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Chapter 1

Microbial diversity and syntrophic acetate degradation to methane in a high-temperature petroleum reservoir

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Abstract

The results of our investigation on the microbial community of the high-temperature Dagang oilfield (P.R. China) are summarized. Detailed experimental data are provided on syntrophic acetate degradation by thermophilic associations, on the isolation of pure cultures from these associations, their physiological characteristics, and reconstruction of microbial interactions during acetate degradation to methane. The microbial community of the high-temperature Dagang oilfield was investigated by culture-based, radioisotope, and 16S rRNA gene techniques. Cultivable microorganisms (aerobic oil-oxidizing, anaerobic fermentative, sulfate-reducing, and methanogenic) were found in formation water. Methanogenic enrichments were obtained in media both with H_2+CO_2 and acetate. The process of methane production in formation waters was also registered by radioisotope methods with $Na_2{}^{14}CO_3$ and $^{14}CH_3COONa$. However, pure cultures of thermophilic aceticlastic methanogens were not obtained. The 16S rRNA gene analysis of the formation water and

methanogenic enrichments revealed that H_2-utilizing methanogens of the genus *Methanothermobacter* were predominant in the archaeal libraries (97% of archaeal clones). Phylotypes of acetate-utilizing methanogens were detected in the libraries only after the acetate content in formation water was increased. Bacterial phylotypes belonged to the known fermentative (*Thermoanaerobacter*, *Thermoterrabacterium*, *Thermovenabulum*, *Fervidobacterium*, *Thermotoga*, *Dictyoglomus*, *Pedobacter*, and *Dysgonomonas*), sulfate-reducing (*Thermodesulfovibrio* and *Desulfotomaculum*), and syntrophic (*Thermacetogenium* and *Thermovirga*) bacteria. Pure cultures of *Methanothermobacter* sp. and *Thermoanaerobacter ethanolicus* were isolated from thermophilic methanogenic enrichments grown on acetate. The binary culture combining both strains carried out the reaction of syntrophic acetate degradation to methane. The data obtained show that syntrophic associations may contribute significantly to methane production in high-temperature petroleum reservoirs. One of important ecological function of fermentative bacteria of the *Thermoanaerobacter–Caldanaerobacter* group is their ability to carry out syntrophic acetate oxidation to H_2 and CO_2, which is then reduced to methane by hydrogenothrophic methanogens.

Keywords: high-temperature petroleum reservoirs, thermophiles, 16S rRNA gene clone library, methanogenesis, syntrophic acetate degradation

1. Introduction

The presence of microorganisms in petroleum reservoirs has been established about 100 years ago. Anaerobic microorganisms reducing sulfate, thiosulfate, Fe(3+), or elemental sulfur, as well as fermentative bacteria, acetogens, and methanogens have been isolated from petroleum reservoirs [1]. The aerobic bacteria isolated from water-flooded oilfields are considered contaminants which arrived from the surface in the course of drilling or with injected water. Abundant data are available concerning the microorganisms of low-temperature (20–45ºC) petroleum reservoirs. The composition of microbial communities in high-temperature reservoirs and methanogenesis under such conditions have been less extensively studied [2-9].

Methanogenesis in oilfields was first reported by Kuznetsov [10]. Belyaev and coworkers isolated the first pure methanogenic cultures from petroleum reservoirs [11-14]. Methanogens oxidizing hydrogen in the course of CO_2 reduction to methane are common in the oilfields with temperatures of 20 to 80°C. Thermophilic H_2-uilizing methanogens isolated from high-temperature petroleum reservoirs of Western Siberia, the North Sea and California belonged to species *Methanothermobacter thermautotrophicus* (previous name *Methanobacterium thermoautotrophicum*), *Methanothermococcus thermolithotrophicus* (previous name *Methanococcus thermolithotrophicus)* and *Methanothermobacter thermoaggregans* (previous name *Methanobacterium thermoaggregans)*. As to organotrophic methanogens, which utilize methanol, acetate, or methylated amines and are often capable of growth on H_2+CO_2, they have been isolated only from oilfields with the temperatures below 50ºC [1,13,14]. However, in the oilfields with the temperatures ranging from 60 to 80°C, methane production from both

bicarbonate and acetate was detected by radioisotope methods [4,6,15-17]. It is an indication of the presence of thermophilic prokaryotes performing acetate degradation to methane in high-temperature oilfields.

Davydova-Charakhch'yan and coauthors [13] were the first to show syntrophic associations to be responsible for methanogenic acetate decomposition in high-temperature oilfields and to isolate the terminal component of this association, a lithotrophic methanogen *M. thermautotrophicus* (formerly *M. thermoalcaliphilum)*. Nilsen and Torsvik [18] observed methane formation in enrichment cultures with acetate at the temperatures of 70, 80, and 92°C, but attempts to isolate acetate-utilizing methanogens were also unsuccessful.

The first molecular studies of microbial diversity of high-temperature oilfields based on 16S rRNA gene analysis were carried out in California [2,3], Western Siberia [4], and China [5,19,20]. Formation water was found to contain 16S rRNA genes of the known thermophilic (*Thermococcus, Thermotoga, Petrotoga, Thermoanaerobacter, Methanothermobacter, Methanococcus*, and *Methanoculleus*) and mesophilic microorganisms, as well as of uncultured archaea and bacteria. In the 16S rRNA gene clone library from a petroleum reservoir in California, the 16S rRNA gene of a novel, probably aceticlastic methanogen of the order *Methanosarcinales* was revealed [2]. Pure cultures of thermophilic aceticlastic methanogens have not yet been isolated from oilfields.

Microbiological, radioisotope, molecular, and biogeochemical techniques have been used to investigate microbial diversity and activity in the oilfields. In most works, these techniques were applied separately; the structure of the microbial community as a whole was therefore not revealed and its geochemical activity was not assessed properly.

The problem of energy recovery from oilfields in the form of methane, based on accelerating natural methanogenic biodegradation is widely discusses in literature. At the high-temperature Dagang oilfield in China, trails of a biotechnology for enhanced oil recovery based on injection of a water-air mixture and of nitrogen and phosphorus mineral salts were carried out [6,7,21,22]. As a result of the trial, 36 thousand tons of additional oil was recovered. Moreover, fundamental studies of the distribution and activity of microorganisms were carried out. When studying the 16S rRNA genes clone library of methanogenic enrichments growing in the medium with acetate, we did not find any phylotypes of aceticlastic methanogens [6,23]. These data did not agree with the numerous results of radioisotope analysis which confirmed formation of $^{14}CH_4$ from $^{14}CH_3COONa$. It was suggested that syntrophic associations were responsible for acetate degradation to methane in the oilfield.

In our previous studies [19,23-25], for the first time pure cultures of both an acetate-oxidizing bacterium (*Thermoanaerobacter ethanolicus*) and an H_2-utilizing methanogen (*Methanothermobacter* sp.) were isolated from thermophilic syntrophic methanogenic enrichments

grown on acetate. For the reconstruction of syntrophic growth on acetate, pure cultures of the methanogen (*M. thermautotrophicus* or *M. wolfeii)* and the acetate-oxidizing bacterium (*T. ethanolicus*) were combined. The binary *Methanothermobacter-Thermoanaerobacter* culture was found to degrade acetate to methane. Methane formation by combined cultures was observed in their subsequent transfers to fresh acetate-containing medium. A range of results of this study was not presented in detail [25].

The aim of our work was to review our data on syntrophic degradation of acetate by thermophilic microorganisms and to present detailed experimental data on the characterization of the microbial community of the high-temperature Dagang oilfield (P.R. China) and on isolation of pure cultures of microorganisms from thermophilic methanogenic enrichments growing on acetate, studying their physiological characteristics and reconstruction of microbial interactions during syntrophic acetate degradation to methane.

2. Materials & Methods

2.1 Reservoir Description and Sample Collection

The Dagang oilfield is situated in the Hebei province (P.R. China). The studied sandstone oil-bearing horizons of the Kongdian bed were located at the depth of 1206–1435 m below sea level; the temperature was 59°C. The natural formation water had a low salinity (5612 mg L^{-1}) 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%) [5].

Production fluids were collected directly from the production wells into sterilized bottles and immediately prepared for chemical analyses, radioisotope and culture studies. The bottles were filled completely with the oil-water-gas mixture and stored at 4°C prior to analyses. For DNA isolation, water was separated from crude oil by decantation at room temperature. Triton X100 (0.1%) and *n*-hexane were added to the samples. The water phase after hexane extraction was fixed with ethanol (1:1 vol/vol) and kept at 4°C prior to analyses.

2.2 Culture Media and Growth Conditions

A technique for cultivation of strictly anaerobic microorganisms [26] was applied for the enumeration of microorganisms. The numbers of cultivable anaerobic bacteria were estimated by serial decimal dilutions of water samples in test tubes with various enrichment media designed to promote growth of specific functional groups. Anaerobic bacteria were cultivated in test tubes with pure argon as the gas phase; an exception was the medium with H_2/CO_2 for methanogens. Anaerobic organotrophic bacteria with a fermentative type of metabolism were assayed by detection of the H_2 in the highest dilution in the medium with peptone (4 g L^{-1}) and glucose (10 g l^{-1}). Sulfate-reducing bacteria were detected by measuring an increase in sulfide

content in medium B containing sodium lactate (4 g L^{-1}) and reduced with $Na_2S \times 9H_2O$ (0.2 g L^{-1}) [27]. Methanogens were determined by measuring an increase in CH_4 in the media [28] with acetate (2.2 g L^{-1}) or H_2/CO_2 (80:20 % vol/vol), supplemented with $Na_2S \times 9H_2O$ (0.5 g L^{-1}), microelements and yeast extract (1 g L^{-1}). Descriptions of the media were given earlier [4].

Aerobic bacteria were cultivated in Hungate tubes with air as the gas phase. Viable aerobic organotrophs were enumerated in the medium composed of bacto-tryptone (5.0 g L^{-1}), yeast extract (2.5 g L^{-1}), glucose (1.0 g L^{-1}), and distilled water (1 L, pH 7.0). All media were inoculated with samples of formation water using syringes and were incubated at 60° for 30 days. Samples from all the tubes were examined by phase-contrast microscopy.

The content of microbial metabolites (sulfide, CH_4, CO_2, and H_2) in the media and the chemical composition of the formation water were analyzed as described previously [4].

2.3 Isolation and Identification of Pure Cultures

Methanogenic enrichments were obtained by inoculation of mineral medium [28] with various substrates and inhibitors: acetate (40 mM); acetate with ampicillin (2 mg mL^{-1}), acetate with 2-bromo-ethane sulfonate (10 mM); H_2/CO_2; and H_2/CO_2 with ampicillin, methanol (5 mL L^{-1}), propionate (20 mM) or/and benzoate (20 mM).

Pure cultures of methanogens were obtained in a liquid medium [28] supplemented with ampicillin (2 mg mL^{-1}), with H_2/CO_2 mixture as the carbon and energy source. Mineral medium [28] supplemented with sodium pyruvate (40 mM) was used for isolation of anaerobic bacteria from methanogenic enrichments growing on acetate. The isolates were identified by 16S rRNA gene sequencing as described below.

The isolates of H_2-utilizing methanogens (strains KZ3-1 and KZ24a) and members of the genus *Thermoanaerobacter* (strains 1017-7b and 1017-7d) were incubated separately or in binary cultures in the medium [28] with sodium acetate (80 mM) at 60°C. Methane formation was determined by gas chromatography after 14 days of cultivation. The acetate-grown enrichment 24A was used as the positive control.

2.4 Radioisotope Methods

The rates of sulfate reduction and methanogenesis in formation waters were determined by radioisotope methods using labelled $Na_2{}^{35}SO_4$, $^{14}CH_3COONa$, and $Na_2{}^{14}CO_3$ as described previously [4]. Radiotracer analysis was used to determine the rates of methane formation from 2-$^{14}CH_3COONa$ by a binary culture of *Methanothermobacter* sp. KZ3-1 and *Thermoanaerobacter* sp. 1017-7b. The strains were incubated separately (controls) and together in the mineral medium [28] in the presence of 2-$^{14}CH_3COONa$ (70 μg/L) and unlabeled CH_3COONa

(200 mg L^{-1}). The experiment was carried out for 24 h at 60°C. The rate of methane formation was calculated according to the equation: I=r·C/(R·T), where r is the overall count for 60 s, C is the substrate concentration (μg L^{-1}), R is activity of the labeled 2-$^{14}CH_3COONa$ (MBq mL^{-1}), and T is the incubation time (24 h).

2.5 Construction and Analysis of 16S rRNA Gene Clone Libraries

DNA Extraction. Total DNA was extracted from formation water and cultures using DiatomTM DNAprep kit ("BioKom", Russia). Microbial biomass from approximately 2 liters of the water phase of production fluids was collected by centrifugation (Beckman JA10, 8000 g, 1 h). The cell pellet was suspended in MilliQ water and was twice frozen with liquid molecular nitrogen and thawed (at 65°C). The cell lysate was then supplemented with guanidine hydrochloride and incubated at 65°C for 1 h. The sorbent (Diatomid/silica) was added to the cell lysate. After DNA sorption, the supernatant was removed and the adsorbent was washed using a buffer (pH 7.0) and 70% (vol/vol) ethanol. The total DNA preparation was dissolved in MilliQ water and used for 16S rRNA gene amplification.

16S rRNA Gene Amplification and Cloning. 16S rRNA genes were amplified by PCR using primers specific for *Bacteria* (8-27f [5'-AGAGTTTGATCCTGGCTCAG-3'], 519r [5'-G(T/A)ATTACCGCGGC(T/G)GCTG-3'] and 1492r [5'-TACGGYTACCTTGTTACGACTT-3']) [29], and *Archaea* (A109F [5'-ACG/TGCTCAGTAACACGT-3'], A1041r [5'-GGCCATGCACCWCCTCTC-3']) [30]. The final 50-μl reaction mixture contained 5 μl of template DNA, 0.5 μM of each primer, 1× DNA polymerase buffer, 2.5 mM MgCl2, 0.1 mM of each deoxynucleoside triphosphate, and 1 U of Taq polymerase (Perkin-Elmer). Polymerase chain reaction cycles were performed on a Mastercycler (Eppendorf, Germany) as follows: after 5 min of initial denaturation at 94°C, nucleic acids were amplified for 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 30 s – 1.5 min of elongation at 72°C) followed by a final extension step at 72ºC for 8 min. Archaeal 16S rDNA were amplified at 35 cycles of PCR. PCR products were checked on 0.8% (wt/vol) agarose gel stained with ethidium bromide. Amplicons were cloned with a pGEM-T (pGEM-T Easy Vector Systems, Promega, USA) according to the manufacturer's instructions.

As a result of the PCR analysis, 785 archaeal and 503 bacterial clones were selected.

Sequencing and Phylogenetic Analysis. Inserts of selected clones were amplified by PCR with T7 and SP6 plasmid primers. The same primers were subsequently used to sequence bacterial PCR products. Archaeal PCR products were sequenced with the A109f primer. The sequencing was performed on an ABI 3100 Avant Genetic Analyzer with the BigDye Terminator V3.1 (Applied Biosystems, USA).

Sequence data were aligned using the CLUSTALW v.1.75 package [31], with clones having similarities of 97% or above grouped into operational taxonomic units (OTUs). The

clones were homology-searched using BLAST and the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov). Chimeras were detected using the CHIMERA-CHECK program from the Ribosomal Database Project (http://rdp.cme.msu.edu). The sequences were edited using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/ BioEdit/bioedit.html).

Nucleotide Sequence Accession Numbers. The sequences identified in this study were submitted to the GenBank database under the following accession numbers: DQ657903, and DQ657904 (pure cultures), DQ097666–DQ097668, DQ097671, FJ898357–FJ898360, and FJ898362–FJ898364 (clones).

2.6. Design of Specific Primers

To analyze the predominant phylotypes in the clone libraries from formation water, we designed two specific primers [25]. Primers M400r (GAAAAGCCACCCCGTTAAGA) and Sph196r (CTCGGCGATAAATCTTTGGAC) were developed to detect *Methanothermobacter* sp. and *Sphingomonas* sp., respectively. The primers were designed using the BioEdit and Lasergene v. 5.06 software packages. Melting temperature was analyzed with Oligo v.6 program and used in the PCR procedure. The specificity of the primers was then tested by using DNA extracts from strains and clones with 16S rDNA inserts representing different species. Each specific primer gave positive PCR results for the corresponding target 16S rDNA and negative PCR results for nontarget 16S rDNA.

3. Results

3.1. Physicochemical characteristics and microbial processes in formation water of the Dagang oilfield

The natural formation water of the Kongdian bed of the Dagang oilfield belonged to the sodium hydrocarbonate type; it had low salinity (5.6–6.7 g L^{-1}), was slightly alkaline (pH 7.1–7.6), and contained less than 5 mg of acetate and 433–670 mg of hydrocarbonate per 1 L (Table 1, well 1066-1). Sulfate concentration did not exceed 26 mg L^{-1}. H_2S was not detected.

Since 1975, the North block of the Kongdian bed of the Dagang oilfield was exploited with the water-flooding method for the enhancement of oil recovery. In 2001–2007, MEOR (microbial enhancement of oil recovery) biotechnology was applied at the North block. The biotechnology involved injection of a water-air mixture with dissolved nitrogen and phosphorous mineral salts for the stimulation of the indigenous microorganisms. During the periods of biotechnological treatment, bicarbonate content in formation waster increased from 0.4–0.6 to 0.7–1.8 g L^{-1}, sulfate, from 0 to 12–72 mg L^{-1}, acetate, from 5 to 160.7 mg L^{-1}, formate, from 0 to 67.4 mg L^{-1}, and *iso*-butyrate, from 0 to 98.2 mg L^{-1} [5,6,25]. The chemical characteristics of formation waters in the course of the biotechnological treatment (January, 2002) are given

in Table 1.

In waters of the North block the number of cultivable fermentative bacteria varied from 10 to 10^7 cells mL^{-1}, of sulfate reducers, from 0 to 10^7 cells mL^{-1}, and of methanogens, from 0 to 10^4 cells mL^{-1} (Figure 1a, January, 2002). Aerobic bacteria were present in low numbers in the zone of production wells. The numbers of methanogens obtained on media with H_2+CO_2 were usually 10 times higher than those obtained on media with acetate. Microscopic analysis revealed a dominance of rod-shaped cells growing on both media for methanogens.

The rates of anaerobic processes in formation waters. Thermophilic sulfate reduction and methanogenesis were registered in the formation waters of the Dagang oilfield (Table 1). In January 2002, the rate of sulfate reduction was in the range 0.06–205.8 μg S^{2-} L^{-1} day^{-1}. The rates of methanogenesis from labeled carbonate and 2-^{14}acetate ranged from 0.037 to 6.776 and from 0.002 to 3.16 μg CH_4 L^{-1} day^{-1}, respectively. The maximal values of methanogenesis rates from labeled carbonate and acetate in waters from production wells of the Kongdian bed for the period 06.2001–12.2007 are given in Figure 1b.

3.2. Archaeal 16S rRNA Gene Clone Libraries from Formation Waters

The archaeal clone libraries obtained from the DNA of methanogenic enrichments and formation water collected outside (well 1066-1) and inside the zone of the biotechnological treatment (North Block), contained 785 clones (Table 2, Figure 2).

Our preliminary study revealed predominance of the members of the genus *Methanothermobacter* in combined first generation methanogenic enrichments [5]. Screening of the clones using primers M400r and A109f, as well as direct DNA sequencing of the clones revealed that methanogens of the order *Methanobacteriales* predominated in these communities (97% of all archaeal clones); H_2/CO_2 is the main growth substrate for these organisms. During the trial of the MEOR technology, acetate content in formation water reached 65–160 mg L^{-1} [5,6]. During that period, in the archaeal library from the production waters (785 clones) the phylotypes related to organotrophic methanogens *Methanosaeta thermophila* (3 clones), *Methanomethylovorans thermophila* (2 clones), and uncultured members of the order *Methanomicrobiales* (2 clones) were detected. The results of phylogenetic analysis of archaeal 16S rRNA gene sequences are presented on Figure 2.

3.3. Bacterial 16S rRNA Gene Library from Formation Waters

Bacterial 16S rRNA gene library constructed using the DNA of formation water from the zone of the biotechnological treatment (North Block) contained 453 clones (Figure 3). Preliminary sequencing revealed predominance of bacteria of the genus *Sphingomonas*. Screening of the clones using the *Sphingomonas*-specific primer revealed 333 clones belonging to

this genus. Sequencing of the DNA of the remaining bacterial clones revealed 30 phylogenetic groups belonging to *Proteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. Representatives of the genera *Sphingomonas*, *Afipia*, *Labrys*, *Phyllobacterium*, *Bradyrhizobium*, *Variovorax*, *Curvibacter*, *Polaromonas*, *Thauera*, *Hydrogenophilus*, *Delftia*, *Leptothrix*, *Achromobacter*, and *Pseudomonas* were revealed in the microbial community. Members of these genera are mesophilic aerobic organotrophs, some bacteria (*Thauera*, *Hydrogenophilus*) are able to grow by nitrate reduction; they probably arrived into the reservoir with cooled injected water. The group of clostridia comprised thermophilic bacteria with fermentative metabolism (*Thermoanaerobacter*, *Thermoterrabacterium*, *Thermovenabulum*), sulfate reducers (*Thermacetogenium*, and *Desulfotomaculum*), and syntrophic bacteria (*Thermovirga*). Thermophilic bacteria with fermentative metabolism belonging to other phylogenetic subdivisions (*Fervidobacterium*, *Thermotoga*, *Dictyoglomus*, *Pedobacter*, and *Dysgonomonas*) were also revealed. Bacteria of the genera *Fervidobacterium* and *Thermotoga* are common in high-temperature oilfields of France, United States, Africa, Japan, and Western Siberia [3,4,32-35]. The phylotype of a sulfate-reducing bacterium *Thermodesulfovibrio* (*Nitrospirae*) was also found in the community. A related phylotype was reported in oilfield formation water [36] and in methanogenic enrichment [5].

3.4. Characterization of the Thermophilic Association Producing Methane from Acetate

Methane formation was observed on the mineral medium [28] with various substrates (acetate, propionate, butyrate, ethanol, or benzoate) inoculated with formation water. Amendment of the medium with ampicillin and/or penicillin resulted in inhibition of growth and methanogenesis from acetate (Figure 4). After treatment with 2-bromo-ethane sulfonate (BES), a specific inhibitor of methanogenesis, methane formation also ceased. These findings demonstrated that both archaeal and bacterial components of the enrichments were necessary for acetate degradation and methane production. Microscopy of methanogenic enrichments growing on acetate revealed rod-shaped cells of different sizes. No cells were found resembling the acetate-utilizing methanogens of the genera *Methanosarcina* or *Methanothrix* (formerly *Methanosaeta*). An enrichment, which exhibited steady methane formation for 3 years, was able to produce $^{14}CH_4$ from $^{14}CH_3COONa$. Addition of unlabeled CH_3COONa (200 mg L^{-1}) resulted in a 4000-fold increased methanogenesis rate (from 0.22 to 880.4 μg CH_4 L^{-1} day^{-1}).

3.5. Isolation of Pure Cultures of Anaerobic Microorganisms from Methanogenic Enrichments

From first-generation methanogenic enrichments grown on media with acetate and H_2+CO_2, two pure cultures of H_2-utilizing methanogens were isolated (strains KZ24a and KZ3-1, respectively). Strain KZ24a was most closely related to the species *Methanothermobacter wolfeii* (DSM 2970, AB104858); another strain, KZ3-1, to *Methanothermobacter*

thermautotrophicus (Delta H, AE000666) (99% similarity of 16S rRNA genes). The 16S rRNA gene of the strain KZ3-1 contained the signature nucleotides (at positions A164, A166, and C188) characteristic of *M. thermautotrophicus* Delta H, and the gene of the strain KZ24a contained the signature nucleotides (C195, A233, C261, T1218, T1274 и T1314) characteristic of *M. wolfeii* [37].

The cells of strains KZ3-1 and KZ24a were nonmotile straight rods with sizes of 0.35–0.5×3–7 μm and 0.35–0.5×2–5 μm, respectively. Both strains grew within the temperature range from 37 to 65°C. The optimum growth temperatures for strains KZ3-1 and KZ24a were 60 and 65°C, respectively. Salinity optimum for both strains was 0–1.0% NaCl; higher NaCl concentrations (1.5–4%) inhibited methane formation. Both strains utilized H_2/CO_2 as the source of carbon and energy; acetate, pyruvate, propionate, methanol, ethanol, and 3-ethylamine were not utilized. Weak growth of strain KZ3-1 occurred on formate. Both strains were resistant to ampicillin and penicillin (2 mg mL^{-1}). Amendment of the medium with BES (2–10 mM) inhibited growth and methanogenesis.

Attempts to isolate thermophilic acetate-utilizing methanogens from enrichment cultures obtained from the Dagang oilfield were unsuccessful.

For isolation of the bacterial component of the thermophilic methanogenic association, which was responsible for acetate oxidation, we used sodium pyruvate as a substrate. Since our previous analysis of the 16S rRNA gene clone libraries obtained from first-generation methanogen cultures [5] revealed a phylotype distantly related to *Thermacetogenium phaeum*, syntrophic acetate degradation was accepted as a working hypothesis. This bacterium is capable of syntrophic acetate degradation to methane in association with H_2-utilizing methanogens. Hattori and co-authors [38] used sodium pyruvate as a substrate for the isolation of *T. phaeum* from a sewage treatment methanogenic reactor. We also used sodium pyruvate, planning to isolate *T. phaeum*.

On media with pyruvate, ten strains of anaerobic rod-shaped motile spore-forming bacteria were isolated from the acetate-utilizing methanogenic association obtained from production well 1017-7. All strains were phylogenetically related to *Thermoanaerobacter ethanolicus* (99.6% similarity of 16S rRNA genes). Two strains, 1017-7b and 1017-7d, were chosen for detailed study. Both strains grew at the temperatures from 40 to 70°C, with an optimum at ~60°C. No growth occurred at 35 and 75°C. Both strains grew at pH range from 5.8 to 8.5 and NaCl concentration in the medium from 0 to 2% with an optimum at 0.5%; higher NaCl concentrations (4–8%) inhibited growth. The strains fermented glucose, fructose, mannose, galactose, ribose, lactose, sucrose, maltose, cellobiose, starch, and pyruvate; growth did not occur on media with acetate, lactate, methanol, ethanol, glycerol, arabinose, raffinose, rhamnose, trehalose, and mannitol. Yeast extract (0.5 g L^{-1}) was required as a growth factor. When

grown with glucose, the strains reduced thiosulfate, sulfite, and sulfur to sulfide; these traits were not previously known for this species. Sulfate, nitrate, and nitrite were not reduced.

3.6. Reconstruction of Syntrophic Acetate Degradation to Methane by the Binary Culture

For the reconstruction of syntrophic growth on acetate (80 мМ), pure cultures of the methanogen *Methanothermobacter* (strains KZ3-1 and KZ24a) and *Thermoanaerobacter* (strains 1017-7b and 1017-7d) were combined. Methane was produced by all four *Thermoanaerobacter – Methanothermobacter* binary cultures (strains 1017-7b + KZ3-1, 1017-7d + KZ3-1, 1017-7b + KZ24a, and 1017-7d + KZ24a), while no individual strain was capable of methanogenesis under these conditions. The experiment with radiolabeled $^{14}CH_3COONa$ confirmed that methane was produced from acetate only by binary cultures (Figure 5a). In this experiment, the methanogenic association 24Ac, which grew steadily on acetate, was used as the positive control. The sulfate-reducing bacterium *Desulfotomaculum kuznersovii* 17^T, which is known to be capable of sulfate-reducing growth on acetate, is often detected in the course of molecular investigation of the microorganisms of high-temperature oilfields. While we expected replacement of sulfate by a biological electron acceptor to result in acetate degradation, the binary culture of *D. kuznetsovii* 17^T and *Methanothermobacter wolfeii* KZ24a was, however, unable to produce methane. Conversion of acetate to methane at 60°C by the *M. thermautotrophicus – T. ethanolicus* co-culture is shown on Figure 5b.

The rates of methane formation from labeled 2-$^{14}CH_3COONa$ by the *M. thermautotrophicus* KZ3-1 – *T. ethanolicus* 1017-7b binary culture and by the 24A enrichment determined in the Zeikus mineral medium supplemented with unlabeled CH_3COONa (200 mg L^{-1}) were similar: 143.75 and 148.34 μg CH_4 L^{-1} day^{-1}, respectively. No formation of methane from 2-$^{14}CH_3COONa$ occurred in pure cultures of strains KZ3-1 and 1017-7b.

3.7. 16S rRNA Clone Gene Libraries from the Methanogenic Association 24A grown on acetate

Two 16S rDNA clone libraries were constructed to examine the phylogenetic diversity of the acetate-degrading methanogenic association 24A. All 96 archaeal clones belonged to one phylotype, *Methanothermobacter thermautotrophicus*. Bacterial clones (46) formed 4 closely related phylotypes belonging to "*Thermoanaerobacter tengcongensis*" MB4 (41 of 46 clones), "*Thermoanaerobacter subterraneus*" (T) SEBR 7858 LA61 (2 clones), *Thermoanaerobacter keratinophilus* 2KXI (2 clones), and *Thermotoga subterranea* SL1 (1 clone). Bacteria "*T. tengcongensis*" and "*T. subterraneus*" are in fact subspecies of the species classified as *Caldanaerobacter subterraneus* [39]. Thus, both H_2-utilizing methanogens of the genus *Methanothermobacter* and anaerobic organotrophic bacteria of the *Thermoanaerobacter – Caldanaerobacter* group are probably responsible for methane formation from acetate in the thermophilic methanogenic association.

4. Discussion

Investigation of formation water from the Dagang high-temperature oilfield by microbiological, radioisotope, and molecular biological methods revealed a diverse, geochemically active microbial community. This community included aerobic organotrophs, as well as anaerobic fermentative bacteria and archaea, sulfate-reducing and syntrophic bacteria, and methanogenic archaea.

In almost sulfate-free waters of the Dagang oilfield, methanogenesis is the main terminal process. Inoculation of formation water into the media with acetate or H_2+CO_2 resulted in methane production. Radioisotope methods also revealed methanogenesis in formation water supplemented with each of the labeled substrates ($^{14}CH_3COONa$ and $Na_2{}^{14}CO_3$). The rates of methanogenesis before the biotechnological treatment were comparable to the values reported for the Mykhpayskoe, Talinskoe, and Samotlor high-temperature oilfields (Western Siberia) and for the Liaohe oilfield (P.R. China), which are also operated with water-flooding [4,5,40]. Injection of the water-air mixture into the oilfield resulted in a significant increase of the rates of methanogenesis from labeled carbonate and from 2-^{14}C-acetate.

These data disagreed with the results of molecular investigation of methanogenic enrichments obtained from the Dagang oilfield [5]. On media with either H_2/CO_2 or acetate, the archaeal component of the enrichments belonged to *Methanothermobacter* sp. (*M. thermautotrophicus*), which consumes hydrogen and carbon dioxide as basic substrates. The genes of acetate-utilizing archaea were not revealed. Phylotypes of *Thermococcus* spp. were also found among archaeal 16S rRNA genes. Bacterial 16S rRNA genes were related to those of the orders *Thermoanaerobacteriales* (*Thermoanaerobacter*, *Thermovenabulum*, *Thermacetogenium*, and *Coprothermobacter* spp.), *Thermotogales*, *Nitrospirales* (*Thermodesulfovibrio* sp.), and *Planctomycetales*. The library was found to contain the 16S rRNA gene with low similarity (95%) to the 16S rRNA gene of *Thermacetogenium phaeum*, a bacterium capable of oxidizing acetate in the course of syntrophic growth with H_2-utilizing methanogens.

Special attention was paid to acetate-utilizing methanogenic associations. Two mechanisms of acetate degradation to methane are theoretically possible. At high acetate concentrations, thermophilic methanogens *Methanosarcina thermophila* and *Methanothrix* (*Methanosaeta*) *thermophila* can carry out direct methane formation by detachment of the methyl group [41,42]. These aceticlastic methanogens, however, have not yet been isolated from high-temperature oilfields.

The mechanism of methane formation from acetate by mesophilic syntrophic associations of an acetate-oxidizing organism and a H_2-utilizing methanogen was originally proposed in 1936 by Barker and Van Niel [see for review 41]. “Oxidation” of acetate to CO_2 and H_2 occurs at the first stage; CO_2 is then reduced to methane with hydrogen. The process of syn-

trophic thermophilic acetate degradation resulting in methane formation was first observed in the course of operating a laboratory reactor for decomposition of lignocellulose-containing solid waste [43,44]. At low acetate concentrations in the system, syntrophic methane production occurs, rather than aceticlastic methanogenesis [42]. Moreover, at high temperature syntrophic acetate oxidation is thermodynamically more preferable [41]. Thermophilic syntrophic methanogenic degradation of propionate, butyrate, and long-chain fatty acids was described. It was shown that the microbes involved in syntrophy have evolved molecular mechanisms to establish specific partnerships and interspecies communication, resulting in efficient metabolic cooperation [45].

Our analysis of archaeal 16S rRNA genes (785 clones) obtained from formation water and methanogenic enrichments of the Dagang oilfield demonstrated that H_2-utilizing methanogens of the order *Methanobacteriales* (*Methanothermobacter* sp., 97% of the total number of clones) predominated in the microbial community. No phylotypes of acetate-utilizing methanogens were revealed in the primary methanogenic enrichments and in the original formation waters. Five phylotypes of methanogens of the order *Methanosarcinales* were found only under considerably increased acetate concentrations in formation water.

The sequences related to the A1m_OTU3 [DQ097668] phylotype of a methanogen from the Kongdian oilfield [5] were subsequently revealed in the course of molecular investigation of gas-associated formation water of a gas-producing well in a natural high-temperature gas reservoir in Japan, as well as in the microbial community of formation water from the high-temperature Shengli oilfield and in enrichment culture derived from this source and producing methane from oil [7,9,46]. Thermophilic hydrogenotrophic methanogens isolated from these environments were assigned to two closely related new species, *Methanothermobacter crinale* and *Methanothermobacter tenebrarum* [7,47]. When analyzing the GenBank data, Cheng and co-authors [7] showed that the phylotypes related to those of A1m_OTU3 and *M. crinale* (GU357468) were also present in the 16S rRNA libraries obtained from various thermophilic anaerobic reactors treating glucose and mixtures of acetate, propionate, and sucrose; propionate and/or acetate; acetate and butyrate, and swine manure; sewage sludge; and a hot-rot compost suspension. The phylotype related to those of *M. crinale* and A1m_OTU3 predominated also in the clone library of the thermophilic crude-oil-degrading methanogenic consortium isolated from the production water of oil reservoirs by Gieg and co-authors [48]; they also suggested that this methanogen could be involved in syntrophic decomposition of crude oil. Syntrophic acetate oxidation as the main methanogenic pathway in a high-temperature Yabase oilfield (Japan) was also shown [49].

In our studies pure cultures of H_2-utilizing methanogens (*M. thermautotrophicus* and *M. wolfeii*) and acetate-oxidizing bacteria (*T. ethanolicus*) were isolated from thermophilic syntrophic methanogenic enrichments. None of the pure cultures was capable of growth on ac-

etate. The binary *Methanothermobacter – Thermoanaerobacter* culture was found to degrade acetate to methane, and its composition remained stable during subsequent transfers to fresh acetate-containing medium. The mesophilic association of *Clostridium ultunense* and an H_2-utilizing methanogen is known to lose its capacity for syntrophic growth after transfers [50]. The thermophilic syntrophic association of a methanogen and *T. phaeum* also retained its ability to form methane from acetate after a series of transfers [38].

Molecular investigation of the new thermophilic methanogenic association 24A, which was maintained on acetate for a long time, revealed that, apart from *Thermoanaerobacter* species (*T. ethanolicus*), *Caldanaerobacter* species (*C. subterraneus*) may also act as an acetate-oxidizing component. *C. subterraneus* uses carbohydrates as fermented substrates, with acetate, L-alanine, H_2, and CO_2 as the major end products [39]. These bacteria are capable of thiosulfate reduction to sulfide. In the presence of thiosulfate, the glucose metabolic profile of *C. subterraneus* changed: acetate production increased and L-alanine was not produced anymore [39]. Ability to degrade acetate was not included in the description of *C. subterraneus* and *T. ethanolicus*. Taking into account similar physiological characteristics of these species and the presence of *C. subterraneus* 16S rRNA gene in the library of associations degrading acetate to methane, we can propose that the bacterium *C. subterraneus* is capable of syntrophic growth on acetate in the presence of hydrogenotrophic methanogens.

Over 30 phylogenetic groups of thermophilic and mesophilic bacteria were revealed in formation water. The representative phylotypes belonged to the classes of *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. The phylotypes of a range of fermentative and syntrophic bacteria closely related to *Thermovirga lienii* (fam. *Syntrophomonadaceae)* and *T. phaeum* were detected. Representatives of these two metabolic groups could be involved in syntrophic acetate degradation to methane.

Anaerobic organotrophic bacteria, including the members of the *Thermoanaerobacter – Caldanaerobacter* group, are common in the oil-bearing horizons. These bacteria were usually isolated on media with peptone, starch, or glucose; since these substrates do not occur in formation waters, the ecological function of these fermentative bacteria remained unclear.

In our studies, it was shown for the first time that bacteria of the *Thermoanaerobacter – Caldanaerobacter* group in the presence of hydrogenothrophic methanogens are capable of syntrophic acetate oxidation to H_2 and CO_2, which is then reduced to methane. The aceticlastic pathway of methanogenesis is probably not pronounced at high temperature so that direct decomposition of acetate to methane contributes but insignificantly to methanogenesis. In high-temperature oilfields with low concentrations of acetate and sulfate in formation water, other acetate-oxidizing prokaryotes, such as *Coprothermobacter* [51] or sulfate-reducing bacteria

[52], may form acetate-degrading methanogenic associations with hydrogenotrophic methanogens; the syntrophic mechanism of the terminal stage of biodegradation of the oil organic matter is in this case preserved.

Our work suggests important biogeochemical conclusions. Since methane formation in the presence of acetate may be carried out either by aceticlastic methanogens or by syntrophic associations, quantitative assessment of the rates of these processes requires experiments with labeled acetate and no antibiotic, as well as with a penicillin group antibiotic suppressing the growth of bacteria involved in syntrophic acetate oxidation. The difference between these values will indicate the rate of aceticlastic methanogenesis.

Thus, our investigation made it possible to specify the composition of the microbial trophic chain, to estimate the geochemical activity of microorganisms, and to obtain new information concerning the terminal stages of oil biodegradation in high-temperature oilfields.

5. Tables

Table 1: Chemical characteristics and rates of microbial processes in formation waters of the Dagang oilfield

Well No.	Total salinity, mg L^{-1}	K^++Na^+, mg L^{-1}	Mg^{2+}, mg L^{-1}	Ca^{2+}, mg L^{-1}	Cl^-, mg L^{-1}	SO_4^{2-}, mg L^{-1}	HCO_3^-, mg L^{-1}	Acetate, mg L^{-1}	Methanogenesis rate, μg of CH_4 L^{-1} day^{-1}		Sulfate reduction rate, μg of S^{2-} L^{-1} day^{-1}	δ $^{13}C/CH_4$, ‰
									From $Na_2{}^{14}CO_3$	From 2-^{14}C-acetate		
63	6797	2458	46	54	3695	41	503	30.7	0.037	0.290	205.804	-42.0
1002-1	6675	2438	31	39	3572	0	595	29.52	0.880	4.370	0.455[a]	-41.9
1008-1	7024	2589	31	36	3618	35	712	97.6	0.700	1.740	9.367	-42.1
1012	6966	2476	32	52	3766	39	610	0.5	0.250	0.002	22.219	-43.3
1012-1	6999	2548	37	49	3783	18	564	4.2	0.060	0.994	1.629	-43.0
1015-1	6425	2349	33	49	3537	0	457	38.2	0.405	0.425	18.950	-41.7
1017-3	6825	2486	24	34	3519	0	762	21.7	0.604	0.943	0.060	-41.7
1017-4	6584	2384	36	54	3537	24	549	0.76	5.116	0.789	11.163	-41.8
1017-5	7138	2609	28	34	3731	35	701	3.6	0.441	0.120	2.776	-42.7
1017-7	7270	2643	27	30	3695	6	869	2.9	6.776	3.160	4.586	-43.5
1050-3	6897	2501	35	54	3811	37	459	29.1	0.040	0.050	87.288	-42.6
1092	6491	2343	36	44	3396	47	625	4.14	0.708	1.96	23.245	-41.6
1066-1[b]	5658	2049	32	73	3069	0	435	1.4	0	0.004	0.010	-42.1

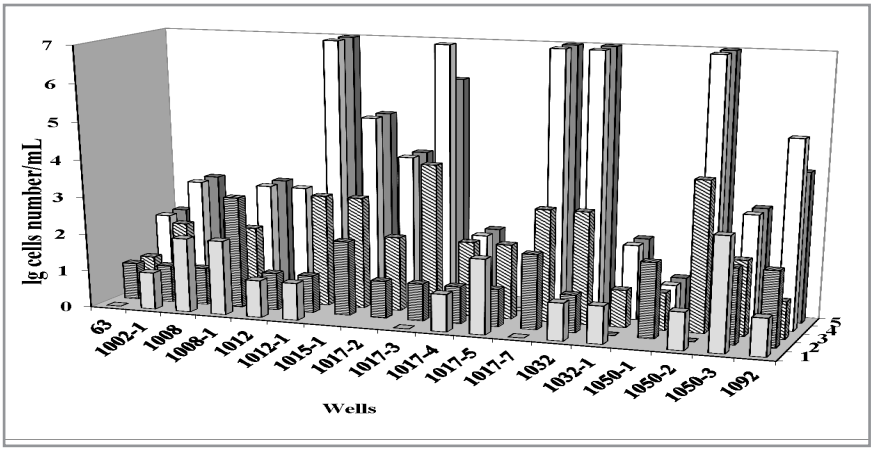
[a] In the absence of sulfate in water the rate of sulfate reduction was calculated using quantity of added label sulfate (20 μg L^{-1}), in the absence of acetate the rate of methanogenesis was calculated using quantity of added label acetate (70 μg L^{-1});
[b] Well located outside of the zone of biotechnological treatment.

Table 2: The total number of sequenced and screened clones of archaeal 16S rRNA genes in the libraries obtained from DNA of methanogenic enrichments and of formation waters from the Dagang oilfield

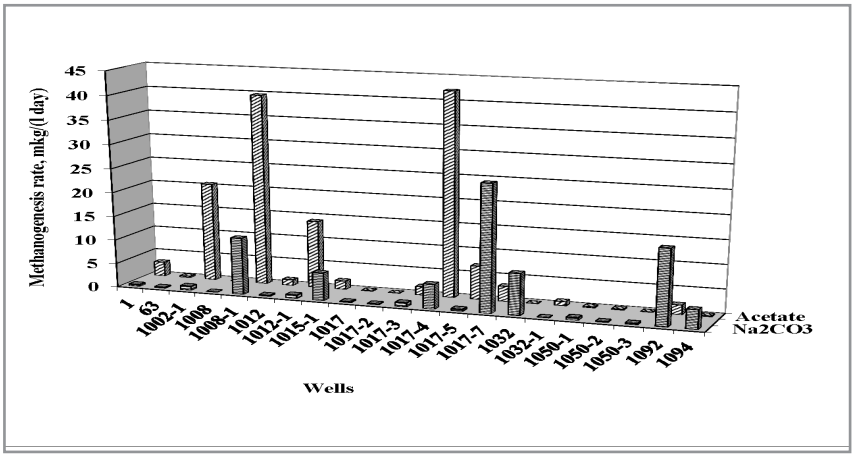
Library of 16S rRNA genes	Number of archaeal 16S rRNA genes in the library	Number of *Methanothermobacter* sp. 16S rRNA genes in the library	References
Combined first-generation methanogenic enrichments	102	101	§
Natural formation water 1066-1	64 + 343*	63 + 343*	#
Formation water from the zone of the pilot trial[#]	38 + 143*	17 + 143*	#
Methanogenic association 24A grown on acetate	41 + 54*	41 + 54*	#
Total number of clones	785	762	

* Clones revealed positive signal by the PCR with the *Methanothermobacter*-specific primer.
§ The data from the paper [5].
# The data from the paper [25].

6. Figures



(a)



(b)

Figure 1: The number of thermophilic microorganisms in formation water from production wells of the Kongdian bed of the Dagang oilfield in January 2002 (a) and maximal values of methanogenesis rates from labeled $Na_2{}^{14}CO_3$ and 2-$^{14}CH_3COONa$ in the waters from production wells during the period 06.2001–12.2007 (b). Designation on Figure 1a: (1), aerobic organotrophs; (2), methanogens, growing on H_2/CO_2; (3), methanogens, growing on acetate; (4), sulfate-reducers; (5), fermentative bacteria.

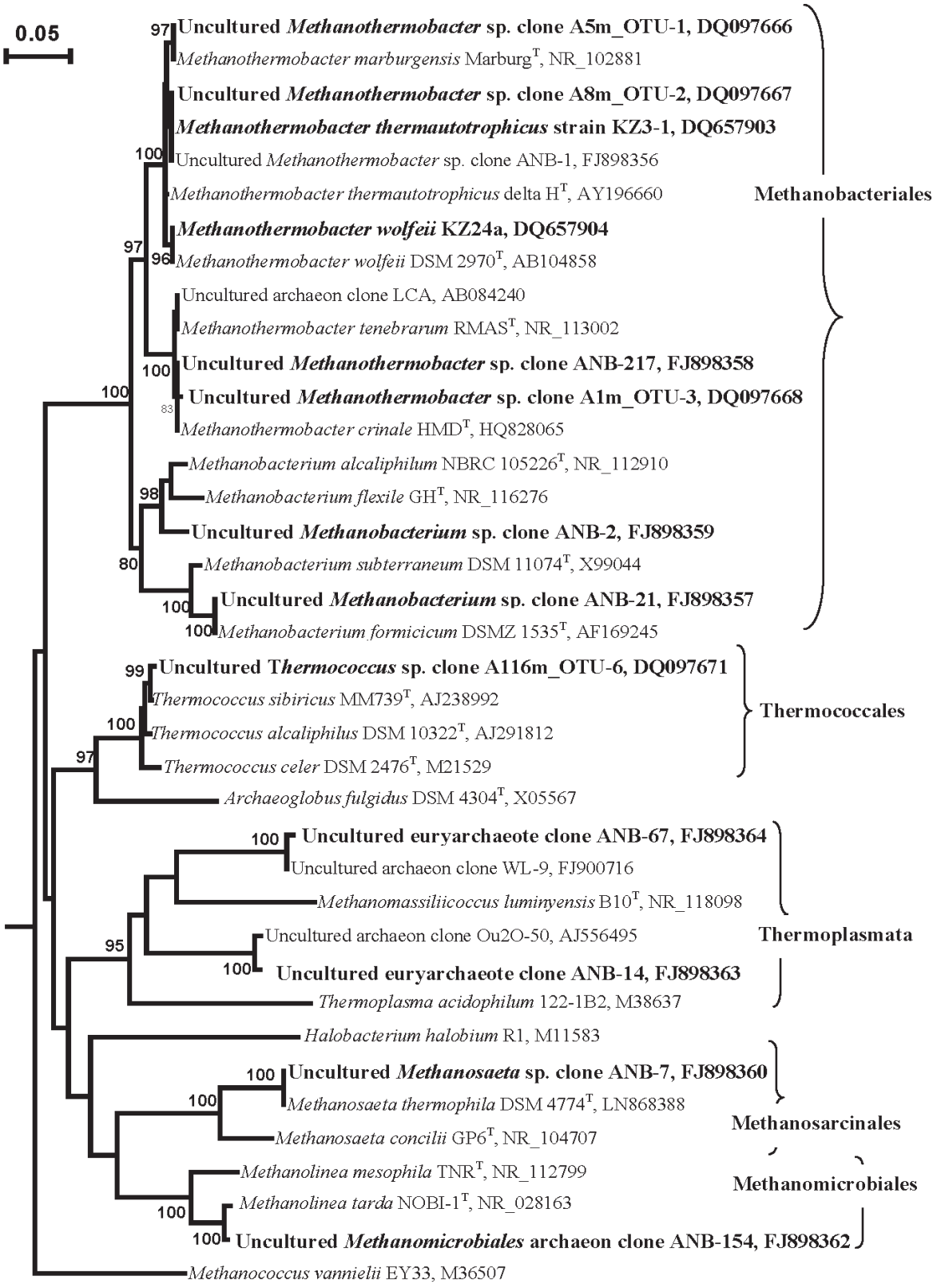


Figure 2: Phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of pure cultures *Methanothermobacter* KZ24a and KZ3-1 and of representatives of the domain *Archaea* revealed in the clone libraries from methanogenic enrichments (clones designated A1m – A116m) and from formation water (North Block, clones designated ANB). The sequences obtained in our studies are in bold. Scale bar, five nucleotide substitutions for each 100 nucleotide base pairs. Numbers at the nodes indicate the percentage of bootstrap values for the clade in 1000 replications; only values above 80% are shown.

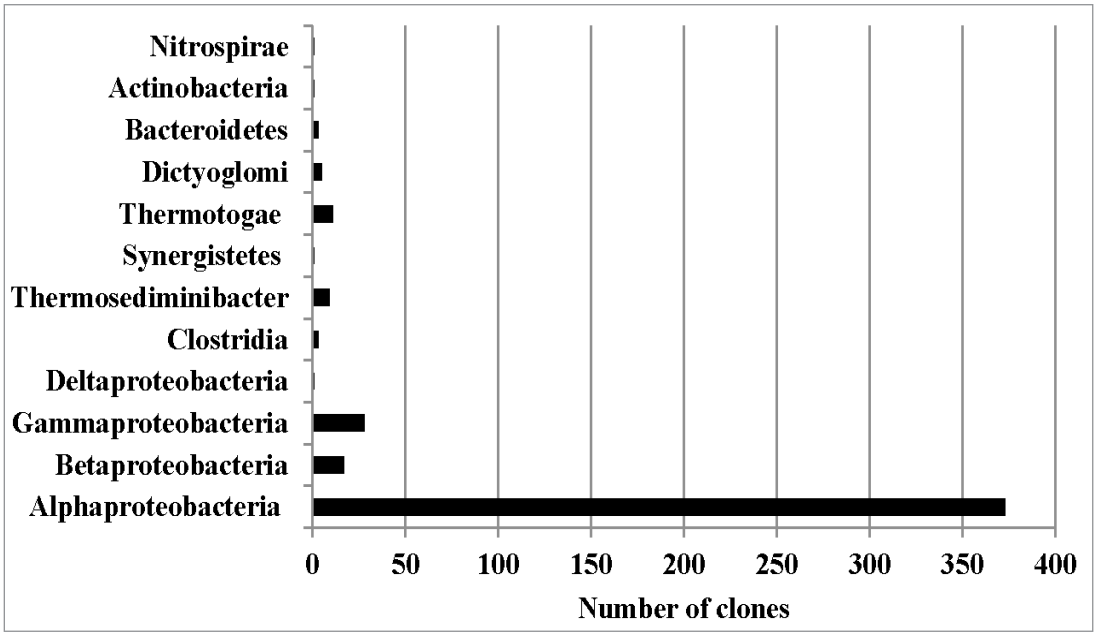


Figure 3: Distribution of 16S rRNA genes of *Bacteria* in clone library of formation water from the zone of the pilot trial of the Dagang oilfield (435 clones total) among high-rank taxa.

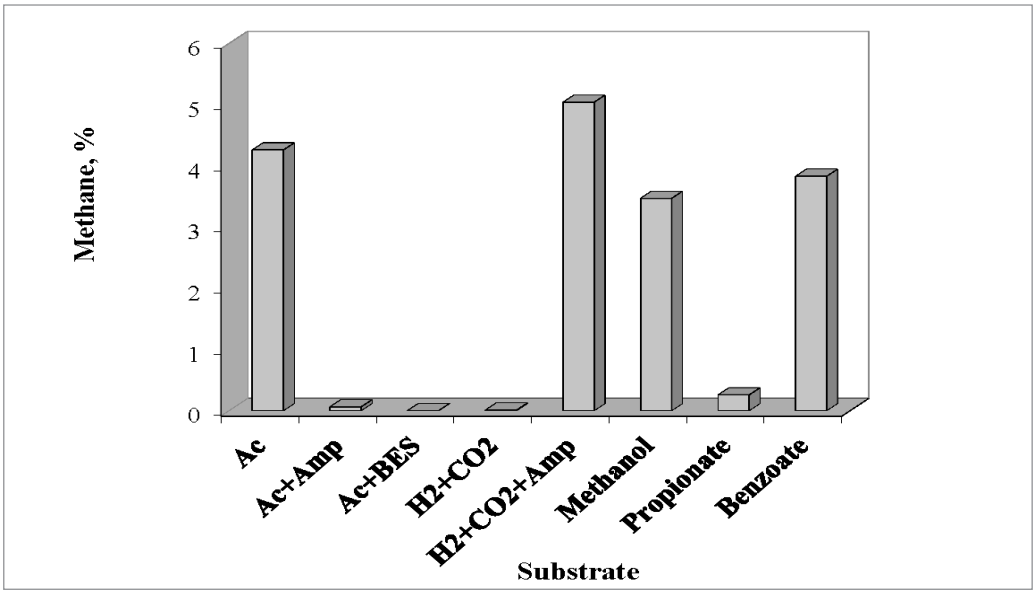
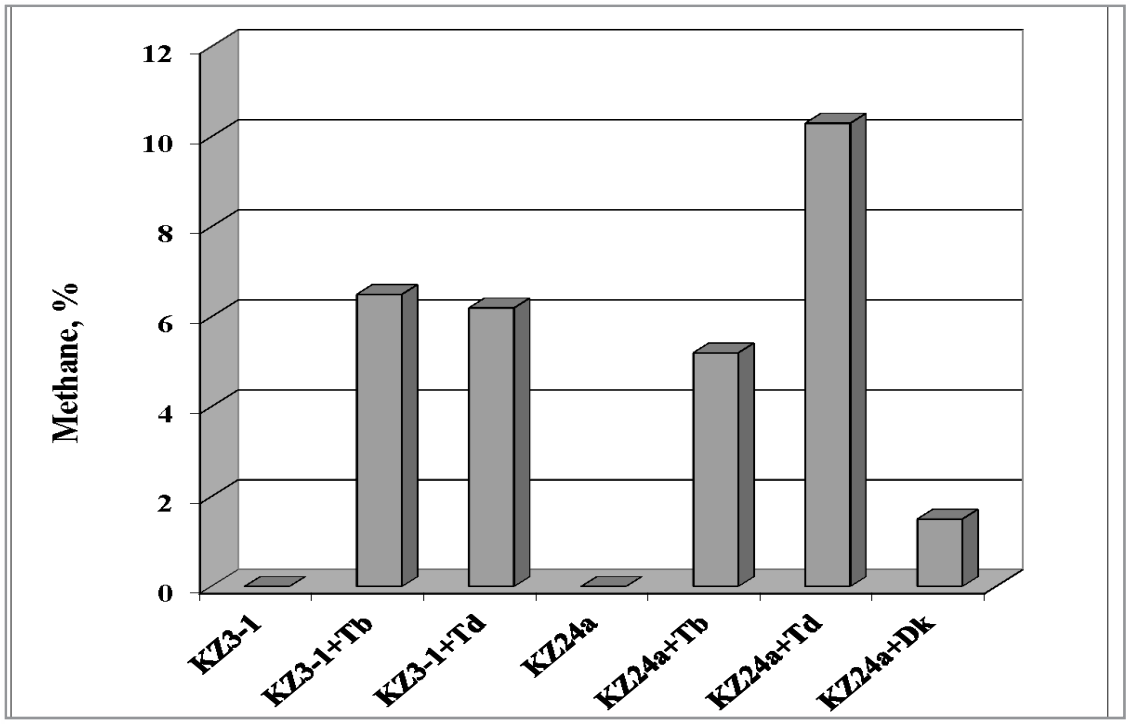
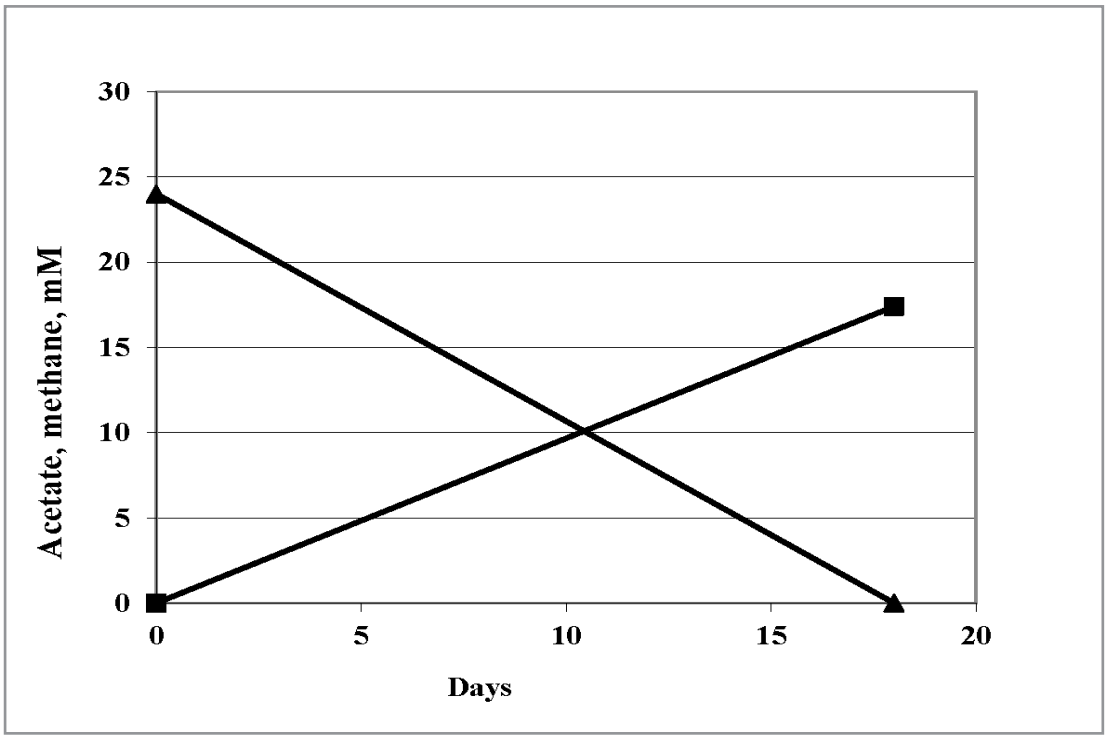


Figure 4: Methane production (%) by enrichments obtained from formation water of the Kongdian bed on media with acetate (Ac), acetate amended with ampicillin (Ac + Amp), acetate amended with 2-bromo-ethane sulfonate (Ac + BES), on H_2+CO_2, H_2+CO_2 with ampicillin (H_2+CO_2 + Amp), methanol, propionate and benzoate.



(a)



(b)

Figure 5: Methane formation in the presence of acetate by pure and binary cultures of the methanogen M. *wolfeii* KZ24a and T. *ethanolicus* strains 1017-7b (Tb) and 1017-7d (Td) and *Desulfotomaculum kuznetsovii* 17^T (Dk) during 18 days of incubation at 60°C (a) and acetate conversion to methane at 60°C the by co-culture of M. *wolfeii* KZ24a – T. *ethanolicus* 1017-7b (b).

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