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EXPERIMENTAL ARTICLES

Analysis of Cultured Methanogenic Archaea from the Tarkhankut Peninsula Coastal Methane Seeps

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Abstract—The major substrates for methanogenesis were used for investigation of cultured methanogenic archaea from coastal methane seeps near the Tarkhankut Peninsula, Black Sea. Analysis of the 16S rRNA gene sequences revealed that growth of the classical methanogenic *Euryarchaeota* occurred in all enrichments but was absent in the controls without the substrates. Enrichments from the seep differed in microbial composition from those from the background point. The most numerous archaea belonged to the genera *Methanolobus* (medium with methanol and hydrogen), *Methanosarcina* (trimethylamine and hydrogen), *Methanococcoides* (trimethylamine), and *Methanococcus* (hydrogen and CO_2). Syntrophic growth of hydrogenotrophic archaea of the genus *Methanogenium* with clostridia and members of the family *Thermotogaceae* probably occurred in enrichments with acetate. Relatively low similarity of the recovered 16S rRNA gene sequences with the closest cultured relatives (94% and lower) indicated that the *Methanogenium* phylotype belonged to a new species. The same was true for the *Methanosarcina* phylotype revealed in the culture with trimethylamine and hydrogen (97% and less similarity of the 16S rRNA gene sequences to those of the closest cultured relatives).

Keywords: Black Sea, methane seep, bottom sediments, methanogens, *Methanococcoides*, *Methanogenium*, *Methanolobus*

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Release of hydrocarbon gases from marine bottom sediments (methane seeps) is an extremely interesting geological phenomenon that has been increasingly attracting researchers' attention over the recent decades. Such gas seepage sites frequently represent sea bottom life oases that differ dramatically from the neighboring sediments in their physicochemical and microbiological characteristics and in the structure of benthic communities (Bernardino et al., 2012). High methane concentrations promote the growth of aerobic methanotrophic bacteria, which consume oxygen and produce additional organic matter that can enter the trophic chains (Ding and Valentine, 2008). The processes of extensive microbial methane oxidation and organic matter transformation are accompanied by a dramatic decrease in the concentrations of oxygen, which is present only in the uppermost sediment layers of methane seeps. Oxygen depletion and high activity of primary degraders lead to activation of sulfate-reducing and methanogenic microorganisms, which play a leading role in the terminal phase of organic matter degradation in marine bottom sediments (Kallistova et al., 2017).

Gas seeps in the Black Sea were discovered in the late 1980s (Polikarpov and Egorov, 1989). By present, researchers have been investigating methane seeps located mainly on the continental slope of the Northwestern Black Sea shelf, as well as deep-water mud volcanoes (Michaelis et al., 2002; Pimenov and Ivanova, 2005). A recent geoacoustic study of the coastal area of the Crimean Peninsula demonstrated that shallow-water methane seeps are commonly present at depths of up to 10 m (Egorov et al., 2011). The methane seeps discovered more than 30 years ago in the Kalamit Bay of the Black Sea near the coast of the Tarkhankut Peninsula are of particular interest. It was found that the maximal depth of oxygen penetration within bottom sediments at gas seep sites did not exceed several millimeters, while sulfide concentrations in subsurface sediments could reach 3 mM (Gulin et al., 2010). Previously, we showed that the local methanogenic communities were dominated by archaea of the families *Methanosarcinaceae* and *Methanomicrobiaceae* (Tarnovetskii et al., 2018). Since metagenomic studies using high-throughput sequencing cannot always provide an adequate estimate of the metabolic potential of microbial communities (Vign-

eron et al., 2015), we undertook a comprehensive investigation of methanogen diversity in coastal seeps of the Tarkhankut Peninsula by combining culturing techniques with molecular analysis.

The main goal of this work was to investigate the microbial potential of gas seeps of the Tarkhankut Peninsula and its development depending on the presence of different methanogenic substrates in the environment. For this purpose, we set up enrichment cultures with selective conditions promoting the growth of different methanogen groups.

MATERIALS AND METHODS

Sampling. The study analyzed bottom sediments of the Tarkhankut Peninsula gas seeps (45°35549 N, 32°73153 E) collected at the depth of 5 m. Samples were collected by a diver into plastic containers in May 2016. For enrichment cultures, the surface layer of bottom sediments (0–10 cm) was used. On the shore, the sediment samples were thoroughly mixed and dispensed into 250-mL glass vials without leaving an air bubble, sealed with rubber stoppers, and transported into the laboratory.

Samples were collected at two sites: at the center of a methane seep and at a reference point located approximately 100 m away from the seep. The sediments were composed of aleuropelitic muds with an admixture of sand and detritus material in the center of the seep and of slightly silted sand at the reference site. Water for sample dilution was collected above the surface of the seep and the reference site using a bathometer.

Enrichment cultures. To start a culture, 10 mL of bottom sediments as inoculation material and 10 mL sea water were placed into a 60-mL vial. The vials were sealed with gas-tight rubber stoppers. Immediately after that, the air phase was removed from the vials and replaced with nitrogen. Selective growth conditions were created by adding different methanogenic substrates: trimethylamine (to the final concentration of 22.5 mM), acetate (15 mM), a mixture of carbon dioxide and hydrogen (85 and 15% of the gas phase, respectively), methanol (20 mM) and hydrogen (15%), or trimethylamine (22.5 mM) and hydrogen (15%). In the control cultures, no substrate was added. Samples from both sites were tested using the same assortment of substrates. Altogether, 12 enrichment cultures were started. The cultures were incubated in a thermostat at 20°C for 2 months. The growth of methanogens was assessed based on methane content in the vials, which was measured at the end of the incubation period using a Kristall gas chromatograph (Chromatec, Russia).

Phylogenetic composition of enrichment cultures determined using high-throughput sequencing of the 16S rRNA genes. DNA was isolated from the inocula (bottom sediment samples) and from enrichment cultures grown for 2 months. The samples or cultures were thoroughly mixed, and 5-mL aliquots were collected into clean tubes and centrifuged for 20 min at 14000 *g*. The sedimented cells were homogenized mechanically and chemically (Lever et al., 2015). For this purpose, the cell pellet was mixed with guanidine hydrochloride and Triton X-100 (to the final concentrations of 800 mM and 0.5%, respectively) with a total volume of 500 μL and a mixture of glass beads 425–600 μm and 106 μm in diameter with a total volume of ~500 μL. The mixture was homogenized for 40 s at 6 m/s using a FastPrep®-24 Instrument (MP Biomedicals, United States) and incubated for 50 min at 50°C. Next, nucleic acids were extracted using the standard phenol–chloroform procedure and precipitated with isopropanol. Libraries of DNA sequences representing the V3–V4 region of the 16S rRNA gene were obtained as described previously (Fadrosh et al., 2014). The fragments were amplified using the following primer system: the forward primer (5'-CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTG-TGCTCTTCCGATCT XXXXXXXXXXXX ZZZZ CCTAYGGGDBGCWSCAG-3') was composed of 5' Illumina Linker Sequence, Index 1, Heterogeneity Spacer (Fadrosh et al., 2014), and the Pro-mod-341F primer sequence (Merkel et al., 2017); the reverse primer (5'-AATGATACGGCGACCACCGAGATCTACACT-CTTTCCCTACACGACGCTCTTCCGATCT XXXX-XXXXXXXX ZZZZ GACTACNVGGGTMTCTAATCC-3') included 3' Illumina Linker Sequence, Index 2, Heterogeneity Spacer, and Pro-mod-805R primer sequence (Merkel et al., 2017). The amplicons were separated by electrophoresis in 2% agarose gel, excised with a scalpel, and purified using the Cleanup Standard kit (Evrogen, Russia). Sequencing was performed in a MiSeq system (Illumina, United States) using the reagent kit that enabled the reading of 300 bases at either end of the amplicon. Demultiplexing was performed with the relevant software scripts of QIIME v. 1.9.0 (Caporaso et al., 2010). Subsequent sequence editing and analysis were also performed with QIIME v. 1.9.0. The data were filtered to select those with the minimal read quality score of 30 and the minimal read length of 350 bp. Chimeric reads were identified using the identify_chimeric_seqs.py script with the USEARCH v. 6.1544 algorithm (Edgar, 2010) and the Silva 123 database of reference 16S rRNA reads (Quast et al., 2013). The table of operational taxonomic units (OTUs) was constructed using the pick_open_reference_otus.py script. Sequences with an identity level of at least 97% were grouped into OTUs (Schloss and Handelsmann, 2006) using USEARCH v. 6.1544 (Edgar, 2010) and the Silva 123 database (Quast et al., 2013). Representative sequences were selected using UCLAST (Edgar, 2010). Alpha diversity analysis and construction of rarefaction curves were performed using the core_diversity_analyses.py script to obtain a normalized sample of 17000 reads per specimen. The

Table 1. Methane production, sequencing results, and alpha diversity estimates in enrichment cultures from samples collected at the seep and reference sites

Conditions of enrichment culture growth	Methane, %	Number of reads	Number of OTUs	Relative abundance of archaea, %	Shannon	Chao1	Saturation, %
		non-normalized libraries			libraries normalized by CSS		
		Seep					
Control (no substrate)	0.0	17184	1776	2.3	4.62	3171	56.0
Trimethylamine and hydrogen	64.7	5590	465	19.2	5.94	795	58.5
Methanol and hydrogen	30.4	28753	2484	27.5	8.22	3714	66.9
Hydrogen and carbon dioxide	14.8	7140	1085	16.2	6.94	1946	55.8
Acetate	8.9	19269	1992	36	7.42	3019	66.0
Trimethylamine	33.3	45684	2362	76.5	4.38	3713	63.6
Inoculum	0.0	68528	2303	2.7	8.4	2306	99.9
		Reference site					
Control (no substrate)	0.0	22288	2944	18.4	9.5	3808	77.3
Trimethylamine and hydrogen	51.8	26323	513	68.2	3.00	764	67.1
Methanol and hydrogen	20.3	20248	1050	64.1	5.10	2028	51.8
Hydrogen and carbon dioxide	8.7	26124	560	49.6	4.02	759	73.8
Acetate	8.1	29479	1839	69.2	5.25	3090	59.5
Trimethylamine	14.8	35974	1536	85.5	2.57	2449	62.7
Inoculum	0.0	74917	2717	0	8.32	2923	93.0

species of methanogenic archaea were identified using the BLAST online service. To calculate the alpha diversity parameters, the table of OTUs was normalized by cumulative sum scaling (CSS) technique using the QIIME script.

The obtained arrays of sequencing data were deposited into the NCBI database under the accession no. PRJNA549749.

RESULTS

Methane production. At the start of enrichment cultures, the gas phase was replaced with nitrogen, so it may be assumed that the initial methane content in the gas phase was zero. After 2 months of cultivation, the methane content in the gas phase ranged from 8.74 to 64.7%. In the control variants without substrate addition, methane was detected in trace amounts: 0.01 and 0.03% for the seep and the reference sample, respectively. The highest methane content was observed in enrichment cultures with methylated substrates: (1) trimethylamine, 33.3 and 14.8% for the seep and the reference sample, respectively; (2) trimethylamine and hydrogen, 64.7 and 51.8% for the seep and the reference sample, respectively; and (3) methanol and hydrogen, 30.4 and 20.2% for the seep and the reference sample, respectively. In the cultures grown on substrates of acetoclastic and hydrogenotrophic methanogenesis, methane levels were lower. The cultures from the seep and the reference site produced 8 and 8.8% methane, respectively, when grown on acetate and 14.7 and 8.7%, respectively, when grown on hydrogen and carbon dioxide.

Molecular analysis of the composition of microbial communities. Altogether, 427501 16S rRNA gene sequences were obtained with an average length of 406 bp. The phylotype diversity coverage calculated using the Chao1 approach (Chao, 1984) ranged from 55.7 to 99.8% in enrichment cultures from the seep and from 51.7 to 92.9% in the reference cultures. The Shannon diversity index ranged from 3 to 9.5 (Table 1).

The obtained reads were grouped into OTUs with an identity level of 97%. Altogether, 10437 unique OTUs were obtained. In the cultures from the reference site, most sequences were classified as archaeal. Their relative abundance was 49.6–85.5% (Table 1). In enrichment cultures from the seep, the share of archaea was lower: 76.5% in the culture grown on trimethylamine and 19.2–36% in other variants. In the inocula, archaea constituted only 2.7% in the seep sample and less than 1% in the reference sample. In the control cultures without added substrates, the share of archaea was 2.3% for the seep and 18.4% for the reference site.

In nearly all cultures, the dominant groups were known methanogens of the phylum *Euryarchaeota*. In enrichment cultures from seep bottom sediments,

Table 2. Methanogen species predominant in enrichment cultures from the seep and reference sites growing on different substrates

Substrate	Predominant species of archaea	Number of reads in the dominant OTU	Identity to the nearest cultured relative, %
	Seep		
Hydrogen and carbon dioxide	*Methanococcus maripaludis*	517	100
Acetate	*Methanogenium frigidum*	3526	94
	Methanogenium marinum	1446	99
Trimethylamine	*Methanococcoides methylutens*	18221	100
	Methanococcoides alaskense	13167	100
Trimethylamine and hydrogen	*Methanosarcina acetivorans*	279	97
	Methanogenium marinum	187	99
Methanol and hydrogen	*Methanolobus oregonensis*	1542	100
	Methanolobus oregonensis	1402	99
	Reference site		
Hydrogen and carbon dioxide	*Methanogenium marinum*	11268	99
Acetate	*Methanococcoides methylutens*	10265	100
	Methanococcoides alaskense	5778	100
Trimethylamine	*Methanococcoides methylutens*	10265	99
Trimethylamine and hydrogen	*Methanococcoides methylutens*	15982	100
Methanol and hydrogen	*Methanococcoides alaskense*	6357	100
	Methanolobus taylorii	1507	100
	Methanolobus oregonensis	836	98

most reads represented the following microbial groups: uncultured clostridia (17.1 and 7.3%) and *Methanococcus* (7.3%) in variant 1 (hydrogen and CO_2); *Methanogenium* (27.7%) in variant 2 (acetate); *Methanococcoides* (71.1%) in variant 3 (trimethylamine); *Gracilibacteria* (15.8%) and *Methanosarcina* (13.4%) in variant 4 (trimethylamine and hydrogen), and *Methanolobus* (19.5%) in variant 5 (methanol) (Table 2). In the inoculum from the seep, the dominant groups were bacteria of the taxa *Gelria* (8.2%), *Nocardioides* (4.1%), and uncultured *Firmicutes* (5.3%), and the control variant without added substrate was dominated by bacteria of the genus *Sulfurimonas* (70.5%).

All enrichment cultures from the reference site, except for variants 1 (methanol and hydrogen) and 2 (hydrogen and CO_2), were dominated by archaea of the genus *Methanococcoides* (34.9–80.8%). In the culture grown on methanol and hydrogen, the most abundant were members of the genus *Methanolobus* (24.8%), and in the culture grown on hydrogen and CO_2, it was archaea of the genus *Methanogenium* (49.4%). In the inoculum from the reference site, the largest groups were bacteria of the taxa *Defluviicoccus* (16.5%), *Acidimicrobiales* OM1 clade (7.2%), and *Rhodospirillaceae* (6.3%). In the control variant with no substrate added, the most abundant group were archaea of the phylum *Woesearchaeota* (10.5%), while 14.4% of reads could not be attributed to any known taxon.

DISCUSSION

The fact that methane production was observed in all enrichment cultures, but not in the control variants indicates that the microbial community studied is capable of methanogenesis using a broad range of substrates. Under natural conditions, methane production is limited by the amount of substrate available. The highest methane concentrations were reached on media that contained trimethylamine and methanol. Most probably, this was due to reaction stoichiometry and a larger energy gain achieved by methanogens during growth on methylated substrates. These results may also suggest that the community is dominated by microorganisms that employ the methylotrophic methanogenesis pathway, which could explain the low activity levels observed in our previous experiments with labeled carbon dioxide and acetate (unpublished data). Acetoclastic and hydrogenotrophic methanogenesis could be suppressed due to growth of sulfate-reducing bacteria. However, in all experiments the relative abundance of sulfate-reducing bacteria (2.3–9.3%) was lower than that of methanogenic archaea (15.9–82.5%). Therefore, it may be concluded that all substrates were added in large excess, and the compe-

tition between methanogens and sulfate-reducers need not be taken into account.

By analyzing the 16S rRNA gene sequences, we identified the microbial groups that predominated on each substrate. In most cases, these were one or two genera of classic methanogens. Importantly, the dominant microorganisms were different in the cultures obtained from the seep and the reference sample. The only exception were the cultures growing on methanol and hydrogen. These results indicate that the microbial communities of the seep and the reference site differ significantly in their potential.

In contrast to our previous work (Tarnovetskii et al., 2018), in this study we were able to obtain longer sequencing reads that spanned over two variable regions of the 16S rRNA gene. As a result, it was possible not only to analyze the composition of the communities at the genus level, but also to identify the species of microorganisms in the enrichment cultures.

The enrichment culture from the seep sample growing on acetate was dominated by sequences representing the genus *Methanogenium*. Based on the analyzed 16S rRNA gene fragment, the closest cultured species were *M. frigidum* and *M. marinum* classified as hydrogenotrophic psychrophilic methanogens (Franzman et al., 1997; Chong et al., 2002). It should be noted that the relationship to *M. frigidum* was remote (94% identity, Table 2). Most probably, the detected phylotype represents a novel species. This is an interesting result, because only two genera of methanogens are currently known to produce methane from acetate: *Methanosaeta* and *Methanosarcina* (Lyu and Liu, 2018). Acetate can also be oxidized to carbon dioxide and hydrogen by bacteria. This process as such does not provide an energy gain. However, if hydrogen is consumed by methanogens, the equilibrium is shifted and the reaction creates an energy gain (Hattori, 2008). Several group of bacteria are known to participate in syntrophic oxidation of acetate: members of the phyla *Firmicutes* (*Thermacetogenium*, *Clostridium*, *Thermotoga*, *Candidatus* Contubernalis, *Candidatus* Syntrophonatronum and Syntrophaceticus) and *Proteobacteria* (*Desulfomicrobium, Geobacter*) (Hattori, 2008; Westerholm et al., 2010; Kimura et al., 2013; Li et al., 2018; Timmers et al., 2018). In our enrichment cultures growing on acetate, none of bacterial species was clearly dominant. The highest relative abundances were observed for members of the taxa *Thermotogaceae* (3.5%), GoM-GC232-4463-Bac (order *Clostridiales*, 6.2%), livecontrolB21 (*Clostridiales*, 2.1%), *Rhodobacteraceae* (3.5%), and *Desulfobacter* (3.9%). Most probably, in the enrichment culture from the seep sample, typical hydrogenotrophic archaea produce methane in syntrophic association with acetate-oxidizing bacterial partners. Clostridia seem likely candidates for this role. Altogether, the order *Clostridiales* accounted for 14% of the reads. Bacteria of the family *Thermotogaceae* may also be involved in this process. Previously, it was found that *Thermotogaceae* and hydrogenotrophic methanogens dominated in enrichment cultures that utilized long-chain alkanes and acetate (Cheng et al., 2013; Li et al., 2018).

The observed active growth of *Methanococcoides methylutens* and *M. alaskense* in the reference cultures growing on acetate remains unexplained. Archaea of this genus are obligate methylotrophic methanogens. None of the known *Methanococcoides* species can utilize acetate for methane production. Members of this genus are widely present in marine ecosystems, including methane seeps (Lanoil et al., 2005; Omoregie et al., 2009; Roussel et al., 2009; Lazar et al., 2011; Lin et al., 2010; Vigneron et al., 2014). Usually, they can be detected even without preliminary cultivation under selective conditions (Orphan et al., 2001; Pachiadaki et al., 2011). Thanks to their ability to utilize methylated substrates, these methanogens can actively grow at high sulfate concentrations without competitive pressure from sulfate reducers. It is known that trimethylamine is an available substrate in marine ecosystems. In bottom sediments, it can be produced from lignin, pectin, betaine, choline, and trimethylamine-*N*-oxide (TMAO). Betaine and TMAO occur as osmolytes in many marine organisms. It was shown that some sulfate-reducing deltaproteobacteria of the genera *Desulfovibrio*, *Desulfobacterium*, and *Desulfuromonas* can transform glycine and betaine into trimethylamine (Hayward and Stadtman, 1959; Fiebig and Gottschalk, 1983; Heijthuijsen and Hansen, 1989). Some members of *Methanococcoides* can produce methane from choline and betaine without a bacterial partner (Watkins et al., 2014; L'Haridon et al., 2014).

The seep enrichment culture growing on trimethylamine and hydrogen produced large amounts of methane (33%). Some reads in this culture were classified into the genus *Methanosarcina* (13.2%), the members of which can produce methane via the hydrogenotrophic, acetoclastic, and methylotrophic pathways. However, the dominant group were bacteria of the phylum *Gracilibacteria* (15.8%). The first genome of *Gracilibacteria* was obtained from a thermal spring sample using single-cell sequencing (Rinke et al., 2013). Subsequently, two further genomes of *Gracilibacteria* were obtained from human mouth cavity. Members of this phylum can ferment glucose to acetate and are auxotrophic by many amino acids (Espinoza et al., 2018). The role of *Gracilibacteria* in methane production remains unknown. In our experiment, nearly all reads (886 of 908) belonged to the same OTU; at the same time, the abundance of *Gracilibacteria* in the inoculation material was below the detection threshold (0 reads). This suggests high levels of selective pressure and microbial specialization.

In comparison to methylotrophs, hydrogenotrophic methanogens were less diverse. In the cultures of samples from the seep and reference sites grown on CO_2 and hydrogen, the dominant OTUs were the

archaea *Methanococcus maripaludis* (seep) and *Methanogenium marinum* (reference site). Both these genera include typical hydrogenotrophic methanogens that produce methane by utilizing hydrogen or formate and carbon dioxide. *M. maripaludis* is a mesophilic microorganism first isolated from salt marsh sediments (Jones et al., 1983). In the bottom sediments of Tarkhankut rich in organic matter, a possible source of hydrogen are numerous heterotrophic microorganisms described in our previous study (Tarnovetskii et al., 2018). Probably, hydrogenotrophic methanogenesis takes place in the subsurface layers depleted in sulfate. The enrichment culture from the seep sample also contained abundant uncultured clostridia (liveControlB21 and vadinBB60 group); however, nothing is currently known about their physiology.

In the control seep culture (without substrate addition), the highest relative abundance was observed for bacteria of the genus *Sulfurimonas* and archaea of the phylum *Woesearchaeota*. Initially, *Sulfurimonas* were considered human and animal pathogens. Since molecular techniques were introduced into microbial ecology, these microorganisms have been detected in thermal springs, marine, and land ecosystems. Currently, four cultured species of *Sulfurimonas* are known (Han and Perner, 2015). Based on the 16S rRNA gene sequence, the bacterium detected in our experiments exhibited little similarity to any of them, with only 96% homology to the nearest cultured relative. Members of the genus *Sulfurimonas* can utilize sulfur compounds as electron donors. This type of metabolism is quite plausible for microorganisms of seep bottom sediments.

Woesearchaeota is a phylum of uncultured archaea belonging to the superphylum DPANN. Metagenomic studies showed that this group of archaea has a high level of phylogenetic diversity. Presumably, they are heterotrophs growing in anoxic environments. It is possible that some members of *Woesearchaeota* can be engaged in syntrophic relationships with methanogens, providing them with acetate and hydrogen (Castelle et al., 2015; Liu et al., 2018).

Thus, our study demonstrated a high potential of the methanogenic community of coastal methane seeps of the Tarkhankut Peninsula. It was found that the microbial composition of bottom sediments differed significantly between the seep and the reference site. The enrichment cultures grown from the collected samples contained methanogens capable of producing methane via the hydrogenotrophic and methylotrophic pathways. Methane production from acetate occurred most probably via the hydrogenotrophic pathway thanks to syntrophy between methanogens and bacteria of the order *Clostridiales* and the family *Thermotogaceae*.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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