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SHORT COMMUNICATIONS

Detection of Methanotrophic Archaea in Pockmark Sediments (Gdansk Deep, Baltic Sea) by Sequence Analysis of the Gene Encoding the α Subunit of Methyl-Coenzyme M reductase

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Methyl-coenzyme M reductase (MCR) is the key enzyme for methanogenesis and anaerobic methanotrophy [1, 2]. In the course of methanogenesis, it catalyzes, together with coenzyme B (HS–CoB), decomposition of methyl-coenzyme M (CH_3–S–CoM) to methane and heterodisulfide (CoM–S–S–CoB) [2]. Homologues of the gene encoding this enzyme, as well as most of the other enzymes involved in methane formation from CO_2, were found in methanotrophic archaea [3–5]. Moreover, participation of MCR in anaerobic methane oxidation was confirmed by biogeochemical analysis of the Black Sea microbial mats where active methanotrophy occurs [6, 7].

MCR was found only in methanogenic and methanotrophic archaea, while other enzymes of methanogenesis may occur also in other microorganisms utilizing C1 compounds [8]. The genes encoding MCR can be used as molecular markers for efficient determination of specific groups of methanogens and anaerobic methane oxidizers [9]. The highly evolutionarily conservative gene encoding the MCR α subunit (*mcrA*) is often used for this purpose [10]. Comparative phylogenetic analysis revealed significant similarity between topologies of the trees constructed based on 16S rRNA gene sequences and on those of the gene encoding the MCR α subunit [11, 12].

Anaerobic methane oxidation (AMO) is the main process of methane consumption in the ocean and, therefore, an important stage in the global carbon cycle [13]. In spite of significant progress in investigation of activity and distribution of anaerobic methanotrophs (ANMEs), no member of this group has been obtained in pure culture [14]. The presently available data indicate significant evolutionary diversity of methanotrophic archaea. According to the results of investigation of *mcrA* phylogeny, they belong to four clusters: groups a-b (ANME-1), c-d (ANME-2c), e (ANME-2a), and f (ANME-3) [3, 15].

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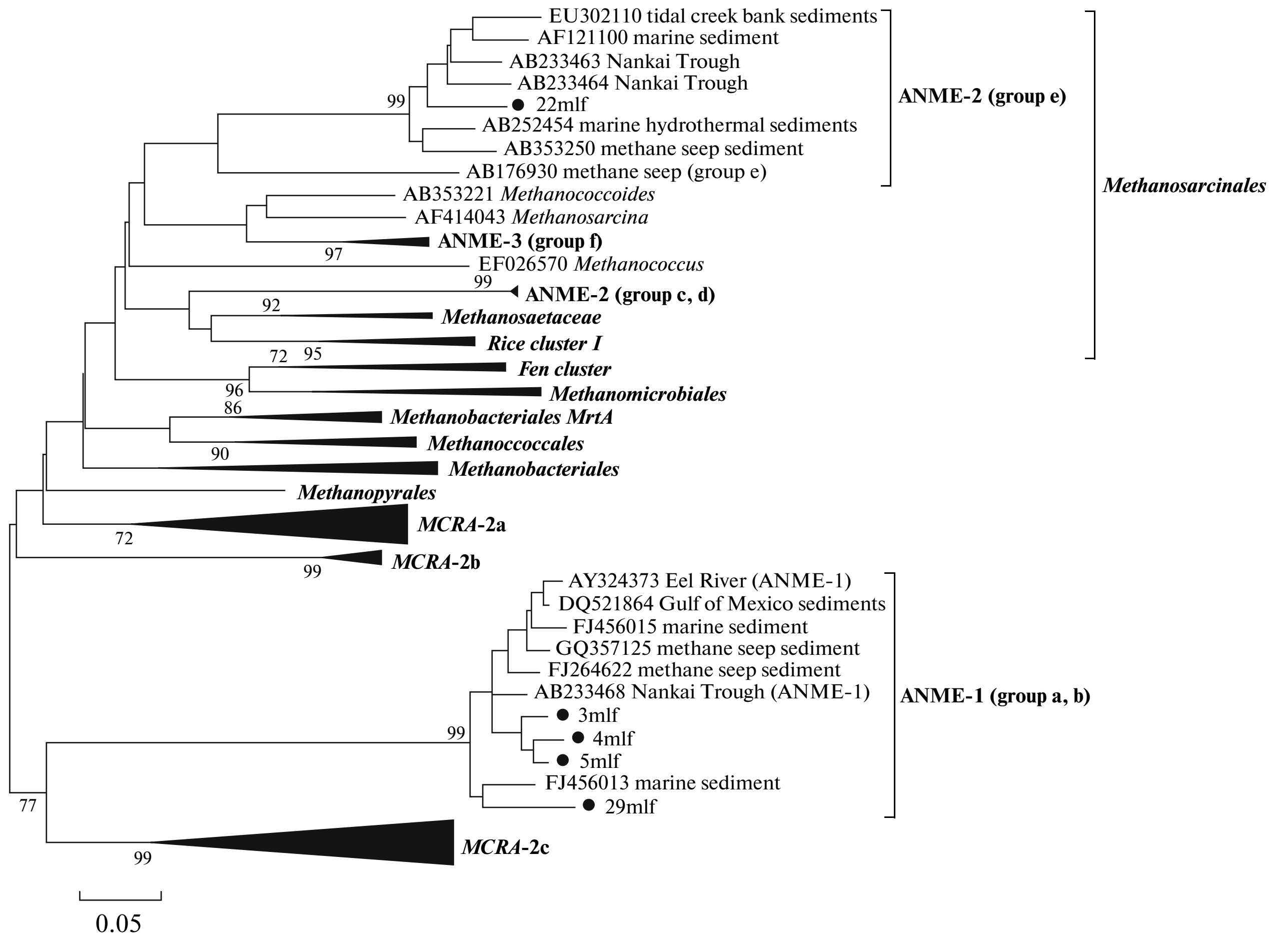
In the Baltic Sea, methane craters (pockmarks) are widespread, with characteristic gas-saturated sediments and elevated methane concentrations in near-bottom water [16]. Using radioisotope techniques, we have previously revealed high rates of AMO coupled to sulfate reduction in the sediments of a Gdansk Deep pockmark [17]. The present investigation of the pockmark sediments concentrated, therefore, on detection of methanotrophic archaea by analysis of the *mcrA* gene sequences.

The bottom sediments were sampled during the 74th expedition of the *Shelf* r/v in September 2009. The station in the vicinity of a pockmark was selected based on the result of echosounding and the data of previous research [17]. Sediment samples were collected with a Niemistö-type hermetic corer. The samples were placed in sterile plastic vials immediately after hauling on board, frozen, and transported to the laboratory (Institute of Microbiology, Russian Academy of Sciences). The sediments of the 25–40 cm horizon were used in the present work. This horizon was previously demonstrated to exhibit the highest AMO rates [17].

The modified phenol–chloroform extraction method [18] was used for DNA isolation. For amplification of the *mcrA* gene fragments, a Terzyc multichannel DNA amplifier (DNA Technology, Russia) and two primer systems (Luton-*mcrA* [19] and ME3MF-ME2r [20]) were used. Amplification was carried out in 20 μl of the mixture containing the following (final concentrations): 1× reaction buffer (Evrogen, Moscow), 200 μM of each deoxyribonucleotide triphosphate, 0.05 μM of each primer, 1 U *Taq* DNA polymerase (Evrogen, Moscow), and 1 μl (1–10 ng) template DNA. Denaturing, annealing, and elongation were carried out at 95, 55, and 72°C, respectively. The duration of these phases was determined experimentally. The PCR products were then separated by denaturing gradient gel electrophoresis as described in [21] with modifications. For the separation of PCR



Phylogenetic tree constructed based on comparative analysis of the fragments (140 residues) of McrA amino acid sequences from the Gdansk Deep pockmark sediments. The sequences obtained in the present work belong to five phylotypes, designated with •. The neighbor-joining algorithm and bootstrap analysis for 100 alternative trees were used. The statistical relevance of the branching order is designated by numerals next to the branching points (only values exceeding 70% are marked). The designations for the phylogenetic lines without cultured representatives were taken from the following publications: (*MCRA*-2a and *MCRA*-2b) [26], (ANME 2 group c, d, e; ANME 1 group a, b) [3]; (ANME 3 group f) [15], (Rice cluster 1 and Fen cluster) [27], and (*MCRA*-2c), Merkel, personal data.

products on a SCIE-PLAS (Yorkshire, United Kingdom), a 30–70% gradient of denaturing agents was used at 70 V and 60°C for 18 h. After electrophoresis, the gel was stained with *SYBR*® Gold (Molecular probes, Leiden, Netherlands) for 40 min in the dark and visualized on a transilluminator. The bands were excised and eluted overnight in a refrigerator with 20 μl of distilled water. PCR with the relevant primer system was then carried out, and the products were purified with the *Wizard* SV Gel and PCR Clean-Up System kit (Promega, United States). The PCR products were sequenced using the Big Dye Terminator v.3.1 kit on an ABI 3730 automatic sequencer (Applied Biosystems Inc., United States) according to the manufacturer's recommendations. The sequencing was carried out in the Bioengineering Center, Russian Academy of Sciences. The sequences were aligned using the MAFFT version 6 online server (http://align.bmr.kyushu-u.ac.jp/mafft/ online/server). The phylogenetic tree was constructed using the MEGA 4 software package. The *mcrA* gene sequences determined in the present work were deposited to GenBank under accession nos. HM013958–HM013962.

For the investigated sample, the PCR products were obtained with both primer systems. After separation by denaturing gradient gel electrophoresis, ten bands were revealed, reamplified, and sequenced. Phylogenetic analysis revealed that all the sequences obtained could be grouped into five phylotypes, according to the similarity of their nucleotide sequences (over 95%). Four phylotypes (Figure) belonged to the ANME 1 group (3mlf, 4mlf, 5mlf, and 29mlf), while one (22 mlf) belonged to the ANME 2 group. The sequences 3mlf, 4mlf, and 5mlf were obtained with the Luton-*mcrA* primer system, while 22mlf and 29mlf were obtained with the ME3MF-ME2r system.

The phylotypes 3mlf, 4 mlf, and 5mlf form a relatively compact cluster inside ANME 1. The clones most similar to the sequences of this cluster have been detected in the sediments of Pacific cold methane seeps near California (United States) [22] and in the Nankai trench (Japan) [23]. The phylotype 29mlf exhibited relatively low similarity (90% in amino acid sequences) with other known ANME 1 clones. The only phylotype of the ANME 2 group was represented by one sequence and exhibited low similarity to the known *mcrA* sequences of the ANME 2 group (79% in amino acid sequences). The clones exhibiting most sequence similarity to the revealed ANME 2 phylotype were detected in the sediments of the Sea of Okhotsk methane seep [24].

Our results demonstrate that, unlike the previously studied sediments of Eckernforde Bay (Germany), where only ANME 2 phylotype was revealed by FISH [25], two groups of methanotrophic archaea, ANME 1 and ANME 2, were present in the pockmark sediments in the Gdansk Deep. Moreover, higher diversity of ANME 1 phylotypes may be an indirect indication of predominance of this microbial group in gas-saturated sediments of the Gdansk Deep pockmarks.

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