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EXPERIMENTAL ARTICLES

Microbial Community of Reduced Pockmark Sediments (Gdansk Deep, Baltic Sea)

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Abstract—The microbial community of reduced pockmark sediments in the Russian sector of the Gdansk Deep, Baltic Sea, was investigated by molecular biological techniques. Fluorescent in situ hybridization was used to determine the numbers of eubacteria, archaea, and sulfate-reducing bacteria. Eubacteria were found to predominate in the upper 10 cm of the sediment (up to 5.3×10^9 cells/g wet sediment), while the number of archaea increased in the 10- to 30-cm layers (up to 2.8×10^9 cells/g wet sediment, which is higher than the number of eubacteria in the same horizons). Analysis of 16S rRNA gene fragments revealed members of the following phyla: *Proteobacteria, Chloroflexi, Firmicutes, Planctomycetales*, and high-G + C gram-positive bacteria. Sulfate-reducing bacteria (SRBs) of the families *Syntrophaceae, Desulfuromonadaceae*, and *Actinobacteria* of the genera *Kocuria* and *Rothia* were the predominant groups. Molecular probes were used to determine predominance of *Desulfovibrionales* in the SRB enrichment cultures obtained from different horizons of pockmark sediments. Three archaeal phylotypes were revealed, belonging to *Euryarchaeota*. One of these fell into the group of uncultured methanotrophic archaea (ANME-1a), while the other two were most closely related to uncultured methanogens.

Keywords: pockmarks, eubacteria, archaea, sulfate-reducing bacteria, anaerobic methanotrophs, methanogens, Baltic Sea.

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Due to its geographic location and specific hydrological regime, the Russian sector of the Gdansk Basin of the Baltic Sea is a zone with increased influx of both natural allochthonous organic matter (OM) and OM and biogenic elements of anthropogenic origin. Since the depths are low, complete decomposition of OM does not occur in the water column, so that the suspension arriving at the bottom contains labile OM, which is actively used by various microbial physiological groups. Intense microbial processes result in deep exhaustion of oxygen and activation of sulfate-reducing and methanogenic microorganisms in the upper sediments and near-bottom water. These organisms are responsible for the terminal phase of OM decomposition with production of biogases, methane and hydrogen sulfide. High methane content promotes formation of ascending flows of hydrocarbon gases from deep, gas-saturated sediment layers. Geoacoustic anomalies are thus formed in the surface silts; they are often associated with pockmarks, characteristic craterlike depressions in the bottom surface. Pockmarks were revealed in different regions of the Baltic Sea within the Gdansk, Arkona, and Gotland depressions [1].

We have previously reported elevated methane content, as well as anomalously high rates of sulfate reduction (SR) and anaerobic methane oxidation (AMO) in the gas-saturated sediments of a pockmark in the Russian sector of the Gdansk Deep [2, 3]. Comparison of integral rates of methanogenesis (MG) and AMO suggested that the high rates of AMO in pockmark sediments resulted from inflow of methane from the deep layers of the sedimentary cover.

High SR and AMO rates were also revealed in gas-saturated sediments of the Eckernförde Bay [4]. Fluorescent in situ hybridization revealed participation of ANME 2 methanotrophic archaea in anaerobic methane oxidation in the Eckernfö]rde Bay. Such organisms were revealed in the sediments of cold methane seeps in different oceanic sites [5].

The goal of the present work was to apply molecular biological techniques to investigation of microbial biodiversity in reduced pockmark sediments.

MATERIALS AND METHODS

Site characterization. Microbial biodiversity in pockmark sediments was investigated during the 95th cruise of the *Professor Shtokman* r/v in July 2008 in the Russian sector of the Gdansk Basin, where physico-

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Table 1. Oligonucleotide probes used for CARD-FISH detection of eubacteria, archaea, and sulfate-reducing bacteria in the sediments

Probe name	Probe structure, 5'–3	Detected group	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Eubacteria*	[9]
ARCH915	GTGCTCCCCCGCCAATTCCT	*Archaea*	[10]
NON338	ACTCCTACGGGAGGCAGC	Negative control	[11]
DSR651	CCCCCTCCAGTACTCAAG	*Desulfobulbaceae*	[12]
DSS658	TCCACTTCCCTCTCCCAT	*Desulfosarcinales*	[12]

Table 2. Oligonucleotide probes used for FISH detection of sulfate-reducing bacteria in enrichment cultures

Probe	Sequence, 5'–3'	Specificity	Reference
Dsv-698	GTTCCTCCAGATATCTACGG	Some *Desulfovibrio*, *Bilophila wadsworthia*, *Lawsonia intracellularis*	[9]
Dsv-1292	CAATCC GGACTGGGACGC	Some *Desulfovibrio* and *Bilophila wadsworthia*	[9]
Dtm-229	AATGGGACGCGGAXCCAT	*Desulfotomaculum* claster 1 and other *Firmicutes*	[15]
Dsv-214	CATCCTCGGACGAATGC	Most *Desulfomicrobium*	[9]

chemical investigation of the bottom sediments and measurements of the key microbial processes (SR, MG, and AMO) were previously carried out [2, 3]. The pockmark chosen for investigation was a crater-like geomorphologic structure on the bottom surface, with 3-m maximal depth in the central part. Pockmark sediments were dark to black aleuro-pelite and pelite silts, water-saturated at the surface and dense in the deeper layers, always with a smell of sulfide. The upper 20-cm horizon of the sediments was enriched with organic carbon (7.6–8.55%). The C_{org} content then decreased with depth to 3.7–4.1% and increased to 5.5–6.9% at the depth of 1–1.2 m. Active destruction processes in the upper 20–30 cm of the sediment resulted in decreased C_{org} content, in an increase in the alkalinity of pore water up to 17–20 mM, and exhaustion of sulfate ions. Simultaneously with decreasing sulfate concentration in the sediments, methane content increased drastically with the maximum of 3.5–3.8 mmol/dm^3 of wet silt at a 24- to 80-cm depth (Figs. 1a–1d) [3].

The sediments were sampled with a Niemistötype hermetic corer and a 12-cm gravity corer. The following horizons were analyzed in order to determine microbial biodiversity, the vertical distribution of microorganisms, and detection of archaea and sulfate reducers: 0–5, 17–24, and 30–36 cm. At these horizons, high rates of microbial processes were detected (Fig. 1) [3].

The total microbial number (TMN) in the sediments was determined by fluorescence microscopy (Olympus, Japan) of DAPI-stained samples using an automated counting system [6].

CARD-FISH. CARD-FISH (catalyzed reporter deposition fluorescent in situ hybridization) [7, 8] was used for detection of eubacteria and archaea in the sediments. Peroxidaze-labeled oligonucleotide probes (Biomers, Germany) specific for the members of the *Bacteria* (EUB338) and *Archaea* (ARCH915) domains for the family *Desulfobulbaceae* (DSR651) and the order *Desulfosarcinales* (DSS658) were used. The probe NON338 exhibiting no complementarity to any locus of the 16S rRNA gene was used as a negative control (Table 1).

Enrichment cultures of sulfate-reducing bacteria (SRBs) from the Baltic Sea sediments were obtained in marine liquid Widdel medium for sulfate reducers, supplemented with vitamins and microelements [13]. The test tubes with enrichments were incubated at 22–23°C for 30 days. Increase in sulfide concentration compared to the control level was used to monitor SRB growth. Sulfide was determined colorimetrically on a Spekol *1* spectrophotometer (λ = 670 nm) with N,N-dimethyl-paraphenylenediamine according to Trüper and Schlegel [14].

Fluorescent in situ hybridization (FISH) was used for molecular monitoring of the SRB enrichment cultures. Bacterial cells were fixed with formaldehyde (at 2% final concentration) and filtered through Millipore GTTP 2500 polycarbonate membranes (0.2 μm). Cy3-labeled oligonucleotide sequences (Syntol, Russia) used as probes are listed in Table 2. Microscopy of the samples was carried out under a Zeiss fluorescence microscope (Germany) equipped with an AxioCam Mrc5 digital camera.

Isolation and purification of total DNA. Genomic DNA was isolated according to the procedure described earlier [16]. DNA preparations were purified using Wizard DNA Clean-Up Resin technology (Promega, United States) according to the manufacturer's recommendations. The degree of purification was assayed by the A_{260}/A_{280} ratio determined using a

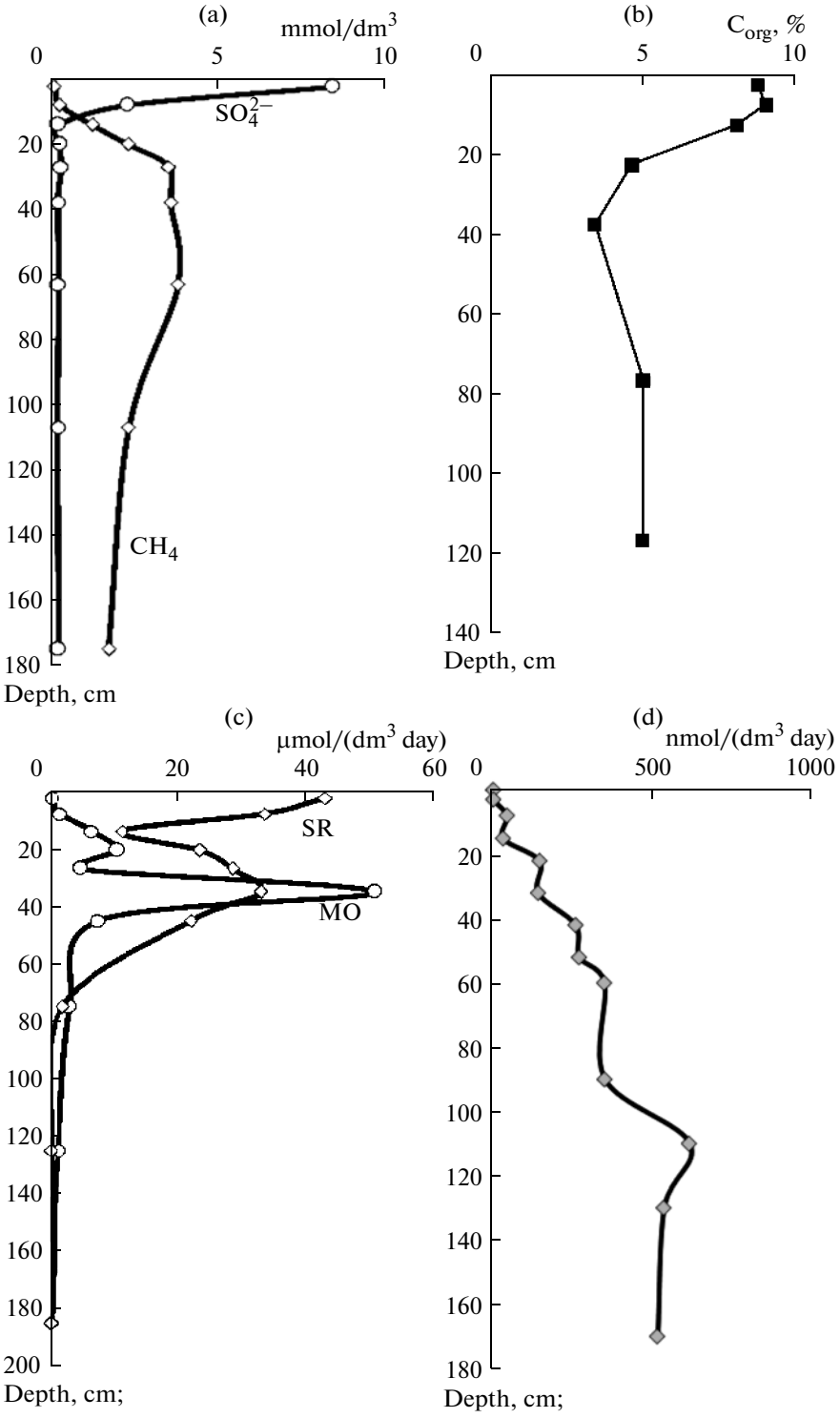

Fig. 1. Profiles of sulfate and methane content (a), C_{org} content (b), rates of sulfate reduction and anaerobic methane oxidation (c), and methanogenesis rates (d) in the pockmark sediments, Gdansk Deep, Baltic Sea.

SmartSpec[TM] Plus spectrophotometer (BioRad, United States).

PCR amplification. Bacterial and archaeal 16S rRNA gene fragments were amplified from the total DNA preparation with a polymerase chain reaction (PCR). The oligonucleotide primers used were complementary to conservative 16S rRNA gene sites in eubacteria: 27F (5'-AGAGTTTGATCMTGGCT-

Table 3. Vertical distribution of TMN, eubacteria, archaea, and sulfate reducers in the sediments (per 1 g of wet silt)

Sediment horizon, cm	TMN, cells/g	Eubacteria, cells/g	Archaea, cells/g	Sulfate reducers, cells/g
0–5	5.28×10^{10}	5.07×10^{9}	3.90×10^{9}	6.54×10^{8}
17–24	3.54×10^{10}	1.38×10^{9}	2.83×10^{9}	n.d.*
30–36	1.70×10^{10}	4.25×10^{8}	9.51×10^{8}	n.d.

* Not determined.

CAG-3') and 1350R (5'-CACGGGCGGTGTGTACAAG-3'). Archaeal 16S rRNA genes were selectively amplified using specific primers A20F (5'-TTCCGGTTGATCCYGCCRG-3') and A958R (5'-YCCGGCGTTGAMTCCAATT-3'). PCR was carried out in a BIS appliance (Russia). The reaction mixture composition and the temperature–time regime were adjusted as described previously [16]. The presence of amplicons and their molecular mass were determined by electrophoretic separation in 1% agarose gel with a DNA marker (Fermentas, Lithuania).

Cloning of PCR products and clone analysis. The amplified 16S rRNA gene fragments were ligated using a pGEM-T Easy Vectors kit (Promega, United States). The competent cells of *E. coli* XL1-Blue were used to obtain recombinants. The transformation was carried out as described in [17]. The clones were selected by "white–blue" screening. The presence of expected inserts in the colonies was determined by amplification of the colony lysates with the primers homologous to the termini of the plasmid polylinker: M13R (5'-CAGGAAACAGCTATGAC-3') and M13F (5'-GTTTTCCCAGTCACGAC-3') using the following protocol: 94°C, 5 min; 57°C, 70 s; 72°C, 90 s (1 cycle), 94°C, 60s; 57°C, 70 s; 72°C, 90 s (30 cycles); and final polymerization at 72°C for 10 min. The fragment of relevant length were excised and eluted.

Sequencing of 16S rRNA gene fragments. Sequencing of the cloned 16S rRNA gene fragments was carried out in the DNA Sequencing Center, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences on an ABI 3130×1 automatic sequencer (Applied Biosystems, United States) according to the manufacturer's recommendations.

Phylogenetic analysis of 16S rRNA sequences. For preliminary phylogenetic screening of the similarity of the sequences obtained to GenBank sequences, the NCBI BLAST software package was used (http://www.ncbi.nlm.nih.gov/blast). For more precise determination of the phylogenetic position of the cloned fragments, the CLUSTAL W software package (http://www.genebee.msu.su/clustal) was used to align their nucleotide sequences with the sequences of various eubacteria and archaea, including the known species and phylotypes of uncultured species, available in the latest version of the GenBank database. Phylogenetic trees were constructed using the neighbor-joining method implemented in the TREECON W software package (http://biocwww.uia.ac.be/u/yvdp/treeconw.httml). Statistical reliability of branching was determined by bootstrap analysis of 100 alternative trees.

Deposition of the sequences. The nucleotide sequences of 16S rRNA gene fragments obtained in the present work were deposited in GenBank under accession nos. HM245630–HM245659.

RESULTS

TMN and numbers of metabolically active microorganisms (CARD-FISH in bottom sediments. The results on determination of TMN and the numbers of metabolically active eubacteria, archaea, and sulfate reducers in the pockmark sediments are presented in Table 3. TMN decreased with depth from 5.3×10^{10} to 1.7×10^{10} cells/g, as did the numbers of eubacteria and archaea. Eubacteria predominated in the upper horizon. Deeper in the sediment, the number of archaea was more than double the number of eubacteria. A positive signal with the SRB probe was obtained only from the upper horizon of the sediment.

Identification of sulfate-reducing bacteria in enrichment cultures. Investigation of SRB diversity revealed predominance of *Desulfovibrionales* in enrichment cultures obtained from the different horizons of pockmark sediments (Table 4). Two probes for *Desulfovibrio* (DSV698 and DSV1292) were used in order to enhance the reliability of our results. Table 5 demonstrates that they exhibited a very high level of hybridization (>50% of all DAPI-stained cells). The authors who suggested these probes demonstrated their particle overlapping, i.e., specificity for the same species [12]. This was observed in our enrichment cultures. Since other probes exhibited low levels of hybridization, *Desulfomicrobium* and *Desulfotomaculum* species were either absent or present as minor components. Low hybridization (<2% of all DAPI-stained cells) may be explained by nonspecific binding of these probes.

Analysis of 16S rRNA clone libraries. Two libraries of bacterial and archaeal 16S rRNA gene fragments were obtained (Table 5). Sequencing of 100 clones resulted in determination of 71 eubacterial nucleotide sequences (1300-bp insert length) and 29 archaeal sequences (900 bp).

A search for the closest relatives among GenBank sequences revealed that the sequences obtained in the present work belonged to 35 phylotypes, which were

Table 4. Genus and species identification of sulfate-reducing bacteria in enrichment cultures from different horizons of pockmark sediments. The number of hybridizing cells is presented as percent of the total number of DAPI-stained cells

SRB species and genera and the relevant probes	Horizon, cm			
	Warp, 0–1	1–5	27–33	170–200
Desulfovibrio spp./Dsv698	34	65	72	26
Desulfovibrio spp./Dsv1292	52	68	50	76
Desulfomicrobium spp./Dsv214	1.4	0	1.4	0.7
Desulfotomaculum spp./Dtm229	3.0	1.1	1.7	2.7
Total cell number, DAPI staining, $\times 10^7$ cells/ml	14	19	16	13

similar to the sequences of the members of *Bacteria*, belonging to *Alpha-, Beta-, Gamma-, Delta-*, and *Epsilonproteobacteria, Firmicutes, Nitrospirae, Planctomycetes,* members of the OP11 group, and gram-positive bacteria with high G + C content, as well as three phylotypes of the phylum *Euryarchaeota*, domain *Archaea*. Among 30 eubacterial phylotypes, seven were bacteria of uncertain taxonomic position, most closely related to uncultured bacteria of unspecified taxonomic position isolated from various environmental sources. The distribution of phylogenetic groups in the sediment horizons is presented in Table 5.

For the upper sediment layer (0–5 cm), 27 eubacterial and 11 archaeal sequences were analyzed. Four phylogenetic groups of eubacteria were found. The *Proteobacteria* were represented by thebeta, delta, and epsilon classes, exhibiting similarity to *Burkholderia fungorum* (98%), an uncultured deltaproteobacterium from the sediments of a North Sea methane seep (97%), *Arcobacter* sp. (98%), sulfate-reducing bacteria *Desulfobacca acetoxidans* (99%) of the order *Syntrophobacterales*, and *Geothermobacter ehrlichii* (94%) of the family *Desulfuromonadaceae*. Members of the *Proteobacteria* constituted 24% of the sequences from this horizon. The *Actinobacteria* were represented by four phylotypes, most closely related to *Kocuria kristinae* (98%), *Rothia terrae* (98%), uncultured *Propionibacterium* (94%), and uncultured *Actinobacteria* (95%). This phylogenetic lineage was more broadly represented (26%). The *Chloroflexi* were represented by a single sequence with high similarity (97%) to an uncultured *Chloroflexi* isolated from a hypersaline microbial mat [18]. The *Nitrospirae* representative was most closely related to an uncultured bacterium (96%) found previously in the Baltic Sea anaerobic sediments [19]. Three phylotypes from the upper sediment layer exhibited high similarity (96–98%) to uncultured bacteria isolated from geographically remote environments: surface sediments of the Santa Barbara Bay (EU181500), sulfide hot spring (AY702840), and gas hydrates of the Kazan mud volcano in the Mediterranean (FJ712478). The ratio of these sequences was 11%. They formed an isolated clade on the phylogenetic tree (Fig. 2).

Archaea of the upper sediments belonged to two phylotypes most similar to uncultured methanogens from gas-saturated near-bottom water in Japan (DQ841227) and subsurface water of a borehole in Olkiluoto, Finland (FJ851598) (Fig. 3). The number of methanogen sequences was 29% of the total clone library for this horizon.

In the 17- to 24-cm horizon, 27 eubacterial and 4 archaeal sequences were analyzed. Eubacteria belonged to five phylogenetic groups. Apart from bacterial groups described for the first horizon, the phylotypes belonging to *Planctomyces* (uncultured *Planctomyces*, 97%) and *Firmicutes* (*Abiotrophia* sp., 99%) were detected. *Nitrospirae* sequences, however, were not found. *Proteobacteria* (26%) and unclassified bacteria (39%) predominated in this horizon. The content of other types of bacteria was lower (3–10%). Importantly, apart from the sequences belonging to uncultured bacteria of unspecified taxonomic position described above, sequences found in a petroleum reservoir (AB186261), Pacific sediments (EU287290), and the Yellow Sea (FJ545475) were found in this horizon.

In the 17- to 24-cm horizon, archaea belonged to the same phylotypes as in the surface sediment layer. Their content, however, was lower (13%).

From the 30- to 36-cm sediment layer, 17 eubacterial sequences of 4 phylotypes and 14 archaeal sequences were retrieved. In this horizon, sequences were found that were not detected in the upper layers: uncultured member of the OP11 phylum (96% similarity). The members of this group have been repeatedly isolated from the sediments of hot springs and methane seeps [20]. Moreover, a phylogenetic group was detected comprising gram-positive bacteria with high G + C content. An SRB sequence was also retrieved that exhibited 98% similarity to strain NaphS3 (EU908726) of the family *Desulfobacteraceae* isolated from the Mediterranean sediments and involved in degradation of aromatic hydrocarbons (2-methylnaphthalene) [21].

The group of uncultured bacteria was represented by the sequences most closely related to uncultured bacteria from Pacific sediments (EU287290), the sediments of freshwater Lake Washington (DQ067029),

Table 5. Distribution of the phylogenetic groups in the sediment horizons

Phylogenetic group	Closest homologue (% similarity)	Number of clones		
		0–5 cm	17–24 cm	30–36 cm
Bacteria				
Proteobacteria	Uncultured	–	3	–
Gammaproteobacteria	*Acinetobacter* sp. (98%), GU570643			
Betaproteobacteria	*Burkholderia fungorum* (97%), FJ796450;	1	–	–
	Burkholderia sp. (99%), FJ603038	–	–	1
Deltaproteobacteria	*Pelobacter* sp. (96%), GQ420895);	–	1	–
	Desulfobacca acetoxidans (99%), AF002671;	1	–	–
	Desulfobaca sp. (95%), EF613372;	–	1	–
	Uncultured *Desulfuromonadaceae* (99%), AM935618;	–	–	1
	Geothermobacter ehrlichii (94%), AY155599;	2	–	–
	Sulfate-reducing bacterium, strain Naph S3 (98%) EU908727;	–	–	2
	Uncultured deltaproteobacterium (96%), AM229511;	1	–	1
	Uncultured deltaproteobacterium (97%) FM179877 (methane seep, North Sea)	1	3	–
Epsilonproteobacteria	*Arcobacter* sp. (98%), FJ968636	3	–	–
Actinobacteria	*Microbacterium oxidans* (99%), FJ169470;	–	1	–
	Kocuria kristinae (98%), EU554443;	3	–	–
	Rothia terrae (98%), DQ822568;	2	–	–
	Uncultured *Propionibacterium* (94%), FJ957480 ;	4	–	2
	Uncultured *Actinobacteria* (95%), EF220354	1	1	1
Chloroflexi	Uncultured *Chloroflexi* (97%), DQ330025	1	1	–
Planctomycetales	Uncultured *Planctomycetes* (97%), CU925984	–	3	–
OP11	Uncultured OP11 (96%), AF027029	–	–	2
Firmicutes	*Abiotrophia* sp. (99%), AM420130	–	1	–
Nitrospirae	Uncultured bacterium (96%), EF460048 (Baltic Sea sediments)	3	–	–
High G + C grampositesites	Uncultured bacterium (99%), AF419679	–	–	1
Bacteria of uncertain taxonomic position	Uncultured bacterium (97%), AY702840 (sulfide hot spring);	1	1	–
	Uncultured bacterium (98%), AB186261 (petroleum reservoir);	–	4	–
	Uncultured bacterium (96%), EU287290 (Pacific sediments);	–	1	1
	Uncultured bacterium (97%), EU181500 (coastal sediments, Santa Barbara Bay);	2	3	–
	Uncultured bacterium (96%), DQ067029 (Lake Washington sediments);	–	–	1
	Uncultured bacterium (97%), FJ545475 (Yellow Sea sediments);	–	2	–
	Uncultured bacterium (98%), FJ712478 (gas hydrates of a Kazan mud volcano, Mediterranean)	1	1	4
Archaea *Euryarchaeota*	Uncultured archaeon (97%), AF419624 (hydrothermal sediments);	–	–	10
	Uncultured archaeon (96%), FJ851598 (underground water, 297-m borehole);	6	1	2
	Uncultured archaeon (97%), DQ841227 (gas-saturated near-bottom water, Japan)	5	3	2
Sequences analyzed		38	31	31

and gas hydrates of the Mediterranean Kazan mud volcano (FJ712478).

Some archaeal sequences from the 30- to 36-cm horizon exhibited 97% similarity to the uncultured archaeon (AF419624) from the active hydrothermal sediments of the Guaymas Basin (Gulf of California). This uncultured organism belongs to the ANME-1a phylogenetic cluster, comprising the phylotypes of uncultured archaea found in methane seeps within the Gauymas hydrothermal basin (Fig. 3). In the 30- to 36-cm horizon, archaea (45%) and bacteria of unclear taxonomic position (19%) predominated.

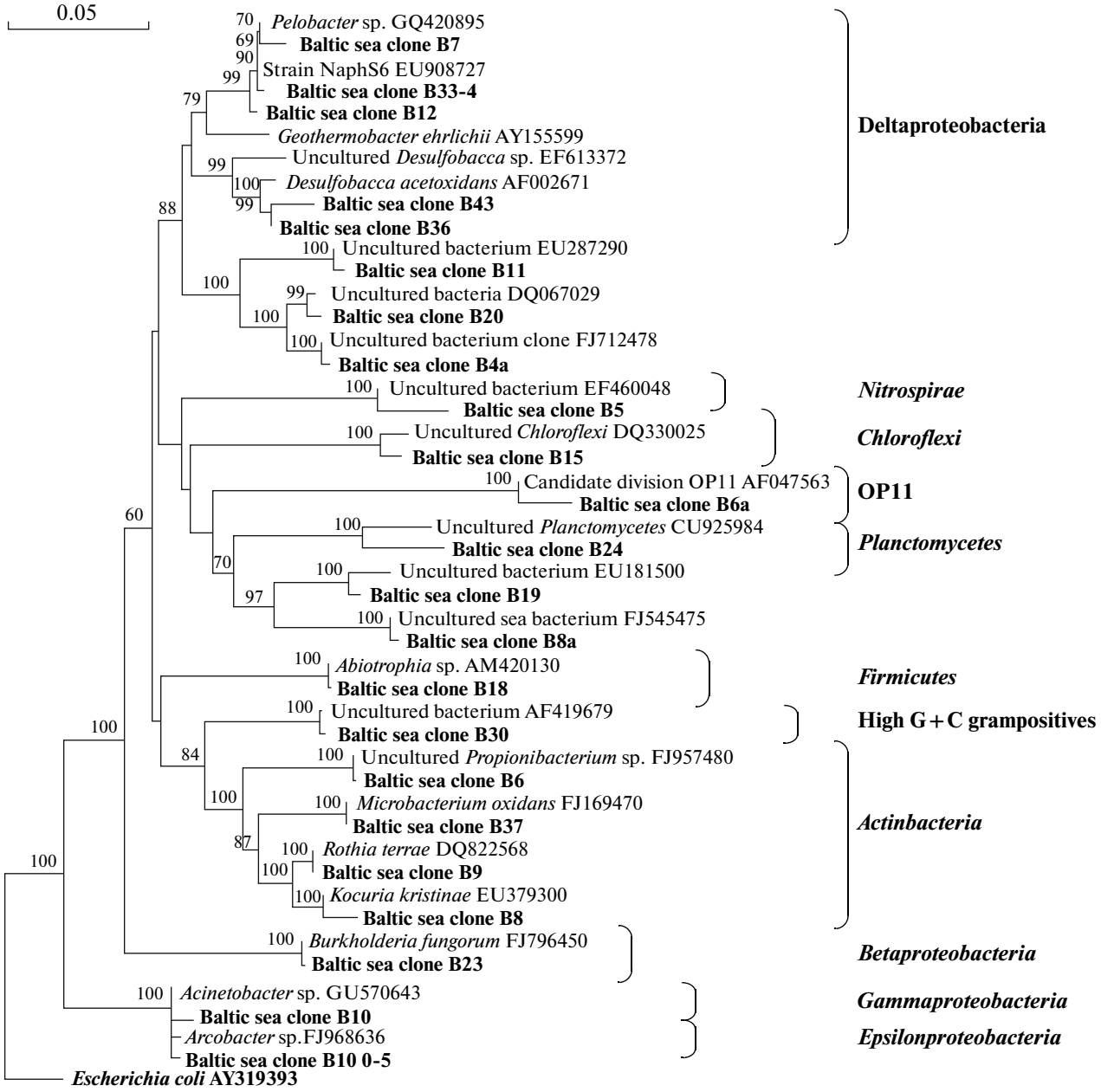

Fig. 2. Phylogenetic tree constructed based on the sequences of eubacterial 16S rRNA gene fragments from the pockmark sediments, Gdansk Deep, Baltic Sea. The tree was constructed using the neighbor-joining algorithm. Statistical reliability of the branching order determined by bootstrap analysis of 100 alternative trees (values over 60% are shown). The scale bar shows evolutionary distances (5 nucleotide replacements per 100 nucleotides). The clones obtained from pockmark sediments are marked by boldface.

DISCUSSION

The results of our investigation demonstrated that the highest total microbial numbers (5.3×10^{10} cell/g wet sediment) occurred in the upper layer (0–5 cm), where the highest OM content was observed (8.55%). Deeper in the sediment, where C_{org} content decreased, TMN was three times lower (1.7×10^{10} cells/g). Only in the surface layer of pockmark sediments did the number of metabolically active eubacteria exceed the number of archaea (Table 3). In the original sample, hybridization with SRB probes was observed only in the upper layer (1.2% of TMN). The absence of a signal in deeper layers is most probably a technical error indicating the necessity for further optimization of the accepted procedure. This suggestion is supported by the results of other investigators, who found the SRB numbers in OM-enriched sediments to be 3–30% of TMN [22]. Since species-specific probes revealed members of the orders *Des-*

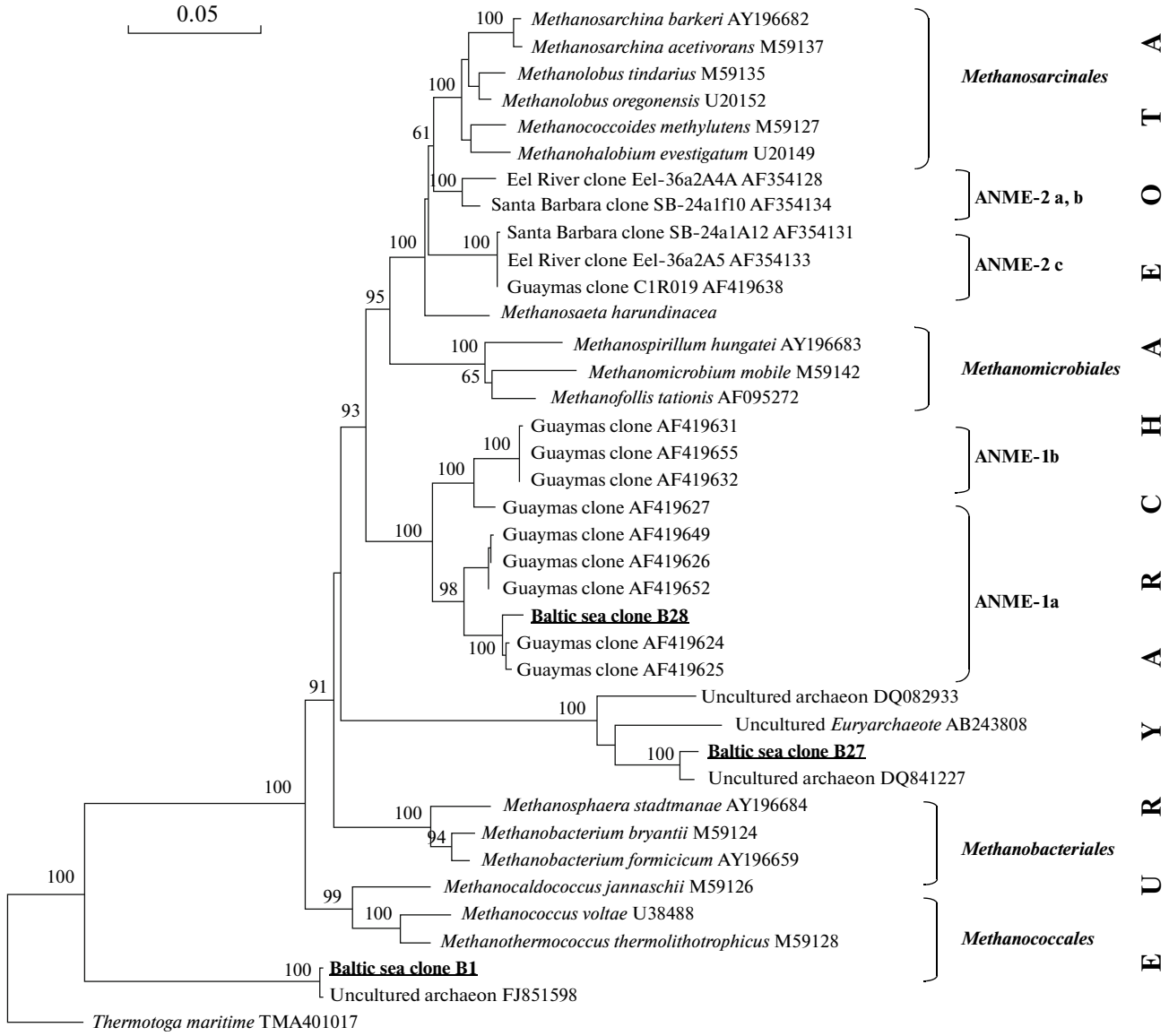

Fig. 3. Phylogenetic tree constructed based on the sequences of archaeal 16S rRNA gene fragments from the pockmark sediments, Gdansk Deep, Baltic Sea, and of the known archaeal species and uncultured ANME phylotypes from the GenBank database. The tree was constructed using the neighbor-joining algorithm. Statistical reliability of the branching order determined by bootstrap analysis of 100 alternative trees (the values over 60% are shown). The scale bar shows evolutionary distances (5 nucleotide replacements per 100 nucleotides). The clones obtained from pockmark sediments are marked by boldface and underlining.

ulfovibrionales and *Clostridiales* in SRB enrichment cultures, the spectrum of probes used in the present work for CARD-FISH detection of sulfate reducers was probably insufficient. Analysis of 16S rRNA gene sequences revealed the SRB sequences most closely related to the families *Syntrophaceae, Desulfuromonadaceae*, and *Desulfobacteraceae*. Thus, different approaches demonstrated the presence of five SRB families in the pockmark sediments. Wide occurrence of different SRB groups with predominance of *Desulfobacteraceae* in the upper layers was also reported for the shallow Kattegat sediments (Aarhus Bay) by analysis of the SRB molecular markers, *dsrAB* (dissimilatory sulfite reductase) genes in the sedimentary columns [22].

In the 30- to 40-cm sediment layer, where the maximums of sulfate reduction and anaerobic methane oxidation were revealed by radioisotope measurements, the sequences of uncultured methanotrophic archaea of the ANME-1a subgroup were detected. Members of the ANME-1 group, which are responsible for anaerobic methane oxidation, were originally found in the sediments of methane seeps on the California coast [23]. Several groups of methanotrophic

archaea (ANME-1, ANME-2, and ANME-3) are now known; their sequences were detected in the sediments of methane seeps of the Pacific and Atlantic Oceans; Mediterranean, Black, and North Seas [5]; and OM-enriched gas-saturated coastal marine sediments [8]. Although these microorganisms have never been isolated in pure cultures, radioisotope, isotopic geochemical, and molecular biological evidence [5, 23, 24] demonstrate that this group of archaea forms microconsortia with sulfate-reducing bacteria, which carry out anaerobic oxidation of methane in marine sediments. In the present work, methanotrophic archaea of the ANME-2 and ANME-3 groups were not detected, although earlier studies [4, 25] demonstrated the presence of ANME-2 archaea in the Baltic coastal sediments. Our recent analysis of the sequences of the gene encoding the α subunit of methyl-coenzyme M reductase (mcrA) retrieved from the same Gdansk Deep pockmark sediments revealed both ANME-1 and ANME-2 sequences, with predominance of the former group [26].

In conclusion, it should be noted that molecular analysis of the microbial community in three sediment horizons with different physicochemical characteristics and rates of microbial processes revealed a broad spectrum of sequences exhibiting high homology (97–98%) to bacteria previously detected in sediments of gas hydrate-containing methane seeps. Members of such phyla as *Proteobacteria* and *Actinobacteria* exhibited the highest diversity. Uncultured forms responsible for anaerobic methane oxidation predominated among archaea.

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REFERENCES

1. *Geokhimiya vod i donnykh osadkov Baltiiskogo morya v raionakh razvitiya gazovykh kraterov i geoakusticheskikh anomalii* (Geochemistry of Waters and Bottom Sediments of the Baltic Sea in the Regions of Development of Gas Craters and Geoacoustic Anomalies), Geodekyan, A.A, Romankevich, E.A, and Trotsyuk, V.Ya., Eds., Moscow: IO RAN, 1997.
2. Pimenov, N.V., Ul'yanova, M.O., Kanapatskii, T.A., Sivkov, V.V., and Ivanov, M.V., Microbiological and Biogeochemical Processes in a Pockmark of the Gdansk Depression, Baltic Sea, *Mikrobiologiya*, 2008, vol. 77, no. 5, pp. 651–659 [*Microbiology* (Engl. Transl.), vol. 77, no. 5, pp. 579–586].
3. Pimenov, N.V., Ulyanova, M.O., Kanapatsky, T.A., Veslopolova, E.F., Sigalevich, P.A., and Sivkov, V.V., Microbially Mediated Methane and Sulfur Cycling in Pockmark Sediments of the Gdansk Basin, Baltic Sea, *Geo-Marine Lett.*, 2010, DOI 10.1007/s00367-010-0200-4.
4. Treude, T., Krüger, M., Boetius, A., and Jørgensen, B.B., Environmental Control on Anaerobic Oxidation of Methane in the Gassy Sediments of Eckernörde Bay (German Baltic), *Limnol. Oceanogr.*, 2005, vol. 50, pp. 1771–1786.
5. Knittel, K. and Boetius, A., Anaerobic Oxidation of Methane: Progress with an Unknown Process, *Annu. Rev. Microbiol.*, 2009, vol. 63, pp. 311–334.
6. Drozdov, V.N., Sergeeva, V.N., Maksimenko, S.Yu., and Zemskaya, T.I., Computer System for Image Analysis of Fluorescently Stained Bacteria, *Mikrobiologiya*, 2006, vol. 75, no. 6, pp. 751–754 [*Microbiology* (Engl. Transl.), vol. 75, no. 6, pp. 751–753].
7. Bobrov, M.N., Harris, T.D., Saughnessy, K.J., and Litt, G.J., Catalyzed Reporter Deposition, a Novel Method of Signal Amplification, *J. Immunol. Methods*, 1989, vol. 125, pp. 279–285.
8. Ishii, K., Muβmann, M., MacGregor, B.J., and Amann, R. An Improved Fluorescence in Situ Hybridization Protocol for the Identification of Bacteria and Archaea in Marine Sediments, *FEMS Microbiol. Ecol.*, 2004, vol. 50, pp. 203–212.
9. Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A., Combination of 16S Ribosomal-RNA-Targeted Oligonucleotide Probes with Flow-Cytometry for Analyzing Mixed Microbial Populations, *Appl. Environ. Microbiol.*, 1990, vol. 56, pp. 1919–1925.
10. Raskin, L., Stromely, J.M., Rittmann, B.E., and Stahl, D.A., Group-Specific 16S Ribosomal-RNA Hybridization Probes to Describe Natural Communities of Methanogens, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 1232–1240.
11. Wallner, G., Amann, R., and Beisker, W., Optimizing Fluorescent in situ Hybridization with Ribosomal-RNA-Targeted Oligonucleotide Probes for Flow Cytometric Identification of Microorganisms, *Cytometry,* 1993, vol. 14, pp. 136–143.
12. Manz, W., Eisenbrecher, M., Neu, T.R., and Szewzyk, U., Abundance and Spatial Organization of Gram-Negative Sulfate-Reducing Bacteria in Activated Sludge Investigated by in situ Probing with Specific 16S rRNA Targeted Oligonucleotides, *FEMS Microbiol. Ecol.,* 1998, vol. 25, pp. 43–61.
13. Widdel, F. and Back, F., The Genus *Desulfotomaculum,* in *The Procariotes,* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., Eds., New York: Springer, 1992, pp. 787–794.
14. Trüper, H.G. and Schlegel, H.G., Sulfur Metabolism in *Thiorhodaceae.* I. Quantitative Measurements in Growing Cells of *Chromatium okenii, Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 1964, vol. 30, pp. 225–238.

15. Hristova, K.R., Mau, M., Zheng, D., Aminov, R.I., Mackie, R.I., Gaskins, H.R., and Raskin, L., *Desulfotomaculum* Genus- and Subgenus-Specific 16S rRNA Hybridization Probes for Environmental Studies, *Environ. Microbiol,* 2000, vol. 2, pp. 143–159.
16. Shubenkova, O.V., Zemskaya, T.I., Chernitsyna, S.M., Khlystov, O.M., and Triboi, T.I., The First Results of an Investigation into the Phylogenetic Diversity of Microorganisms in Southern Baikal Sediments in the Region of Subsurface Discharge of Methane Hydrates, *Mikrobiologiya,* 2005, vol. 74, no. 3, pp. 370–377 [*Microbiology* (Engl. Transl.), vol. 74, no. 3, pp. 314–320].
17. Inoue, H., Nojima, H., and Okayama, H., High Efficiency Transformation of *E. coli* with Plasmids, *Gene,* 1990, vol. 26, pp. 23-28.
18. Ley, R.E., Harris, J.K., Wilcox, J., Spear, J.R., Miller, S.R., Bebout, B.M., Maresca, J.A., Bryant, D.A., Sogin, M.L., and Pace, N.R., Unexpected Diversity and Complexity of the Guerrero Negro Hypersaline Microbial Mat, *Appl. Environ. Microbiolol.*, 2006, vol. 72, no. 5, pp. 3685–3695.
19. Edlund, A., Hardeman, F., Jansson, J.K., and Sjoling, S., Active Bacterial Community Structure along Vertical Redox Gradients in Baltic Sea Sediment, *Environ Microbiol.,* 2008, vol. 10, no. 8, pp. 2051–2063.
20. Teske, A., Hinrichs, K., Edgcomb, V., Comez, A., Kysela, D., Sylva, S.P., Sogin, M.L., and Jannasch, H.W., Microbial Diversity of Hydrothermal Sediments in the Guaymas Basin: Evidence for Anaerobic Methanotrophic Communities, *Appl. Environ. Microbiolol.*, 2002, vol. 68, no. 4, pp. 1994–2007.
21. Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., and Rabus, R., Anaerobic Degradation of Naphthalene and 2-Methylnaphthalene by Strains of Marine Sulfate-Reducing Bacteria, *Environ. Microbiol.,* 2009, vol. 11, no. 1, pp. 209–219.
22. Leloup, J., Fossing, H., Kohls, K., Holmkvist, L., Borowski, C., and Jørgensen, B.B., Sulfate-Reducing Bacteria in Marine Sediment (Aarhus Bay, Denmark): Abundance and Diversity Related to Geochemical Zonation, *Environ. Microbiol.,* 2009, vol. 11, no. (5), pp. 1278–1291.
23. Hinrichs, K.U., Hayes, J.M., Sylva, S.P., Brewer, P.G., and DeLong, E.F., Methane-Consuming Archaebacteria in Marine Sediments, *Nature,* 1999, vol. 398, no. 6730, pp. 802–805.
24. Valentine, D.L. and Reeburgh, W.S., New Perspectives on Anaerobic Methane Oxidation (Mini Review), *Environ. Microbiol.,* 2000, vol. 2, no. 5, pp. 477–484.
25. Meulepas, R., Jagersma, C., Gieteling, J., Buisman, C., Stams, A., and Lens, P., Enrichment of Anaerobic Methanotrophs in Sulfate-Reducing Membrane Bioreactors, *Biotechnol. Bioeng.,* 2009, vol. 104, no. 3, pp. 458–470.
26. Merkel', A.Yu., Chernykh, N.A., Kanapatskii, T.A., and Pimenov, N.V., 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, *Mikrobiologiya,* 2010, vol. 79, no. 6, pp. 852–855 [*Microbiology* (Engl. Transl.), vol. 79, no. 6, pp.].