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EXPERIMENTAL ARTICLES

Physiological and Phylogenetic Diversity of Thermophilic Spore-forming Hydrocarbon-oxidizing Bacteria from Oil Fields

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Abstract—The distribution and population density of aerobic hydrocarbon-oxidizing bacteria in the high-temperature oil fields of Western Siberia, Kazakhstan, and China were studied. Seven strains of aerobic thermophilic spore-forming bacteria were isolated from the oil fields and studied by microbiological and molecular biological methods. Based on the 16S rRNA gene sequences, phenotypic characteristics, and the results of DNA–DNA hybridization, the taxonomic affiliation of the isolates was tentatively established. The strains were assigned to the first and fifth subgroups of the genus *Bacillus* on the phylogenetic branch of the gram-positive bacteria. Strains B and 421 were classified as *B. licheniformis*. Strains X and U, located between *B. stearothermophilus* and *B. thermocatenulatus* on the phylogenetic tree, and strains K, Sam, and 34, related but not identical to *B. thermodenitrificans* and *B. thermoleovorans*, undoubtedly represent two new species. Phylogenetically and metabolically related representatives of thermophilic bacilli were found to occur in geographically distant oil fields.

Key words: oil fields, hydrocarbon-oxidizing bacteria, 16S rRNA, phylogeny, thermophiles, *Bacillus*.

The microflora of high-temperature subterranean ecosystems, oil fields in particular, have attracted a great deal of attention from researchers in connection with the establishment of the lower boundary of the biosphere and the search for new aerobic and anaerobic thermophilic and hyperthermophilic bacteria and archaea [1, 2]. The aerobic oil-oxidizing components of microbial communities have been insufficiently studied [3–5]. Hydrocarbon-oxidizing bacteria initiate oil biodegradation in exploited oil reservoirs [5, 6]. Due to the activity of the microflora, some undesirable phenomena may occur in oil fields: an increase in oil viscosity because of the consumption of light hydrocarbons; the reduced permeability of the oil-bearing rock; and the corrosion of the metallic equipment. However, the growth of hydrocarbon-oxidizing bacteria is accompanied by the formation of some compounds that exhibit oil-displacing properties (CO_2, organic acids, alcohols, biopolymers, surfactants, and others); this may be used as a basis for the development of biotechnologies for the enhancement of oil recovery. Thus, the distribution and biodiversity of oil-oxidizing bacteria in the oil fields is of theoretical and practical interest [3–6].

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The bacteria of the genus *Bacillus* are widespread in nature and play a substantial role in microbial communities [7]. We have previously isolated from oil fields two strains (B and K) of thermophilic hydrocarbon-oxidizing bacilli which were preliminary identified as representatives of the species *B. thermoleovorans*, although they exhibited some pecularities [3, 4]. Species identification in bacilli is difficult because only a few characteristics are used to distinguish their species [7, 8].

The genus *Bacillus* comprises a broad spectrum of rod-shaped aerobic and facultatively anaerobic endospore-forming bacteria [7, 8], including thermophiles and psychrophiles, fresh water inhabitants and halophiles, acidophiles and alkaliphiles, and heterotrophs and autotrophs. Analysis of 16S rRNA sequences revealed several phylogenetic groups within this genus [9–11], some of which have already been described as separate genera: *Alicyclobacillus, Paenibacillus, Halobacillus, Brevibacillus, Aneurinibacillus*, and *Virgibacillus* [7, 10, 12–15]. Nevertheless, further analysis of this heterogeneous genus, its thermophilic representatives in particular, is required.

The known thermophilic species belong to the genera *Alicyclobacillus* (*A. acidocaldarius, A. acidoterrestris*, and *A. cycloheptanicus*) [14] and *Bacillus* (*B. stearothermophilus, B. thermoglucosidasius, B. thermoleovorans,*

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B. kaustophilus, B. thermocatenulatus, B. thermodenitrificans, B. flavothermus, B. coagulans, B. smithii, B. thermoruber, B. thermoamylovorans, B. pallidus, B. thermocloaceae, B. thermosphaericus, and *B. thermoaerophilus*) [7, 11]. All these bacteria grow at temperatures higher than 55°C.

The thermophilic hydrocarbon-oxidizing bacilli that we describe in this paper exhibit some phenotypic distinctions from the known species and seem to belong to new taxa. According to the current recommendations of the International Committee on Prokaryote Systematics, the description of novel bacterial forms should be replenished with data on the primary structure of 16S rRNA. Not only are these data required for elucidation of the taxonomic affiliation; they can also be used for the development of oligonucleotide probes to detect these bacteria in natural environments.

In this work, we studied the distribution of thermophilic hydrocarbon-oxidizing spore-forming bacteria in oil fields and classified new isolates taxonomically on the basis of their phenotypic traits, data from DNA–DNA hybridization, and the results of 16S rRNA gene sequencing.

MATERIALS AND METHODS

Bacterial strains. Aerobic hydrocarbon-oxidizing spore-forming bacterial strains isolated from the oil fields Uzen' (strains U and K), Samotlor (strain Sam), Mykhpaiskoe (strains B and X), Daqing (strain 421), and Liao He (strain 34) were studied. Some phenotypic characteristics of strains B and K were previously described, and these microorganisms were shown to dominate the hydrocarbon-oxidizing microflora of the Mykhpaiskoe and Uzen' oil fields, respectively [4].

Pure cultures were obtained by plating enrichment cultures grown in hexadecane-containing medium onto solid media—potato agar (PA) (strains U, X, and 34) or nutrient agar (NA, Merck) (strain Sam)—incubated at 60°C. Strain 421 was isolated on PA at 40°C. For chemotaxonomic studies, biomass was grown on NA at 60°C for 15 h.

The population density of microorganisms in stratal waters was determined by the most-probable-number method on the media described previously [5]. Aerobic heterotrophic bacteria were enumerated in glucose-containing medium, hydrocarbon-oxidizing bacteria, in hexadecane-containing medium, anaerobic fermentative bacteria, in medium containing peptone and glucose, sulfate-reducing bacteria, in sodium lactate–containing medium, and methanogens, in media with acetate or $H_2 + CO_2$. The plated water samples from the oil fields Uzen', Mykhpaiskoe, Liao He, and Samotlor were incubated at 60°C, whereas those from Daqing, at 40°C.

DNA analysis. DNA from pure cultures of thermophilic bacilli was isolated by the Marmur method. The G+C content of the DNA was measured by the thermal denaturation method with *Escherichia coli* K-12 DNA as a reference [17]. DNA–DNA hybridization was conducted by the method of De Ley *et al.* [18].

Amplification and sequencing of the 16S rRNA gene. The isolated genomic DNA and the following oligonucleotide primers were used in the polymerase chain reaction [19] (in parentheses, the nucleotide numbering is indicated according to *E. coli* numbering):

27f-AGAGTTTGATCCTGGCTCAG (8–27);
357f-CTCCTACGGGAGGCAGGAG (342–357);
342r-CTGCTGCCTCCCGTAG (357–342);
530F-CAGC(C/A)GCCGCGGTAAT(T/A)C (519–536);
519r-G(T/A)ATTACCGCGGC(T/G)GCTG (536–519);
907f-AAACT(C/T)AAA(G/T)GAATTGACG (907–926);
907r-CCGTCAATTC(C/A)TTT(G/A)AGTTT (926–907);
1114f-GCAACGAGCGCAACCC (1114–1130);
1492r-TACGG(C/T)TACCTTGTTACGACTT (1492–1170);
1525r-AGAAAGGAGGTGATCCAGCC (1545–1525).

The reaction mixture 50 μl in volume contained 1 mg of the DNA template, standard concentrations of dNTP (200 μM), equimolar amounts of the primers, the thermostable Taq-DNA-polymerase (Perkin-Elmer), Mg^{2+} ions (20 mM $MgCl_2$) required for the functioning of the latter enzyme, and buffer containing 70 mM Tris–HCl (pH 8.8), 170 mM $(NH_4)_2SO_4$, and 0.1 vol % Tween 20. Thirty amplification cycles of the 16S rRNA gene were conducted on a DNA-Ther-Cyc temperature-controlled automatic cycler (DNK Tekhnologiya) in the following regime: DNA denaturation, 94°C for 1 min; primer annealing, 40°C for 1 min; and elongation, 72°C for 2 min. The specific PCR products were purified by electrophoresis in low-gelling-temperature agarose (Sigma). The specific fragments about 1400 bp long were detected in UV light at 365 nm and cut out together with agarose, which was melted at 70°C for 15–20 min. The Wizard resin was then added to the material, and it was passed through a Promega column.

The nucleotide sequence of the 16S rRNA gene was determined by enzymatic sequencing on an automatic Applied Biosystems DNA Sequencer 373A and using an Applied Biosystems kit (PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit with AmpliTaq DNA Polymerase, FS) according to the recommendations of the manufacturers.

Analysis of 16S rRNA sequences. The results obtained by sequencing of the 16S rRNA gene fragments of the bacteria studied were preliminary analyzed using the software package of the Ribosomal Database Project [20]. For a more accurate determination of the phylogenetic position of the strains studied, their 16S rRNA sequences were manually aligned with the corresponding sequences of the known species of the genus *Bacillus* and other representatives of the

Table 1. Physicochemical and microbiological characteristics of the habitats of thermophilic hydrocarbon-oxidizing bacteria

Characteristics	Strain U	Strain B	Strain Sam	Strain 421	Strain 34
Oil field	Uzen'	Mykhpaiskoe	Samotlor	Daqing	Liao He
Isolation source*	1	1	3	2	2
Horizon	XIV	A_{1-2}	J**	P_1	S_1
Depth, m	1300	1700	2300**	1060	1960
Stratal temperature, °C	55	60	25	46	66
Oil specific gravity, g/cm^3	0.767	0.779	0.855	0.848	
Total water mineralization, g/l;	16.6	18.0	17.98	4.4	3.5
pH	7.4	7.7	7.3	7.5	7.3
O_2, mg/l	0	0.5	0	0	0
H_2S, mg/l	30.6	0	0	0	0
Population density of microorganisms***, cells/ml					
aerobic saprophytes	10^4	10	10^4	2.5×10^2	< 10
hydrocarbon-oxidizers	10^3	< 10	10	250	< 10
fermenters	10^4	10	$>10^2$	10^2	10^3
sulfate-reducers	10^3	10	10^3	2.5×10^2	0
methanogens	< 10	< 10	0	>60	$>10^2$

* 1, stratal water from the near-bottom zone of the injection wells; 2, stratal water from the production wells; 3, oil-free settled water from the oil–water separation system.

** Data on the Jurassic horizon, whose stratal fluids enter the system of oil separation.

*** The population density of microorganisms was determined at 60°C in samples from the Uzen', Samotlor, Mykhpaiskoe, and Liao He oil fields and at 40°C in samples from the Daqing oil field.

phylogenetic subdivision of gram-positive bacteria available from the latest version of the GenBank database. After excluding the aligned positions for which the nucleotides were determined not for all of the compared sequences were excluded, 1297 nucleotides were compared. The phylogenetic tree was constructed using the programs of the TREECON software package [21]. The statistic significance of the branching order was estimated by bootstrap analysis of 100 alternative trees.

RESULTS AND DISCUSSION

Physicochemical and microbiological characteristics of habitats of the thermophilic hydrocarbon-oxidizing bacteria. The physicochemical conditions and population density of microorganisms of some physiological groups were studied during our expedition to the oil fields with a temperature from 40 to 80°C in Western Siberia (Mykhpaiskoe, Samotlor), Kazakhstan (Uzen'), and China (Daqing, Liao He). The samples from which thermophilic and hydrocarbon-oxidizing bacteria were isolated are characterized in Table 1.

Thermophilic microorganisms were revealed in all high-temperature oil fields examined (Uzen', Mykhpaiskoe, Samotlor, and Liao He). They were present in the near-bottom zones of the injection wells, in production wells, and in the oil separation system, wherefrom the settled water is reinjected into the oil stratum. Aerobic saprophytic bacteria, including hydrocarbon-oxidizing ones, contributed to the thermophilic microbial community and were most abundant in the near-bottom zones of the injection wells. Anaerobic microflora mostly consisted of fermentative bacteria (10–10^4 cells/ml), sulfate reducers, and methanogens (up to 10^3 and 10^2 cells/ml, respectively).

Comprehensive analysis of the mesophilic microbial community of the Daqing oil field, having a temperature of 30–45°C in the oil-bearing horizons, also revealed thermophilic bacteria (strain 421).

To obtain pure cultures, enrichments were initiated from the highest dilutions of the stratal water; this allowed us to reveal the predominant hydrocarbon-oxidizing microflora. Stratal water samples were not subjected to pasteurization, because we did not specifically aim at the isolation of spore-forming bacteria. From the oil fields examined, five pure cultures of aerobic thermophilic hydrocarbon-oxidizing bacteria were isolated (strains U, X, Sam, 34, and 421), which became the main subjects of investigation, along with the earlier isolated strains (B and K).

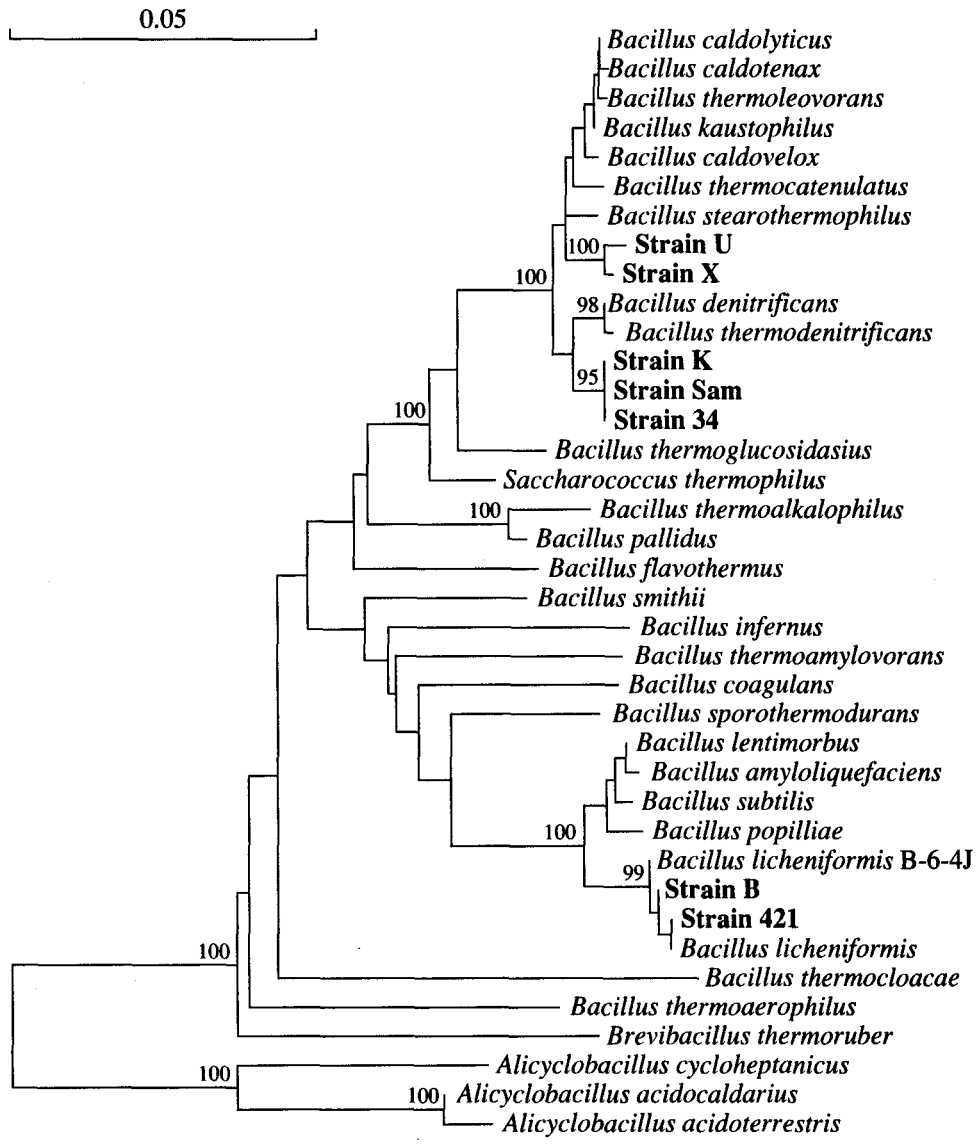
Morphological, physiological, and biochemical properties of the thermophilic bacilli. More than 50 diagnostically valuable phenotypic traits were studied in strains U, X, K, and Sam (Table 2).

Table 2. Some differentiating characteristics of the thermophilic spore-forming hydrocarbon-oxidizing bacteria isolated from oil fields and of the closest species of thermophilic bacilli

Characteristics	Strain U	Strain X	Strain Sam	Strain K	*B. thermo-glucosidasius*	*B. thermo-catenulatus*	*B. thermo-leovorans*
Cell size	0.9–1.3 × × 4.7–8.0	1.0–1.7 × × 5.5–8.5	0.8–1.5 × × 5.5–8.0	1.0–1.5 × × 4.7–7.0	0.5–1.2 × × 3.0–7.0	0.9 × 6–8	1.5 × 3.3
Motility	+	+	+	+	+	+	–
Temperature optimum, °C	55	55–60	55	55–60	61–63	55–60	55–65
NaCl range, %	0–4	0–4	0–5	0–5	<1.5		
Utilize for growth:							
hydrocarbons	+	+	+	+			+
formate	–	–	–	–			
acetate	–	+	+	+			+
propionate	–	+	–	–			+/–
butyrate	–	+	+	+			+
pyruvate	–	+	+	+			+
succinate	+	+	+	+			+/–
fumarate	+	+	+	+			
malate	–	+	–	+			
lactate	–	+	+	+			
arabinose	+	+	–	+	–	–	+/–
galactose	+	+	+	+	–	+	+
glucose	+	+	+	+	+	+	+
fructose	+	+	+	+	+		+/–
lactose	–	–	–	–	–	–	–
sucrose	+	+	+	+	+	+	+
cellobiose	+	+	+	+	+	+	+
glycerol	–	+	+	–	+	+	+/–
inositol	–	–	–	–	–		
sorbitol	–	–	–	–	+		–
methanol	–	–	+	+			
ethanol	–	+	+	+		–	
$NO_3^- \longrightarrow NO_2^-$	+	+	+	+	+	+	
$NO_3^- \longrightarrow N_2$	–	–	+	+	–	+	
G+C, mol %.	51.3	52.3	49.7	51.9	45–46	69	52–58

Table 3. G+C content of DNA and the level of DNA–DNA homology among the hydrocarbon-oxidizing bacteria from the oil fields, *B. thermodenitrificans* DSM 466, and the type strains *B. stearothermophilus* DSM 22^T and *B. thermoleovorans* DSM 5366^T

Strain	G+C, mol %	DNA homology, %								
		22^T	466	5366^T	U	X	K	Sam	34	B
22^T	52.2	100								
466	49.6	32	100							
5366^T	53.7	51	31	100						
U	51.3	38	45	45	100					
X	52.3	33	43	48	80	100				
K	51.9	39	44	41	32	37	100			
Sam	49.7	37	47	45	42	44	93	100		
34	52.3	53	45	48	49		98	91	100	
B	45.4	8	7	6	7	5	10	6	11	100



Phylogenetic tree of the type strains of *Bacillus* species and thermophilic bacteria isolated from oil fields. The tree is based on the results of 16S rDNA sequencing. The scale bar corresponds to five nucleotide substitutions per 100 nucleotides (evolutionary distances). Numerals indicate the statistical significance of the order of branching, determined by "bootstrap" analysis of 100 alternative trees; values less than 95% are not shown.

All bacteria were straight spore-forming rods exhibiting positive Gram staining. Ultrathin sections revealed no outer lipoprotein cell wall layer in any of the strains, which all had the cell wall structure typical of gram-positive bacteria (data not shown). All isolates grew within salinity and pH ranges intrinsic to their habitats and a temperature range from 35 to 70°C. The strains could utilize individual hydrocarbons and oil, as well as the products of oil oxidation—lower alcohols and volatile fatty acids (Table 2). Strains K, 34, and Sam could utilized acetate and reduce nitrates to molecular nitrogen. Acetate and other volatile acids can often be detected in subsurface waters. In some water samples from the Samotlor, Uzen', and Liao He oil fields, the content of acetic acid reached 150–400 mg/l. Nitrates are usually absent from stratal waters except that they occasionally enter the oil strata with injected waters. Thus, the denitrifying capacity provides a certain ecological advantage in the near-bottom zone of the injection wells.

The bacilli that we isolated were almost indistinguishable from the reference strains (Table 2) in a broad spectrum of properties, such as the consumption pattern of sugars, peptone, tryptone, and yeast extract.

Genotypic analysis. We studied the major genotypic characteristics of the thermophilic bacilli isolated. The G+C content of their DNA was consistent with that typical of the genus *Bacillus* (Table 3). As revealed by DNA–DNA hybridization, the isolates were differently related to the type strains of the known species of ther-

mophilic bacilli. Strains U and X and strains K, 34, and Sam constituted two genotypic groups with intragroup DNA homology levels of 80 and 93–96%, respectively. These groups were equidistant from each other and from the reference *Bacillus* strains (32–53% of DNA homology). According to the conventional criteria [22], the relatedness within the genotypic groups was of the intraspecies level, whereas the relatedness between these groups and other bacilli was of the interspecies level. Strains B (45.4 mol % G+C) and 421 (46.3 mol % G+C) exhibited the greatest difference from all the bacilli compared in the nucleotide composition of DNA, and strain B also had the lowest level of DNA homology (no more than 11%) with other bacilli.

Phylogenetic analysis of 16S rRNA gene sequences. The complete nucleotide sequences of the 16S rRNA gene (1500 nucleotides) were determined in strains B, K, U, X, and 34, whereas, in strains 421 and Sam, about 300 nucleotides were determined. All seven strains belonged to the phylogenetic spectrum of the species of the genus *Bacillus*, within the subdivision of gram-positive bacteria with a low content of G+C pairs in DNA.

Strains B and 421 proved to be representatives of the most voluminous species group 1 of the genus *Bacillus* [9], to which the type species of the genus *B. subtilis* also belongs (figure). Within this group, strain B is closest to the type strain of *B. licheniformis* (99.8%). The sequenced region of the 16S rDNA of strain 421 was completely identical (100%) to the 16S rDNA of the latter strain. Therefore, both strains can be classified as *B. licheniformis*. This conclusion is supported by the refined value of the G+C content of strain B DNA (45.4 mol %). The presence of *B. licheniformis* strains in the stratal waters of oil fields is not surprising because they utilize a broad spectrum of substrates, can grow within wide temperature (35–70°C) and salinity (up to 100 g/l) ranges, and are the first to colonize oil-polluted soils and water bodies [7].

Strains X, U, Sam, K, and 34, isolated from geographically distant oil fields, are relatively close to each other phylogenetically. All of them belong to group 5 of the phylogenetic spectrum of bacilli (figure). The similarity level of 16S rRNAs from strains X and U and their similarity levels with 16S rRNAs of the type strains of the closest species *B. stearothermophilus* and *B. thermocatenulatus* were 99.5 and 98.5–98.9%, respectively. The sequences of 16S rRNAs from strains Sam, K, and 34 were identical and their similarity to those of the closest species *B. thermoleovorans* and *B. thermodenitrificans* was 98.6–99.1%. Thus, the results of phylogenetic analysis indicate that strains X and U and strains Sam, K, and 34 belong to new species of thermophilic bacilli.

Our results demonstrate that phylogenetically and metabolically related species of thermophilic bacilli inhabit geographically distant oil fields.

The taxonomic study of the new thermophilic hydrocarbon-oxidizing bacilli is currently being continued.

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