Microbiology, Vol. 71, No. 2, 2002, pp. 196–201. Translated from Mikrobiologiya, Vol. 71, No. 2, 2002, pp. 230–236.

EXPERIMENTAL ARTICLES

Phylogenetic Diversity of the Archaeal Component in Microbial Mats on Coral-like Structures Associated with Methane Seeps in the Black Sea

T. P. Tourova*, T. V. Kolganova, B. B. Kuznetsov**, and N. V. Pimenov***

**Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
e-mail: ttour@biengi.ac.ru
***Bioengineering Center, Russian Academy of Sciences, Moscow*
Received July 24, 2001

Abstract—With the use of molecular ecology methods, the archaeal component of microbial mats on coral-like structures associated with methane seeps occurring at a depth of about 200 m in the Black Sea was investigated without the isolation of pure cultures. Using archaea-specific 16S rDNA–targeted oligonucleotide primes, long fragments of genes were amplified, cloned, and sequenced and their phylogenetic analysis was carried out. It was shown that archaea in microbial mats on coral-like structures are represented by two dominant phylotypes that belong to the kingdoms *Crenarchaeota* and *Euryarchaeota* and are not specifically related to any described archaeal species. The possible role of the revealed archaea in the process of anaerobic methane oxidation is discussed.

Key words: methane seeps, microbial mats, anaerobic methane oxidation, archaea, PCR, 16S rRNA, sequencing, phylogenetic analysis.

It was shown in papers devoted to the process of microbial transformation of organic matter in marine sediments that, together with aerobic methane oxidation due to methanotrophic bacteria, there is also a process of anaerobic methane oxidation [1]. It was proposed that anaerobic methane oxidation includes electron transfer from methane to sulfate and is possibly mediated by several microorganisms including methanogens with reversed metabolism and sulfate reducers [2]. Using biomarkers (specific lipids) and by means of phylogenetic analysis of 16S rRNA genes retrieved from samples taken in the zone of methane seeps in the Pacific Ocean near California coast it was shown that methane consumption may be driven by archaea belonging to the order *Methanosarcinales* [3]. The exact microbiological characteristics of these organisms remain unknown. However, with the use of fluorescent probes, it was definitely shown that these archaea, together with sulfate reducers, are involved in the microconsortia which perform anaerobic oxidation of methane in marine sediments [4]. The presence of such microconsotia consisting of methanotrophic archaea and bacterial sulfate reducers in methane-enriched anaerobic marine ecosystems [4–6] is currently the most reliable evidence in favor of the concept that methane oxidation under anaerobic conditions occurs as reversed methanogenesis coupled to sulfate reduction [1, 2]. At the same time, it cannot be ruled out that the above mechanism of anaerobic methane oxidation is the only one. In this connection, two recent findings deserve attention: occurrence of anaerobic methane oxidation in swampy soils, where sulfate reducers cannot develop due to the lack of sulfates [7], and presence in the zone of methane seeps of microaggregates consisting solely of archaea and involved, according to the data of radioisotopic studies, in anaerobic methane oxidation [6].

The investigations of these seeps were continued during an expedition on board the *Benthos-300* submersible. Numerous coral-like aragonite structures associated with methane seeps were found at a depth of about 200 m. They were covered with slimy microbial mats, whose organic carbon was characterized by an extremely light isotopic composition ($\delta^{13}C$ = –75.6…–83.3 ‰) [8] and thus evidently originated from isotopically light methane. Taking into account that these coral-like structures were situated in an anaerobic H_2S-containing zone, it was proposed that microbial mats on their surface develop at the expense of anaerobic methane oxidation [9].

Using radioisotopic analysis, the processes of anaerobic methane oxidation, sulfate reduction, and methane production from carbonate and acetate were demonstrated in the microbial mats [9]. Cytological studies of the mats revealed rod-shaped cells with squared ends. These cells varied in length and were able to form long multicellular filaments covered with sheaths [9]. Morphologically, these cells were similar to the sheath-cov-

0026-2617/02/7102-0196$27.00

ered anaerobic methanogenic archaea belonging to the genus *Methanosaeta* (formerly *Mehtanothrix*). However, significant differences in the fine structure allowed a suggestion to be made that the organism found might be a new representative of archaea.

The goal of the present work was further investigation of the phylogenetic diversity of archaea in the microbial community of the mats by molecular biological methods without isolation of pure cultures.

MATERIALS AND METHODS

Mat sampling. The samples of microbial mats were obtained during the 45th cruise of the research vessel *Professor Vodyanitskii* by trawling in the region of methane seeps (44°46'680 N; 31°58'970 E; depth, 180–190 m). In this zone, oxygen was chemically undetectable, and the hydrogen sulfide concentration was 0.1 to 0.3 ml/l. The water temperature was 4°C. The thickness of microbial mats was 2 to 3 cm.

Total DNA extraction and characterization. DNA preparations for PCR amplification were carried out according to Promega MiniPrep protocol with minor modifications. DNA concentration in the obtained preparations was 5–7 μ/ml. RNA was present in trace amounts (less then 1% according to electrophoretic data).

PCR. Fragments of 16S rDNA were amplified using archaeal forward primer A8F (5'-TCCGGTTGATCCTGCCGG-3') [10] and universal reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [11], corresponding to the 5' and 3' ends of the 16S rRNA gene. Fragments of the methane monooxygenase gene *pmoA* were amplified using primers PmoA189 (GGNGACTGGGACTTCTGG) and PmoA682 (GAASGCNGAGAAGAASGC) [12]. PCR was carried out on a Cetus 480 (Perkin Elmer, Sweden) using the thermostable BioTaq polymerase (Dialat LTD, Moscow) according to the recommendations of the polymerase manufacturer. Amplification was carried out according to the following protocol: first cycle: 94°C, 3 min; 50°C, 3 min; 72°C, 3min; next 30 cycles: 94°C, 30 s; 40°C, 30 s; 72°C, 30 s; and final polymerization, 7 min at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel. Purification of PCR fragments was carried out using a Wizard PCR Preps kit according to the manufacturer's recommendations.

Cloning and restriction analysis. Amplified 16S rDNA fragments were cloned into the Sma I site of pGEM-3Zf(+) polylinker. *E. coli* DH5α competent cells were used for transformation. Screening for recombinants was performed after the plasmids were extracted from single colonies and treated with *Pvu* II restriction endonuclease in the reaction mixture of the following composition: plasmid DNA, 0.3 μg; *Pvu*II, 3 units; Tris–HCl (pH 7.5), 50 mM; $MgCl_2$, 1mM; NaCl, 50 mM. The mixture was incubated at 37°C for 1 h. Electrophoretic separation of the restriction products was performed in 1% agarose gel at 6 V/cm.

Sequencing of cloned 16S rDNA fragments. Sequencing of the cloned 16S rDNA fragments was carried out according to Promega Silver Sequencing protocol with minor modifications. Electrophoresis was performed on Macrophore (Pharmacia, Sweden) and SQ3 Sequencer (Hoefer, United States) installations at a polyacryl amide gel thickness of 0.19 mm. The universal plasmid primers (T7 and Sp6) and terminal and inner 16S rDNA-targeted primers [10] were used for sequencing.

Phylogenetic analysis. The nucleotide sequences determined were checked for the presence of chimerical artifacts using the CHECK_CHIMERA software of the Ribosomal Database Project [http://rdp.cme.msu.edu]. Preliminary screening for similar sequences was carried out by using the GenBank BLASTA program [http://ncbi.nlm.nih.gov]. For the exact determination of the phylogenetic position of the cloned 16S rDNAs, their sequences were aligned using the CLUSTAL W software [http://www.genebee.msu.su/clusal] with the 16S rDNA sequences of various archaea, including known uncultivated phylotypes available from GenBank. A rooted phylogenetic tree was constructed by the neighbor-joining method realized in the TREECONW software package [13] and using bacterial consensus 16S rDNA sequence as an outgroup. Statistical significance of the branching order was estimated by bootstrap analysis of 100 alternative trees.

Sequence deposition. The sequences of cloned 16S rDNA fragments obtained in this work were deposited in GenBank under the accession numbers AF412942–AF412944.

RESULTS

Detection of functional methanotrophic genes. PCR with the primer pair PmoA189–PmoA682 resulted in the appearance of 490-bp bands, both when total DNA from the microbial mat and DNA from *Methylocystis trichosporium* (positive control) were used as templates (Fig. 1).

Analysis of the cloned 16S rDNA library. Amplified PCR fragments (about 1490-bp-long) of 16S rRNA genes were used to create a clone library. Among the analyzed clones, 30 were found to carry insertions about 1490 base pair long. These clones, according to the results of restriction analysis, were subdivided into 3 groups, which included 26, 3 and 1 clone. For more precise identification, these clones were partially sequenced using the T7 and Sp6 plasmid primers; this allowed us to determine sequences at the 5' end of 16S rDNA, where most variable regions of 16S rDNA are located. It was shown that clones belonging to groups 2 and 3 were chimerical artifacts, so they were not subjected to further analysis. Clones belonging to the largest group 1 were divided into two subgroups: 12 of them had an identical sequence type, and the other 14 clones had two close but distinct sequence types.

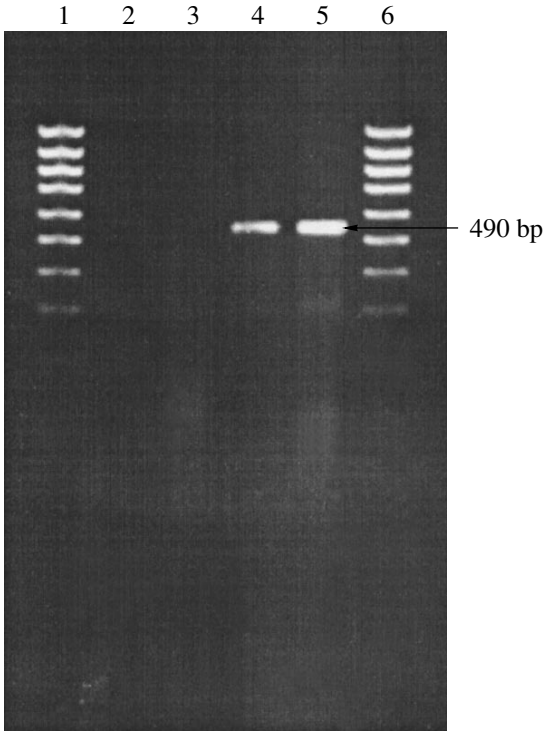

Fig. 1. Detection of methanotrophic functional genes. Lanes 1, 6, ladder GeneRuler 100 bp (Fermentas, Sm#0241); lane 2, control PCR without DNA template; lane 3, control PCR with *E. coli* DNA (50 ng/reaction); lane 4, control PCR with *Methylocystis trichosporium* DNA (50 ng/reaction); lane 5, PCR with DNA extracted from the microbial mat (50 ng/reaction). The arrow shows the band corresponding to the PCR fragment of the *pmoA* gene.

One representative of each sequence type were subjected to complete sequencing.

Phylogenetic analysis. Almost complete sequences were obtained for clones BScra3, BScra15, and BSeua2. These sequences approximately corresponded to positions 1–1500 in *E. coli* numbering. For phylogenetic tree construction, 1197 nucleotides were taken into account (the positions in which not all of the compared molecules were sequenced and the positions with alignment uncertainties were omitted). The phylogenetic tree constructed using the neighbor-joining method is shown in Fig. 2. When alternative algorithms were used, the topology of trees did not vary (data not shown).

The phylotype represented by clone BSeua2 belonged to the kingdom *Euryarchaeota* and showed a high level of 16S rDNA sequence similarity (94.0–98.5%) with members of the phylogenetic cluster called ANME-1 (*an*aerobic *me*thane oxidizers), which includes uncultivated archaea found in methane seep sediments near the California coast [3]. This cluster occupies in the phylogenetic tree position intermediate between *Methanomicrobiales* and *Methanosarcinales* and does not include any known cultivated species.

The phylotypes BScra3 and BScra15 were closely related but not identical (94.5.% of identities out of 1524 aligned nucleotides). Their position in the phylogenetic tree shows that they are members of the kingdom *Crenarchaeota* (Fig. 2). These phylotypes form a phylogenetic cluster (87.2–97.7% of similarity) with phylotypes of uncultivated archaea from the marine benthic group B, which were discovered in deep-sea sediments of the Pacific Ocean [14]. No close relatives of these phylotypes were found among known species of *Crenarchaeota* (71.3–78.3% of 16S rDNA similarity).

DISCUSSION

The results of our study show that the archaeal component of the microbial mats on the surface of coral-like structures associated with methane seeps in the Black Sea differs in its composition from the earlier studied prokaryotic communities of the bottom sediments at methane seeps near the coast of California. [3, 5]. In those communities, the archaeal component was represented by five main groups of phylotypes and included sequences close to cultivated members of the order *Methanosarcinales*, sequences of the ANME-2 group (related to *Methanosarcinales*), of the ANME-1 group, and sequences of low-temperature *Thermoplasmales* and *Crenarchaeota*. Representatives of the ANME-1 and ANME-2 groups were most abundant. In the community that we studied, the archaeal component was much less diverse: we revealed only one phylotype belonging to the *Euryarchaeota* (ANME-1) group and two phylotypes belonging to *Crenarchaeota*.

Phylogenetically, the ANME-1 group is remotely related to methanogens of the orders *Methanosarcinales* and *Methanomicrobiales* and represents a new order, according to the opinion of researchers who were the first to describe it [3]. Proceeding form their phylogenetic position, the archaea of the ANME-1 group are believed to be methanogens; however, this is just a tentative supposition, since this group is so far represented only by phylotypes, and no cultivated species have been isolated. Up to now, representatives of the ANME-1 group have been isolated only from anaerobic ecosystems enriched with methane [3, 5, 15, 16]. It may be assumed that these archaea are specific inhabitants of such ecosystems and play an important role in the processes occurring therein. It remains so far unclear whether the new uncultivated member of this group, phylotype BSeua2 discovered in the present work, is the only representatives of *Euryarchaeota* in the microbial community studied and whether we may associate it with the cell morphotype dominating the community according to our cytological studies [9].

Other representatives of archaea in the ecosystem studied are the two closely related (about 95% 16S rDNA homology) phylotypes BScra3 and BScra15, belonging to another archaeal kingdom, *Crenarchaeota*. The microheterogeneity of the 16S rDNA

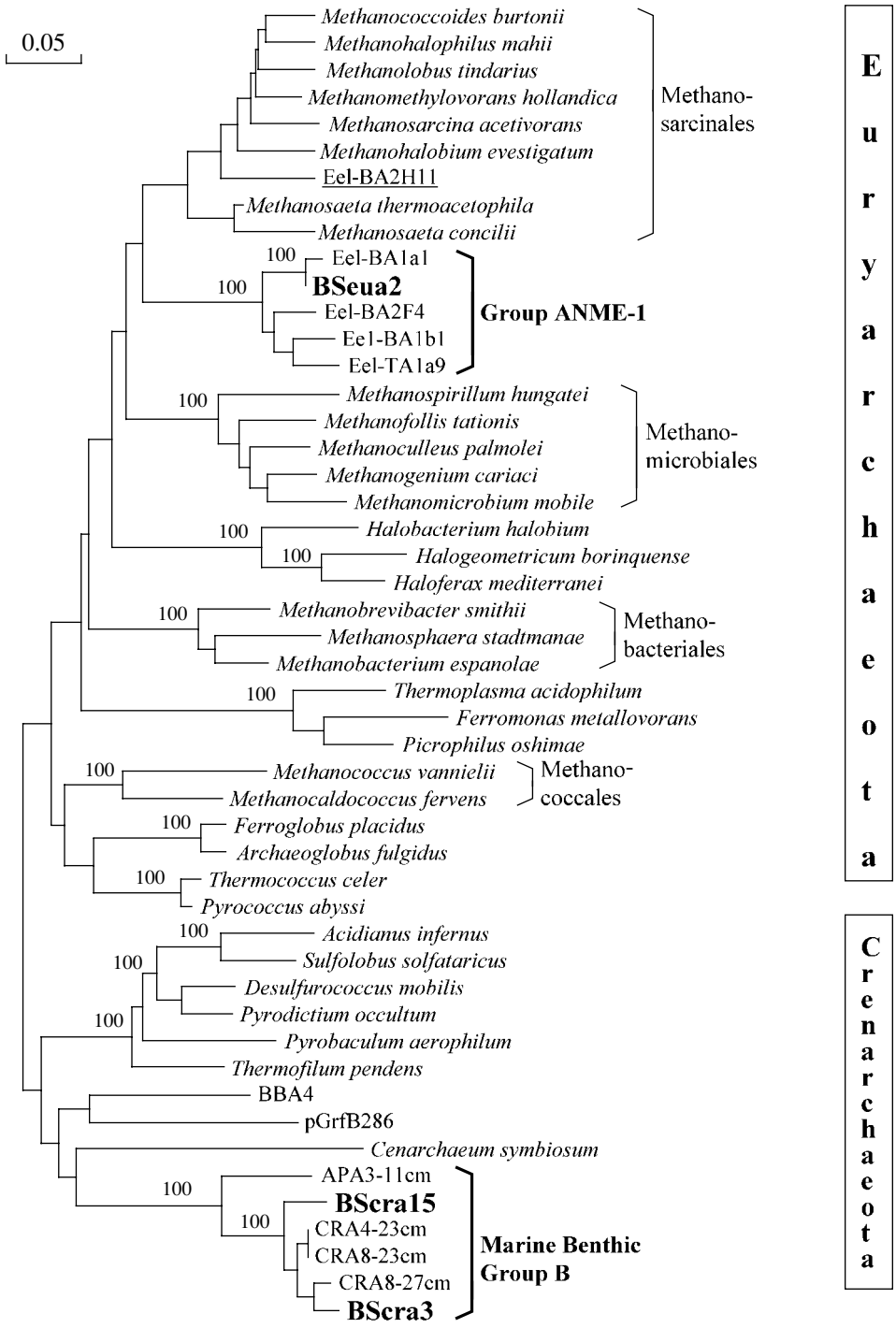

Fig. 2. Phylogenetic analysis of Archaea from the microbial mat on a coral-like structure at methane seeps in the Black Sea. Bootstrap values are shown at branching points; only maximal values (100) were considered significant. Validly described species are represented by sequences of the type strains. Abbreviations for the novel phylotypes of Archaea (given in boldface) were formed by combining for the region of isolation (Black Sea) with the abbreviations of the taxa—eua (*Euryarchaeota*) and cra (*Crenarchaeota*)—followed by the clone number. Uncultured phylotypes from various natural environments are represented by the following abbreviations: BBA (AF004344), a phylotype from continental shelf sediments; pGrfA4 (U59968), a phylotype from freshwater sediments; CRA and APA (AF119128, AF119132, AF119133, AF119137), phylotypes from deep-sea sediments [14]; Eel (AF134380, AF134392, AF134382, AF134387, AF134393), phylotypes from methane seep sediments [3]. The uncultured clone detected in a methane-consuming microbial consortium [4] is underlined.

sequences in the natural prokaryotic communities may be due both to the coexistence of closely related species and to the presence of diverged rRNA operons in the genome of the same organism [17]. However, in our case the degree of divergence is rather high; therefore, the phylotypes discovered are likely to represent two different organisms, whose morphology and physiology remain unknown. The close relatedness of the phylotypes BScra3 and BScra15 to phylotypes revealed in deep-sea sediments of the Pacific Ocean [14] and the presence of similar phylotypes as minor components of the microbial communities of the methane seeps near the coast of California [5] suggest that the organisms of this group may be widespread in various marine near-bottom ecosystems. This group of uncultivated organisms, along with numerous other phylogenetic groups revealed by molecular ecological methods, belongs to low-temperature *Crenarchaeota*, whose only described species is the symbiotic organism *Cenarchaeum symbiosum* [18]. In spite of the broad distribution of free-living low-temperature *Crenarchaeota* in various ecosystems, no pure cultures have been isolated so far, and their morphological and physiological properties remain unknown.

In the ecosystem studied in this work, several anaerobic processes (methanogenesis, methane oxidation, sulfate reduction) have been recorded [9]. The question as to the involvement in these processes of the organisms represented by the phylotype discovered remains debatable. In the present study, we also revealed in the total DNA of the community genes coding for enzymes specific to aerobic methanotrophs. The study of the phylogenetic diversity of microorganisms in the communities associated with methane seeps near the coast of California also revealed a phylotype close to the aerobic methanotroph *Methylobacter luteus* [5]. The presence of aerobic methanotrophs in anaerobic marine near-bottom zones was earlier shown by microbiological and serological methods [8]; probably, this is the result of their sedimentation from the aerobic zone. However, it does not seem possible that these organisms can be active in the zone of coral-like constructions at the methane seeps in the Black Sea: the anaerobic water column in this zone contains free hydrogen sulfide, which rules out the occurrence of aerobic processes [9]. The activity of the corresponding enzymes was not revealed [9]. It should be, however, mentioned that data exist on the exchange of genes involved in anaerobic methanogenesis and aerobic methylotrophy between methanogenic archaea and aerobic methylotrophic bacteria [19]. At the same time, the results of our phylogenetic analysis suggest that in the ecosystem studied the only organism that may be involved in the processes of methanogenesis and anaerobic methane oxidation is represented by the phylotype BSeua2. If it is indeed so, then the mechanism of anaerobic methane oxidation in this ecosystem is dissimilar from that suggested for prokaryotic bottom-sediment microconsortia, where representatives of the ANME-1 group were not revealed [4–6]. Still more hypothetical is the role of the *Crenarchaeota* represented by pylotypes BScra3 and BScra15. It may be supposed that these organisms might be new forms of sulfate reducers.

The answers to the questions posed can be found via further molecular-biological studies of the diversity of the microflora of the Black Sea methane seeps and via the isolation of pure cultures of microorganisms making up the community and via their detailed microbiological characterization.

ACKNOWLEDGMENT

This study was supported by the Russian Foundation for Basic Research (project nos. 99-04-48360 and 00-15-97897) and grant IC15CT96 0107.

REFERENCES

1. Zehnder, A.J. and Brock, T.D., Anaerobic Methane Oxidation: Occurrence and Ecology, *Appl. Environ. Microbiol.*, 1980, vol. 39, pp. 194–204.
2. Hoehler, T.M., Alperin, M.J., Albert, D.B., and Martens, C.S., Field and Laboratory Studies of Methane Oxidation in Anoxic Marine Sediments: Evidence for a Methanogen–Sulfate Reducer Consortium, *Global Biogeochem. Cycles*, 1994, vol. 8, pp. 451–463.
3. Hinrichs, K.-U., Hayes, J.M., Sylva, S.P., Brewer, P.G., and DeLong, E.F., Methane-Consuming Archaebacteria in Marine Sediments, *Nature* (London), 1999, vol. 398, pp. 802–805.
4. Boetius, A., Ravenschlag, K., Schubert, G.J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jorgensen, B.B., Witte, U., and Pfannkuche, O., A Marine Microbial Consortium Apparently Mediating Anaerobic Oxidation of Methane, *Nature* (London), 2000, vol. 407, pp. 623–626.
5. Orphan, V.J. and Hinrichs, K.-U., Ussler III, W., Paull, C.K., Taylor, L.T., Sylva, S.P., Hayes, J.M., and DeLong, E.F., Comparative Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 1922–1934.
6. Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., and DeLong, E.F., Methane-Consuming Archaea Revealed by Directly Coupled Isotopic and Phylogenetic Analyses, *Science*, 2001, vol. 293, pp. 484–487.
7. Salentine, D.L. and Reeburgh, W.S., New Perspectives on Anaerobic Methane Oxidation, *Environ. Microbiol.*, 2000, vol. 2, pp. 477–484.
8. Ivanov, M.V., Polikarpov, G.G., Lein, A.Yu., *et al.*, Biogeochemistry of the Carbon Cycle in the Region of Methane Seeps in the Black Sea, *Dokl. Akad. Nauk SSSR*, 1991, vol. 320, no. 5, pp. 1235–1240.
9. Pimenov, N.V., Rusanov, I.I., Poglazova, M.N., *et al.*, Bacterial Mats on Coral-like Structures at Methane Seeps in the Black Sea, *Mikrobiologiya*, 1997, vol. 66, no. 3, pp. 412–428.
10. Kolganova, T.V., Kuznetsov, B.B., and Tourova, T.P., Designing and Testing Oligonucleotide Primers for Amplification and Sequencing of Archaeal 16S rRNA Genes, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 283–286.

11. Edwards, U., Rogall, T., Bloeker, H., Ende, M.D., and Boeettge, E.C., Isolation and Direct Complete Nucleotide Determination of Entire Genes, Characterization of Gene Coding for 16S Ribosomal RNA, *Nucleic Acids Res.*, 1989, vol. 17, pp. 7843–7853.

12. Holmes, A.J., Costello, A., Lidstrom, M.E., and Murrel, J.C., Evidence That Particulate Methane Monooxygenase and Ammonia Monooxygenase May Be Evolutionary Related, *FEMS Microbiol. Lett.*, 1995, vol. 132, pp. 203–208.

13. Felsenstein, J., PHYLIP, Phylogenetic Inference Package (Version 3.2), *Cladistics*, 1989, vol. 5, pp. 164–166.

14. Vetriani, C., Jannasch, H.W., MacGregor, B., Stahl, D.A., and Reysenbach, A.-L., Population Structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments, *Appl. Environ. Microbiol.*, 1999, vol. 65, no. 10, pp. 4375–4384.

15. Thomsen, T.R., Finster, K., and Ramsing, N.B., Biogeochemical and Molecular Signatures of Anaerobic Methane Oxidation in a Marine Sediment, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 1646–1656.

16. Takai, K. and Horikoshi, K., Genetic Diversity of Archaea in Deep-Sea Hydrothermal Vent Environments, *Genetics*, 1999, vol. 152, pp. 1285–1297.

17. Schleper, C., DeLong, E.F., Preston, C.M., Feldman, F.A., Wu, K.-Y., and Swanson, R.V., Genomic Analysis Reveals Chromosomal Variation in Natural Populations of the Uncultured Psychrophilic Archaeon *Cenarchaeum symbiosum, J. Bacteriol.*, 1998, vol. 180, pp. 5003–5009.

18. Preston, C.M., Wu, K.Y., Molinski, T.F., and DeLong, E.F., A Psychrophilic Crenarchaeon Inhabits a Marine Sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov, *Proc. Natl. Acad. Sci. USA*, 1996, vol. 93, pp. 6241–6246.

19. Christoserdova, L., Vorholt, J.A., Thauer, R.K., and Lidstrom, M.E., C_1 Transfer Enzymes and Coenzymes Linking Methylotrophic Bacteria and Methanotrophic Archaea, *Science*, 1998, vol. 281, pp. 99–102.