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EXPERIMENTAL ARTICLES

Characterization of the Aerobic Hydrocarbon-Oxidizing Enrichments from a High-Temperature Petroleum Reservoir by Comparative Analysis of DNA- and RNA-Derived Clone Libraries

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Abstract—Enrichment cultures of aerobic hydrocarbon-oxidizing bacteria obtained from the injection and production wells of the Dagang oil field (China) were studied by molecular biological and microbiological methods. This work is the first to report simultaneous isolation of DNA and RNA from enrichment cultures of microorganisms from oil strata with further construction of clone libraries of 16S rRNA genes and 16S crDNA (complementary rDNA). Comparative analysis of the DNA- and RNA-derived clone libraries made it possible to determine the total genomic diversity of microorganisms, as well as to reveal metabolically active microorganisms in these cultures. Phylotypes of bacteria of the genus *Geobacillus* were found to be dominant in the DNA and RNA clone libraries of the enrichment cultures from the production well. Phylotypes of bacteria belonging to *Geobacillus, Pseudomonas, Tepidiphilus*, and other genera were detected in the DNA and RNA libraries obtained from the culture from the injection well. Phylotypes of bacteria of the genus *Geobacillus* were predominant in the RNA library and represented the second-largest group (after pseudomonads) in the DNA library. In the RNA libraries of the *alkB* genes of both enrichments, three homologs close to *alkB-geo1, alkB-geo2*, and *alkB-geo4* of bacteria of the genus *Geobacillus* were detected. The occurrence pattern of the *alkB* transcripts, ribosomal RNA, and the 16S rRNA genes of bacteria of the genus *Geobacillus* indicates the predominance and functional activity of geobacilli in the enrichment cultures of hydrocarbon-oxidizing bacteria from high-temperature petroleum reservoir.

Keywords: Geobacillus, Shewanella, Pannonibacter, thermophiles, *alkB* genes, 16S rRNA genes, 16S crDNA, n-alkane oxidation, oil fields.

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Water injected into oil fields exploited with waterflooding contains dissolved oxygen, thereby creating conditions favoring oil oxidation in the near-bottom zone [1–3]. In this zone, aerobic hydrocarbon-oxidizing bacteria are the primary constituents of the microbial trophic chain involved in oil biodegradation. The products of oil oxidation enter the zone of production wells and serve as substrates for anaerobic microorganisms. In the near-bottom zone of injection wells, high numbers of microorganism belonging to various physiological groups and the highest rates of sulfate reduction and methanogenesis were detected [1–3]. In water-flooded high-temperature oil strata, bacteria of the genus *Geobacillus* may presumably be the primary aerobic degraders of the organic matter of oil. However, there is no direct evidence yet that these bacteria are responsible for oil biodegradation. A number of *Geobacillus* species (*G. subterraneus, G. stearothermophilus, G. jurassicus, G. thermoglucosidasius, G. uzenensis, G. thermodenitrificans*, etc.) have been isolated from oil strata [3–7]. Under laboratory conditions, it was demonstrated that bacteria belonging to this genus utilize, above all else, the aliphatic components of oil.

It was found that, in mesophilic hydrocarbon-oxidizing bacteria, it is the alkane hydroxylase enzymatic complex encoded by the *alk* genes that is responsible for the oxidation of n-alkanes [8–10]. The main enzyme of this complex, alkane monooxygenase, binds oxygen atoms directly to the end atoms of n-alkanes. Two other proteins, rubredoxin and rubredoxin reductase, provide energy for alkane monooxygenase via electron transfer from NADH. The *alkB* genes of thermophilic bacteria were studied using *Geobacillus* strains as an example [11]. A total of eight homologs of the *alkB* gene were found in the genomic DNA of eleven *Geobacillus* strains. In some *Geobacillus* strains, three to seven homologs of the *alkB* gene

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were revealed; two of them were found to be universal for all strains.

The diversity of microorganisms inhabiting the waters of the production wells of high-temperature oil strata was studied using molecular biological techniques [12–14]. No *Geobacillus* phylotypes were detected in the clone libraries of 16S rRNA genes constructed on the basis of DNA from the stratal waters of oil fields. cDNA fragments of the *alkB* gene of *Geobacillus thermoleovorans* T70 were detected in the enrichment cultures obtained from soil samples on medium with n-hexadecane [15]. The clone libraries constructed on the basis of DNA from oil-polluted soils and other natural environments contained *alkB* genes as well [10].

The amounts of ribosomes and enzymes in metabolically active cells are significantly larger than in resting cells [16]. Analysis of the 16S rRNA genes and functional genes obtained on the basis of the total RNA of the community allows researchers to reveal the constituents of this community that actively synthesize proteins and, thus, to reveal functionally active groups of microorganisms within the community [17]. This approach was used for studying microbial communities inhabiting freshwater sediments [18], soils polluted with polychlorinated biphenyl [16], marine gas hydrates [17], and other habitats. This work is the first study of the oil field microbial community that uses comparative analysis of clone libraries of the 16S rRNA genes and *alkB* genes constructed on the basis of DNA and RNA.

The goal of the work was investigation of biodiversity and identification of the microorganisms responsible for n-alkane degradation in enrichment cultures of aerobic thermophilic hydrocarbon-oxidizing bacteria from the Dagang oil field by microbiological methods, as well as by the construction of DNA and RNA-derived clone libraries of 16S rRNA and *alkB* genes.

MATERIALS AND METHODS

Obtaining enrichment cultures. Enrichment cultures of aerobic hydrocarbon-oxidizing bacteria were obtained from the high-temperature Kondian bed of the Dagang oil field (Hebei Province, China). The studied sandstone oil-bearing horizons occurred 1206–1435 m below sea level and had a temperature of 59°C. The oil had a density of about 0.900 g/cm^3 and contained saturated hydrocarbons (53%), aromatic compounds (20%), and resins and asphaltenes (21.15%). The formation waters were of hydrocarbonate–sodium type and had a mineralization of 5612 mg/1. Detailed characteristics of the bed are presented in our previous publications [3, 5].

The enrichment cultures were obtained by inoculating stratal water into mineral medium [2] containing a mixture of C_{12}–C_{22} *n*-alkanes (0.5 vol %). The stratal water from production well 1017-3, as well as from injection well 1098, operated in a regime of backflow (a 8-m^3 sample), was used as inoculum. Dissolved oxygen (0.2–0.8 mg/l) and various microorganisms enter the stratum with injection water [3]. Inoculated media were incubated without agitation at 60°C. The biomass grown after the third transfer of the enrichment cultures of hydrocarbon-oxidizing bacteria (designated as 1017-3 and 8m3) into the medium with n-alkanes was used to isolate nucleic acids.

Pure cultures were isolated from the enrichments by plating onto Plate Count Agar (PCA, Sigma) and solid and liquid Raymond mineral agar supplemented with a paraffin mixture (C_{12}–C_{22}, 5 ml/l) as described earlier [5]. Pseudomonads were isolated using media with glycerol (10 ml/l) as the growth substrate and levomycetin (10 μg/ml). Inoculated media were incubated at 37 and 60°C for 1–5 days. Fourteen strains of aerobic organotrophic bacteria were isolated and purified by successive transfers of individual colonies. The strains were maintained on PCA solid medium or in liquid medium containing a mixture of C_{12}–C_{22} *n*-alkanes. Growth in oil-containing medium was assessed by monitoring the concentration of n-alkanes in degraded oil and comparing it to the control as described in [19]. The thermophilic strains 1017 and 8m3 were incubated at 60°C; the mesophilic strains M-8m3-3 and M-8m3-2 were incubated at 37°C. The control, which was represented by uninoculated oil-containing medium, was incubated under the same conditions. Inoculated media were incubated for 7 days.

Identification of pure microbial cultures. Pure microbial cultures isolated from the enrichments of aerobic hydrocarbon-oxidizing bacteria were identified using molecular biological techniques. DNA extraction, as well as amplification and sequencing of the 16S rRNA gene fragments of pure cultures, was carried out as described in [5]. The full-length 16S rRNA gene of the studied microorganisms was amplified with the primers 8f-1492r and used as a template for DNA sequencing with the primers 8f, 519r, 519f, and 1492r. To identify *Pseudomonas* strains, *gyrB* gene fragments were amplified using the primers Up-1-deA–Up-2R [20]; sequencing of the PCR products was performed using the primers Up-1S and Up-2Sr, complementary to the 5' ends of the degenerate primers Up-1-deA and Up-2R [20]. *Geobacillus* strains were identified using a specially designed direct primer, gyrBs-G(gyrl-G), in combination with the primer Up-2Sr [21].

DNA and RNA extraction from enrichment cultures. Total DNA from the enrichment cultures 1017-3 and 8m3 was isolated using a Diatom^{TM}DNAprep kit (Biokom, Moscow) according to the manufacturer's protocol with minor modifications. Biomass from cultures (10 ml) was harvested by centrifugation, and the cell sediment was resuspended in Milli-Q water and then subjected twice to a freezing–thawing procedure

(freezing under liquid nitrogen and thawing at 65°C). Then, guanidine hydrochloride was added, and the mixture was thermostated at 65°C for 1 h. The resulting lysate was supplemented with Diatomid/silica carrier. After DNA sorption, the supernatant was removed and the sorbent was washed with guanidine hydrochloride, a salt buffer, and 70% ethanol. The DNA preparation was dissolved in 100 μl Milli-Q water and used as a template for PCR.

Total RNA was isolated using TRIzol (TRIzol Reagent, Invitrogen) according to the manufacturer's protocol. Culture growth was terminated by rapid cooling on ice and subsequent cell precipitation by centrifugation at 10000 *g* for 5 min. The precipitated cells were supplemented with 1 ml of TRIzol and incubated at room temperature for 5 min. Then, 0.2 ml of chloroform was added, and the cell debris was precipitated by centrifugation (12000 *g*) at 2–8°C for 15 min. RNA from the water phase was precipitated with isopropanol; the sediment was washed with 75% ethanol and dried at room temperature. RNA was dissolved in water and treated with RNase-free RQ1 DNase (Promega, United States). The reaction mixture (10 μl) contained the following: 1× buffer (40 mM Tris–HCl, pH 8.0; 10 mM $MgSO_4$; and 1 mM $CaCl_2$), 2–5 μg of RNA, and 2 U of enzyme. The reaction mixture was incubated at 37°C for 30 min; the reaction was terminated by heating at 65°C. For reverse transcription, RNA was precipitated with 70% ethanol and 0.3 M sodium acetate. The total RNA preparations were stored at –70°C.

During all stages of the experiments with RNA and cDNA, plastic labware and pipettes pretreated with diethylpyrocarbonate (DEPC) were used; the solutions were also treated with 0.05% DEPC overnight at room temperature and then autoclaved at 0.5 atm for 30 min. All working surfaces were also pretreated with an anti-RNase solution (AmBion, United States).

The obtained preparations of genomic DNA and total RNA were assayed by electrophoresis in 0.8–1.5% agarose gel. The nucleic acid concentration was determined on an ND-1000 spectrophotometer (NanoDrop, United States) according to the manufacturer's recommendations.

Reverse transcription. The first cDNA chain was synthesized using the Sileks kit for reverse transcription (Sileks, Moscow). The reaction mixture (25 μl) contained the following: 2–5 μg of total RNA, 15–20 nmol of hexanucleotide primers, 1× buffer (70 mM Tris–HCl, pH 8.3; 16.6 mM $(NH_4)_2SO_4$, and 7.5 mM $MgCl_2$), 6 mmol of dNTPs, and 100 U of M-MLV reverse transcriptase. The primers were preannealed with total RNA at 70°C for 5 min. Then, buffer, dNTPs, and the M-MLV reverse transcriptase were added; the resultant mixture was incubated at 25°C for 10 min and at 42°C for 60 min. The reaction was terminated by heating at 70°C for 10 min, and then the mixture was cooled on ice. The resultant cDNA was used immediately as a template for PCR with universal primers.

DNA amplification. PCR amplification of *alkB* genes was performed using degenerate oligonucleotide primers: the forward Alk-BFB primer (5'-GGT ACG GSC AYT TCT ACR TCG A-3') and the reverse Alk-BRB primer (5'-CGG RTT CGC GTG RTG RT-3') [11]. The reaction mixture contained the following: 50–80 ng of DNA; 5 pmol of each primer; dNTPs, 200 μM each; 0.5 U of the thermostable Taq DNA-polymerase; and 1× Taq buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl; and 2 mM $MgCl_2$). Amplification was performed in a Mastercycler (Eppendorf, Germany). The PCR cycle parameters were as follows: polymerase activation (94°C for 3 min); then, 35 cycles of DNA denaturation (94°C for 0.5 min), primer annealing (60°C for 1 min), and extension (72°C for 0.5 min); and final extension (72°C for 8 min).

The 16S rRNA gene fragments of representatives of the domain *Bacteria* were amplified using the universal primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 519r (5'-G(T/A)ATTACCGCGGC(T/G)G-CTG-3') [22, 23]. There were 30 PCR cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.5 min. The 16S rRNA gene fragments of archaea were amplified using the primers A109F (5'-ACG/TGCTCAG-TAACACGT-3') and A1041r (5'-GGCCATGCAC-CWCCTCTC-3') [24]. Analysis of the obtained DNA fragments was carried out by electrophoresis in 1% agarose gel stained with ethidium bromide. The PCR products were purified by DNA precipitation with a mixture of ethanol and 0.75 M ammonium acetate at room temperature.

Cloning and sequencing of PCR products. The amplified *alkB* and 16S rRNA gene fragments (about 500 bp) were cloned into the plasmid vector pTZ57RT (Fermentas, Lithuania). Clones containing DNA inserts of the expected size (~500 bp) were sequenced using the plasmid primers M13D and M13R. Sequencing was performed on a 3730 DNA Analyzer sequencer using a *BigDye* Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States).

Phylogenetic analysis. Preliminary analysis of the obtained nucleotide sequences was performed using the NCBI BLAST software (www.ncbi.nlm.nih.gov/blast/). The obtained nucleotide sequences were aligned with the corresponding sequences retrieved from the GenBank database using the CLUSTALX 2.0 software (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/ [25]). Phylogenetic trees were constructed by the neighbor-joining method [26] implemented in the TREECONW software package (http://bioinformatics.psb.ugent.be/psb/Userman/treeconw.html) [27].

The obtained nucleotide sequences were deposited in the GenBank under the following accession numbers: FJ895350–FJ895365 and FJ895368–FJ895376

(cloned 16S rRNA genes); FJ895366, FJ895367, FJ895377, and FJ895378 (*alkB* genes); and HQ324905–HQ324913 (16S rRNA genes of pure cultures).

RESULTS AND DISCUSSION

Determination of the diversity of microorganisms in the enrichment cultures of hydrocarbon-oxidizing bacteria by microbiological methods. From the enrichment cultures 8m3 and 1017-3 grown at 60°C in media with C_{12}–C_{22} *n*-alkanes, 14 pure cultures of mesophilic and thermophilic aerobic organotrophic bacteria were isolated using PCA medium and media with n-alkanes at incubation temperatures of 37 and 60°C.

The obtained pure cultures were identified by analysis of the 16S rRNA gene sequences (600–1500 nucleotides). Spore-forming rods grew at 60°C on PCA medium. Two identical strains, G1017_C12 and G1017_C14 (100% identity of the 16S rRNA gene sequences), isolated from the enrichment culture 1017-3, were found to be close relatives of *G. stearothermophilus* (99.0% identity with 16S rRNA gene of the type strain DSM 22^T) (Fig. 1). Strain G8m, isolated from enrichment culture 8m3, was found to be closely related to *Geobacillus pallidus* (99% identity of 16S rRNA genes).

In the course of incubation on media with *n*-alkanes and on PCA at 37°C, 11 strains of mesophilic bacteria were isolated; the two different enrichments yielded different strains. Two strains, M-8m-1 and M-8m-3, isolated from culture 8m3, were found to be identical (100% identity of the 16S rRNA gene sequences). They were affiliated with the species *Shewanella putrefaciens* (99.8% 16S rRNA gene identity), whereas strain M-8m-2 was affiliated with *Pannonibacter phragmitetus* (99% identity). Eight 100% identical strains, P-1017-1–P-1017-8, were isolated from the enrichment 1017-3. They were closely related to *Pseudomonas putida* (100% identity). In pure culture, the maximum growth temperature of *P. putida* strains was 37°C, the maximum growth temperature of *S. putrefaciens* M-8m-l and M-8m-3 was 39°C, and the maximum growth temperature of *Pb. phragmitetus* M-8m-2 was 44°C. All isolates grew on oil, utilizing the n-alkane fraction. Figure 2 shows the contents of n-alkanes in oil degraded by thermophilic and mesophilic microorganisms. It can be seen that the studied *Geobacillus* strains, G1017_C12 and G8m, as well as *Pb. phragmitetus* M-8m-2 and *S. putrefaciens* M-8m-3, grew on oil, utilizing *n*-alkanes with carbon chains of various lengths. Earlier, bacteria of the genus *Shewanella* were reported to be present in stratal waters of oil fields and in microbial communities involved in the biodegradation of oil, long-chain *n*-alkanes (C_{24} to C_{34}), and aromatic compounds [28, 29]. Our *Shewanella* isolates grew on oil, utilizing a wide range of n-alkanes with a carbon chain length of C_{14}–C_{34} (Fig. 2d). It remains unclear how mesophilic microorganisms survived three culture transfers at 60°C.

Diversity of the 16S rRNA genes in the clone libraries obtained from enrichment cultures. The total DNA and RNA were extracted from the thermophilic hydrocarbon-oxidizing enrichment cultures obtained from the near-bottom zone of injection well 1098 and from production well 1017-3 (cultures 8m3 and 1017-3, respectively) of the Dagang oil field. Our work is the first to use this approach to reveal metabolically active microorganisms in oil strata. The RNA isolated was used as a template for reverse transcription and synthesis of the first cDNA chain. The total DNA extracted from the enrichment cultures 8m3 and 1017-3 and the cDNA obtained on the basis of their total RNA were used for amplification of the 16S rRNA gene fragments with primers universal for the *Archaea* and *Bacteria* domains. We were able to obtain PCR products only with the bacterial primers. The obtained PCR products were cloned, and two clone libraries were constructed for each culture.

The DNA clone library (16S rRNA genes) and the RNA clone library (16S rRNA) of the culture 8m3 consisted of 89 and 55 clones, respectively; the clone libraries of the culture 1017-3 consisted of 75 and 48 clones. Designations for the clone libraries are given in Tables 1 and 2. The bacterial community of the culture 8m3 obtained from the near-bottom zone was more diverse than that of the culture 1017-3 obtained from the production well; a significant difference in the clone library compositions was observed as well. In the DNA library of the culture 8m3, phylotypes of bacteria of the genus *Pseudomonas* were predominant (47.8% of the total number of clones in the DNA clone library); the second-largest group (26%) was phylotypes of the thermophilic bacterium of the genus *Tepidiphilus*; the third group (19%) was represented by phylotypes of bacteria of the genus *Geobacillus*. However, geobacilli were predominant (54.2%) in the RNA library, the percentage of pseudomonads decreased to 32%, and members of the genus *Tepidiphilus* were not detected at all. Phylotypes of various mesophilic bacteria belonging to the genera *Acinetobacter*, *Gordonia, Carnobacterium*, and *Caulobacter* were minor components (one or two clones) (Table 1).

By contrast, geobacilli prevailed in the DNA and RNA libraries of the enrichment culture from production well 1017-3 (98.7 and 79.1%, respectively); pseudomonads constituted 1.3 and 12.5% of the total number of clones in these libraries. Phylotypes of the minor constituents represented by mesophilic bacteria of the genera *Pseudomonas*, *Williamsia, Gordonia*, etc., were detected only in the RNA library of culture 1017-3 (Table 2).

Thus, the investigation of the microbial diversity based on the genomic DNA from the bacterial community showed that geobacilli and pseudomonads were the main constituents of the studied enrichment

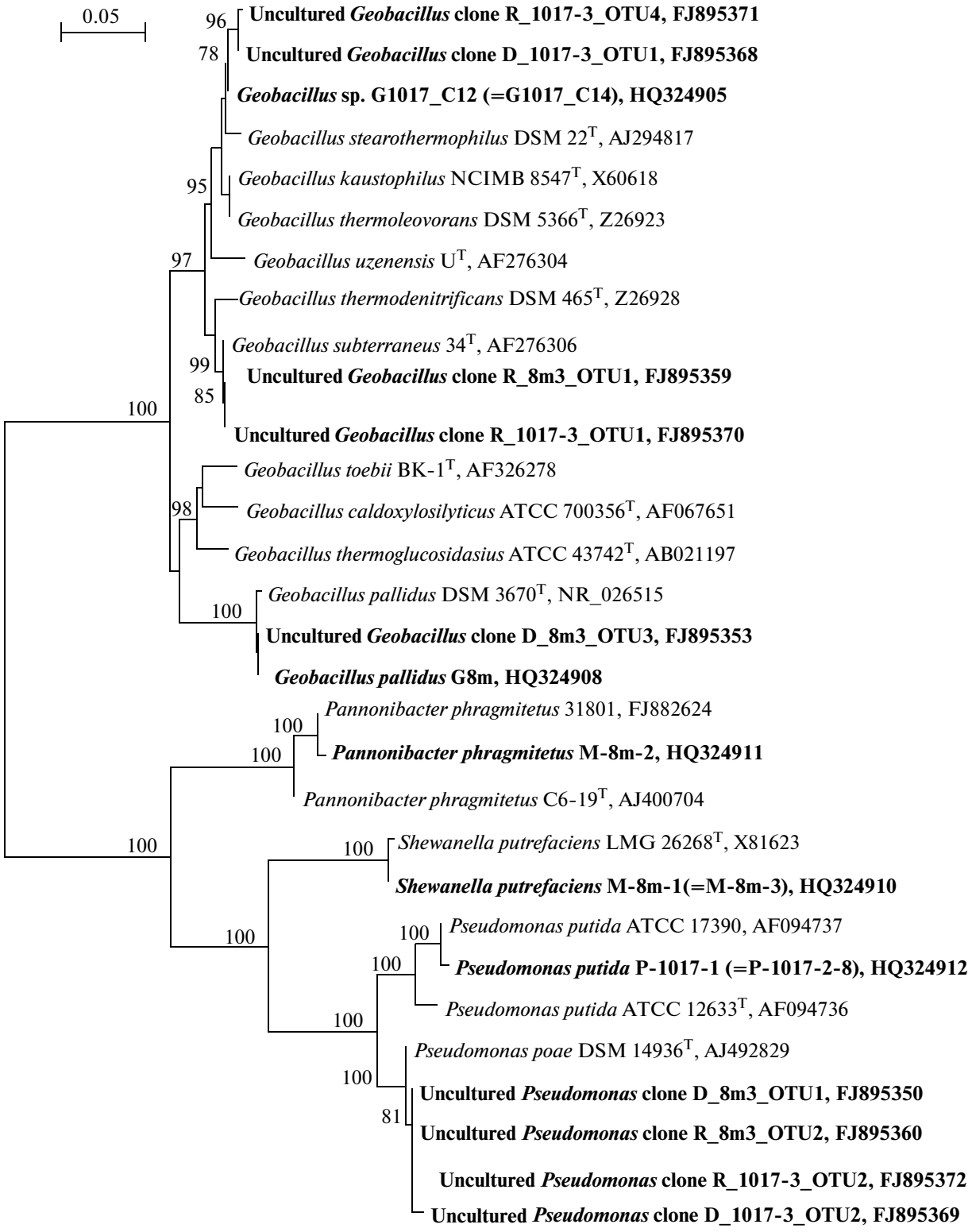

Fig. 1. Phylogenetic tree of the 16S rRNA genes of pure cultures isolated from the Dagang oil field, as well as of the 16S rRNA genes and 16S crDNAs from the clone libraries constructed on the basis of the total DNA and RNA from the enrichment cultures of hydrocarbon-oxidizing bacteria. The bar shows evolutionary distance, corresponding to five substitutions per 100 nucleotides. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees (only bootstrap values above 75% were considered significant). R in the clone names indicates that these clones were detected in the RNA library; D indicates that the clones were detected in the DNA library.

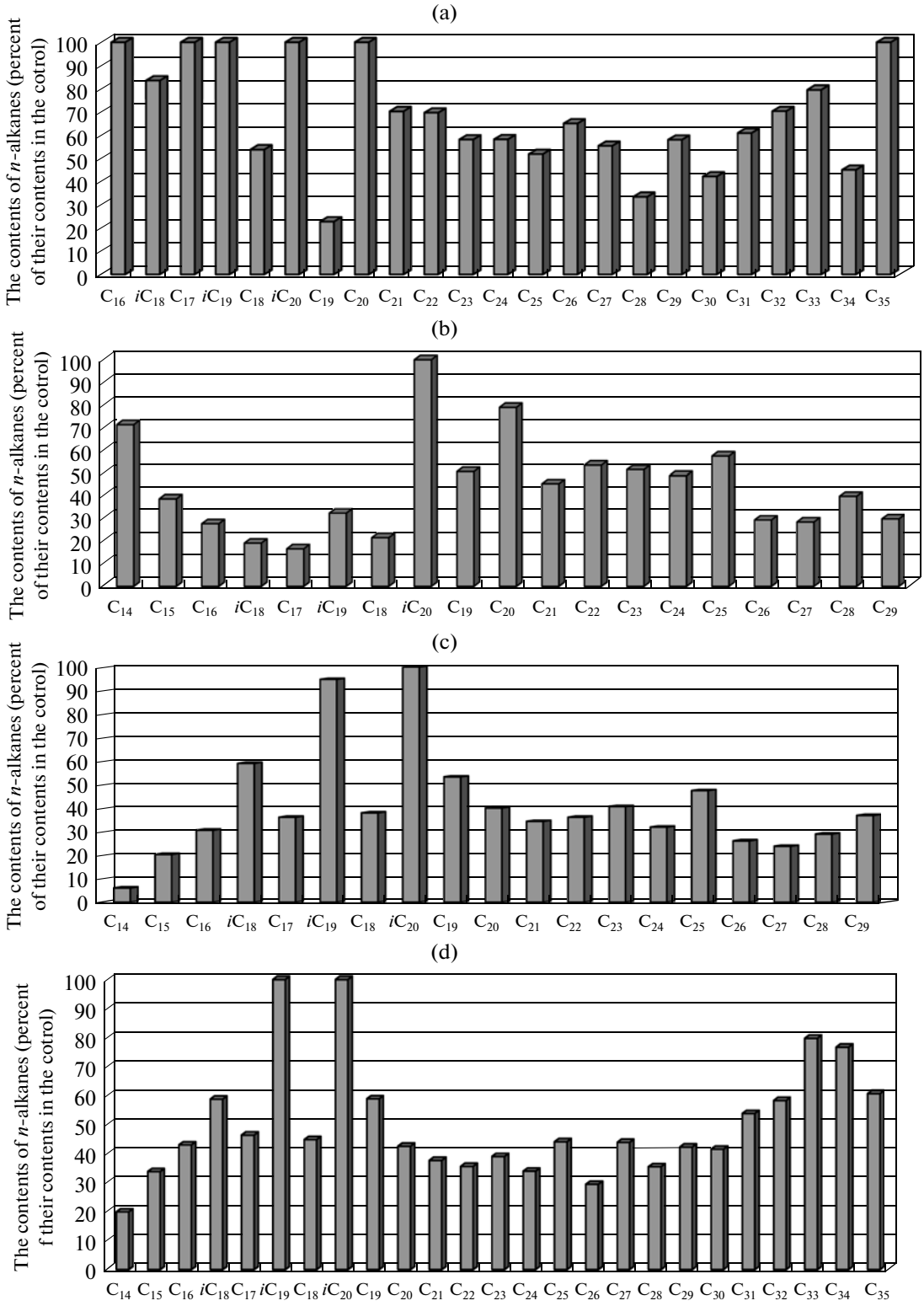

Fig. 2. The contents of n-alkanes (expressed as percent of their contents in the control) in oil degraded by the thermophilic strains *Geobacillus stearothermophilus* G1017_C12 (a) and *G. pallidus* G8m (b), as well as by the mesophilic strains *Pannonibacter phragmitetus* M-8m-2 (c) and *Shewanella putrefaciens* M-8m-3 (d). The thermophilic and mesophilic strains were incubated at 60 and 37°C, respectively, for 7 days. The controls (uninoculated oil-containing medium) were incubated under appropriate conditions.

Table 1. Diversity of the 16S rRNA genes, 16S crDNA, and cDNA of the *alkB* genes in the clone libraries constructed on the basis of enrichment culture 8m3

OTU (representative clone)	Clone number	Length, bp	Closest relative in GenBank	% identity	GenBank accession number
16S rRNA gene clone library (DNA library)					
D_8m3_OTU1	42	525	*Pseudomonas poae* ST250	99	EU350370
D_8m3_OTU2	20	501	*Tepidiphilus margaritifer* N2-214^T	98	AJ504663
D_8m3_OTU3	17	541	*Geobacillus pallidus* DSM 3670	98	Z26930
D_8m3_OTU4	3	444	*Tepidiphilus margaritifer* N2-214^T	97	AJ504663
D_8m3_OTU5	2	476	*Carnobacterium divergens* CWBI B1439	98	EU128490
D_8m3_OTU6	2	509	*Gordonia sputi* DSM 43896^T	99	X80634
D_8m3_OTU7	2	451	*Caulobacter crescentus* CB15	95	AE005673
D_8m3_OTU8	1	469	*Acinetobacter johnsonii* UFV-E05	99	EF114343
Total	89				
16S crDNA clone library (RNA library					
R_8m3_OTU1	26	542	*Geobacillus subterraneus* 34^T	99	AF276306
R_8m3_OTU2	18	525	*Pseudomonas poae* ST250	99	EU350370
R_8m3_OTU3	8	531	*Escherichia coli* ATCC 8739	99	CP000946
R_8m3_OTU4	1	502	*Thermoanaerobacterium thermosaccharolyticum* GD17	97	EF680277
R_8m3_OTU5	1	474	*Sphingomonas rhizogenes* RSB-1	99	AY962684
R_8m3_OTU6	1	514	*Lactococcus lactis* ATCC 19257	99	AB008215
Total	55				
cDNA of the *alkB* gene clone library (RNA library)					
AR_8m3_OTU1	27	483	*alkB-geo2*	99	EF534177
AR_8m3_OTU2	14	483	*alkB-geo4* (=*alkB3 Nocardia* sp. H17-1)	99	EF534179

Table 2. Diversity of the 16S rRNA genes, 16S crDNA, and the cDNA of the *alkB* genes in the clone libraries constructed on the basis of enrichment culture 1017-3

OTU (representative clone)	Clone number	Length, bp	Closest relative in GenBank	% identity	GenBank accession number
16S rRNA gene clone library of the (DNA library)					
D_1017-3_OTU1	74	540	*Geobacillus stearothermophilus* ARM 1	98	EF025325
D_1017-3_OTU2	1	471	*Pseudomonas poae* ST250	99	EU350370
Total	75				
16S crDNA clone library (RNA library)					
R_1017-3_OTU1	35	541	*Geobacillus subterraneus* 34^T	99	AY608959
R_1017-3_OTU2	6	526	*Pseudomonas poae* ST250	99	EU350370
R_1017-3_OTU3	1	531	*Escherichia coli* BL21(DE3)	99	CP001509
R_1017-3_OTU4	3	541	*Geobacillus stearothermophilus* ARM 1	99	EF025325
R_1017-3_OTU5	1	505	*Williamsia muralis* MA140-96^T	98	Y17384
R_1017-3_OTU6	1	499	*Gordonia sputi* DSM 44019^T	99	X80627
R_1-17-3_OTU7	1	404	*Leuconostoc citreum* KM20	98	DQ489736
Total	48				
cDNA of the *alkB* gene clone library (RNA library)					
AR_1017-3_OTU1	48	486	*alkB-geo4* (=*alkB3 Rhodococcus* sp. Q15)	99	EF534179
AR_1017-3_OTU2	3	482	*alkB-geo1* (=*alkB4 Rhodococcus* sp. Q15)	98	EF534176

cultures. At the same time, analysis of the RNA clone libraries revealed geobacilli to be the predominant functionally active components of both enrichments. High temperatures are likely to inhibit the activity of pseudomonads, known to be capable of utilizing oil and various hydrocarbons. A large amount of thermophilic bacteria of the genus *Tepidiphilus*, not known to utilize hydrocarbons [30], was detected in enrichment 8m3. However, these microorganisms did not exhibit functional activity. In order to confirm the above conclusions, analysis of the *alkB* gene libraries of both enrichments was performed.

Diversity of *alkB* genes in the *alkB* gene clone libraries obtained from enrichment cultures. Preparations of total DNA and cDNA obtained from both enrichment cultures were used as templates for PCR amplification with degenerate primers targeting the *alkB* gene. We failed to obtain PCR products with the total DNA of either of the enrichments. The specific yield of *alkB* gene fragments was observed only when cDNA was used as the template. This indicates that the *alkB* gene expression occurred during growth on hydrocarbons. On the basis of the obtained cDNA, two *alkB* clone libraries, consisting of 41 and 51 clones, were constructed for the cultures 8m3 and 1017-3. In the clone library of the culture 8m3, homologs of the *alkB-geo2* and *alkB-geo4* genes were detected, and homologs of the *alkB-geo1* and *alkB-geo4* genes were detected in the clone library of the culture 1017-3. The level of identity between the obtained sequences and the corresponding *alkB* homologs found in *Geobacillus* representatives was 98–99%.

Earlier, we showed that the *alkB-geo4* and *alkB-geo1* homologs are universal for all the *Geobacillus* strains studied [11]. Phylogenetic analysis showed that these homologs are not specific for *Geobacillus* strains; they are close to the corresponding *alkB3* and homologs found in the genomes of some *Rhodococcus erythropolis* strains [11]. Moreover, an *alkB3* homolog was detected in *Pseudomonas frederiksbergensis* (AY452488). However, in the 16S rRNA gene libraries of the studied cultures, no phylotypes of these microorganisms were detected. At the same time, *alkB-geo2* homologs have been detected only in *Geobacillus* strains. Thus, the results of the analysis of the *alkB* gene diversity confirmed the conclusion from the 16S rRNA gene analysis about the predominant role of geobacilli in the hydrocarbon-oxidizing activity of the studied cultures.

The results of comparative phylogenetic analysis of the 16S rRNA gene sequences of cultivable and uncultivable bacteria from the studied enrichment cultures only partially coincided (Fig. 2). The D_1017-3_OTU1 and R_1017-3_OTU4 phylotypes, prevailing in the DNA library and detected in the RNA library, respectively, of the culture 1017-3 may be associated with strains *G. stearothermophilus* G1017_C12 and G1017_C14. The phylotype D_8m3_OTU3 from the DNA library of the culture 8m3 can be associated with *G. pallidus* G8m. At the same time, we failed to isolate any pure *Geobacillus* cultures corresponding to the *Geobacillus* phylotypes R_8m3_OTUl and R1017-3OTU1 prevailing in the two RNA libraries. Moreover, although phylotypes closely related to *Pseudomonas Poae* were predominant in the DNA library of the culture 8m3 and were present in other clone libraries of both enrichment cultures, we failed to isolate a pure culture of an analogous bacterium. The strains isolated from the enrichment culture 1017-3 belonged to the species *P. putida*, which is known for its ability to degrade oil and hydrocarbons of various classes. Since this species belongs to the group of closely related pseudomonads with a high similarity level between their 16S rRNA gene sequences, we carried out an additional identification of the isolates on the basis of their *gyrB* genes, which is a method with a higher resolution ability. As a result, the level of similarity between the *gyrB* gene sequence of the new isolate P-1017-1 and the sequences of various strains *P. putida* strains available in the GenBank database reached 95%, which confirmed the identification.

The ability of members of the genus *P. putida* to degrade oil and various hydrocarbons is determined by species-specific *alkB* genes, the sequence structure of which differs considerably from the *alkB* homologs of geobacilli [31]. However, the in clone libraries of the studied enrichment cultures, no *alkB* genes of *P. putida* were detected, probably due to the fact that the oligonucleotide primers used for PCR amplification were modified in order to enhance their specificity to geobacilli [11]. No data exist on the capacity of pseudomonads of the species *P. poae* for hydrocarbon oxidation. The mechanism responsible for the ability of mesophilic pseudomonads to survive numerous transfers at 60°C remains unclear. It is likely that mesophilic pseudomonads are able to survive and participate in oil biodegradation in the near-bottom zones of injection wells, where the temperature is lower due to the influx of cold injection water. The 16S rRNA genes of mesophilic bacteria have been detected in high-temperature oil strata by other researchers as well [12, 14].

No concordance was observed between the mesophilic strains M-8m-1 and M-8m-3 isolated from the enrichment culture 8m3 and the minor phylotypes detected in the two enrichment cultures. Thus, the results obtained show that the methods we used complement each other and provide a more comprehensive analysis of microbial communities.

Earlier, a number of thermophilic hydrocarbon-oxidizing bacteria belonging to different species of the genus *Geobacillus* were isolated from the Dagang oil field (*G. subterraneus* strains 31, 32, 44, 45, and 47; *G. stearothermophilus* 46; *G. jurassicus* DS1^T and DS2; and *G. thermoglucosidasius* 3Feng) [5, 6]. Thus, the results of the previous and present microbiological studies correlate with the results of molecular biologi-

cal analysis of ribosomal and functional genes and demonstrate the predominance of geobacilli and their major role in the hydrocarbon-degrading activity of the enrichment cultures originating from the high-temperature horizons of the Dagang oil field.

The lack of species specificity of the *alkB* genes found in geobacilli does not allow them to be used as a sole phylogenetic marker for taxonomic analyses, as well as for assessment of the diversity of geobacilli in natural ecosystems. Nevertheless, this analysis can be useful for the elucidation of the genetic determinants of alkane biodegradation and, in combination with ribosomal gene analysis and traditional microbiological methods, can yield valuable data on the diversity of metabolically active microorganisms in the microbial communities of oil fields.

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