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SHORT COMMUNICATIONS

Molecular Detection of Methanogenic Archaea in the Black Sea Oxidized Waters

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Methanogenic archaea are traditionally considered strictly anaerobic microorganisms. However, some methanogens, which possess the enzymatic mechanisms of antioxidative defense, may survive at low oxygen concentrations [1] and retain their activity in the anoxic microniches formed within oxic habitats (e.g., diatom shells, copepod fecal pellets, and other detrital microparticles) [2, 3]. Earlier, radioisotope analysis revealed methanogenesis not only in anoxic waters, but also in the oxidized surface waters of the Black Sea [4]. The presence of viable archaea in the Black Sea surface waters and in the chemocline zone was originally demonstrated using fluorescence in situ hybridization (FISH) [5]. Subsequent FISH investigation of the Black Sea oxic waters revealed the presence of physiologically active methanogenic archaea of subgroups I (genera *Methanobacterium, Methanobrevibacter*, and *Methanosphaera*) and II (order *Methanomicrobiales*) [6].

The goal of the present work was to detect methanogenic archaea in the oxic water column and in the chemocline zone of the Black Sea using PCR analysis.

Water samples from the depths from 30 to 200 m were collected with a submerged pump in July, 2010, on board the *Ashamba* research vessel (Southern Branch of Shirshov Institute of Oceanology, Russian Academy of Sciences) at a 1000-m deep station (44°29.85′ N, 37°55.24′ E, 9 miles from the Blue Bay town of the Gelendzhik region).

Since methanogenic archaea are known to exist in the water column not only as free-living cells, but may also be associated with microparticles [2, 3], the water samples for PCR detection (5 L each) were filtered sequentially through GF/C coarse-pore glass fiber filters and through 0.22-μm bacterial filters (Millipore, United States). The filters were stored in the TE buffer (pH 8.0) : ethanol mixture (1 : 1) at 4°C. After the filters were ground in liquid nitrogen, DNA was extracted using the Genomic DNA Purification Kit (Fermentas, Lithuania). The oligonucleotide primers [7, 8] (Syntol, Russia) used for PCR are listed in the table. PCR amplification was performed in the final reaction volume of 25 μL, which contained ~25 ng DNA template, 400 μM dNTP (Fermentas), 500 nM each primer (Syntol), and 2.5 U *Taq* DNA polymerase, in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, United States) under the recommended cycling parameters [7, 8].

The 16S rRNA gene fragments were separated by denaturing gradient gel electrophoresis (DGGE) in 6.5% polyacrylamide gel with a 40 to 60% denaturing gradient of urea and formamide on a DCode Universal Mutation Detection System (Bio-Rad, United States) in 0.5× TAE buffer at 60°C and constant voltage of 200 V during 6 h. The bands were excised from the gel, and DNA was eluted with sterile water at 4°C and reamplified. The DNA was then purified in a 1% agarose gel using the DNA Gel Extraction Kit (Fermentas). Purified DNA (5–20 ng) was used as a template for sequencing with the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) in a 3130 Genetic Analyzer (Applied Biosystems) with 36-cm capillaries and POP6 polymer. The resulting nucleotide sequences of the 16S rRNA gene fragments were compared with the known sequences using the BLAST software package.

PCR analysis revealed the presence of both associated and free-living archaea (ARCH344 and ARCH915 primers) in the samples collected at all studied depths of the Black Sea water column. With the primers to the 16S rRNA gene of methanogenic archaea (Met146F and Met1324R), free-living methanogens were detected at all depths, with the exception of 200 m, while the particle-associated methanogens were detected at all depths, with the exception of 100 and 200 m. However, upon PCR with more specific primers to the *mcrA* gene (mcrA-F and mcrA-R), the positive signal was revealed only for the free-living methanogens at three depths (100, 165, and 180 m).

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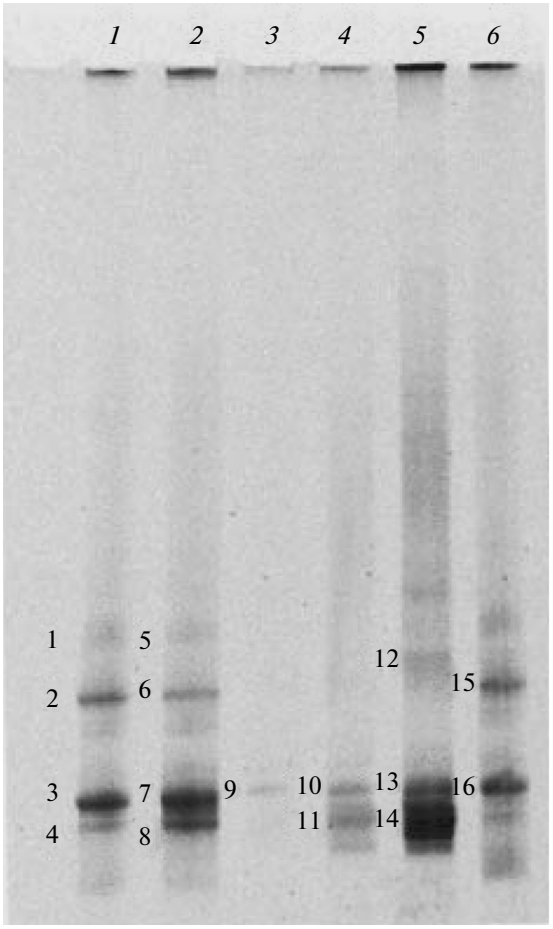
Oligonucleotide PCR primers used in the study

PCR primers	Phylogenetic specificity	Nucleotide sequence (5' ⟶ 3')	Target 16S rDNA fragment	T_a, °C
ARCH344F ARCH915R	*Archaea*	ACGGGGTGCAGGCGCGA GTGCTCCCCCGCCAATTCCT	344–360 934–915	57
McrA-F McrA-R	Methanogenic archaea (the *mcrA* gene encoding the α subunit of methyl-coenzyme M reductase)	GGTGGTGTAGGATTCACACAAT ACGCAACAGC TTCATTGCGTAGTTAGGGTAGTT	–	52
Met146F Met1324R	Methanogenic archaea	GGCATAACCTCGGGAAAC GCGAGTTACAGCCCACAA	146–163 1341–1324	40

The obtained DGGE profiles of the archaeal communities for different hydrological zones were noticeably different in the quantity and distribution patterns of archaeal 16S rRNA gene fragment bands, as well as in the intensities of the bands (figure). Sequence analysis of the 16S rRNA gene fragments isolated and reamplified from the individual DGGE bands demonstrated the following maximum homology of the nucleotide sequences: for band 1 (30 m, GenBank accession no. JQ732776), 99% similarity with uncultured representatives of marine *Thaumarchaeota* (family *Nitrosopumilaceae*) and *Crenarchaeota*; for bands 2 (30 m, JQ732775), 4 (30 m, JQ732773), 7 (100 m, JQ732772), 10 (145 m, JQ732770), and 11 (165 m, JQ732769), 98–99% similarity to uncultured marine *Crenarchaeota*; for band 3 (30 m, JQ732774), 99% similarity to uncultured marine archaea; and for band 8 (100 m, JQ732771), 98% similarity to uncultured *Thaumarchaeota*. The results of DGGE/sequence analysis indicate that the oxygen-containing water column and the chemocline zone of the Black Sea were dominated by uncultured *Crenarchaeota* and *Thaumarchaeota*, while some apparently minor methanogens could not be detected by DGGE with the primers specific to the 16S rRNA gene fragment of the *Archaea* domain.



DGGE profile of archaeal communities from the upper part of the Black Sea water column (*1–6*, DNA from the samples obtained at 30, 100, 145, 165, 180, and 200 m, respectively).

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