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EXPERIMENTAL ARTICLES

Microbial Diversity in Formation Water and Enrichment Cultures from the Gangxi Bed of the Dagang Terrigenous Oilfield (PRC)

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Abstract—Microbial diversity and biogeochemical processes of the Gangxi bed with low-mineral water and a temperature gradient from 35 to 54°C were studied. The 16S rRNA gene clone libraries (over 800 clones) were obtained from microbial DNA isolated from formation water and from the primary enrichment cultures for fermenting, sulfate-reducing, methanogenic, and aerobic organotrophic prokaryotes. While both sulfate reduction and methanogenesis were registered in formation water by radioisotope techniques, the genes of sulfate-reducing prokaryotes were not revealed in the 16S rRNA gene clone library from formation water. The 16S rRNA genes of *Methanobacterium congolense* and *Methanococcus vannielii* predominated among archaeal sequences retrieved from formation water, while the genes of *Methanothermobacter thermoautotrophicus*, *Methanomethylovorans thermophila*, and *Methanoculleus* sp. predominated in the combined library from enrichment cultures. In the library of *Bacteria* 16S rRNA genes from formation water, the genes of thermophilic fermentative bacteria of the family *Thermoanaerobacteriaceae* predominated; the remaining sequences belonged to mesophiles (genera *Brevundimonas*, *Sphingomonas*, *Oxalicibacterium*, and *Stenotrophomonas*), the phylum *Chloroflexi*, and unidentified bacteria. The combined library from enrichment cultures, contained, apart from the sequences of the family *Thermoanaerobacteriaceae*, the genes of fermentative bacteria (genera *Anaerobaculum*, *Coprothermobacter*, *Thermanaerovibrio*, *Soehngenia*, *Bacteroides*, and *Aminobacterium* and the order *Thermotogales*), of aerobic hydrocarbon-oxidizing bacteria (genera *Pannonibacter* and *Pseudomonas*), and of sulfate reducers (genera *Desulfomicrobium*, *Thermodesulfovibrio*, and *Desulfotomaculum*). High coverage was shown for bacterial (97.6%) and archaeal (100%) clone libraries, indicating that a significant portion of the microbial diversity in the studied communities was revealed.

Keywords: oilfields, sulfate reduction, methanogenesis, molecular ecology, 16S rRNA gene clone libraries of *Bacteria* and *Archaea*, formation water, primary enrichment cultures, statistical analysis of clone libraries

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Investigation of oilfield microbiota started in 1926, when sulfate-reducing bacteria were first detected in oilfield water [1, 2]. Cultural, biogeochemical, and molecular biological methods were used to isolate oilfield microorganisms. These methods were mostly used separately; the overall composition of the microbial communities of oilfields and their geochemical activity remained therefore obscure.

With the usage of traditional microbiological methods, were revealed in formation water anaerobic fermentative; acetogenic; iron-, thiosulfate-, sulfur-, and sulfate-reducing; and methanogenic prokaryotes [1, 2]. Aerobic bacteria found in oil reservoirs are considered contaminants brought with injected water.

Methods for quantitation of the rates of sulfate reduction and methanogenesis using radioactively labeled $Na_2^{35}SO_4$, $^{14}CH_3COONa$, and $NaH^{14}CO_3$ were developed in Russia by Ivanov and Belyaev. Application of these methods showed that methanogenesis was predominant in oil reservoirs with low-sulfate water, whereas sulfate reduction dominated in high-sulfate ones [3].

Molecular methods based on sequencing of the 16S rRNA genes have been applied to microbial communities of oil reservoirs since 1996 [4]. Ample information has been obtained on the taxonomic diversity of microorganisms inhabiting the waters of terrigenous oilfields in Canada [4], United States [5], Russia [6], China [7–10], and deposits in the North Sea [11]. Molecular and cultural studies of oil reservoir microorganisms provided concordant results. They pointed to a great taxonomic and functional diversity of prokaryotes in the ecosystem.

Analysis of the 16S rRNA gene clone libraries of formation water microorganisms often reveals a single predominant phylotype [10, 12]. For example, phylotype of bacteria of the genus *Arcobacter* predominated in the *Bacteria* libraries from the low-temperature Pel-

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ican Lake oilfield in Canada, and the phylotypes of *Archaea* belonged to methanogens of the orders *Methanomicrobiales* and *Methanosarcinales* [12]. The 16S rRNA genes of various taxa were detected by analysis of clone libraries from enrichment cultures: *Firmicutes, Deltaproteobacteria, Epsilonproteobacteria, Spirochaetes*, and *Euryarchaeota*. They included genes of homoacetogenic, acetogenic, denitrifying, and sulfate-reducing bacteria, as well as acetoclastic methanogens. Investigation of microbial communities of formation water and enrichment cultures is necessary for understanding the relationships and ecological functions of various components of the community.

Simultaneous application of cultural, radioisotope, and molecular techniques made it possible to reveal predominance of hydrogenotrophic methanogens in the high-temperature Kondian deposits in the Dagang oil field [7–9]. Acetate degradation with methane production was carried out by a syntrophic association of organotrophic thermophilic anaerobic bacteria *Thermoanaerobacter ethanolicus* and methanogenic *Methanothermobacter thermautotrophicus*, none of which is able to grow on acetate in pure culture. It was proven that in high-temperature oilfields bacteria of the genera *Thermoanaerobacter* and *Caldanaerobacter* participated in the terminal stages of oil degradation in cooperation with methanogens.

An important problem in the assessment of molecular cloning data is clone library representativeness. Many scientists apply curves of the expectancy of the number of phylotypes in a random sample from a library depending on the sample size [13]. The leveling-off of the curve is considered a criterion of sample sufficiency. However, it is practically impossible to determine the original sample size due to significant variation in the species richness of natural communities.

It is assumed that examination of 10000 nucleotide sequences is required for assessment of soil biodiversity [14]. This task can be solved by high-throughput sequencing. Other scientists believe that 1000 clones are sufficient for assessment of a microbial community biodiversity [15].

The first statistical evaluation of clone libraries of 16S rRNA genes of microorganisms from the water-flooded oilfield (Qinghuang Unit) was done by Chinese scientists [10]. A total of 388 bacterial and 220 archaeal sequences were analyzed, and the coverage was 90.7 and 92.3%, respectively. Thus, a significant portion of the microbial diversity formation water was revealed. Nevertheless, the asymptotic shape of distribution curves points to incomplete detection of microbial phylotypes, and further studies are required.

The goal of the present work was to determine the population sizes of cultivated microorganisms and the rates of sulfate reduction and methanogenesis in formation water samples from the Gangxi bed of the Dagang oilfield (35–54°C), to obtain clone libraries of the 16S rRNA genes based on DNA of microorganisms from formation water and enrichment cultures, and to carry out their comparative phylogenetic and statistical analysis.

MATERIALS AND METHODS

The Gangxi bed. Formation water was sampled from the production wells in the Gangxi bed of the Dagang oilfield, China, in 2005. Water samples were obtained from depths of 810–879 m, where the temperature was 42°C. The water was of the hydrocarbonate-sodium type. Since 1999, the bed had been subjected to water-flooding with coproduced oilfield water separated from oil. The main parameters of the Gangxi bed are reported in [16].

Sampling. Oilfield water was collected at well mouths into sterile vials and tightly sealed with no gaseous phase. To determine microbial population sizes, the samples were plated onto nutrient media under laboratory conditions within 4–6 h after sampling. Radioisotopes were added to determine the rates of microbial processes. A 1-L sample of formation water from well 37-7-2 was fixed at the sampling site with an equal volume of 97% ethanol and stored in a refrigerator until molecular studies.

Media and bacterium enumeration. The population sizes of microorganisms of the main metabolic groups were determined by inoculation of formation water into liquid nutrient media in a series of tenfold dilutions. The numbers of aerobic organotrophs were determined in liquid medium containing the following (g/L distilled water): Bacto tryptone, 5.0; yeast extract, 2.5; glucose, 1.0; sodium chloride, 5.0; pH 7.0. Anaerobic organotrophs with fermentative metabolism were enumerated in medium with peptone (4 g/L) and glucose (10 g/L) supplemented with Na_2SO_4 (2 g/L), $MgSO_4$ (1 g/L), and Mohr's salt (0.5 g/L). Sulfate-reducing bacteria were enumerated in medium B with sodium lactate (4 g/L) supplemented with microelements and reduced with 200 mg/L $Na_2S \cdot 9H_2O$. Methanogens were determined in Zeikus media with acetate (2 g/L) and $H_2 + CO_2$ (4 : 1, vol/vol) supplemented with microelements and yeast extract (1 g/L). The composition of the media was reported in [16].

The media were dispensed into Hungate tubes. The gaseous phases were as follows: for aerobic bacteria, air; for hydrogen-consuming methanogens, $H_2 + CO_2$ (4 : 1, vol/vol) mixture; and for other microbial groups, purified argon. The cultures were incubated at 42°C for 30 days and then examined under a phase contrast Olympus microscope. The growth of aerobic bacteria was assessed as biomass increment. The growth of anaerobic organotrophs, sulfate-reducing bacteria, and methanogens was determined by hydrogen production, sulfide production, and methane production in a series of dilutions, respectively. Methods for chemical analyses of formation water, gases (CH_4,

CO_2, and H_2), and volatile fatty acids have been reported in [6, 16].

Radioisotope determination of the rates of anaerobic processes. The rates of sulfate reduction and methanogenesis were determined by radioisotope techniques with labeled compounds $Na_2^{35}SO_4$, $NaH^{14}CO_3$, and $^{14}CH_3COONa$ as described in [16]. The composition of stable carbon isotopes in mineral carbonates ($\delta^{13}C/\Sigma(CO_2 + HCO_3^- + CO_3^{2-})$) dissolved in formation water and of methane carbon ($\delta^{13}C/CH_4$) in accompanying gas was determined by the Craig method [17] with a Delta Advantage mass spectrometer (Finnigan, United States) with the accuracy ±0.1‰.

DNA extraction. One liter of oilfield water from well 37-7-2 fixed with ethanol was treated by repeated extraction with hexane and phase separation in order to remove oil. The cells from the aqueous phase were collected by centrifugation, and total DNA was extracted from the cell pellet with an ExtraGene kit according to the manufacturer's recommendations. In a similar way, DNA was extracted from first generation enrichment cultures of formation water microorganisms in the media for aerobic organotrophs, fermentative microorganisms, sulfate reducers, and methanogens. In each case, the first tube with 1 mL of formation water and 9 mL of nutrient medium was used. Samples of the medium for aerobic organotrophs inoculated with formation water from each of four production wells (37-7-2, 37-7, 38-8-1, and 39-6-3) were bulked (4 × 10 mL) prior to DNA extraction. Each of the first generation enrichment cultures in media for fermentative, sulfate-reducing, and methanogenic (with acetate or $H_2 + CO_2$) microorganisms was bulked into four tubes and centrifuged. The biomass pellets were used for DNA extraction. We assumed that the use of the total enrichment culture biomass for each metabolic group would provide for better representation of prokaryotic diversity in the oilfield, although it probably did not match the composition of formation water microbial community from a single production well.

A cell pellet was resuspended in guanidine hydrochloride and incubated at 65°C for 60 min. GlassMilk sorbent was added to the lysate, the supernatant was discarded, and the sorbent was washed with guanidine hydrochloride, buffered saline, and 70% ethanol. The purified DNA preparation was dissolved in 50–100 μL of Milli-Q water and used as a template for amplification of the 16S rRNA genes.

Amplification of the 16S rRNA genes. Total DNA samples isolated from formation water and five enrichment cultures were used in the preparation of 12 clone libraries of the 16S rRNA genes of *Bacteria* and *Archaea*.

Polymerase chain reaction was conducted in an iCycler thermocycler (BioRad, United States). The 16S rRNA genes were amplified with universal primers: bacterial with 8-27f [5'-AGAGTTTGATCCTGGCTGAG-3'] and 519r [5'-G(T/A)ATTACCGCGGC(T/G)GCTG-3'] [18] and archaeal with A109f [5'-ACG/TGCTCAGTAACACGT-3'] and A1041r [5'-GGCCATGCACCWCCTCTC-3'] [19, 20]. The reaction volume was 40 μL. The mixture contained 1 × *Taq* buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$), 200 μM of dNTPs, 5 pM of each of the corresponding 5' and 3' primers, 1 U of *Taq* DNA polymerase, and template DNA. The PCR program with bacterial primers was conducted as follows: a predenaturation step for 3 min at 94°C followed by 30 cycles; denaturation at 94°C for 30 s; annealing at 50°C for 30 s; and elongation at 72°C for 30 s. Postextension was performed at 72°C for 7 min. Amplification with archaeal primers was done in a similar way, with the elongation time at 72°C extended to 1.5 min and the numbers of elongation cycles equaling 30 for DNA from primary cultures and 35 for DNA from formation water. The amplificates were resolved by electrophoresis in 1.0–1.5% agarose gel with ethidium bromide.

Cloning and sequencing of PCR products. PCR products of the 16S rRNA gene fragments from bacteria (~500 bp) and from archaea (~900 bp) were purified and cloned with a TA cloning vector kit (Promega, United States) according to the manufacturer's recommendations. Clones with inserts were sequenced with plasmid primers M13D or M13R. The sequencing was conducted in an automated Applied Biosystems DNA Sequencer ABI 3100 Avant Genetic Analyzer with a Dyenamic Terminator Cycle Sequencing Ready Reaction kit (Amersham, United States) following the manufacturer's recommendations.

A total of 12 clone libraries were constructed: two libraries of 16S rRNA clones of bacteria (bC) and archaea (aC) from formation water DNA and ten minilibraries for first generation enrichment cultures (five bacterial and five archaeal). The libraries of the 16S rRNA gene clones of bacteria and archaea from the medium for aerobic organotrophs inoculated with formation water were designated as bG and aG, respectively; those of fermentative prokaryotes, bF and aF; of sulfate-reducers, bR and aR; of methanogens grown with $H_2 + CO_2$, bZ and aZ; and of methanogens grown with acetate, bM and aM.

Phylogenetic analysis of the sequences. Preliminary analysis of the nucleotide sequences of the 16S rRNA genes was performed with the Blast program hosted by the NCBI GenBank (http://www.ncbi/nlm/nih.gov). The sequences were aligned with BioEdit (http://jwbrown.mbio. ncsu.edu/BioEdit/bioedit.html). To eliminate chimeras, the sequences were checked with the CHECK_CHIMERA resource from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu). Sequences with the similarity level of at least 98% were combined into phylotypes, or operational taxonomic units (OTUs), at the species level

and with the similarity of at least 95%, at the genus level.

The resulting OTUs were subject to further analysis. Phylogenetic trees were constructed by the neighbor-joining method [21] with TREECONW software package (http://bioinformatics.psb.ugent.be/psb/Userman/treeconw.html) [22] using the reference sequences from RDP (http://rdp.cme.msu.edu) and GenBank databases.

The 16S rRNA gene sequences obtained in this study were submitted to GenBank, accession nos. GU129032–GU129130.

Statistical analysis of clone libraries. The representativeness of the 16S rRNA gene libraries obtained in this study, that is, the sufficiency of the clone sample size for reliable analysis of the community structure, was assessed from their coverage calculated according to Good [23] and by construction of the curves of the number of phylotypes in a library vs. sample size in clones (rarefaction curves). The following expression was used for calculation of coverage according to Good: $(1 - n/N) \times 100$, where n is the number of unique clones and N is the total number of clones in the library) [23]. Rarefaction curves were constructed with the aRarefactWin 1.3 program [http://www.uga.edu/strata/software/anRareReadme.html].

The representativeness of phylotypes in a library was assessed from the ratio of phylotypes to clones. The diversity and species richness of microorganisms in the libraries were analyzed by calculating the Shannon-Weaver and Simpson diversity indices and species richness estimators Chao 1 [24] and ACE with the EstimateS program (version 8, R.K. Colwell, http://purl.oclc.org/estimates). The dominance index was calculated as the ratio between the number of clones of the predominant phylotype and the total number of clones.

RESULTS

Physicochemical Parameters and Microorganisms of Formation Water from the Gangxi Bed

Oilfield water and gas were sampled from four production wells of the Gangxi bed. The salinity of formation water varied from 4200 to 8061 mg/L. It contained 1266 to 2628 mg/L hydrocarbonates and 24 to 106 mg/L sulfates, and its pH varied from 6.5 to 7.2. Acetate concentrations were within 0.5–15 mg/L (Table 1). The number of culturable aerobic organotrophs was below 10^2 cells/mL. Anaerobic microorganisms with fermentative metabolism were predominant in the samples, and their numbers were 10^3–10^7 cells/mL. The numbers of sulfate-reducing prokaryotes and methanogens were below 10^2 cells/mL (Table 1). The samples from wells 37-7 and 38-8-1 had higher abundance of microorganisms and sulfate reduction rates: 61.26 and 494.8 μg S^{2-} L^{-1} day^{-1}, respectively. The rates of hydrogenotrophic methanogenesis exceeded those of aceticlastic methanogenesis in three samples out of four, although the numbers of methanogens in the medium with acetate were higher than in the medium with $H_2 + CO_2$.

The carbon isotope ratio ($\delta^{13}C$) in formation water carbonates $\Sigma(CO_2 + HCO_3^- + CO_3^{2-})$ varied from 7.5 to 9.7‰. The $\delta^{13}C$ of methane carbon in accompanying gas samples had similar values: from −51.9 to −52.8‰. The predominant fraction of the associated gas was methane: 75.7–88.1%. The concentration of carbon dioxide was high in the accompanying gas from several wells, 10.7–23.2%, indicated oil biodegradation in the bed.

Construction of the 16S rRNA Gene Clone Libraries and Their Statistical Evaluation

We analyzed 806 cloned 16S rRNA sequences of microorganisms from formation water and of enrichment cultures. The results are shown in Table 2 and Fig. 1. The coverage of archaeal clones in the library was greater than that of bacterial clones. This fact agrees with the data reported by other researchers [10]. The clone libraries were representative. They showed that the coverage of bacterial 16S rRNA genes in the library from formation water was greater than that in the combined library from enrichment cultures, in spite of the greater number of analyzed clones in the libraries from enrichment cultures. As for archaea, the coverage values were approximately equal and close to the maximum value.

The representation of the phylotypes of archaea and bacteria was poor: 0.0126 and 0.088, respectively. Hence, the diversity of the prokaryotic community in the oil bed under study was relatively low. The diversity of bacterial phylotypes was notably greater than that of archaeal ones in all libraries.

Archaea. The library of archaeal 16S rRNA genes obtained with the DNA template from formation water included 170 sequences. Only 81 of them had individual sequence types (Fig. 1a, Table 2). The cloned archaeal sequences from formation water were distributed into three phylotypes at the species level. According to Chao 1 and ACE estimators, this result corresponded to the maximum value, 100%. The diversity index values were relatively low, and the most abundant phylotype showed a significant degree of dominance.

The numbers of cloned sequences of *Archaea* (217) in the libraries from enrichment cultures varied from 22 to 63. The number (104) of unique sequence types in the libraries varied from 15 to 34. At the species level, the archaeal communities in enrichment culture libraries contained one to six phylotypes, nine in total, and the largest numbers of phylotypes were detected in the aM and aZ libraries, obtained from methanogenic enrichment cultures. As confirmed by the asymptotic shape of the rarefaction curves (Fig. 1a), the maxi-

Table 1. Physicochemical parameters and microorganisms in formation water samples from the Gangxi oil bed

Parameters, content in formation water	Water samples from production wells			
	37-7	37-7-2	38-8-1	39-6-3
$Na^+ + K^+$, mg/L	2707	1932	1586	1334
Ca^{2+}, mg/L	12	74	12	10
Mg^{2+}, mg/L	67	43	67	86
Cl^-, mg/L	2628	1526	1094	970
HCO_3^-, mg/L	2196	1902	1967	1266
CO_3^{2-}, mg/L	345	471	345	510
SO_4^{2-}, mg/L	106	65	24	24
Total salinity, mg/L	8061	6013	5095	4200
Acetate, mg/L	0.5	10	9.5	15
$\delta\ ^{13}C/\Sigma(CO_2 + HCO_3^- + CO_3^{2-})$, ‰	9.7	7.5	8.8	8.7
$\delta\ ^{13}C/CH_4$,‰	–52.1	–51.9	–52.2	–52.8
% CO_2 in gas	18.9	23.2	10.7	15.0
% CH_4 in gas	79.9	75.7	88.1	83.2
Sulfate reduction rate, μg S^{2-} L^{-1} day^{-1}	61.26	0.66	494.8	0.058
Methanogenesis rate, μg CH_4 L^{-1} day^{-1}				
From $NaH^{14}CO_3$	0.66	0.31	0.49	0
From $^{14}CH_3COONa$	0	0.002	0.228	0
Microorganisms number, cells/mL				
Aerobic organotrophs	<10	10	10^2	10^2
Fermentative	10^4	10^3	10^7	10^5
Sulfate-reducing	10^2	10	10^3	10
Methanogens (medium with $H_2 + CO_2$)	10	10	10^2	10^3
Methanogens (medium with acetate)	10^2	10^3	10	10^2

mum coverage values of archaeal sequences were found in the library from formation water, in the libraries of each of the enrichment cultures except for aZ (where it was less than in the others), and in the combined sequences from all enrichment culture libraries (Fig. 1a).

The representation level of phylotypes in each enrichment culture library varied broadly: 0.037–0.14. The corresponding value for the total library was close to the lower boundary of the range: 0.044. These values significantly exceeded the value for formation water (0.018) and the integrated value for all libraries (0.026). Hence, enrichment cultures varied significantly in the degree of archaeal representation, and a significant portion of the phylotypes present in several libraries belonged to predominant ones.

This assumption was confirmed by calculation of the Shannon and Simpson diversity indices for archaea, because their values in the libraries of each enrichment were different, but the overall degree of diversity increased significantly for combined data on all the cultures in comparison to the indices for formation water community. The index of dominance was relatively high for the formation water library (0.55) and even higher (0.59–1.0) in the clone libraries from each enrichment culture. However, the values slightly decreased after pooling of the data; thus, the ratios of the dominating components were different in different enrichment cultures.

Bacteria. The library of cloned 16S rRNA genes of *Bacteria* constructed with formation water DNA as a template contained 146 sequences, of which 102 had individual sequence types. At the species level, the formation water bacterial library was represented by nine phylotypes. Although it was much higher than the number of phylotypes obtained for archaeal library, the representation level according to Chao 1 and ACE was slightly below the maximum: 90% of the predicted

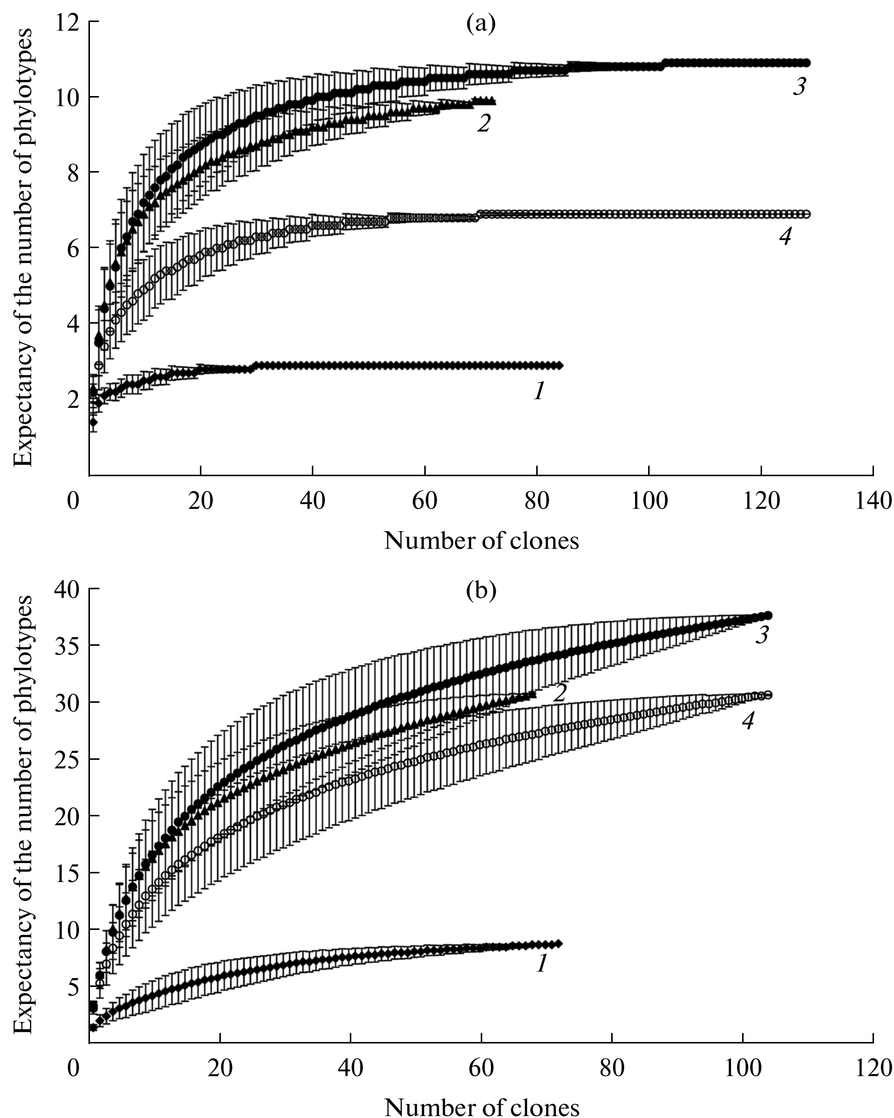

Fig. 1. Rarefaction curves constructed from comparison of the numbers of the 16S rRNA genes and phylotypes found in clone libraries of archaea (a) and bacteria (b) (Table 3). Curve designations: (1) aC and bC, formation water; (2) aEnr and bEnr, total for first generation enrichment cultures; (3) Total_98 and bTotal_98, total for all OTUs at the species level (≥98% similarity); (4) aTotal_95 and bTotal_95, total for all OTUs at the genus level (≥95% similarity).

values. Low indices of bacterium diversity in the oilfield water library and, correspondingly, the high index of dominance indicated that the community was represented mainly by its predominant species.

The libraries of the 16S rRNA gene clones of *Bacteria* constructed from enrichment cultures contained 44 to 63 cloned sequences, totaling 273. Of them, 36 to 56 (229 in total) were individual sequence types. Each enrichment culture library contained more bacterial phylotypes than archaeal ones: 7 to 14, totaling 30. The coverage values in the enrichments libraries varied insignificantly: 92.1–97.7%. In the combined set of sequences from all enrichment culture libraries, the degree of coverage was 97.6%, being within the same range of values, slightly less than the coverage of bacteria in the formation water library (98.6%) (Fig. 1b).

The representation values of bacterial phylotypes were much higher than those of archaea. However, they were less variable in each library (0.11–0.23) and in the combined library of enrichment cultures (0.11). The values for formation water (0.062) and the combined library (0.088) were lower than those for enrichment culture libraries. Apparently, the proportions of predominant bacterial phylotypes among those shared by several libraries were less than for archaeal phylotypes.

The same was confirmed by calculation of diversity indices. They were higher for bacteria than for archaea. For both bacteria and archaea, the indices

Table 2. Phylogenetic diversity of bacterial and archaeal 16S rRNA genes in clone libraries from enrichment cultures and formation water of the Gangxi oil bed

Phylogenetic group	Representative clone of the phylotype	Phylogenetically closest sequences in GenBank	Similarity of 16S rRNA genes, %	Number of similar clones of the phylotype						Combined enrichment culture libraries	Combined enrichment culture and formation water libraries
				formation water community	formation water community						
Libraries of *Archaea*					aC	aF	aR	aZ	aM	aENR	aTotal
Euryarchaeota											
Methanobacteriales	1aC	*Methanobacterium congolense* DSM 7095^T	98–99	94	4	27	8	34		73	167
	15aZ	*Methanobacterium* sp.	95					3		3	3
	190aC	*Methanobacterium* sp. SA-12	98	7				1		1	8
	26aM	*Methanobacterium formicicum* DSM 1535^T	99		13				6	19	19
	laR	*Methanothermobacter thermautotrophicus* ΔH^T	99		5		8	5	5	23	23
	40aM	*Methanothermobacter* sp.	95						2	2	2
Methanococcales	4aC	*Methanococcus vannielii* SB	98–99	69							69
Methanomicrobiales	llaR	*Methanofollis liminatans* DSM 4140^T	99				44		37	81	81
	56aR	*Methanoculleus* sp. ZC-2	98				1	1	5	7	7
Methanosarcinales	47aM	*Methanomethylovorans thermophila* DSM 17232^T							8	8	8
Number of archaeal 16S rRNA genes				170	22	27	61	44	63	217	387
Libraries of *Bacteria*				bC	bG	bF	bR	bZ	bM	bENR	bTotal
Firmicutes											
Thermoanaerobacterales	7bC, 37bG	*Thermoanaerobacterium* sp.	90	89	5	16				21	110
	lbC, 79bG	*Thermoanaerobacterium* sp.	90	36	2					2	38
	46bZ	*Thermosediminibacter*	90					1		1	1
	35bG	*Thermoanaerobacter*	90		26					26	26
	3bG	*Coprothermobacter proteolyticus* DSM 5265^T	98		7	11			3	21	21

Table 2. (Contd.)

Phylogenetic group	Representative clone of the phylotype	Phylogenetically closest sequences in GenBank	Similarity of 16S rRNA genes, %	Number of similar clones of the phylotype						Combined enrichment culture libraries	Combined enrichment culture and formation water libraries
				Formation water community	Formation water community						
Clostridiales	73bG	*Desulfotomaculum* sp.	91		1					1	1
	3bR	*Soehngenia saccharolytica* DSM 12858^T	100				7	13	4	24	24
	20bF	*Soehngenia saccharolytica* DSM 12858^T	97			1		2	3	6	6
	2bZ	*Soehngenia* sp.	93					18		18	18
Bacillales	12bR	*Bacillus boroniphilus* DSM 17376^T	98				1			1	1
	7bM	*Exiguobacterium* sp.	95						12	12	12
Alphaproteobacteria											
Rhodobacterales	45bF	*Pannonibacter phragmitetus* DSM 14782^T	98		2	2				4	4
Caulobacterales	224bC	*Brevundimonas aurantiaca* DSM 4731^T	'99–100	4							4
Sphingomonadales	228bC	*Sphingomonas* sp.	93	3							3
Betaproteobacteria, Burkholderiales	159bC	*Oxalicibacterium* sp.	94	1							1
Gammaproteobacteria											
Xanthomonadales	117bC	*Stenotrophomonas maltophilia* ATCC 19861^T	98	5							5
Enterobacteriales	181bC	*Raoultella*, *Enterobacter*, *Klebsiella*	99–100	4							4
Pseudomonadales	29bG	*Pseudomonas stutzeri* ATCC 17588^T	96		1					1	1
Deltaproteobacteria, Desulfovibrionales	2bR	*Desulfomicrobium thermophilum* DSM 16697^T	98				21		1	22	22
	44bR	*Desulfomicrobium* sp.	96				1		2	3	3

Table 2. (Contd.)

Phylogenetic group	Representative clone of the phylotype	Phylogenetically closest sequences in GenBank	Similarity of 16S rRNA genes, %	Number of similar clones of the phylotype						Combined enrichment culture libraries	Combined enrichment culture and formation water libraries
				Formation water community	Formation water community						
Synergistetes, Synergistales	IbR	*Anaerobaculum thermoterrenum* DSM 13490^T	98–99				20	4	11	35	35
	52bF	*Anaerobaculum mobile* DSM 13181^T	98–99		9	3	1			13	13
	7bZ	*Thermoanaerovibrio acidaminovorans* DSM 6589T	98–99		2			2	3	7	7
	llbR	*Aminobacterium mobile* DSM 12262^T	88				2			2	2
Bacteroidetes, Bacteroidales	26bG	*Bacteroides* sp.	92		3	4	3	3	4	17	17
	23bM	*Proteiniphilum acetatigenes* JCM 12891^T	97, 95					1	3	4	4
	22bG	Uncultured bacterium *Bacteroidetes*	89		3	7		2	1	13	13
Chloroflexi	257bC	Uncultured bacterium *Chloroflexi*	83	1							1
Thermotogae, Thermotogales	17bR	Uncultured bacterium *Thermotogales*	99				1	5	5	11	11
Deferribacteres, Deferribacterales	23bG	*Calditerrivibrio nitroreducens* Yu37-1^T	91		1					1	1
Spirochaetes, Spirochaetales	44bG	*Spirochaeta stenostrepta* DSM 2028^T	96		1					1	1
Nitrospirae, Nitrospirales	33bR	*Thermodesulfovibrio islandicus* DSM 12570^T	95				2			2	2
Unknown group 1	187bC	Uncultured bacterium, clone D144		3							3
Unknown group 2	33bM	Uncultured bacterium, clone GZKB57							2	2	2
Unknown group 3	28bM	Uncultured bacterium, clone ET5-2							2	2	2
Number of bacterial 16S rRNA genes				146	63	44	59	51	56	273	419

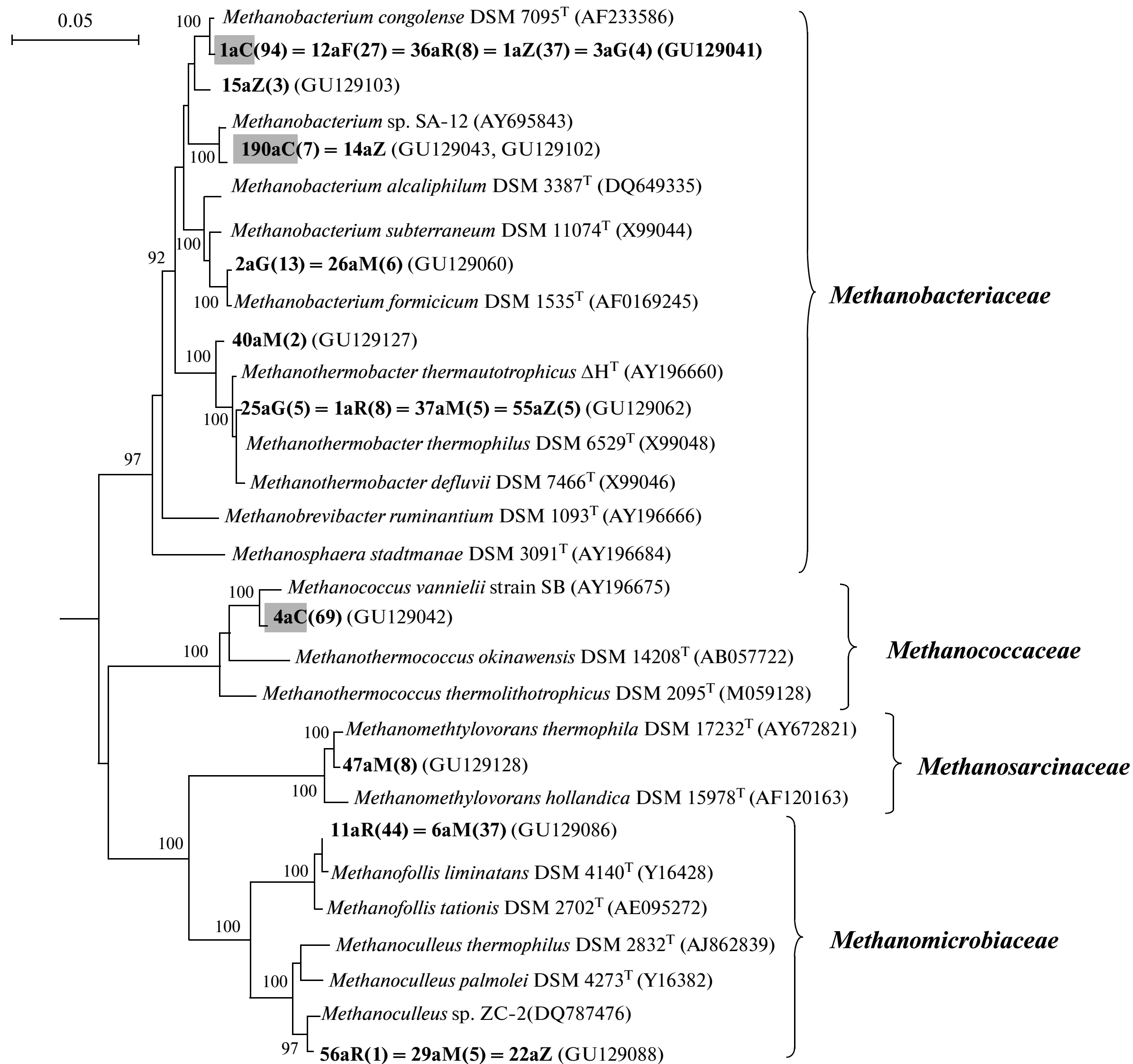

Fig. 2. Phylogenetic tree of the 16S rRNA gene sequences of *Archaea* in clone libraries obtained from DNA isolated from formation water and enrichment cultures. Letters in phylotype identifiers indicate in what 16S rRNA library they were detected: C, formation water; G, enrichment culture for aerobic organotrophs; F, fermentative; R, sulfate-reducing; Z, methanogens grown on $H_2 + CO_2$; and M, methanogens grown on acetate. Gray filling indicates the phylotypes found in the library from formation water. The scale bar indicates the evolutionary distance corresponding to 5 nucleotide substitutions per 100 bp. The numerals indicate the branching order reliability according to bootstrap analysis of 100 alternative trees. Values exceeding 75% are considered significant.

were different in the libraries of each enrichment, but the overall level of bacterial diversity in each library and in the data integrated over the libraries increased significantly in comparison to that in the formation water library. The bacterial indices of dominance for each enrichment culture (0.21–0.41) were notably lower than the value for formation water library (0.61). Thus, the communities of enrichment cultures were more leveled than the formation water community. Integration of data on enrichment cultures lowered the dominance index for bacteria to 0.15, to a greater degree than for archaea. This fact pointed to a considerable variation in the composition of predominant phylotypes among the libraries.

Phylogenetic Diversity of the 16S rRNA Genes of Archaea and Bacteria in the Clone Libraries of Formation Water and Enrichment Cultures

The results concerning phylogenetic analyses of libraries from enrichment cultures and formation water from well 37-7-2 are presented in Table 3.

Clone libraries of archaeal 16S rRNA genes constructed from microbial DNA isolated from formation water and enrichment cultures contained 170 and

Table 3. Compositions of 16S rRNA gene clone libraries of *Archaea** and *Bacteria*** from DNA isolated from enrichment cultures and formation water of the Gangxi bed; statistical analysis

Data type	Formation water libraries (C)		Primary enrichment culture libraries												Overall diversity in the libraries from formation water and enrichment cultures (Total)***	
			Aerobic organotrophs (G)		Fermentative (F)		Sulfate-reducing (R)		Methanogens on $H_2 + CO_2$ (Z)		Methanogens on acetate (M)		Combined enrichment culture libraries (Enr)			
Library	a*C	b**C	aG	bG	aF	bF	aR	bR	aZ	bZ	aM	bM	aEnr	bEnr	aTotal	bTotal
Number of clones (N)	170	146	22	63	27	44	61	59	44	51	63	56	217	273	387	419
Number of individual sequence types (ST)	81	102	16	56	15	36	32	48	23	42	34	47	104	221	182	320
Number of OTUs (≥98% similarity)	3	9	3	14	1	7	4	10	5	10	6	13	9	30	10 (7)***	37 (31)
Representation of phylotypes, OTU/clone	0.018	0.062	0.14	0.22	0.037	0.16	0.066	0.17	0.11	0.20	0.10	0.23	0.041	0.11	0.026 (0.018)	0.088 (0.074)
Coverage according to Good, %	100	98.6	100	92.1	100	97.7	98.4	93.2	95.5	96.1	100	96.4	99.5	96.7	100	97.6
Predicted number of phylotypes Chao 1	3	10	3	18	1	7	4	14	7	11	6	15	9	39	10 (7)	48 (43)
Min–max Chaol (95% COI)	3–3	9–23	3–3	15–40	1–1	7–15	4–4	11–39	5–27	10–18	6–6	13–35	9–9	32–70	10–10	39–85
Predicted number of phylotypes ACE	3	10	3	16	1	7	4	12	6	10	6	14	9	40	10 (7)	45 (43)
Shannon diversity index (H)	0.82	1.19	0.96	2.01	0.0	1.63	0.84	1.64	0.64	1.84	1.30	2.30	1.52	2.83	1.59 (1.34)	2.85 (2.51)
Evenness, (H/H_{max})	0.75	0.54	0.87	0.76	1.0	0.84	0.60	0.71	0.40	0.80	0.73	0.90	0.69	0.83	0.69 (0.69)	0.79 (0.73)
Simpson diversity index (I/D)	2.13	1.18	2.45	4.91	1.0	4.59	1.82	4.01	1.41	5.03	2.66	9.50	3.56	12.81	3.64 (2.97)	11.04 (7.32)
Index of dominance	0.55	0.61	0.59	0.41	1.0	0.36	0.72	0.36	0.84	0.35	0.59	0.21	0.37	0.15	0.43 (0.51)	0.22 (0.31)

* Archaeal clones.
** Bacterial clones.
*** The values for OTUs with sequence similarity ≥95% are shown in parentheses.

217 clones, respectively. All archaeal sequences cloned belonged to the phylum *Euryarchaeota*, orders *Methanobacteriales, Methanococcales, Methanomicrobiales*, and *Methanosarcinales* (Table 3, Fig. 2). Ten archaeal phylotypes were recognized at the species level in all libraries, of which only two phylotypes were common for formation water and enrichment culture libraries.

The archaeal sequences in the aC library of the formation water community formed three phylotypes. They all belonged to hydrogenotrophic methanogens *Methanobacterium congolense* (representative clone 1aC, 55% of the total composition of the aC library, 99% similarity to the genes of the type strain of the species), *Methanobacterium* sp. (190aC, 4% of the total composition, no more than 95% similarity to type strains of described species of the genus), and *Methanococcus vannielli* (4aC, 41% of the total composition, 97–98% similarity). The last phylotype was detected only in the aC library from formation water.

The phylogenetic diversity indices of archaea and predominant phylotypes in enrichment culture communities were different in different cultures, but only methanogens of the phylum *Euryarchaeota* were detected. The highest diversity was found in the aZ and aM libraries of methanogenic enrichment cultures. These libraries contained seven of nine phylotypes found in the total library of enrichment cultures. The following archaeal genera were found in the enrichment culture libraries: *Methanobacterium, Methanothermobacter* (family *Methanobacteriaceae*), *Methanofollis, Methanoculleus* (*Methanomicrobiaceae*), and *Methanomethylovorans* (*Methanosarcinaceae*). The single phylotype 47aM of the aM library (13% of its composition) obtained from the culture grown on acetate belonged to the species *Methanomethylovorans thermophila* (99% similarity), which does not grow on hydrogen or acetate.

Methanobacterium congolense sequences, dominating in the formation water library, were also found in four of five enrichment culture libraries: 12aF, 36aR, 1aZ, and 3aG. They constituted 35% of the total number of clones in the total library of enrichment cultures (Table 3, Fig. 2). Sequences of *Methanothermobacter thermautotrophicus* (99% similarity) were also found in four of five libraries (25aG, 1aR, 37aM, and 55aZ), although in lesser quantities, 11% of the total composition of aEnr. In addition, other methanogens were detected in some libraries: *Methanofollis liminatans* (98–99% similarity, 11aR and 6aM, 34% of the aEnr library), *M. formicicum* (99% similarity, 26aM and 2aG, 9% of aEnr), and *Methanoculleus* sp. (>95% similarity; 56aR, 22aZ, and 29aM; 3% of aEnr). The aZ library also contained *Methanobacterium* sp. phylotypes (no more than 95% similarity) as minor components, represented by one to three clones. One of them (14aZ) was identical to the phylotype 190aC from the formation water library and another (15aZ) was unique for enrichment cultures. Most sequences belonged to methanogens able to grow at the temperatures typical of the oil bed under study: 35–54°C.

Clone libraries of bacterial 16S rRNA genes from formation water and enrichment cultures contained 146 and 273 clones, respectively. The cloned sequences of the bC library from formation water formed nine phylotypes belonging to the phyla *Firmicutes; Alpha-, Beta-*, and *Gammaproteobacteria; Chloroflexi*; and a group of uncultured bacteria (Table 3, Fig. 3a). Two phylotypes predominated in the library: 1bC and 7bC. They were similar to each other (96% similarity). They constituted 85% of the total number of clones in bC. Both phylotypes showed no more than 90% similarity to the genes of type strains of described *Thermoanaerobacterium* species. Other phylotypes included one to five clones, belonging to proteobacterial genera *Brevundimonas, Sphingomonas, Oxalicibacterium, Stenotrophomonas*, the family *Enterobacteraceae*, uncultured bacteria of the phylum *Chloroflexi*, and unidentified bacteria.

Clone libraries of enrichment cultures showed much greater phylogenetic diversity of bacterial phylotypes than the formation water library. Sequences of all enrichments libraries (bEnr) formed 30 phylotypes belonging to the phyla *Firmicutes, Sinergistetes, Bacteroidetes, Proteobacteria, Thermotogae, Nitrospira, Spirochaetes*, and *Deferribacteres*, and to two clusters of unidentified uncultured bacteria (Table 3, Fig. 3b).

Sequences of the 16S rRNA genes of the phylum *Firmicutes* constituted 62% in the total library of enrichment cultures. A phylotype of the family *Thermoanaerobacteriaceae* predominated in the bG (35bG, 41%) and bF (1bF, 36%) libraries. Its similarity to the genes of species of known genera did not exceed 90%. Phylotype 34bG was similar to it (97%). It constituted 3% in the bG library. This library also contained phylotypes 37bG (6%) and 79bG (1.6%), identical to the phylotypes of *Thermoanaerobacterium* sp. dominating in the formation water library. Sequences of the anaerobic organotrophic bacterium *Soehngenia saccharolytica* (100% similarity) were found in the libraries bZ (1bZ, 25%), bM (4bM, 5%), and bR (3bR, 13%). Similar *Soehngenia* sp. phylotypes (97% similarity) were found in bZ (15bZ, 3%), bM (1bM, 4%), and bF libraries (20bF, 2%). Phylotype (2bZ, 35%), dominating in the bZ library, was also identified as *Soehngenia* sp., although its similarity to the gene of the *S. saccharolytica* type strain did not exceed 93%. Sequences of *Coprothermobacter proteolyticus* (98% similarity), abundant in bF (50bF, 25%) were minor in bM (58bM, 5%) and bG (3bG, 11%). Several *Firmicutes* were unique in the total enrichment culture community. The 7bM phylotype of facultative anaerobes of the genus *Exiguobacterium* sp. (95% similarity) constituted 21% of the bM library. Minor components included sequences of *Bacillus boroniphilus* (98% similarity, 12bR, 1.6%), a sulfate reducer *Desulfotomaculum* sp. (≤91% similarity, 73bG, 1.6%) and an uniden-

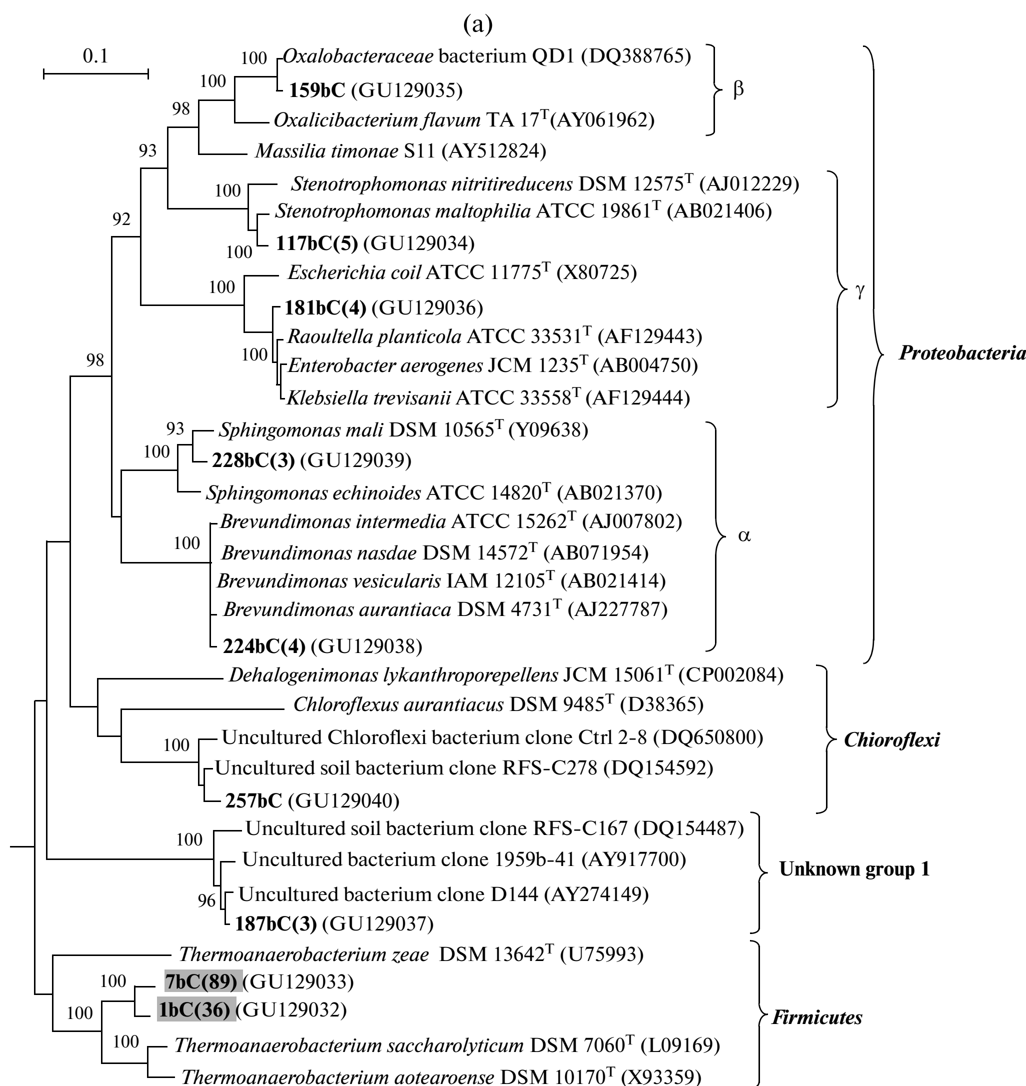

Fig. 3. Phylogenetic tree of 16S rRNA genes of *Bacteria* in clone libraries obtained from DNA isolated from oilfield water (a) and enrichment cultures (b). Gray filling indicates the phylotypes common for both libraries. The scale bar indicates the evolutionary distance corresponding to 5 nucleotide substitutions per 100 bp. Numerals indicate the faithfulness of branching according to bootstrap analysis of 100 alternative trees. Values exceeding 75% are considered significant. Phylotype designations follow Fig. 2.

tified bacterium of the family *Thermoanaerobacteraceae* (46bZ, 2%).

Sequences of the bacterial phylum *Sinergistetes* constituted 13.6% in the total enrichment culture library. They had 98–99% similarity to the genes of anaerobic organotrophic bacteria *Anaerobaculum thermoterrenum* (1bR, 34%; 3bM, 20%; and 12bZ, 8%), *Anaerobaculum mobile* (52bF, 7%; 16bR, 1.7%; and 5bG, 14%), and *Thermoanaerovibrio acidaminovorans* (25bM, 5%; 7bZ, 4%; and 46bG, 3%). The phylotype 11bR showed ≤88% similarity to the genes of the bacterial genus *Aminobacterium* and constituted 3% of all clones in the bR library.

The *Bacteroidetes* phylotype present in all enrichment culture libraries (28bF, 9%; 24bR, 5%, 5bM, 5%, 9bZ, 6%; and 26bG, 5%) showed 92% similarity to the gene of the type strain of *Proteiniphilum acetatigenes*. Thus, it belonged to this genus or to a closely related one. The phylotypes from bM (23bM, 5%) and bZ (65bZ, 2%) showed 97% and 95% similarity, respectively, to *P. acetatigenes*. They were also identified as *Proteiniphilum* sp. The *Bacteroidetes* phylotype found in bF (78bF, 16%), bZ (3bZ, 4%), and bG (22bG, 5%) was the farthest from *P. acetatigenes* (89% similarity).

The 16S rRNA gene sequences of proteobacteria constituted 5.2% in the total enrichment culture library. Phylotype 2bR was identified as the thermophilic sulfate-reducing deltaproteobacterium *Desulfomicrobium thermophilum* (98% similarity). It constituted 36% in the library from the enrichment culture of sulfate reducers and was present in the methanogenic library as a minor component (8bM, 4%). A

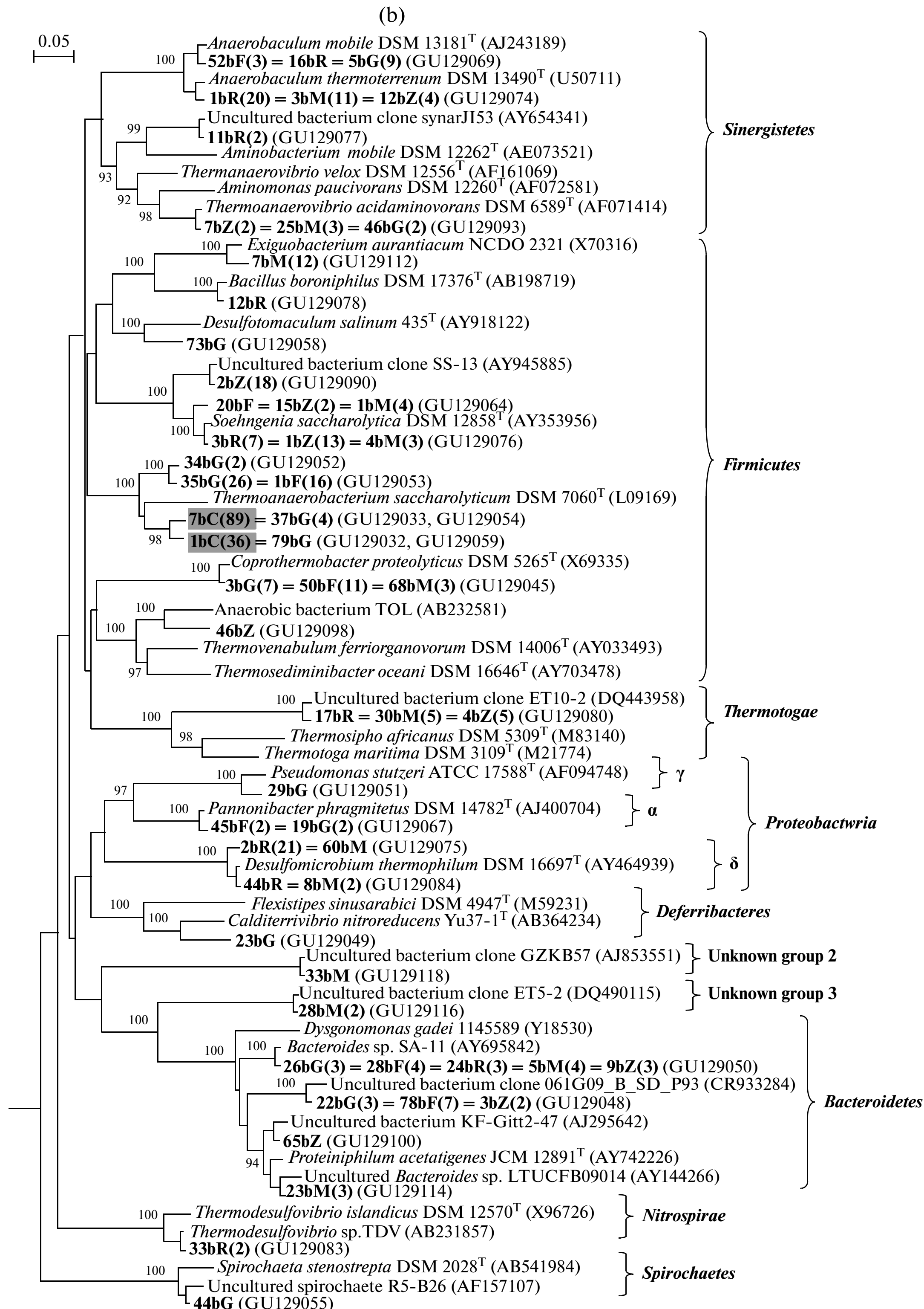

Fig. 3. Contd.

Desulfomicrobium sp. phylotype (≤96% similarity) was present in the same libraries (44bR, 1.7% and 60bM, 1.8%). Other minor phylotypes belonged to the gammaproteobacterium *Pseudomonas stutzeri* (96% similarity, 29bG, 1.6%) and the alphaproteobacterium *Pannonibacter phragmitetus* (98% similarity, 45bF, 4.5% and 19bG, 3%), which was formerly isolated from the Dagang oil field [25].

The phylotype of the phylum *Thermotogae* found in three libraries (17bR, 1.7%; 30bM, 9%; and 4bZ, 10%) showed 99% similarity to sequences of uncultured bacteria of the phylum. Several unique bacterial phylotypes in clone libraries of enrichment cultures belonged to the phyla *Deferribacteres* (23bG, 1.6%, 91% similarity to the gene of *Calditerrivibrio nitroreducens*), *Nitrospirae* (33bR, 3%, 95% similarity to *Thermodesulfovibrio islandicus*), and *Spirochaeta* (44bG, 1.6%, 96% similarity to *Spirochaeta stenostrepta*). We also found phylotypes 28bM (2.8%) and 33bM (7%); they belonged to two phyla of unidentified bacteria.

DISCUSSION

We analyzed the physicochemical conditions, abundance of culturable microorganisms, and rates of sulfate reduction and methanogenesis in formation water from the terrigenous Gangxi bed of the Dagang oilfield. The phylogenetic diversity of microorganisms in formation water and first generation enrichment cultures of the major metabolic groups was also investigated.

The Gangxi oil-bearing horizons have a notable incline, and their temperature varies over its area from 35 to 54°C. In formation water samples taken from the bed sulfate reduction and methanogenesis were registered and culturable anaerobic fermentative, sulfate-reducing, and methanogenic enrichments growing at 42°C were isolated.

With the usage of molecular biological techniques, the presence of mesophilic and thermophilic prokaryotes in formation water and enrichment cultures were revealed.

Although the application of molecular techniques based on determination of nucleotide sequences of 16S rRNA genes makes it possible to determine the phylogenetic position of the constituents of a microbial community, it is seldom sufficient to determine their biological functions. Methanogenic archaea are among the rare microbial groups, for which their phylogenetic position indicates their pathways of energy production.

In spite of the relatively low temperature in the studied zone of the bed Gangxi, 16S rRNA genes of thermotolerant hydrogenotrophic methanogen *Methanobacterium congolense* dominated among the archaeal sequences cloned from the formation water community. They constituted 55% of all genes in the aC library. This species can grow within a broad temperature range from 25 to 50°C, with the optimum being at 37–42°C. Another dominant archaeon was mesophilic methanogen *Methanococcus vannielii*.

Phylotypes of thermophilic methanogens (*Methanothermobacter thermautotrophicus, Methanomethylovorans thermophila*, and *Methanoculleus* sp.) dominated over the mesophilic ones in the combined enrichment culture library. Mesophilic hydrogenotrophic methanogens of the genus *Methanofollis* grow at temperatures within 20–45°C [26], and the thermophilic methanogen *Methanoculleus thermophilus* (close to phylotype 56aR), grows at 37–65°C [27]. The thermophilic methanogen *Methanomethylovorans thermophila* grows at 42–58°C, the optimum temperature being 50°C. It consumes methanol and methylated amines but not $H_2 + CO_2$, formate, acetate, or propanol [28]. Nevertheless, its phylotype was detected in the library of the methanogenic culture grown on acetate. It is unclear what supported its growth in the enrichment culture.

The 16S rRNA genes of thermophilic fermentative bacteria of the family *Thermoanaerobacteriaceae* dominated among the cloned sequences of the bacterial community of formation water, comprising 85.6% of all genes in the bC library. Other phylotypes in bC library included mesophilic aerobic organotrophic proteobacteria of the genera *Brevundimonas, Sphingomonas, Oxalicibacterium*, and *Stenotrophomonas*; uncultured bacteria of the phylum *Chloroflexi*; and unidentified bacteria. Although culturable sulfate-reducing prokaryotes and process of sulfate reduction were registered in formation water, no sequences of known sulfate reducers were found in the formation water library.

At the beginning of the Gangxi bed exploitation, formation water contained little (44–61 mg/L) or no sulfates. Injection of air–water mixture and hydrogen peroxide solution resulted in the increase of the sulfate content, sulfate reduction rate, and the numbers of sulfate-reducing bacteria in formation water [16]. However, sequences of sulfate reducers belonging to known thermophilic bacteria *Desulfomicrobium thermophilum* and *Thermodesulfovibrio* sp. were found only in the libraries of enrichment cultures of sulfate-reducing and methanogenic prokaryotes. The presence of sulfate reducers in the sulfate-free medium for methanogens appears to be related to their versatile metabolism. These bacteria are known to produce energy not only by sulfate reduction. In the absence of sulfates, they are capable to ferment organic substrates and perform interspecies electron transfer, thereby acting as fermentative or syntrophic bacteria.

Metabolic versatility and ability to grow within a broad range of temperatures are also characteristic of fermentative microorganisms of oil fields, as was repeatedly noted in studies of pure and enrichment cultures [1].

The greatest number of bacterial phylotypes (14 of 30 in the combined enrichment culture library) was found in the culture of aerobic organotrophs grown with tryptone, glucose, and yeast extract. Oxygen of the gas phase was entirely exhausted by aerobic microorganisms of the microbial community grown without additional aeration. The succession of the microbial community under anaerobic conditions resulted in formation of a phylogenetically diverse consortium, including both anaerobic and aerobic prokaryotes.

This medium revealed even greater diversity of anaerobic bacteria than the medium with peptone and glucose for anaerobes (bF library). The phylotypes of aerobic bacteria in bG belonged to the hydrocarbon-oxidizing gammaproteobacterium *Pseudomonas stutzeri* and alphaproteobacterium *Pannonibacter phragmitetus*. A pure culture of a facultative anaerobe *P. phragmitetus* was formerly isolated from the Kondian bed of the Dagang oil field. It could grow under aerobic conditions on *n*-alkanes or crude oil [25]. The anaerobic phylotypes in bG library belonged to fermentative bacteria of the genera *Thermoanaerobacter, Thermoanaerobacterium, Anaerobacterium, Coprothermobacter*, and *Thermanaerovibrio*; sulfate reduces of the genus *Desulfotomaculum*; and a spirochaete *Spirochaeta* sp.

Sequences of fermentative bacteria of the genera *Anaerobaculum, Soehngenia, Bacteroides, Proteiniphilum*, and *Aminobacterium* were detected in enrichment culture libraries of sulfate-reducing (bR) and methanogenic (bM) prokaryotes. It is known that anaerobic organotrophic bacteria of the genera *Thermoanaerobacterium, Thermoanaerobacter*, and *Anaerobaculum* are capable of fermenting organic substrates with reduction of thiosulfate, sulfite, or sulfur to sulfide. Microorganisms of these genera were most frequently isolated from oilfields [1, 5, 8, 9, 29]. Bacteria of the genus *Thermoanaerobacter* can also grow autotrophically on hydrogen, reducing oxidized iron [30]. Most oil components are not fermented under anaerobic conditions without electron acceptors. The role of fermentative prokaryotes in oil degradation in the oilfields is poorly understood.

Phylotypes of obligate syntrophic bacteria were absent from the libraries under study. As noted above, the study of microorganisms from the high-temperature Kondian bed of the Dagang oilfield showed that acetate degradation to methane was performed by a syntrophic association of acetate-oxidizing microorganisms and H_2-consuming methanogens. Acetate is oxidized to H_2 and CO_2 by microorganisms of the group *Thermoanaerobacter–Caldanaerobacter* in the presence of methanogens of the genus *Methanothermobacter*, which consume molecular hydrogen [7–9]. The results suggest that the role of syntrophic prokaryotes in the Gangxi bed, as in Kondian, is played by fermentative bacteria of the order *Thermoanaerobacteriales* or by sulfate-reducing bacteria, which also can transfer electrons to acceptor microorganisms, e.g., methanogens present in the bed.

No sequences of the bacterial genus *Soehngenia* were found in the formation water library, but they were present in the libraries obtained from enrichment cultures with lactate, acetate, and $H_2 + CO_2$ supplemented with yeast extract. *Soehngenia* grow at temperatures within 15–40°C, with the optimum at 30–37°C. They consume a variety of sugars, organic acids, and yeast extract [31]. They can disproportionate benzaldehyde to benzoate and benzyl alcohol, although this process does not support growth. It is likely that their metabolism in the oil bed is associated with conversion of aromatic oil components or microbial biomass.

According to statistical analysis of the 16S rRNA gene libraries of bacteria and archaea from formation water and enrichment cultures of Gangxi bed, 387 cloned archaeal and 419 bacterial sequences formed 10 and 37 phylotypes, respectively, at the species level. The total number of individual sequence types and phylotypes was less than their total numbers for each particular clone library, owing to the presence of identical sequence types and phylotypes. The presence of similar but not identical sequences is revealed, as a rule, during analysis of the 16S rRNA gene libraries, pointing to sequence microvariability. The overall coverage of total archaeal libraries with reference to predicted values was the greatest, whereas for bacteria it was 82 and 77% of the predicted ACE and Chao 1 indices, respectively. It was confirmed by the shape of the corresponding rarefaction curves (Figs. 1a, 1b).

Determination of the community composition at the genus level yielded slightly lesser numbers of bacterial and archaeal phylotypes: 7 and 31, respectively (Table 3). The changes in the indices of coverage, diversity, and dominance were insignificant, too. Thus, the diversity of the community members at the intrageneric level was poor.

To sum up, studies of formation water and enrichment cultures of the major metabolic types in formation water of the Gangxi bed revealed a diverse microbial community. It contained aerobic organotrophic bacteria, including those oxidizing hydrocarbons; anaerobic fermentative bacteria capable of syntrophic growth; and sulfate-reducing and methanogenic prokaryotes. The integrated cultural, radioisotopic, and molecular study of formation water and enrichment cultures provided a more comprehensive insight in the functional and phylogenetic diversity of the microbial community inhabiting the oil bed.

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