cocci 1 μm in diameter, single or in pairs, and short rods (0.5–0.7 × 2 μm (Fig. 5). Morphology of the cells in this enrichment resembled that of the known *Methanosaeta* species [26]. The work on isolation of pure archaeal cultures by serial dilutions is currently in progress.

DISCUSSION

Our research revealed that enrichment cultures inoculated with microbial communities from different horizons of the sediments of the Posolsky Bank methane seep and incubated at 4°C for prolonged periods produced both methane and ethane. Production of these gases was least intense in the case of communities from the surface and subsurface sediments. Potential activity of the methanogenic microbial community increased with depth, and a switch occurred from aceticlastic to hydrogenotrophic methanogenesis, which was in agreement with earlier data [27].

Low rates of methanogenesis in subsurface sediments of the studied area may result from activity of sulfate-reducing bacteria (SRB), which compete with methanogens for hydrogen produced at the first stage of OM diagenesis in upper sediments. This suggestion agrees with our previous findings [15], where high sulfate reduction rates were revealed in the same sediment layers as in the present work.

Ethane content among gaseous hydrocarbons released by the enrichments varied from 1.5 to 26.8%, with the highest values observed for the vials inoculated with sediment samples collected in the GH layer or immediately above it. In spite of significant acetate concentration in the pore water of the site, hydrogenotrophic methanogenesis prevailed in enrichment cultures. These results are in agreement with the hypothesis proposed by Xie et al. [28] concerning the possible ethane precursors. If acetate (CH_3COO^-) were such a precursor, higher ethane production would have occurred in acetate-containing media. However, cultivation of the enrichments with high concentrations of acetate resulted mostly in methane formation. According to [28], conversion of ethylene to ethane occurred in ethylene-enriched bottom sediment samples, with organisms related to methanogenic *Methanocalculus* spp. probably responsible for this process.

Accumulation of considerable amounts of ethane in methanogenic enrichment cultures indicates that the presence of methane homologues in natural habitats may be also a result of microbial processes. To obtain additional reliable information concerning the origin of methane, geochemical and isotopic research of methane and its homologues is required. Apart from investigation of the patterns of ethane formation by methanogenic cultures, we are planning to determine the changes in $\delta^{13}C$ values of methane and ethane in enrichment cultures grown on various substrates.

The presence of members of the phyla *Euryarchaeota* and *Crenarchaeota* (domain *Archaea*) in ethane-producing mixed cultures may indicate their possible involvement in ethylene reduction to ethane in Lake Baikal sediments.

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