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EXPERIMENTAL ARTICLES

Phylogenetic Diversity of Aerobic Saprotrophic Bacteria Isolated from the Daqing Oil Field

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Abstract—A diverse and active microbial community in the stratal waters of the Daqing oil field (China), which is exploited with the use of water-flooding, was found to contain aerobic chemoheterotrophic bacteria (including hydrocarbon-oxidizing ones) and anaerobic fermentative, sulfate-reducing, and methanogenic bacteria. The aerobic bacteria were most abundant in the near-bottom zones of injection wells. Twenty pure cultures of aerobic saprotrophic bacteria were isolated from the stratal waters. Under laboratory conditions, they grew at temperatures, pH, and salinity values typical of the stratal water from which they were isolated. These isolates were found to be able to utilize crude oil and a wide range of hydrocarbons, fatty acids, and alcohols. Phylogenetic analysis carried out with the use of complete 16S rRNA sequences showed that the isolates could be divided into three major groups: gram-positive bacteria with a high and a low G+C content of DNA and gram-negative bacteria of the γ-subclass of the *Proteobacteria*. Gram-positive isolates belonged to the genera *Bacillus, Brevibacillus, Rhodococcus, Dietzia, Kocuria, Gordonia, Cellulomonas*, and *Clavibacter.* Gram-negative isolates belonged to the genera *Pseudomonas* and *Acinetobacter.* In their 16S rRNA sequences, many isolates were similar to the known microbial species and some probably represented new species.

Key words: aerobic bacteria, oil-bearing strata, 16S rRNA, phylogenetic analysis.

The microflora of oil deposits has been the subject of investigation for more than 75 years [1, 2]. The main regularities of microbial distribution in these ecosystems have been established [2–6] and the role of water-flooding, which is used to enhance oil recovery, in the formation of microbial communities in oil reservoirs has been cleared up [3–6]. The elaboration of new culture, radioisotopic, and molecular biological methods stimulated researchers' interest in the microflora of oil- and water-bearing strata [2, 7, 8].

It is known that oil-bearing strata are predominantly anoxic environments, due to which they are dominated by anaerobic fermentative, sulfate-reducing, and methanogenic bacteria [3, 6, 9]. However, aerobic bacteria are also present in oil-bearing strata, into which they arrive with injection water and, presumably, drilling mud [3–5, 9, 10]. The most favorable growth conditions for aerobic bacteria are created in the near-bottom zone of the injection wells, since injection water contains dissolved oxygen. Hydrocarbon- and oil-oxidizing bacteria are important members of the oil-transforming aerobic–anaerobic microbial trophic chain. Aerobic bacteria were found in oil-bearing strata at temperatures ranging from 20 to 70°C and pH ranging from 6.0 to 8.4 [3–6, 9–11]. The growth substrates of these bacteria are nutrient agar, peptone, glucose, sucrose, yeast extract, liquid hydrocarbons, crude oil, and methane. Some of these bacteria (such as methane-oxidizing bacteria) are specific and some (saprotrophic, hydrocarbon-oxidizing, and oil-oxidizing bacteria) are nonspecific [1, 10–12]. Aerobic bacteria are more abundant in sandstone oil-bearing collectors than in carbonate oil-bearing collectors containing hydrogen sulfide [13].

The taxonomic study of aerobic bacteria from oil-bearing strata showed that mesophilic aerobic bacteria belong to the genera *Pseudomonas, Rhodococcus, Brevibacterium, Micrococcus*, and *Arthrobacter*, methane-oxidizing bacteria belong to the genera *Methylomonas, Methylobacter, Methylococcus, Methylosinus*, and *Methylocystis* [1, 11–13], and thermophilic hydrocarbon-oxidizing bacteria belong to the genera *Bacillus* and *Geobacillus* [9, 11, 14].

Only a few works deal with the diversity of aerobic microorganisms in particular subsurface ecosystems. For instance, water-bearing strata in South Carolina at a depth of 200–300 m were found to contain aerobic bacteria, protozoans (amoebae and flagellates), fungi, and algae [7, 15, 16]. The abundance and activity of

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Table 1. Physicochemical and microbiological characteristics of the Daqing oil field

Characteristic	Well number and the volume of back-flushed water in m^3				
	11-5-27, 150 m^3	10-1-310, 40 m^3	12-1-26	2-32	11-5-263
Analyzed water*	1	1	2	2	2
Total mineralization, g/l	2.9	2.69	3.78	4.7	4.4
pH	7.5	7.3	7.5	7.4	7.4
O_2, mg/l	0.8	0.03	0	0	0
H_2S, mg/l	8.5	10	0	0	0
SO_4^{2-}, mg/l	36	36	12	12	12
Acetate, mg/l	61	7	38	408	100
Saprotrophic aerobes, cells/ml	2.5×10^3	2.5×10^2	25	25	2.5×10^2
Hydrocarbon-oxidizing aerobes, cells/ml	25	2.5×10^2	2.5	25	25
Fermentative anaerobes, cells/ml	0.6	6.0	6.0	6.0	0.6
Sulfate-reducing anaerobes, cells/ml	2.5×10^2	2.5	2.5	2.5	25
Methanogens, cells/ml:					
$H_2 + CO_2$	25	$>2.5 \times 10^2$	0	1.3	25
acetate	60	$>2.5 \times 10^2$	0.6	25	60
Sulfate reduction rate, μg S^{2-}/(l day)	16.63	1027.84	0.980	ND**	0.38
Methanogenesis rate, μl CH_4/(l day)	0.815	127.02	3.26	ND	0

* 1, stratal water from the near-bottom zone of the injection wells; 2, stratal water from the production wells.
** ND stands for "no data available."

microflora in these strata are controlled by the availability of water and nutrients, pH, the concentration of metal ions, hydrodynamic communication with the ground surface, the lithology of bearing rocks, and so on [15–18]. The aerobic bacteria of sandy rocks were found to be dominated by gram-negative bacteria, while clay rocks, by gram-positive bacteria [7, 15–18]. The microflora of core samples analyzed by plating onto solid media gave rise to a small number (from 11 to 62) of colonial morphotypes, 80% of which represented gram-negative rod-shaped bacteria [7]. The phylogenetic analysis of aerobic chemoheterotrophic bacteria isolated in pure cultures showed that 75% of them belonged to eight genera *Arthrobacter, Micrococcus, Terrabacter, Sphingomonas, Comamonas, Alcaligenes, Acinetobacter,* and *Pseudomonas* [7, 16]. The aerobic bacteria that were isolated from ground waters in the Washington district belonged to the genera *Arthrobacter, Bacillus, Staphylococcus, Micrococcus, Rhodococcus, Sphingomonas, Variovorax,* and *Pseudomonas.* In general, subsurface isolates were not taxonomically diverse but exhibited a broad metabolic diversity [18].

The pure cultures that were isolated from ground waters collected at depths of 160–180 m were found to be phylogenetically close to bacteria of the genera *Acinetobacter, Comamonas,* and *Aeromonas* [19]. The fact that some bacteria occur in lithologically different and geographically distant rocks suggest that they are common species of subsurface microbial communities.

The aim of the present study was to investigate the diversity of aerobic saprotrophic bacteria, including hydrocarbon-oxidizing ones, isolated from the Daqing oil field, to study their metabolic characteristics, and to analyze their taxonomic status based on 16S rRNA sequence data.

MATERIALS AND METHODS

Characterization of the Daqing oil field. Aerobic bacteria were isolated from the stratal waters of the Daqing oil field located in the Heilongjian province, China. Temperature in the investigated oil reservoirs was 40 to 46°C. Oil from this field contained 87.4% hydrocarbons, 11.5% resins, and 1% bitumen and had a density of 0.797 g/cm^3. The coproduced gas contained 87.5% methane, 8.3% the sum of ethane, propane, and butane, 3.1% nitrogen, and 0.75% CO_2. The original formation water had pH 7.2–7.8, a low content (7.45 g/l) of minerals (predominantly, $NaHCO_3$) and commonly did not contain sulfates or hydrogen sulfide. In the water samples studied, sulfates were present at small concentrations (12–36 mg/l). Hydrogen sulfide (8.5–10.0 mg/l) was found only near in the near-bottom zone of the injection wells. To enhance oil recovery, pressure in the oil field was maintained by reinjecting coproduced water (i.e., the water that remained after oil separation) containing up to 0.8 mg/l of dissolved oxygen.

Table 2. Phenotypic and genotypic characteristics of aerobic bacteria isolated from the formation water of the Daqing oil field

Strain	Cell shape	Spores	G+C, mol %	16S rRNA similarity, %	The closest species according to RDP
32a	Rods	+	45.2	100.0	*Bacillus subtilis*
32e	Rods	+	47.1	99.1	*Bacillus subtilis*
62	Rods	+	36.9	100.0	*Bacillus cereus*
421	Rods	+	46.3	100.0	*Bacillus licheniformis*
13	Rods	+	55.2	98.5	*Brevibacillus parabrevis*
14	Rods	+	66.6	98.2	*Brevibacillus parabrevis*
32c	Rods–cocci	–	ND	99.4	*Arthrobacter oxydans*
32d	Rods–cocci	–	67.6	99.4	*Dietzia maris*
263	Rods–cocci	–	67.8	98.3	*Dietzia natronolimnea*
32f	Cocci	–	69.8	100.0	*Kocuria erythromyxa*
14H	Cocci–rods	–	66.5	100.0	*Rhodococcus ruber*
33	Cocci–rods	–	66.2	100.0	*Rhodococcus ruber*
41	Cocci–rods	–	66.5	99.1	*Rhodococcus ruber*
23	Rods	–	71.4	98.0	*Cellulomonas cellulans*
310-2-3	Rods	–	71.7	99.0	*Clavibacter michiganensis*
321	Cocci	–	ND	97.2	*Gordonia aichiensis*
1a	Cocci	–	40.5	99.7	*Acinetobacter calcoaceticus*
1b	Rods	–	ND	100.0	*Pseudomonas putida*

Note: ND stands for "not determined."

Table 3. Utilization of various substrates by aerobic bacteria isolated from the Daqing oil field

Substrate	*Bacillus subtilis* 32a	*Bacillus cereus* 62	*Bacillus licheniformis* 421	*Brevibacillus parabrevis* 13	*Arthrobacter oxydans* 32c	*Cellulomonas cellulans* 23	*Dietzia natronolimnea* 263	*Gordonia rubropertinctus* 321	*Kocuria erythromyxa* 32d	*Rhodococcus ruber* 14H
Formate	+	–	–	±	–	–	–		–	–
Acetate	–	+	+	+	+	–	+	+	+	+
Propionate	–	–	+	+	+	+	+	+	–	+
Butyrate	+	±	+	+	–	+	–	+	–	+
Methanol	–	–	–	±	–	–	–	–	–	±
Ethanol	–	+	+	±	–	+	+	+	+	+
Fructose	+	+	+	+	+	–	+	+	+	+
Phenol	–	–	–	–	–	–	–	–	–	+
Benzoate	–	–	+	–	–	+	+	+	–	+
Glutamate	+	–	+	–	–	–	–	+	–	–
Alanine	+	–	–	+	–	–	–	–	–	+
Serine	–	–	+	+	–	–	–	+	±	+
Pyruvate	+	+	–	+	–	+	+	+	–	+
Lactate	+	±	+	+	+	+	–	+	–	+
Malate	±	+	–	–	–	–	+	+	+	+
C_{14}	+	–	–	±	+	–	+		–	+
C_{16}	–	+	+	+	–	+	+	+	±	+

Note: All strains were able to grow on glucose, sucrose, potato agar, peptone, and yeast extract. "+," "–," and "±," stand for "good growth," "no growth," and "poor growth," respectively.

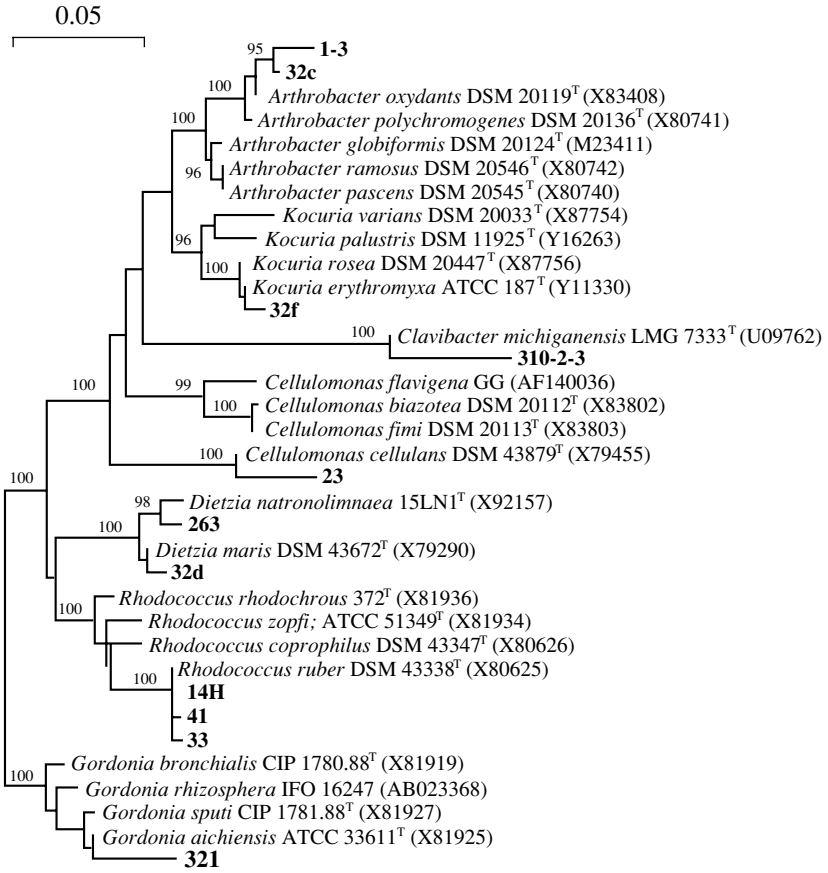

Fig. 1. The position of the gram-positive non-spore-forming bacteria isolated from the Daqing oil field on the phylogenetic tree of actinobacteria. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Numbers near internal branches refer to the bootstrap replications (out of 100 resampling) confirming the grouping of the species to the right of the branch. Bootstrap values of less than 95% are not shown.

Isolation of bacteria. Bacteria were isolated from the formation water that was effluent from depths of 1008–1068 m through production wells and injection wells operating in a backflow regime. The stratal water was plated onto solid media, such as Plate Count Agar purchased from Sigma (United States), potato agar (PA), and mineral Raymond medium supplemented with a mixture of liquid paraffins (C_{12}–C_{22}) in an amount of 10 ml/l [6]. The plates were incubated aerobically at 40°C for 7 days until separate colonies had been formed. The colonies differed in color, shape, consistency, and size. Twenty strains of aerobic saprotrophic bacteria were isolated in pure cultures using the method of successive transfer onto fresh media. The abundance of aerobic and anaerobic bacteria in the stratal water was determined by the dilution method using the media described previously [6].

DNA analysis. The DNA samples isolated from pure bacterial cultures by the Marmur method [20] were analyzed for the G+C content from the melting profiles in dilute buffers using the DNA of *Escherichia coli* K-12 as the standard [21].

Amplification, sequencing, and analysis of 16S rRNA genes. The 16S rRNA genes of pure bacterial cultures were amplified and sequenced using the known primers [22], an automatic DNA Sequencer 373A, and a Ready Reaction Dye Terminator Sequencing Kit with AmpliTaq DNA polymerase purchased from Applied Biosystems (N 402080).

The 16S rRNA gene sequences were preliminarily analyzed using the database and software of the Ribosomal Database Project (RDP) (http:\\rdp.cme.msu.edu) and then aligned manually using the respective sequences of allied reference strains. The unrooted phylogenetic trees of bacteria were constructed with the aid of the TREECON software package [23].

RESULTS AND DISCUSSION

Microbiological characterization of the stratal waters from the Daqing oil field. The microbial community of these stratal waters contained bacteria that are typical of such ecosystems, i.e., hydrocarbon-oxidizing, fermentative, sulfate-reducing, and methanogenic bacteria (Table 1). In the stratal water that was

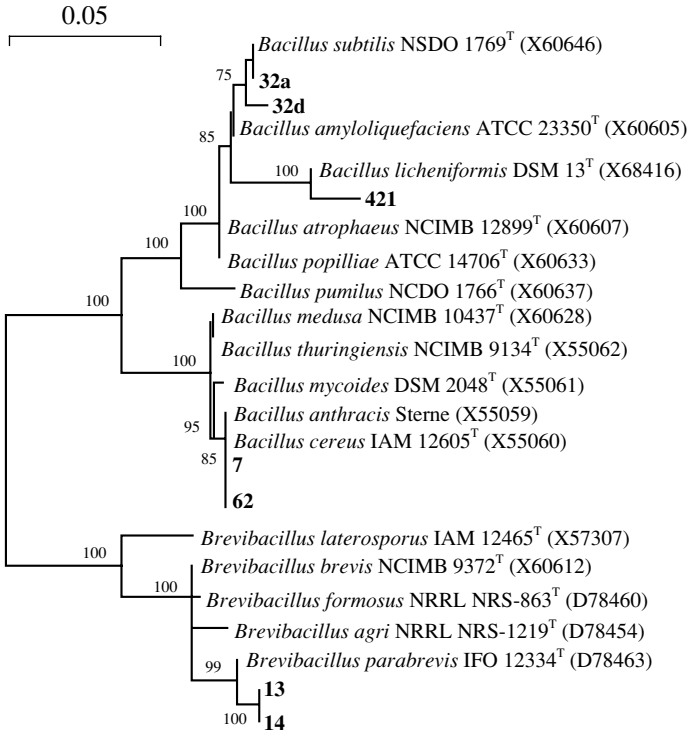

Fig. 2. The position of the gram-positive spore-forming bacteria isolated from the Daqing oil field on the phylogenetic tree of bacilli. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Numbers near internal branches refer to the bootstrap replications (out of 100 resampling) confirming the grouping of the species to the right of the branch. Bootstrap values of less than 95% are not shown.

effluent from the near-bottom zone of injection wells 11-5-27 and 10-1-310, the number of aerobic and anaerobic bacteria reached 10^3–10^5 cells/ml (in the water effluent from production wells, the number of bacterial cells was lower). Among cultivated aerobic saprotrophs, the fraction of hydrocarbon-oxidizing bacteria typically varied from 1 to 10%, but reached 100% in the samples from wells 2-32 and 10-1-310. The anaerobic microflora was represented by fermentative bacteria (several cells/ml), as well as by sulfate-reducing and methanogenic bacteria, whose number varied from single cells to several hundred cells per milliliter. Sulfate reduction and methanogenesis were most active in the water in the near-bottom zone of injection wells (Table 1). In spite of a relatively low concentration of sulfates, the sulfate reduction rate in the water effluent from injection well 10-1-310 was as high as 1027.84 μg S^{2-}/(l day). At the same time, the sulfate reduction rates in the water effluent from production wells were low and comparable with the rates observed for other oil fields, Romashkinskoe and Bondyuzhskoe [3, 10, 12].

General characteristics of aerobic saprotrophic bacteria isolated from the Daqing oil field. The results of investigation of the morphology, utilizable substrates, and the G+C content of the DNA of 20 pure cultures of aerobic saprotrophic bacteria isolated from the stratal water are presented in Tables 2 and 3. It can be seen that the aerobic microflora of the stratal water was represented by different spore-forming and non-spore-forming bacteria. The majority of the isolated strains could grow on sucrose, potato agar, peptone, yeast extract, acetate, ethanol, and fructose. Some strains were found to be able to utilize crude oil, a mixture of liquid C_{12}–C_{20} *n*-alkanes, lower alcohols, and volatile fatty acids (Table 3). The bacteria could grow at neutral pH values and at temperatures from 20 to 45°C, except that strains 421 and 33 could grow at 55 and 50°C, respectively. The medium salinity that was beneficial for bacterial growth was below 40 g NaCl/l. Six of the twenty strains were unable to oxidize hexadecane but could oxidize lower fatty acids (C_2–C_4) and alcohols.

Phylogenetic analysis of the 16S rRNA gene sequences of the aerobic bacteria isolated from the Daqing oil field. The complete nucleotide sequences of the 16S rRNA genes, which contain about 1500 nucleotides, were determined for strains 32f and 321. For the other strains studied, only about 350 nucleotides, corresponding to the *E. coli* 16S rRNA positions from 20 to 400, were sequenced. This region of the 16S rRNA genes is the most informative for preliminary phylogenetic analysis, which by itself showed that the aerobic microbial community of the stratal water included representatives of three bacterial groups, namely, gram-positive bacteria with a high (Fig. 1) and a low (Fig. 2)

G+C content of DNA and gram-negative bacteria of the γ-subclass of the *Proteobacteria*.

The first bacterial group, which included representatives of the genera *Rhodococcus, Dietzia, Kocuria, Gordonia, Cellulomonas,* and *Clavibacter*, was the most abundant. Strains 14H, 33, and 41 of this group had 16S rRNA gene sequences that are practically identical (99.1–100% 16S rRNA similarity levels) to that of the typical species of the genus *Rhodococcus, R. ruber.* This allowed these three strains to be assigned to the species *R. ruber.*

The coryneform strains 321 and 263 were the most close to the species *Gordonia aichiensis* (97.2% 16S rRNA similarity level) and *Dietzia natronolimnea* (98.3% 16S rRNA similarity level), respectively. These similarity levels, however, were not sufficiently high to assign strains 321 and 263 unambiguously to the species mentioned. At the same time, the high similarity (99.4%) of the 16S rRNA genes of strain 32d to those of the species *D. maris* suggested that strain 32d belongs to this species.

The other coryneform bacteria were affiliated with the *Arthrobacter* group. Strain 32c was assigned to the species *Arthrobacter oxydans* (99.4% similarity level). Strains 1-3, 23, and 310-2-3 were the most close to the species *A. oxydans* (97.7% similarity), *Cellulomonas cellulans* (98.0% similarity), and *Clavibacter michiganensis* (99.0% similarity), respectively. These three strains presumably represent new species of the genera *Arthrobacter, Cellulomonas,* and *Clavibacter.* Strain 32f was assigned to the species *Kocuria erythromyxa* (100% similarity).

The second most abundant phylogenetic group of the bacteria isolated from the Daqing oil strata included spore-forming bacilli. Strains 32a, 32e, 62, 7, and 421 turned out to be members of the largest bacilli group 1 (Fig. 2). Within this group, strains 32a and 32e were very close to the type species of the genus *Bacillus, B. subtilis* (99.1–100% similarity), strain 421 was found to be identical to the type strain *B. licheniformis* DSM 13^T (100% similarity), and strains 62 and 7 were identical to the type strain *B. cereus* IAM 12605^T (100% similarity). These similarity levels of the 16S rRNA genes correspond to the intraspecies similarity levels of *B. subtilis, B. cereus,* and *B. licheniformis* (99.8–100%). This fact, together with the closeness of the G+C contents of the DNA of strains 32a, 32e, 62, 7, and 421 (Table 2) to those of *B. subtilis* (42.9–47.5 mol %), *B. cereus* (31.7–40.1 mol %), and *B. licheniformis* (42.9–49.9 mol %) allow these five strains to be assigned to the respective bacilli species.

Strains 13 and 14 were the most close to the species *Brevibacillus parabrevis* (98.2–98.5% similarity), a member of the phylogenetic bacilli group 4, which is presently affiliated with the genus *Brevibacillus.* However, this similarity level is not sufficiently high to assign these strains unambiguously to the species mentioned.

The aerobic gram-negative bacteria isolated from the stratal waters of the Daqing oil field were found to belong to the γ-subclass of the *Proteobacteria.* Strains 1b and 1a were virtually identical to the species *Pseudomonas putida* (100% similarity) and *Acinetobacter calcoaceticus* (99.7% similarity) and, hence, may be considered representatives of these species.

To conclude, molecular biological analysis allows the bacteria that were isolated from the Daqing oil strata to be classified into three major groups, coryneform bacteria (the species *Rhodococcus ruber, Dietzia maris,* and *Kocuria rosea*), bacilli, and γ-proteobacteria. These bacteria were also found in other oil fields [15, 16]. At the same time, three isolates belonging to the genera *Clavibacter, Gordonia,* and *Brevibacillus* have not been previously detected in oil fields and may represent new species of these genera.

The data presented give an idea of the diversity of aerobic microorganisms in the flooded Daqing oil field. The occurrence of the aerobic bacteria in this oil field is obviously due to their ability to utilize oil hydrocarbons and their oxidation products (lower alcohols and volatile organic acids) at temperatures, pH values, and salinity typical of this environment.

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