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EXPERIMENTAL ARTICLES

Phylogenetic Diversity and Activity of Anaerobic Microorganisms of High-Temperature Horizons of the Dagang Oil Field (P. R. China)

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Abstract—The number of microorganisms of major metabolic groups and the rates of sulfate reduction and methanogenesis processes in the formation waters of the high-temperature horizons of Dagang oil field have been determined. Using cultural methods, it was shown that the microbial community contained aerobic bacteria oxidizing crude oil, anaerobic fermentative bacteria, sulfate-reducing bacteria, and methanogens. Using cultural methods, the possibility of methane production from a mixture of hydrogen and carbon dioxide ($H_2 + CO_2$) and from acetate was established, and this result was confirmed by radioisotope methods involving $NaH^{14}CO_3$ and $^{14}CH_3COONa$. Analysis of enrichment cultures 16S rDNA of methanogens demonstrated that these microorganisms belong to *Methanothermobacter* sp. (*M. thermautotrophicus*), which consumes hydrogen and carbon dioxide as basic substrates. The genes of acetate-utilizing bacteria were not revealed. Phylotypes of the representatives of *Thermococcus* spp. were found among archaeal 16S rDNA. 16S rRNA genes of bacterial clones belong to the orders Thermoanaerobacteriales (*Thermoanaerobacter*, *Thermovenabulum*, *Thermacetogenium*, and *Coprothermobacter* spp.), Thermotogales, Nitrospirales (*Thermodesulfovibrio* sp.) and Planctomycetales. 16S rDNA of a bacterium capable of oxidizing acetate in the course of syntrophic growth with H_2-utilizing methanogens was found in high-temperature petroleum reservoirs for the first time. These results provide further insight into the composition of microbial communities of high-temperature petroleum reservoirs, indicating that syntrophic processes play an important part in acetate degradation accompanied by methane production.

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Key words: oil reservoir, thermophilic bacteria, sulfate reduction, methanogenesis, 16S rDNA, phylogeny, syntrophy, methanogenic community.

INTRODUCTION

The presence of microorganisms in oil fields was established about 80 years ago [1]. A number of reviews on petroleum microbiology with detailed descriptions of microorganisms of major physiological groups were published [2, 3]. Molecular-biological, ecological, cultural, and biogeochemical methods were used in clarifying various aspects of diversity and activity of microorganisms inhabiting oil reservoirs [3–8]. In the majority of studies, these methods were applied separately, precluding interpretations of the structure of microbial community as a whole and estimations of its geochemical activity. The conventional analysis methods of microbial communities (inoculation into elective nutrient media) make it possible to isolate and take into account only cultivated forms of microorganisms. Thus, the existence of anaerobic microorganisms of different metabolic groups such as bacteria reducing sulfate, thiosulfate, Fe^{3+}, or elemental sulfur; fermentative bacteria; acetogens, and methanogens) was established in different oil-bearing horizons [1–3].

Methanogenic microorganisms of petroleum reservoirs are the least investigated group of prokaryotes. The first pure cultures of methanogens from oil strata were isolated by Belyaev and coauthors [2, 4, 9, 10].

Methanogens, which oxidize hydrogen into methane, simultaneously with carbon dioxide reduction, are common inhabitants of petroleum reservoirs with the temperature in the range from 20 to 80°C [1–3, 11, 12]. Mesophilic hydrogen-utilizing methanogens belong to *Methanobacterium bryantii*, *M. ivanovii*, and *"Methanoplanus petrolearius"* [2, 3, 9, 10]. Hyperthermophilic

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methanogens growing at 110°C [13] have not been found in petroleum reservoirs. Lithotrophic methanogens responsible for methane production in high-temperature oilfields of the United States, the North Sea and Western Siberia are represented by *Methanococcus thermolithotrophicus* and different species of the genus *Methanothermobacter* (classified previously with the genus *Methanobacterium*), such as *M. thermautotrophicus* (= *M. thermoalcaliphilum*) and *M. thermoaggregans* [2, 3, 9, 11, 12].

Organotrophic methanogens utilizing acetate, methanol, and methylated amines (which also often grow on mixtures of hydrogen and carbon dioxide) have thus far been isolated from oilfields with temperatures not in excess of 50°C [2, 3]. Organotrophic mesophilic methanogens are represented by *Methanosarcina* spp. (*M. mazei* and *M. siciliae*), "*Methanohalophilus euhalobius*" (= *Methanococcoides euhalobius*), and *Methanocalculus halotolerans* [2, 3, 10, 11]. Mesophilic filamentous microorganisms, such as *Methanosaeta* (*Methanothrix*) spp., were found in methanogenic enrichment cultures from oilfields of Apsheron peninsula [14].

Davydova-Charakhch'yan and coauthors [9] were the first to describe thermophilic syntrophic degradation of acetate with methane production. A binary association including the lithotrophic methanogen *M. thermautotrophicus* (= *M. thermoalcalifilum*) as the terminal component was isolated from a high-temperature oilfield (Mykhpay) using an acetate-containing medium [2, 3, 9]. *M. thermautotrophicus* was also revealed in Samotlor strata, but acetoclastic methanogens growing at 60 and 80°C were not found [15]. Hyperthermophilic acetate-utilizing methanogens are hitherto unknown [2, 3, 16].

Nevertheless, methane production from $NaH^{14}CO_3$ or $^{14}CH_3COONa$ was detected by using radioisotope methods, in strata with the temperature range of 60 to 80°C, which points to the existence of thermophilic prokaryotes effecting these processes in oil reservoirs [3, 6, 7, 15].

In recent years, molecular-biological methods have been increasingly applied for studying of oil strata microbial communities [2, 3, 8, 15–17]. Analyses of nucleic acids isolated from natural samples, PCR amplification of 16S rDNA fragments followed by detailed studies of amplicons using molecular-genetic methods, give a thorough understanding of the composition of microbial communities. Such approaches are widely used for studying microbial communities of thermal springs (including submarine) and subsurface ecotopes; Orphan and coauthors applied similar methodology to California oil strata having a temperature in the range from 50 to 125°C [16, 17]. The formation water was shown to contain 16S rRNA genes of (1) thermophilic microorganisms belonging to the genera *Thermococcus*, *Petrotoga*, *Thermoanaerobacter*, *Methanothermobacter* (= *Methanobacterium*), *Methanococcus*, and *Methanoculleus*; (2) mesophilic bacteria of the genera *Desulfothiovibrio*, *Aminobacterium*, *Acidaminococcus*, *Pseudomonas*, *Halomonas*, *Acinetobacter*, *Sphingomonas*, *Methylobacterium*, *Desulfomicrobium*, and *Methanosarcina*; and (3) new archaeae and bacteria. Autotrophic methanogens of the genera *Methanothermobacter* (= *Methanobacterium*) (growing at 60–70°C), *Methanococcus* (growing at 60–80°C), and *Methanoculleus* (growing at 60–70°C) were isolated from a Californian oilfields using cultural methods. The 16S rRNA gene of a new acetoclastic methanogen (forming separate cluster inside the order Methanosarcinales) was detected in the strata of the Monterrey oil formation [16].

In this work, we sought to study the phylogenetic structure, number, and activity of the microbial community of the high-temperature horizons of Dagang oil field (Kongdian bed, China). The following tasks were fulfilled for this purpose: (1) measurement (using cultural methods) of the number of bacteria of major metabolic groups; (2) estimation of the contemporaneous rates of biogenic methanogenesis and sulfate reduction (using radioisotope methods); (3) elucidation of the phylogenic diversity of the microorganisms, particularly methanogens (using analytical molecular-biological methods of enrichment-culture 16S rRNA genes of methanogens and the natural bacterial community of the formation water).

MATERIALS AND METHODS

Characterization of Dagang oil field. Microorganisms of the Kongdian bed of the Dagang oil field (Hebei province, China) were studied. Sandstone oil-bearing horizons of the Kongdian bed had a temperature of 59°C. Dagang oil had a density of about 0.900 g/cm^3 and contained 53% saturated hydrocarbons, 20% aromatic compounds, and 21.15% resins and asphalthenes. The formation water of sodium hydrocarbonate type had a low salinity (5612 mg/l) and a pH of 7.1–7.6. Accompanying gas contained methane (95–98%), its higher homologues (0.8–1.8%), nitrogen (0.5–3.3%), and carbon dioxide (0.06–0.77%). The coproduced formation water of this oil field (separated from the oil produced), containing up to 0.8 mg/l dissolved oxygen and was 40–50°C, was injected into a formation to maintain the formation pressure.

Medium composition and methods of calculation of bacteria. The number of the microorganisms of major metabolic groups was determined by inoculating the formation water into a liquid media using a method of tenfold dilutions (in duplicates); the results were calculated using the McCready method of the most probable number. The number of hydrocarbon-oxidizing bacteria was determined using an R medium supplemented with a mixture of C_{10}–C_{22} *n*-alkanes (2% w/v) [5]. Anaerobic fermentative bacteria were estimated using the medium supplemented with peptone (4 g/l) and glucose (10 g/l) [15]. Sulfate-reducing bacteria were deter-

mined by the increase in H_2S (using Postgate's B medium [15] with sodium lactate (4 g/l) supplemented with microelements and reduced with 200 mg/l $Na_2S \cdot 9\ H_2O$). Methanogens were calculated by the methane increase in the Zeikus media with acetate (2 g/l) or $H_2 + CO_2$ (4 : 1), supplemented with microelements and yeast extract (1 g/l) (references to the description of the media were cited in a preceding publication [15]).

The inoculated media were incubated at 60°C for 7–14 days; the absence of growth was recorded after 30 days of incubation. All cultures were studied using an Olympus microscope supplemented with a phase-contrast device. The content of microbial metabolites (such as H_2S, CH_4, CO_2, and H_2) in a media and the chemical composition of the formation water were analyzed as described previously [15].

Radioisotope methods for determining the rates of anaerobic processes. The rates of sulfate reduction, lithotrophic, and acetoclastic methanogenesis were determined by radioassays using labeled compounds ($Na_2{}^{35}SO_4$, $NaH^{14}CO_3$ and $^{14}CH_3COONa$), as described previously [6].

Cloning and sequencing 16S rRNA genes. Isolation of nucleic acids. The samples of formation water and the primary cultures thereof in media for methanogens were used in molecular-biological studies of methanogens. The primary cultures of the formation water samples from wells 1017-7, 1008-1, 1017-2, 1017-4 and 1094-1 in the Zeikus media for methanogens ($H_2 + CO_2$ and acetate) were combined; the cell suspension (100 ml) was centrifuged at 5000 rpm for 30 min. The total DNA was isolated from the bulk biomass obtained using Diatom™ DNAprep kit for DNA isolation (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). For total DNA isolation, cell sediment was slurried with guanidine hydrochloride at 65°C for an hour. The carrier (Diatomid/silica) was added to the cell lysate. After DNA sorbtion, the supernatant was eliminated and the sorbent, washed using a buffer and 70% ethanol. Total DNA preparation was dissolved in 100 μl H_2O (MQ) and used for 16S rRNA gene amplification.

16S rRNA gene amplification. Isolated DNA was used for PCR with universal primers for Bacteria and Archaea. The PCR mixture (50 μl) contained a Taq polymerase buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl; and 0.1 vol. % Tween 20), 2 mM $MgCl_2$, 200 μM deoxyribonucleoside triphosphates, 5 pmol 5'- and 3'-terminal primers, 10–50 ng DNA, and a 0.1 μM thermostable DNA-polymerase (Taq, Perkin-Elmer). A bacterial 16S rRNA gene was synthesized using the forward primer 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') corresponding to positions 8–27 of *Escherichia coli* 16S rRNA gene and the reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3') corresponding to positions 1510–1492 of the *E. coli* 16S rRNA gene [18, 19]. A fragment of archaeal 16S rRNA gene was amplified using the forward primer A109F (5'-ACG/TGCTCAGTAACACGT-3') corresponding to positions 109–125 of 16S rRNA gene of *E. coli* and the reverse primer A1041r (5'-GGCCATGCACCWCCTCTC-3') corresponding to positions 1058–1041 of the 16S rRNA gene of *E. coli* [18, 20].

DNA was amplified using the iCycler thermocycler model from BioRad. DNA amplification with bacterial primers used the following program: activation of polymerase (3 min at 94°C); 30 cycles of DNA denaturation (0.5 min at 94°C), primer annealing (0.5 min at 50°C) and chain extension (1.5 min at 72°C); and final chain extension (7 min at 72°C). Amplification of DNA with archaeal primers used the same program with the exception of DNA chain extension, which was at 72°C for 1 min (35 cycles).

Amplicons were separated using 0.8% agarose gel, which was stained with ethidium bromide, and visualized in UV light. For PCR product purification, fragments were cut out from the gel, and the DNA was extracted using a V-gene DNA Gel Extraction Kit (V-gene Biotechnology Ltd., Hangzhou 310022, China).

Cloning of 16S rRNA genes. Eubacterial 16S rRNA gene (1490 bp that practically corresponds to the full length of the gene) and a 1000-bp fragment of archaeal 16S rRNA gene were purified and cloned into the plasmid vector pGEM-T (Promega) using a special kit (TA cloning vector kit). The recombinant plasmids obtained were introduced into the *E. coli* Z85 cells. After inoculation of the transformants on plates, white colonies were selected and analyzed for the presence of inserts of requisite size using PCR with universal plasmid primers SP6 and T7. Plasmid DNA of the clones selected was isolated using the Birnboim method [21], with minor modifications. Purified preparations of plasmids containing cloning fragments of archaeal and bacterial 16S rDNAs were used for DNA sequencing.

Sequencing of cloned 16S rDNAs. As a result of the PCR analysis of 400 clones, 122 unique clones, which contained inserts of the 16S rDNA fragment of the required size were selected. The clone libraries obtained consisted of 102 and 20 clones with inserts of 16S rDNA of Achaea and Bacteria, respectively. The sequencing was performed on an ABI 3100 Avant Genetic Analyzer using Amersham Dyenamic Terminator Cycle Sequencing Ready Reaction kits, in compliance with the recommendations of the manufacturers. The sequencing of the DNA fragments of the clones selected used the primers A109f and 8-27f for archaeal and bacterial DNA, respectively.

Phylogenetic analysis of 16S rRNA gene sequences. The preliminary analysis of the nucleotide sequences obtained was accomplished using BLAST software and the database of NCBI GenBank, where sequences of reference organisms are deposited. BioEdit software edited the sequences (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were aligned with those of the closest

related species of microorganisms using CLUSTALW software (v 1.75). Unrooted phylogenetic trees of the microorganisms examined were constructed using methods realized in the TREECONW software package (http://bioc.www.uia.ac.be/u/yvdp/treeconw.html) and reference sequences from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu).

The sequences used in constructing phylogenetic trees were deposited in GenBank under the numbers DQ097666–DQ097681.

RESULTS

Physicochemical and microbiological characterization of the formation waters of the Dagang oil field. The Kongdian area is exploited using waterflooding to maintain formation pressure. The coproduced formation waters (40–50°C) were separated from oil and used for injection. Oil-bearing horizons had the temperature of about 60°C. The original formation waters of sodium hydrocarbonate type had low salinity (5.6–6.7 g/l), were slightly alkaline (pH 7.1–7.6), and contained less than 5 mg/l of acetate and 433–670 mg/l of hydrocarbonate. The sulfate concentration did not exceed 27 mg/l. H_2S (less than 2 mg/l) was detected only in the water from the near-bottom zone of injection wells and was not found in the water of production wells.

The samples of formation water were analyzed in June 2002 during biotechnology testing for the enhancement of oil recovery. This biotechnology is based on increasing the activity of stratal microorganisms, which was achieved by injecting oxygen (as a water–air mixture) and nitrogen and phosphorus salts through the injection wells. The appearance of these oxygen and biogenic compounds in the stratum stimulated biodegradation of residual oil in the zone of injection wells. Products of oil oxidation migrated throughout the stratum with the water flow, increasing the content of hydrocarbonate and volatile acids in the water of a number of production wells (1002-1, 1008-1, 1017-3, 1017-7, 1094-1) (Table 1).

Microbiological analysis of the samples of formation water from the production wells demonstrated the presence of aerobic and anaerobic thermophilic microorganisms (Figs. 1a-1b). Note that the formation waters containing a higher concentration of oil biodegradation products were characterized by higher numbers of microorganisms. The water from production wells contained little, if any, aerobic hydrocarbon-oxidizing bacteria; only in three samples (wells 1008, 1008-1, 1012-1) the number of bacteria was 10^2–10^3 cell/ml, reaching 10^5 cell/ml in the near-bottom zone of injection well 1098 (sample 24 m^3). The numbers of fermentative and sulfate-reducing bacteria before the biotechnology testing did not exceed 10^3 cell/ml (for each group); in June 2002, they increased to 10^7–10^8 cell/ml and 10^4 cell/ml, respectively. The number of methanogens growing on $H_2 + CO_2$ increased from 10^2–10^3 to 10^3–10^4 cell/ml (wells 1002-1, 1017-3, 1017-7, and 1094-1) (Fig. 1b). The number of methanogens, determined using the acetate-containing medium was generally an order of magnitude lower than that of H_2-utilizing methanogens. Microscopic analysis demonstrated an absolute dominance of rod-shaped cells growing on both media used for methanogens.

The rates of anaerobic processes in formation waters. Thermophilic processes of bacterial sulfate reduction and methanogenesis were detected in the Kongdian area. Sulfate-reducing bacteria inhabited this stratum, despite the low concentration of sulfates in the formation waters; the rate of sulfate reduction before the biotechnology testing was 0.002–18 $\mu g\ S^{2-}\ L^{-1}\ day^{-1}$. The rates of methanogenesis from labeled bicarbonate and acetate were about 0–1.595 and 0–5.61 $\mu g\ CH_4\ L^{-1}\ day^{-1}$, respectively.

During the activation period of stratal microflora, the rate of sulfate reduction in most samples did not exceed 29.97 $\mu g\ S^{2-}\ L^{-1}\ day^{-1}$; in two of the wells, (1008-1 and 1092), it increased to 215.629 $\mu g\ S^{2-}\ L^{-1}\ day^{-1}$. The rates of methanogenesis from hydrocarbonate and acetate increased to 15.668 and 42.255 $\mu g\ CH_4\ L^{-1}\ day^{-1}$, respectively (Table 1). In the sample from well 1094-1, methane production from acetate was exceptionally high and reached 1143.479 $\mu g\ CH_4\ L^{-1}day^{-1}$ (in this well, oil production also increased).

During the biotechnology testing, a moderate increase in the contents of CO_2 (0.5–1.7%) and methane (3–5%) in the gas was detected.

Thus, the use of cultural methods and radioassays allowed us to demonstrate that thermophilic microbial communities, producing methane from $H_2 + CO_2$ or acetate, inhabited the Dagang oil field.

Characterization of methanogen enrichment cultures by 16S rRNA gene analysis. Total DNA from the primary cultures of formation water (in the Zeikus media with $H_2 + CO_2$ or acetate) was used for 16S rRNA gene amplification and construction of the 16S rDNA clone library. A near-complete sequence of the full-length eubacterial 16S rRNA gene (1490 bp) and a 1000-bp fragment of archaeal 16S rDNA were cloned into a pGEM-T vector. The PCR analysis of 400 clones using universal plasmid primers revealed 122 unique clones containing inserts of 16S rRNA fragments of the corresponding sizes. The clone libraries obtained were designated A1 (Archaea) and B1 (Bacteria); they comprised 102 and 20 clones containing the 16S rDNA inserts of archaea and bacteria, respectively.

The sequencing of 16S rDNA fragments of archaeal and bacterial clones used the A109f and 8-27f primers, respectively. This made it possible to determine the nucleotide sequences corresponding to the 5'-areas of 16S rRNA genes, which carry variable regions and are the most informative with respect to phylogenetic relations. The primary analysis of the obtained nucleotide sequences was carried out using the Blast software

Table 1. Chemical composition of formation water and rates of sulfate reduction and methanogenesis processes in the formation water of the Kongdian bed

Well number	Mineralization, mg/l	HCO_3^-, mg/l	SO_4^{2-}, mg/l	Acetate, mg/l	Rate of methanogenesis, μg CH_4 L^{-1} day^{-1}		Rate of sulfate reduction, μg S^{2-} L^{-1} day^{-1}
					from $NaH^{14}CO_3$	from $^{14}CH_3COONa$	
1098* – 8 m^3	6314	534	12	16	0.693	228.475	291.296
1098* – 24 m^3	6279	488	12	30	0.635	40.200	20.123
Production wells:							
01	6283	412	30	22.6	0.048	0.052	5.179
1002-1	6827	747	0	74.4	0.151	20.55	0.009
1008	7064	610	12	1.0	0	0.003	0.187
1008-1	6616	747	12	160.7	0.569	39.680	215.629
1012	6852	610	47	70.8	0	1.184	5.49
1012-1	6897	579	30	70.8	0.039	14.054	0.574
1015-1	6544	564	0	52.2	0	0.716	0.012
1017	6553	488	0	11.6	0.167	0.04	0
1017-2	7038	1830	0	2.5	0.190	0.011	0
1017-3	6786	732	0	86.6	0.873	1.768	0.02
1017-4	6522	503	24	64.2	0.161	42.255	0.795
1017-5	7005	625	35	119.9	0.231	6.969	12.029
1017-7	7185	808	6	0.5	0.378	0.162	1.445
1032	6737	457	0	5	0.04	0.006	0
1032-1	6003	579	12	7.4	0	0.640	0.044
1050-1	6320	457	53	70.6	0.559	0.236	16.306
1050-2	6688	457	0	55.6	0.03	0.192	0
1050-3	6938	640	0	2.8	0.2	0.018	0
1092	6459	579	47	65.5	15.668	0.169	159.698
1094	6767	640	35	26	1.04	0.175	0.217
1094-1	6080	1.052	47	53.4	12.066	1143.479	29.970
1066-1**	5681	33.6	0	457	0	0.086	No data***

* The water sample, from the near-bottom zone of the injection well, was obtained when the well switched to the well-spring mode of operation.

** Formation water sample from a well situated outside the zone of biotechnologic influence.

*** No data.

package in the database of NCBI GenBank with sequences of the referent organisms. The analyzed archaeal sequences consisted of 424–696 bp and belonged to the subdivision Euryarchaeota; 101 clones belonged to the order Methanobacteriales, and 1 clone, to the order Thermococcales. The sequences of archaeal 16S rDNAs were divided into six operative taxonomic units (OTUs), each of which the similarity of 16S rDNA exceeded 97.0% (Table 2). 16S rRNA genes of methanogens were divided into five OTUs (OTU 1–OTU 5), comprising 31, 21, 45, 1, and 3 clones, respectively. The sixth OTU was represented by a single clone, A116m, which had a similarity of 99.1% to the species *Thermococcus sibiricus*. One representative clone was selected from each OTU of methanogens (A5m, A8m, A1m, A4m, and A10m from OTU 1–OTU 5, respectively) for the construction of the phylogenetic tree. The fragments 700–845 bp of the 16S rRNA gene of these clones were analyzed. Methanogens from all OTUs were phylogenetically close to the representatives of the genus *Methanothermobacter*, particularly *M. thermautotrophicus* and the non-cultivated species were 97–100% similar. The clone of OTU 4 was phylogenetically close to *M. thermautotrophicus* GC-1 and to the clone E21A1 from the methanogen enrichment culture, obtained from the formation waters of oil-bearing horizons of Monterrey [16]. Molecular hydrogen is the main substrate for methane production of the representatives of the order Methanobacteriales. It is note-

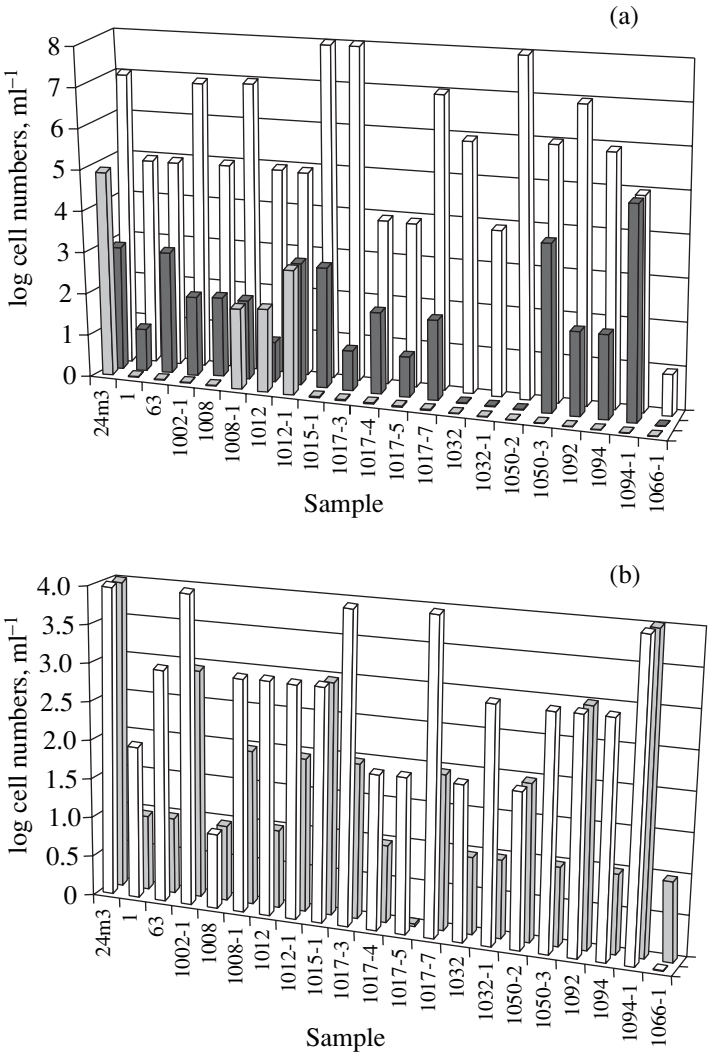

Fig. 1. Number of aerobic and anaerobic thermophilic microorganisms in the formation water of the Kongdian bed. A: number of hydrocarbon-oxidizing ▒, sulfate-reducing ■, and fermentative □, bacteria; B: number of methanogens, calculated in media with $H_2 + CO_2$ and acetate; □, H_2-utilizing methanogens, ▒, acetate-utilizing methanogens.

worthy that the phylotypes of methanogens that utilize acetate were not found in the cultures studied.

In contrast to the archaeal component, the bacterial component of the community studied was more variable, (even though it was represented by a smaller number of clones) (21). The cloned fragments of 16S rDNA formed ten OTUs (OTU 7–OTU 16), in which similarity of nucleotide sequences exceeded 98.4% (Table 2). The size of the analyzed fragments of bacterial 16S rRNA gene, which were used for the construction of the phylogenetic tree, was in the range from 1197 bp (clone B21, OTU 10) to 1545 bp (clone B14, OTU 8). Bacterial phylotypes belonged to the orders Thermoanaerobacteriales, Thermotogales, Nitrospirales and Planctomycetales. Fermentative bacteria were an important component of the metabolic spectrum of the bacteria representing the methanogen community. The clone B6 (OTU 7) belonging to the group of gram-positive bacteria with low G + C content was most closely related to *Thermanaeromonas toyohensis* (92.8% similarity), a species that was isolated previously from subsurface water-bearing thermal horizons. The obtained phylotypes of the order Thermoanaerobacteriales were close to the genera *Thermoanaerobacter* (clone B20 of OTU 8), *Coprothermobacter* (clones B12 and B9 of OTU 9), *Thermovenabulum* (clones B5, B14, and B8) and *Thermacetogenium* (clones B11, B16, and B18 of OTU 13). The sequences of 16S rRNA genes from clones B14, B5, and B8 were different (96.5–97.2% similarity) and exhibited a relationship to self-dependent operating units (OTU 10, OTU 11, and OTU 12) of *Thermovenabulum* sp.; they likely belong to a new species. This genus includes thermophilic iron-reducing bacteria isolated from Kamchatka hot springs. Clones B9 and B12 constituted a self-dependent unit (OTU 9) phylogenetically close to the genus *Coprothermobacter* (94.7–

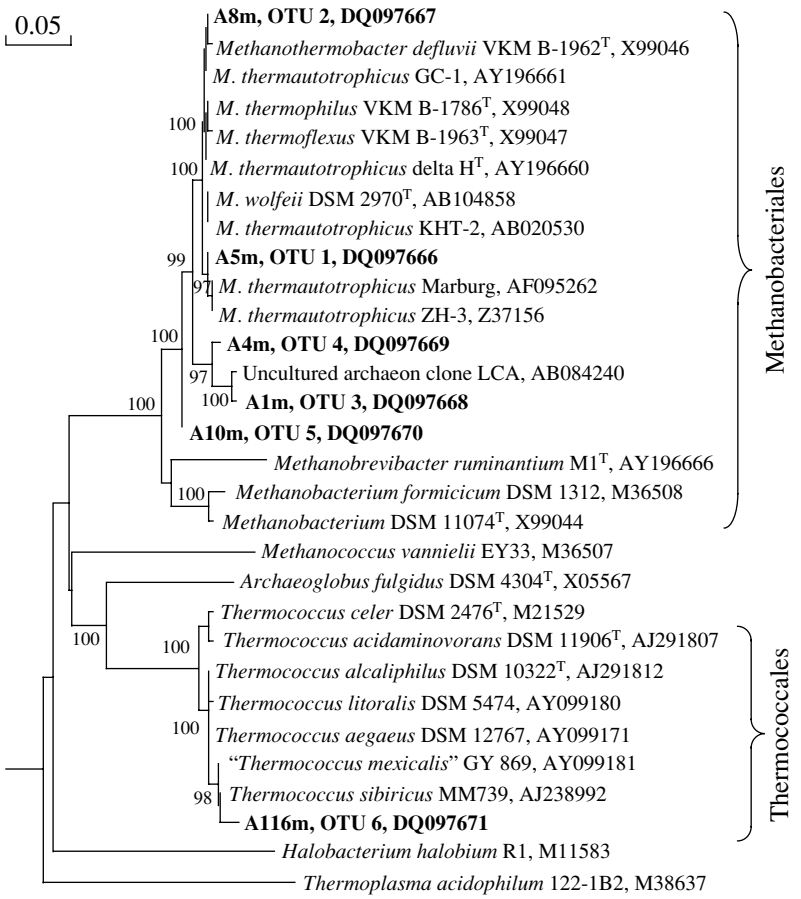

Fig. 2. Phylogenetic tree of 16S rRNA genes of archaea, found in enrichment cultures of methanogens of the Kongdian bed of the Dagang oil field. The scale shows the evolutionary distance corresponding to the 5 base replacements for every 100 bases. Numbers are values of the reliability of embranchment, determined using "bootstrap" analysis of 100 alternative trees (values in excess of 90% are significant).

97.5% similarity), representatives of which were also isolated from thermal ecosystems. Clones B10, B13, B17, and B2 (OTU 14) constituted a separate branch inside the order Thermotogales.

Sulfate-reducing bacteria constituted another important metabolic group, widely represented among bacterial phylotypes. 16S rRNA genes of these sulfate-reducing bacteria (clones B19, B15, and B7) belonged to a monophyletic group of the genus *Thermodesulfovibrio* inside the branch delta-Proteobacteria (OTU 15). Moreover, phylotypes of representatives of the order Planctomycetales (clones B21, OTU 16) were also found in hot springs.

Among the information obtained as a result of the analysis of the bacterial clone library, of key importance was the identification of a phylotype related to the bacterium *Thermacetogenium phaeum* (which was isolated previously from a sewage-purifying methanogenic reactor) [23]. This bacterium is capable of syntrophic degradation (with H_2-utilizing methanogens) of acetate to methane. The process of methane production in such binary association consists of two reactions. Oxidation of acetate to H_2 and CO_2 occurs in the first reaction. Hydrogen is then converted to methane in the second reaction with the participation of the second microorganism. The forward reaction is thermodynamically disadvantageous under standard conditions, similar to the cases of anaerobic syntrophic oxidation of fatty acids. Syntrophic degradation of acetate is possible only under the conditions of combined growth of acetate-oxidizing and hydrogen-utilizing microorganisms. The bacterium *T. phaeum*, which is capable of growing on acetate, reducing sulfates, and producing hydrogen sulfide, behaves like a typical sulfate-reducing bacterium.

In this work, 16S rRNA gene of a bacterium related to *T. phaeum* (the acetate-oxidizing component of syntrophic binary associations) was identified in subsurface environments for the first time.

Phylogenetic analysis of archaeal 16S rRNA genes represented in the microbial community of the formation water of Kongdian area. In order to check the results obtained on the diversity of methanogens in enrichment cultures from the Dagang oil field,

Table 2. Phylogenetic diversity of 16S rRNA genes of methanogen cultures from the Kongdian bed of the Dagang oilfield

Domain, order, phylogenetic group	Type clone	Phylogenetically close organisms or Genbank clones	Similarity of sequences	Quantity of related OTU clones	Operation taxonomic unit (OTU)
Archaea					
Methanobacteriales	A5m	*M. thermautotrophicus*	99	31	OTU 1
	A8m	*M. thermautotrophicus* GC-1	99	21	OTU 2
	A1m	Non-cultivated archaea, clone LCA and clone Ou2I-42 (*M. thermautotrophicus*)	99	45	OTU 3
	A4m	*M. thermautotrophicus* GC-1, clone from the enrichment culture E21A1 (*M. thermautotrophicus*)	97	1	OTU 4
	A10m	*M. thermautotrophicus* GC-1	99	3	OTU 5
Thermococcales	A116m	*Thermococcus sibiricus*	99	1	OTU 6
Bacteria					
Thermoanaerobacteriales	B6	Gram-positive bacteria SA-5 with a low G + C value (*Thermanaeromonas toyohansis* ToBE^T)	97	1	OTU 7
	B20	*Thermoanaerobacter ethanolicus* (ATCC 33223)	99	1	OTU 8
	B9	*Coprothermobacter* sp. P1	97	2	OTU 9
	B5	*Thermovenabulum ferriphilus*	92	1	OTU 10
	B14	*T. ferriphilus*	93	1	OTU 11
	B8	*T. ferriphilus*	92	1	OTU 12
	B11	*Thermacetogenium phaeum* PB^T	95	3	OTU 13
Thermotogales	B17	Non-cultivated Thermotogales, clone W28	97	4	OTU 14
Nitrospirales	B19	Non-cultivated bacteria, clone GAB-B04 (*Thermodesulfovibrio* sp. TGE P1)	97	3	OTU 15
Planctomycetales	B21	Unknown Planctomycetales, OPB17	99	1	OTU 16

we collected a sample of formation water from well 1066-1 (situated on the Kongdian bed in a region hydrodynamically isolated from the zone of testing of the biotechnology for enhancement of oil recovery). The formation water was used for extracting DNA and subsequent molecular-biological studies. We constructed another library (A2), comprising 452 archaeal clones. The size of the inserts of archaeal 16S rRNA gene was about 1000 bp.

16S rRNA genes from the first 60 archaeal clones were sequenced. The phylogenetic analysis demonstrated that 55 out of the 60 sequences obtained were more than 99% similar to the 16S rRNA gene of the methanogen *M. thermautotrophicus*. Using a special program (MegAlign DNA), we constructed the primer M400r (5'-GAAAAGCCACCCCGTTAAGA-3') specific for this species to simplify the procedure for clone identification by PCR.

Clones from the archaeal library (397) were analyzed, using PCR with the primers M400r and A109f, for the presence of *M. thermautotrophicus*. The majority of the clones (343) gave a positive signal, indicating that they belonged to the species *M. thermautotrophicus*. The rest of the clones (those that did not give the signal with the primers used) did not belong to this species. The nucleotide sequences of these clones were thoroughly analyzed and proved to be chimeric; they did not resemble the 16S rRNA gene. Thus, the molecular-biological analysis of the formation water of the Kongdian bed confirmed the study results of the enrichment cultures of methanogens and demonstrated absolute domination of the of H_2-utilizing methanogens (*M. thermautotrophicus*) genes among the archaeal clones.

DISCUSSION

The study of biodiversity, number, and the activity of microorganisms of the high-temperature horizons of the Kongdian bed of the Dagan oil field, which was performed using cultural, radioisotopic, and molecular-biological methods, revealed a geochemically active community represented by diverse bacteria. Microorganisms of different metabolic groups (such as fermentative anaerobes), sulfate-reducing bacteria, and methanogens) were found in the microflora. The rates of contemporaneous biogenic processes of sulfate reduction and methanogenesis were estimated by radioassays; it was shown that these rates were comparable to those measured in other high-temperature oil strata (such as Mykhpaiskoe, Talinskoe and Samotlor in Western Siberia, and the Chinese Liaohe) exploited

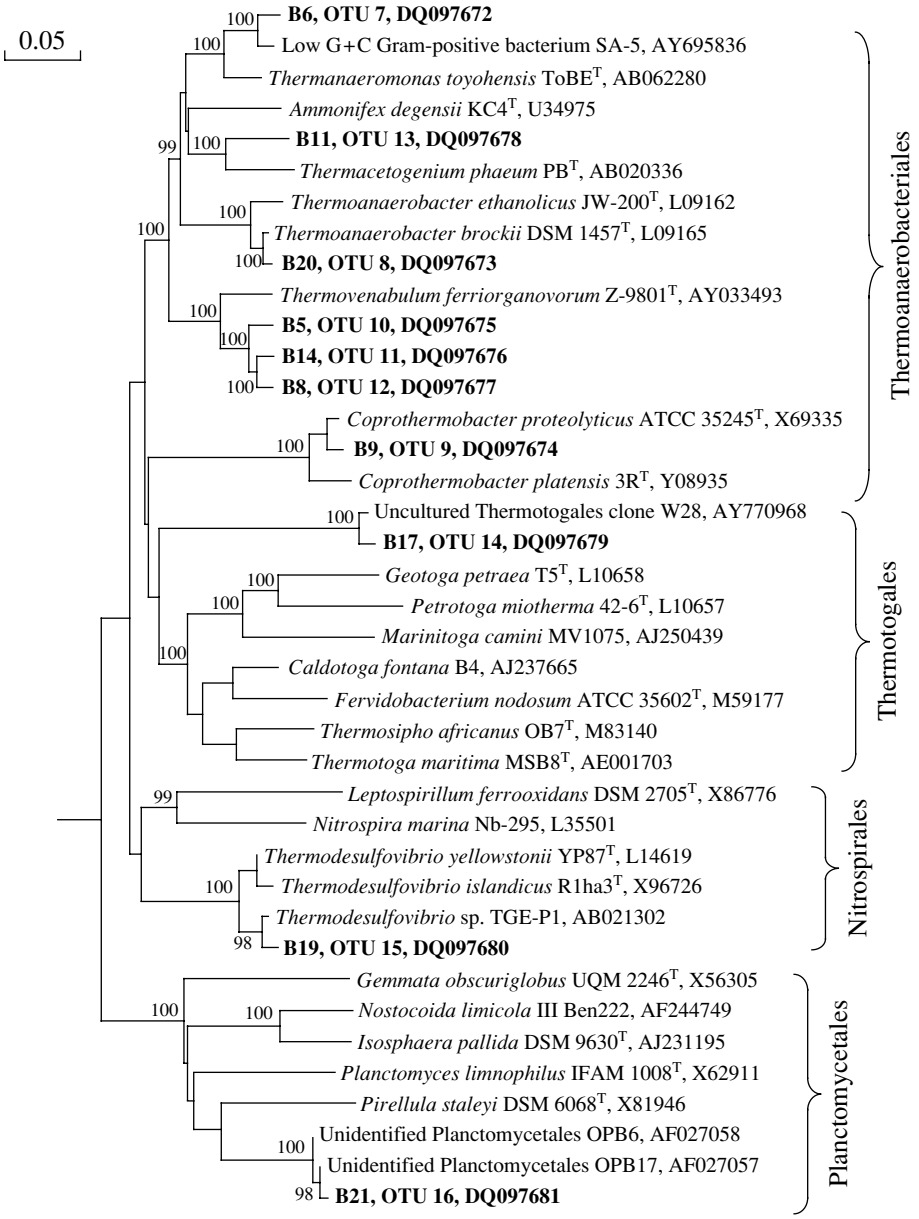

Fig. 3. Phylogenetic tree of bacterial 16S rRNA genes found in the enrichment cultures of methanogens of the Kongdian bed of the Dagang oil field. The scale shows the evolutionary distance corresponding to 2 base replacements for every 100 bases. Numbers are values of the reliability of embranchment, determined using "bootstrap" analysis of 100 alternative trees (values in excess of 90% are significant).

using water flooding [3, 6, 7, 15]. Significant differences in the rates were observed between the zones of the Kongdian bed, into which nitrogen and phosphorus salts were injected and acetate and other volatile acids produced in the course of oil biodegradation were present.

Methanogens were found in media containing acetate and hydrogen–carbon dioxide mixture using cultural methods. Methanogenesis from both $^{14}CH_3$-COONa and $NaH^{14}CO_3$ was detected using radioisotopic methods also.

It was shown by molecular-biological methods that thermophilic bacteria and archaea were present in the primary cultures of methanogens. Analysis of more than 550 clones of archaea demonstrated the presence of nucleotide sequences of 16S rRNA genes of *M. thermautotrophicus* (of the order Methanobacteriales), which uses $H_2 + CO_2$ as the main substrate for methanogenesis. The phylotypes of methanogens capable of utilizing acetate were not found. Among the archaeal clones, 16S rDNA of *Thermococcus sibiricus*, a hyperthermophilic species with an fermentative type of metabolism, was found. Bacterial clones belonged to the orders Thermoanaerobacteriales (the genera *Thermoanaerobacter*, *Thermovenabulum*, *Coprothermobacter*, and *Thermacetogenium*), Thermotogales,

Nitrospirales (the genus *Thermodesulfovibrio*), and Planctomycetales. Note that all members of the order Thermotogales produce acetate during growth, which in turn may serve as a substrate for syntrophic microorganisms. The 16S rRNA gene of a bacterium closely related to *Thermacetogenium phaeum*, which was capable of oxidizing acetate during syntrophic growth with H_2-utilizing methanogens, was found in high-temperature oil reservoirs for the first time.

Thus far, all pure cultures isolated from different (geographically separated) high-temperature oil fields have been represented by hydrogen-utilizing methanogens of the genera *Methanothermobacter*, *Methanococcus*, and *Methanoculleus* [2, 3, 12]. Bacterial consortia from high-temperature oil reservoirs of Alaska (Kuparuk) and the North Sea (Ninian) also produced methane from H_2 + CO_2, but not from acetate [24]. Acetate was found in oil strata of the North Sea in concentrations reaching 20 mM [25]; methane production was observed in acetate-containing enrichment cultures at 70, 80, or 92°C (note that pure cultures consuming acetate were not isolated) [12]. Hydrogen-utilizing (but not acetoclastic) methanogens dominated in the Samotlor oil field [15].

Results of studies of methanogens from high-temperature oil fields, obtained by other authors [9, 12, 24], together with our molecular-biological data on the Dagan oil field suggest that acetate is degraded not by methanogens directly, but rather, in syntrophic associations with an acetate-oxidizing component. The identification of 16S rRNA gene of a bacterium closely related to *T. phaeum* confirms this assumption.

We believe that bacterium *T. phaeum* and microorganisms with a similar type of metabolism (e.g., sulfate-reducing bacteria of the genus *Desulfotomaculum*), which may use acetate and other fatty acids under the conditions of sulfate deficiency in strata, are capable of triggering syntrophic growth using H_2-utilizing methanogens as biological acceptors of electrons. The prevalence of the order Methanobacteriales phylotypes in the association studied led us to conclude that thermophilic H_2-utilizing methanogens dominated in the subsurface ecosystem of the Kongdian bed.

Thus, phylotypes of the thermophilic bacterial association of the Kongdian bed belonged to four main metabolic groups of microorganisms: methanogenic archaea, bacteria and archaea with a fermentative type of metabolism, sulfate-reducing, and syntrophic bacteria. These results expand the known concept of the composition of the bacterial community of oil reservoirs and provide evidence of the important role of syntrophic processes of the destruction of acetate, associated with methane production.

The phenomenona of (1) the absence of acetate-utilizing methanogens in samples of formation water and enrichment cultures, obtained from the Dagan oil field, and (2) syntrophic degradation of acetate by acetate-oxidizing bacteria and hydrogen-utilizing methanogens call for further studies (to be conducted in other high temperature oil reservoirs).

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