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EXPERIMENTAL ARTICLES

The Phylogenetic Diversity of Aerobic Organotrophic Bacteria from the Dagang High-Temperature Oil Field

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Abstract—The distribution and species diversity of aerobic organotrophic bacteria in the Dagang high-temperature oil field (China), which is exploited with water-flooding, have been studied. Twenty-two strains of the most characteristic thermophilic and mesophilic aerobic organotrophic bacteria have been isolated from the oil stratum. It has been found that, in a laboratory, the mesophilic and thermophilic isolates grow in the temperature, pH, and salinity ranges characteristic of the injection well near-bottom zones or of the oil stratum, respectively, and assimilate a wide range of hydrocarbons, fatty acids, lower alcohols, and crude oil, thus exhibiting adaptation to the environment. Using comparative phylogenetic 16S rRNA analysis, the taxonomic affiliation of the isolates has been established. The aerobic microbial community includes gram-positive bacteria with a high and low G+C content of DNA, and γ and β subclasses of *Proteobacteria.* The thermophilic bacteria belong to the genera *Geobacillus* and *Thermoactinomyces*, and the mesophilic strains belong to the genera *Bacillus, Micrococcus, Cellulomonas, Pseudomonas*, and *Acinetobacter.* The microbial community of the oil stratum is dominated by known species of the genus *Geobacillus* (*G. subterraneus, G. stearothermophilus*, and *G. thermoglucosidasius*) and a novel species *"Geobacillus jurassicus."* A number of novel thermophilic oil-oxidizing bacilli have been isolated.

Key words: aerobic bacteria, *Geobacillus*, oil oxidation, subsurface thermal ecosystems, 16S rRNA, phylogeny.

Over the last few decades, the microbial communities of subsurface ecosystems have attracted a great deal of attention from researchers [1–4]. The main emphasis has been placed on anaerobic microorganisms: oil strata, as a rule, lack dissolved oxygen and are primarily anaerobic habitats. A great variety of anaerobic microorganisms have been isolated from oil fields, including organotrophic bacteria, sulfate and sulfur reducers, iron and manganese reducers, and methanogens [1, 2]. However, the microbial communities of oil fields also include aerobic microorganisms introduced with the injection water, drilling solution, or, possibly, by natural flows of subsurface water [1, 5–10]. Aerobic microflora have generally been studied in oil fields with temperatures ranging from 20 to 40°C. Using traditional approaches, mesophilic representatives of the genera *Pseudomonas, Rhodococcus, Brevibacterium, Micrococcus, Arthrobacter, Methylomonas, Methylobacter, Methylococcus, Methylosinus*, and *Methylocystis* have been isolated [1, 5–10]. An active and diverse microbial community has also been found in the Daqing oil field (China) [3, 4]. In this case, molecular methods (analysis of sequences of 16S rRNA genes from pure cultures) demonstrated the taxonomic affiliation of the isolated bacteria to the genera *Bacillus, Brevibacillus, Rhodococcus, Dietzia, Kocuria, Gordonia, Cellulomonas, Clavibacter, Pseudomonas*, and *Acinetobacter.* The bacteria were found to grow in the temperature, pH, and salinity ranges characteristic of the oil field and assimilate individual hydrocarbons, crude oil, sugars, alcohols, and fatty acids.

Only a small number of studies have characterized the distribution of aerobic organotrophic bacteria in high-temperature oil fields: thermophilic bacteria were isolated from formation waters and identified as belonging to the genera *Bacillus* and *Geobacillus* [11, 12], and aerobic thermophilic bacteria were found in the cores of hydrocarbon rocks at depths of 2 km in Virginia (United States) [13]. As well as the ecological aspects of the problem, the taxonomic aspects are also of great importance. The thermophilic hydrocarbon-oxidizing bacteria found in various geographically dis-

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tant oil fields mostly belong to the genus *Geobacillus.* However, it is yet to be determined to what extent representatives of this species are typical of the microbial communities of subsurface ecosystems and how great their diversity is.

Currently, comparative 16S rRNA analysis is an obligatory stage in all investigations of prokaryote taxonomy. International banks of nucleotide sequences of 16S rRNA are available on-line. The simplest approach in an ecological investigation is to use a sequence analysis of 16S rRNA genes to make a fast and precise identification of strains isolated under different conditions from different ecosystems without their preliminary characterization by traditional phenotypic methods. It is known that the 16S gene contains both variable and conservative regions. For a phylogenetic analysis and determination of the preliminary taxonomic position of an organism, it is sufficient to determine the first 300–350 nucleotides of its 16S rRNA gene, where most of the variable regions are located [14].

The aims of the present work were to isolate aerobic organotrophic bacteria, including hydrocarbon-oxidizing species, from the Dagang high-temperature oil field and to study their phylogenetic and genotypic diversity, physiological properties, and ability to utilize oil and its components as substrates.

MATERIALS AND METHODS

Characteristics of the Dagang oil field. Aerobic bacteria were isolated from the formation waters of the Dagang oil field (Kondian bed, Hebei Province, China). The sandy oil-bearing horizons had a temperature of 59°C. The oil had a density of 0.9 g/cm^3 and contained 53% saturated hydrocarbons, 20% aromatic compounds, and 21.15% gums and asphaltenes. The formation waters were of a hydrocarbonate–sodium type and had a mineralization of 5612 mg/l and pH 7.1–7.6. The oil gas contained methane (95–98%), its higher homologues (0.8–1.8%), N_2 (0.5–3.3%), and CO_2 (0.06–0.77%). For the maintenance of stratal pressure, waste water produced during oil separation was injected back into the stratum. The waste water contained up to 0.8 mg/l of dissolved oxygen and had a temperature of 35–45°C.

Strain source. Samples were taken from oil-production wells and the near-bottom zones of injection wells operated on a regime of back flow. Formation waters from the oil-bearing horizons from depths of 1206–1435 m were plated onto the following agar media: Plate Count Agar (PCA, Sigma); potato, milk, and blood agars; and Raymond mineral agar supplemented with a mixture of liquid paraffins (C_{12}–C_{22}, 5 ml/l) [15]. The plates were incubated at 30 and 60°C under aerobic conditions for 2–7 days, until colony formation. The colonies that differed in shape, color, size, and consistency were then taken for further investigation. Twenty-two strains of predominant aerobic organotrophic bacteria were isolated and purified by subsequent subculturing.

The cultures were maintained on PCA and potato agar. Substrate utilization was studied using Adkins mineral medium [16]. The substrates were added in the following concentrations: sugars, 0.5%; organic salts, 0.25% (except for butyrate, which was added in a concentration of 0.1%); amino acids, 0.2%; alcohols, 0.2%; hydrocarbons (C_6, C_8, C_{10}, C_{14}, and C_{16}), 0.5%; and oil, 0.5%. The cultures were then incubated without agitation at 40 and 60°C for 3–5 days.

The cell morphology in live specimens was examined under a Jenaval phase-contrast microscope (magnification, 800×) and, in whole-cell specimens, under a JEM 100C electron microscope (magnification, 10000×). Gram staining was performed using an NH90 Sigma Diagnostics kit.

Biomass yield was judged from the optical density measured on a Specord UV VIS spectrophotometer in a 1-cm cuvette at 600 nm. An inoculated mineral medium not supplemented with a substrate was used as the control.

DNA analysis. DNA was extracted using the Marmur method [17]. The G+C content of the DNA was determined according to the thermal denaturation method, using the DNA of *Escherichia coli* K-12 as a standard [18].

Amplification, sequencing, and analysis of the 16S rRNA genes. Amplification and sequencing of the 16S rRNA genes of pure cultures were performed on the automatic DNA Sequencer 373A using universal bacterial primers [19] and a no. 402080 Applied Biosystems kit (Ready Reaction Dye Terminator Sequencing Kit with AmpliTaq DNA Polymerase, FS).

A preliminary analysis of the sequences of the 16S rRNA genes was conducted using data and software of the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu). A sequence analysis was conducted on the basis of the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/blastn). Unrooted phylogenetic trees were constructed according to the different methods implemented in the TREECONW software package (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html).

RESULTS AND DISCUSSION

Twelve strains of aerobic thermophilic bacteria and ten strains of mesophilic bacteria were isolated from the formation water of the Dagang oil field. Mesophilic microorganisms are known to occur in the near-bottom zones of injection wells in high-temperature oil fields. Flooding with cold water lowers the temperature in these near-bottom zones to 40–45°C and, thus, creates conditions favoring the development of mesophilic species.

In order to determine the taxonomic position of the isolated bacteria, morphological, physiological, biochemical, and molecular approaches were used. According to data obtained using light and electron

Table 1. Phenotypic and genotypic characteristics of the aerobic bacteria isolated from the Dagang oil field

Strains	Cell shape	Spore formation	Motility	G+C, mol %	Similarity of 16S rRNA gene fragments, %	Closest species according to BLAST (GenBank)
DS1, DS2	Rods	+	+	53.8–54.5	98.4–99.1	*Geobacillus uzenensis*
46, 49	Rods	+	+	53.1–53.3	98.2–99.1	*G. stearothermophilus*
3 Feng	Rods	+	+	46.1	100	*G. thermoglucosidasius*
31, 32, 44, 45, 47	Rods	+	+	52.6–53.0	99.5–100	*G. subterraneus*
29, 30	Rods	+	-	ND	100.0	*Thermoactinomyces sacchari*
6, 7	Rods	+	+	43.0–43.4	99.8	*Bacillus cereus*
10	Rods	+	+	41.3	98.9	*Oceanobacillus iheyensis*
4	Cocci	–	–	68.3	99.8	*Micrococcus luteus*
8	Coccobacilli	–	–	64.4	99.8	*Mycobacterium mucogenum*
11	Rods	–	+	71.2	99.2	*Cellulomonas cellulans*
34, 35	Rods	–	–	40.0	95.9–98.6	*Acinetobacter shindleri*
5	Rods	–	+	62.9	99.8	*Pseudomonas stutzeri*
33	Rods	–	+	65.2	92.8–96.4	β-*Proteobacterium*

Note: ND stands for not determined.

microscopy, the isolated bacteria had the form of rods or cocci, were spore-forming or non-spore-forming, occurred as single cells or were integrated in chains, and were immotile or motile with flagella (Table 1). Most of the strains stained Gram-positive, but four strains stained Gram-negative.

Phylogenetic analysis of the 16S rRNA gene sequences of aerobic bacteria isolated from the Dagang oil field. The complete 16S rRNA gene sequences of strains DS1, 10, 33, 46, and 49 (about 1500 nucleotides) were determined. For the other strains, 450 nucleotides, approximately corresponding to *E. coli* positions 30 to 500 were analyzed. The initial fragment of the 16S rRNA gene sequence includes variable regions and is the most informative with respect to preliminary phylogenetic analysis. Preliminary screening within the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blastn) showed that the aerobic community included representatives from diverse phylogenetic subdivisions of bacteria: two lineages of gram-positive bacteria (with a high and low G+C content of DNA) and γ and β subclasses of *Proteobacteria*.

Most of the aerobic thermophilic isolates, including strains 46, 49, DS1, 3 Feng, 31, 32, 44, 45, and 47, belonged to the phylogenetic spectrum of species of the genus *Geobacillus*, within the subdivision of gram-positive bacteria with a low G+C content (Table 1, Fig. 1).

The complete sequences of the 16S rRNA genes of strains 46 and 49 were characterized by high levels of similarity with the sequence of the type strain of *G. stearothermophilus* (99.1 and 98.2%, respectively). The sequences of the 16S rRNA gene fragments of strains 31, 32, 44, 45, and 47 were practically identical to that of the type strain of *Geobacillus subterraneus* (99.5–100% similarity), and the sequence of strain 3 Feng was identical to that of the type strain of *G. thermoglucosidasius* (100% similarity). On this basis, the isolates were identified as representatives of the aforementioned species. Strains DS1 and DS2 displayed the highest similarity with the species *G. uzenensis* (98.4–99.1%).

Thermophilic strains 29 and 30 belonged to the genus *Thermoactinomyces*, within the subdivision of gram-positive bacteria with a low G+C content (Fig. 2). These strains fell into a cluster with *T. sacchari* and had a 100% sequence similarity with the type strain of this species. Thus, strains 29 and 30 were identified as *T. sacchari.*

The aerobic mesophilic bacteria isolated from the Dagang oil field included representatives of various phylogenetic subdivisions; seven strains were found to belong to known species (Fig. 2).

Spore-forming strains 6, 7, and 10 belonged to group 1 of the phylogenetic spectrum of bacilli [20]. Strains 6 and 7 were most closely related to the type strain of *Bacillus cereus* (99.8% 16S rDNA similarity), whereas strain 10 was closest to *O. iheyensis*, a species of the novel genus *Oceanobacillus*, which is currently represented only by the type strain (98.9% similarity).

Strains 4, 8, and 11 belonged to the group of gram-positive bacteria with a high G+C content of DNA. Strain 4 fell into a cluster with *Micrococcus luteus* strains (including the type strain), exhibiting a high level of sequence similarity with them (99.6–99.8%). Strains 8 and 11 were characterized by high sequence similarities with *Mycobacterium mucogenicum* (98.8–99.8%) and *Cellulomonas cellulans* (98.9–99.2%), respectively.

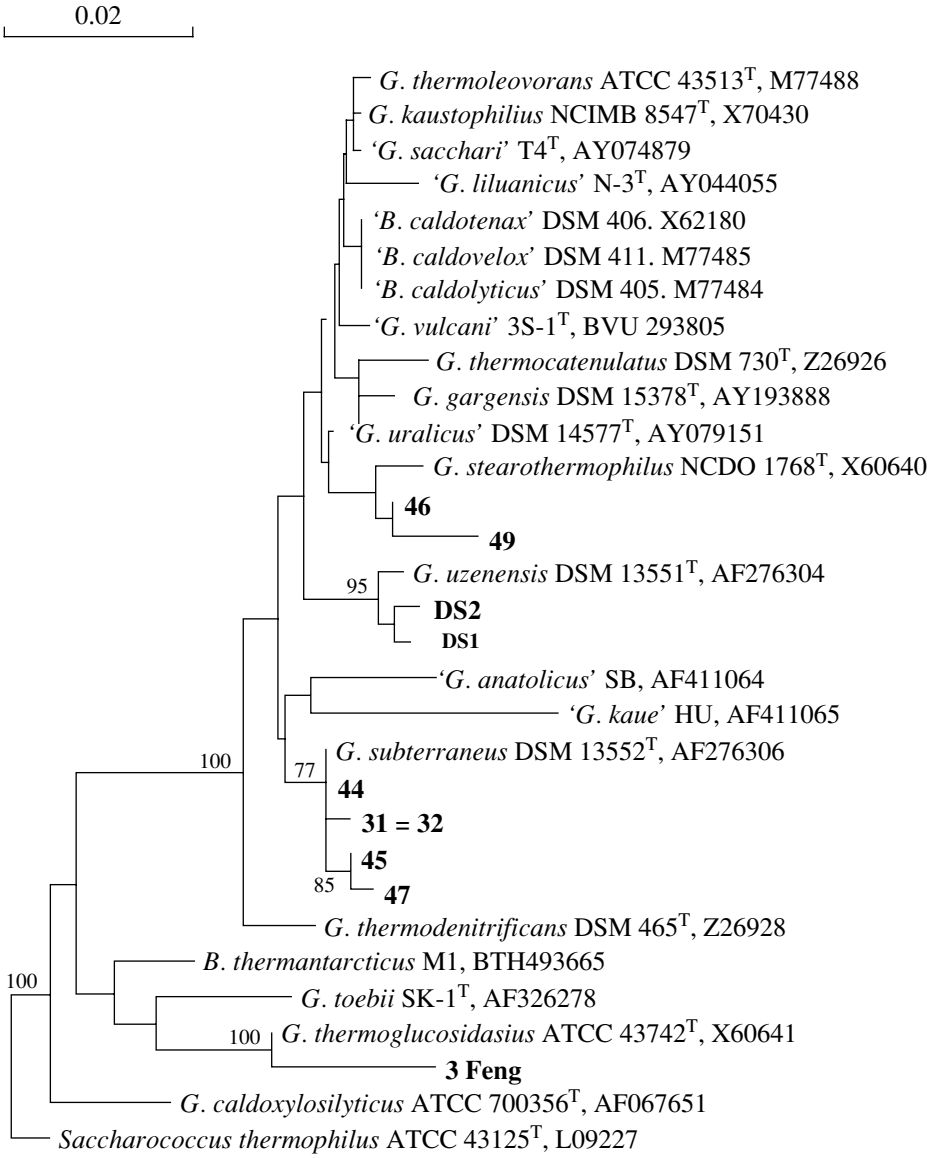

Fig. 1. Phylogenetic positions of the aerobic thermophilic spore-forming bacteria isolated from the Dagang oil field in relation to other members of the genus *Geobacillus*. The bar represents 2 nucleotide substitutions per 100 nucleotides. The numerals represent the statistical confidence of the branching determined using the bootstrap method (values greater than 95% were considered significant).

Strain 5 belonged to the γ subclasses of *Proteobacteria* and clustered with strains of the heterogeneous species *Pseudomonas stutzeri.* Its sequence similarity with one of the genomovars of this species was very high (99.8%).

Strains 34 and 35 also belonged to the γ subclass of *Proteobacteria* and clustered with species of the genus *Acinetobacter*, being closest to several strains (including the type strain) of the species *A. haemolyticus.* However, the similarity level was not high enough (only 95.9–98.0%) to identify these isolates as *A. haemolyticus* representatives. It can be suggested that these isolates comprise a novel species of the genus *Acinetobacter.*

Mesophilic strain 33 belonged to the β subclass of *Proteobacteria* and fell into the family *Oxalobacteriaceae*, where it comprised an individual branch with a relatively low level of sequence similarity to representatives of the described genera of this family (92.8–96.4%). It may be suggested that strain 33 represents a new genus of the aforementioned family.

Genotypic analysis of thermophilic spore-forming bacteria of the genus *Geobacillus*. The results of the analysis of the 16S rRNA gene sequences indicate that the thermophilic microorganisms found in the high-temperature Dagang oil field are relatively phylogenetically homogeneous. A predominant majority of the isolates belonged to the genus *Geobacillus*. When there is a high similarity between 16S rRNA gene sequences,

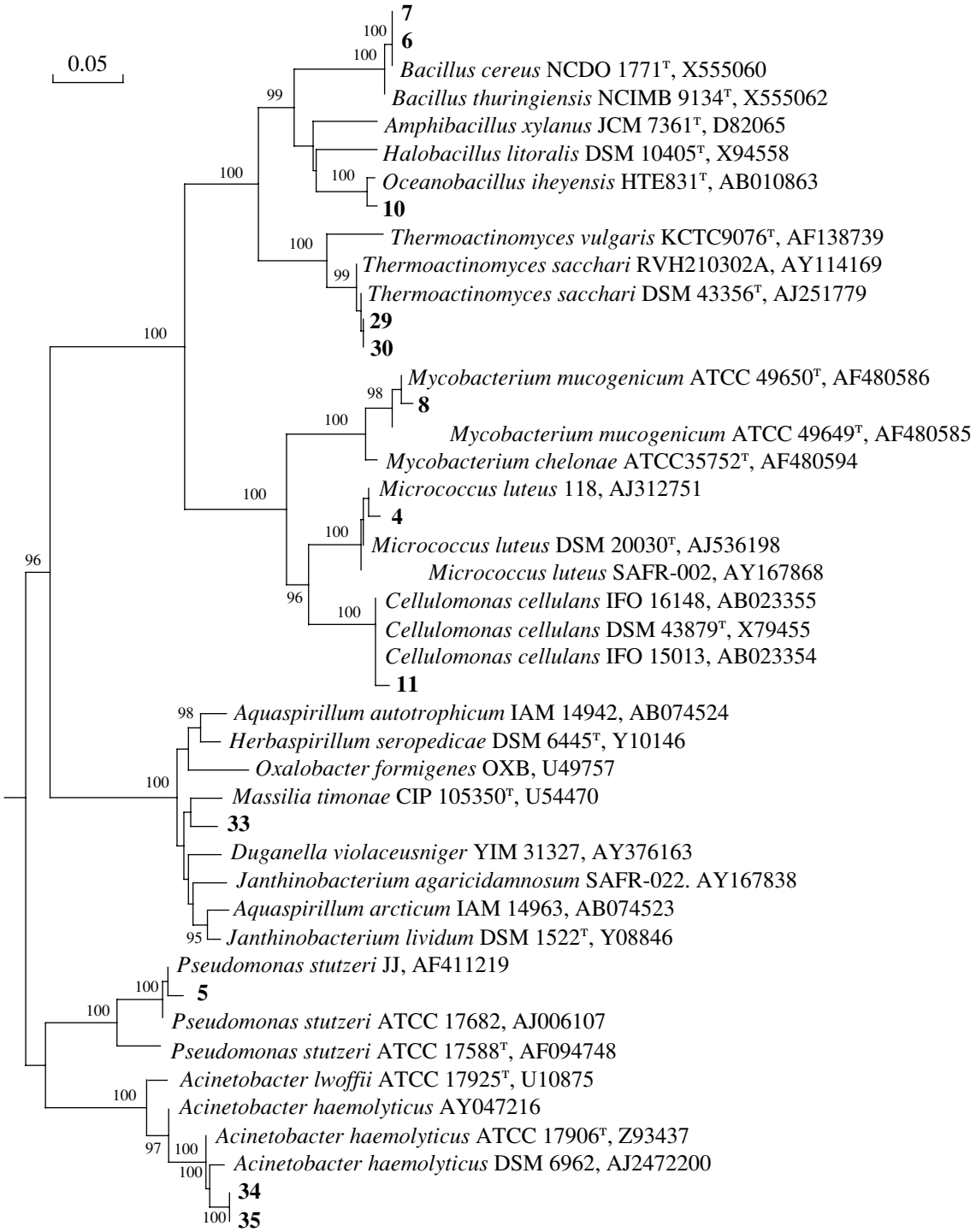

Fig. 2. Phylogenetic position of the aerobic mesophilic and thermophilic microorganisms isolated from the Dagang oil field in relation to other eubacteria. The bar represents 5 nucleotide substitutions per 100 nucleotides. The numerals represent the statistical confidence of the branching determined using the bootstrap method (values greater than 95% were considered significant).

DNA–DNA hybridization with the type strains of known species is obligatory for species identification of the organism studied. We conducted DNA–DNA hybridization of the thermophilic isolates with phylogenetically close species of the genus *Geobacillus* obtained from the German (DSMZ) and Russian (VKM) collections of microorganisms. It is known that the levels of DNA–DNA hybridization between strains of one species, strains of different species belonging to one genus, and strains of different genera should be, respectively, no less than 70%, 30–70%, and less than 30% [21].

Table 2. G+C contents of DNA and levels of DNA–DNA reassociation for the thermophilic spore-forming bacteria isolated from the Dagang oil field and phylogenetically close species of the genus *Geobacillus*

Species, strains	G+C of genome DNA, mol %	Reassociation (%) with DNA from														
		1	2	3	4	5	7	8	9	10	11	12	13	14	15	16
1. *G. stearothermophilus* 22^T	52.2	100														
2. *G. thermodenitrificans* 466	49.6	32	100													
3. *G. thermoleovorans* 5366^T	53.7	51	31	100												
4. *G. thermocatenulatus* B–1259^T	55.2	37	47	51	100											
5. *G. uzenensis* DSM 13551^T	50.4	38	45	45	54	100										
6. *G. uzenensis* B-2228	51.5	33	43	48	51	80										
7. *G. subterraneus* DSM 13552^T	52.3	53	45	48	50	49	100									
8. '*G. uralicus*' B-2276^T	54.5							100								
9. Strain 46	53.1	76							100							
10. Strain 49	53.3	78	33	53	39	39	51	48		100						
11. Strain DS1	54.5	44	33	53	51	49	53	39	47		100					
12. Strain DS2	53.8										82	100				
13. Strain 44	52.6						94				55		100			
14. Strain 45	53.0						93				53		93	100		
15. Strain 47	52.8												93	95	100	
16. Strain 31	52.9						92				58		95	97		100
17. Strain 32	52.8						91						91	92		98
18. *E. coli* K-12	51.7															

The affiliation of strains 31, 32, 44, 45, and 47 with the species *Geobacillus subterraneus* was confirmed by the high level of DNA–DNA hybridization between the isolates and with the type strain of *Geobacillus subterraneus* (91–98%) (Table 2).

The levels of DNA–DNA hybridization between strains 46 and 49 and type strain *G. stearothermophilus* DSM 22^T (76–78%) confirmed the affiliation of these strains to the species *G. stearothermophilus* (Table 2).

According to the existing criteria, the level of DNA–DNA homology between strain DS1 and strain DS2 (82%) conforms to an intraspecific level of relatedness, whereas the level of DNA–DNA homology between these strains and *G. uzenensis*, as well as other representatives of the genus *Geobacillus* (39–53%), conforms to an interspecific level of relatedness. Thus, strains DS1 and DS2 can undoubtedly be identified as representatives of the same novel species of the genus *Geobacillus*. Such an identification is also confirmed by the results of chemotaxonomic and phenotypic studies (data not presented), which allowed the classification of strains DS1 and DS2 into the novel species *Geobacillus jurassicus.*

General characteristics of the aerobic organotrophic bacteria from the Dagang oil field. With the aim of understanding which physiological characteristics determine the survival and metabolic activity of the isolates under the conditions of the high-temperature oil stratum, we studied spectra of the substrates utilized and the ranges of temperature and salinity adequate for their growth. The range of carbon and energy sources tested (at optimal temperatures and salinity) included soluble organic compounds (sugars, amino acids, organic salts, and alcohols) and poorly soluble hydrocarbons and oil.

The most extensive study was carried out on thermophilic strains 46 and 49 of the species *G. stearothermophilus* and strain 3 Feng belonging to *G. thermoglucosidasius* (this was the first time that representatives of these species had been isolated from an oil field); strains 31 and 32 of the large cluster of the species *G. subterraneus*; strains DS1 and DS2, representing a novel taxon, and some mesophilic strains.

The isolates displayed organotrophic properties and grew on a wide range of substrates, including sugars (glucose, sucrose, and fructose); compound protein substrates (peptone and tryptone); and milk, potato, and blood agars. Some strains utilized lower alcohols (ethanol), the salts of volatile fatty acids (formate, acetate, propionate, and butyrate) and organic acids (malate,

Table 3. Phenotypic characteristics of the aerobic bacteria isolated from the Dagang oil field

Characteristic	*G. jurassicus* DS1T	*G. jurassicus* DS2	*G. subterraneus* 31	*G. subterraneus* 32	*G. thermoglucosidasius* 3 Feng	*G. stearothermophilus* 46	*G. stearothermophilus* 49	*Bacillus cereus* 7	*Oceanobacillus* sp. 10	*Acinetobacter* sp. 34	*Micrococcus luteus* 4	*Pseudomonas stutzeri* 5	β-*Proteobacterium* 33
Substrates used:													
formate	–	–	–	–	–	–	–	ND	+	+	ND	+	+
acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
propionate	–	–	–	–	–	+	–	–	–	+	+	+	–
butyrate	+	+	+	+	+	+	+	+	–	+	+	+	+
methanol	–	–	–	–	–	–	–	+	–	–	–	–	–
ethanol	+	+	–	–	–	+	+	+	–	–	+	+	–
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
phenol	–	–	–	–	–	–	+	–	–	–	–	+	–
benzoate	+	+	+	+	–	–	–	+	–	+	–	+	+
alanine	–	–	–	–	+	–	–	+	–	–	+	–	–
serine	–	–	+	+	–	–	–	+	–	–	+	–	–
glutamate	–	–	–	–	ND	+	+	+	+	–	+	+	–
lactate	+	+	+	+	+	+	+	–/+	+/–	+	+	+	+/–
malate	+	+	+	+	+	+	+	+	+	+	+/–	+	+
pyruvate	+	+	+	+	+	+	+	+	+/–	–	+	+	+
C_6	+	+	–	–	+	–	–	ND	–	–	ND	–	–
C_{14}	+	+	+/–	+/–	–	–	–	+	–	–	+	+	–
C_{16}	+	+	+/–	+/–	–	–	–	+	–	+	–	+	–
Temperature range, °C	45–65	45–65	43–65	43–65	60–70	43–70	43–70	10–45	25–45	25–45	20–45	18–45	10–45
Temperature optimum, °C	58–60	58–60	60	60	65	65	60–65	37	37	37	30	37	37
Salinity range, % NaCl	0–5	0–5.5	0–2	0–4	0–2	0–1.5	0–3	0–4.5	0–10	0–10		0–8	0–5.5

Note: "+" indicates growth, "–" indicates no growth, "+/–" indicates weak growth (OD_{600}, 0.05–0.06), and ND stands for not determined. All the strains grew on peptone, yeast extract, and potato media.

lactate, and pyruvate), individual hydrocarbons, and oil (Table 3).

The thermophilic isolates grew at temperatures ranging from 43 to 65–70°C, but the optimal temperature corresponded to that of their habitat, i.e., 55–60°C. The largest biomass yield was detected in the absence of NaCl, and no growth was detected at NaCl concentrations of higher than 3–5.5%, which demonstrates the freshwater nature of the isolates. Strains 31, 32, 44, 45, 47, DS1, and DS2 grew on crude oil, utilizing mainly saturated hydrocarbons.

Most of the mesophilic strains, isolated from the near-bottom zones of injection wells, grew in a wide salinity range (0 to 8–10% NaCl) and at temperatures ranging from 20 to 40°C, with the temperature optimum at 30–37°C. These properties indicate the allochthonous nature of the mesophilic isolates.

Thus, the results obtained in the present work show that the microbial community of the high-temperature

Dagang oil field includes mesophilic and thermophilic bacteria. The thermophilic bacteria were represented by a wide range of species of the genus *Geobacillus* and actinobacteria of the genus *Thermoactinomyces* (the latter were isolated from an oil field for the first time). The mesophilic bacteria belonged to the genera *Micrococcus, Mycobacterium, Cellulomonas, Pseudomonas, Acinetobacter* and to the β subclass of *Proteobacteria.* Presumably, the mesophilic species were introduced into the oil stratum from the surface, whereas the thermophilic species were of indigenous origin.

The thermophilic isolates were well adapted to the temperature and salinity of their habitat. Their distribution within the oil stratum was determined by the ability to utilize a wide range of substrates, including organic salts, alcohols, individual hydrocarbons, and oil.

The ability of thermophilic microorganisms to oxidize hydrocarbons requires further investigation, since active oil degraders and surfactant producers could be used for the enhancement of oil recovery and decontamination of the equipment used in wells of asphaltic–gum–paraffin deposits, as well as for the bioremediation of oil-polluted ecosystems.

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