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EXPERIMENTAL ARTICLES

A Hydrocarbon-Oxidizing Acidophilic Thermotolerant Bacterial Association from Sulfur Blocks

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Abstract—A stable bacterial association isolated from a sulfur block sample of the Astrakhan gas processing complex was able to utilize *n*-alkanes as the sole carbon and energy source at low pH. Hydrocarbon-dependent growth occurred at pH 1.6–5.5 (optimum at pH 2.5) and 20–50°C (optimum at 30–35°C). Analysis of the 16S rRNA gene fragments isolated from the total DNA of the enrichment by PCR–DGGE revealed the nucleotide sequences most closely related to extreme acidophilic chemolithotrophs *Acidithiobacillus thiooxidans* and *Sulfobacillus* sp. (98–99% similarity) and the sequences exhibiting high similarity to those of slowly growing actinobacteria *Mycobacterium europaeum* and *M. parascrofulaceum* (98%). Capacity of any of these organisms for hydrocarbon oxidation has not been reported previously. The taxonomic position of the 16S rRNA gene fragments from the enrichment culture suggests that this bacterial association is a unique microbial community, in which development of acidophilic hydrocarbon-oxidizing bacteria is mediated by a localized pH decrease in the sulfur blocks resulting from elemental sulfur oxidation due to massive development of chemolithotrophic sulfur-oxidizing bacteria.

Keywords: sulfur block, acidic environment, hydrocarbon oxidation, enrichment culture, PCR–DGGE analysis

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The largest Russian open site for sulfur storage is located at the Astrakhan gas-processing complex (AGC). Sulfur blocks are a unique environment characterized by almost complete absence of carbon (elemental sulfur is 99.8% pure), significant diurnal and seasonal fluctuations of temperature, and high acidity in local microzones. Organic admixtures present in the Astrakhan sulfur are mostly hydrocarbons and mercaptans from the gas condensate, which is the source for sulfur isolation. This "natural" background remaining after rectification impairs the sulfur quality and may theoretically act as an additional source of organic matter for the microbial population of sulfur blocks.

Microbial communities of this extreme ecosystem are poorly studied, and the possibility of their activity, including their participation in the oxidation of sulfur and its organic admixtures, seems problematic. Recent research of the sulfur blocks in Alberta (Canada) indicated, however, the presence of scanty and sporadic, albeit viable, populations of conventional heterotrophic microorganisms growing on tryptone–soy agar, of mycelial fungi, and of heterotrophic and autotrophic acidophilic sulfur-oxidizing bacteria [1]. The possible coexistence of acidophilic bacteria of the oxidative branch of the sulfur cycle and hydrocarbon-oxidizing microorganisms was not investigated.

Hydrocarbon contamination of the objects with strongly acidic pH is not, however, a rare phenomenon (it occurs in acidic wastewaters of mine pumping and opened coal storage sites, those resulting from oil spillage treatment, as well as acidic soils in the vicinity of coal mines). Bioremediation of such environments is of significant interest [2]. Investigation of microbial metabolism of hydrocarbons is, however, mostly focused on this process under neutral conditions.

Several bacterial isolates capable of growth on individual hydrocarbons at pH below 4.0 have been isolated [3–6]. Among five strains for which the phylogenetic position was determined, four belong to the *Proteobacteria* (genera *Acidocella* and *Acidisphaera*) [4, 5], while one strain belonged to the phylum *Actinobacteria* (genus *Mycobacterium*) [6]. The latter organism, most closely related to *M. montefiorense* was obtained in pyrene-degrading enrichment and was in fact never isolated in pure culture, since it did not form colonies on solid media. However, the results of microscopy and molecular biological investigation made the authors suggest that these bacteria were the only component of the culture responsible for the deg-

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radation of polycyclic hydrocarbons [6]. *M. montefiorense* belongs to the phylogenetic group of slow-growing mycobacteria.

Diversity and functional activity of the hydrocarbon-oxidizing microbial populations under acidic conditions were also investigated (mostly by molecular ecological techniques) in the samples of acidic soils associated with natural oil seepages at the surface [7], as well as in drainage waters and soils surrounding open coal quarries and coal piles [8].

The goal of the present work was to isolate and characterize the biological properties and taxonomic composition of the acidophilic hydrocarbon-oxidizing microbial association from the AGC sulfur blocks by the cultural and molecular techniques.

MATERIALS AND METHODS

Sampling, medium, and cultivation conditions. The samples of elemental sulfur from sulfur blocks were collected in September 2009 from the U-150 and U-250 storehouses at the AGC open sulfur storage site. The samples were aseptically collected into sterile plastic Falcon vials. The bacterial association described in the present work was obtained from the sample collected from 5 cm depth at the top of the sulfur block of the U-150 warehouse.

Humidity of the sulfur samples was determined as a weight loss after drying to constant weight for 20 h at 105°C. Aquatic pH was measured in a sulfur suspension (sulfur in distilled water 1 : 2.5) with a pH 410 pH meter (Akvilon, Russia).

Sulfur (10 g) was placed in sterile flasks with 100 mL of liquid medium for the isolation of acidophilic bacteria [9] without any organic components and homogenized on a shaker at 150 rpm for 1 h. Sulfur particles were then allowed to precipitate, and the supernatant was used for inoculation of enrichment cultures (10% vol/vol). The cultures were grown under static conditions in 250-mL vials with 45 mL of the same mineral medium supplemented with cycloheximide (up to 100 mg/L) to inhibit fungal growth and a mixture of *n*-alkanes (C_{14}–C_{17} or C_{12}–C_{19}, 0.5% vol/vol) as a source of carbon and energy. The initial pH was 2.7–3.0, the cultivation temperature was 28–30°C. Inoculated media without *n*-alkanes were used as the control. Efficiency of the antibiotic was confirmed by the absence of yeast and fungal growth on solid media—Sabouraud with 2% glucose and malt extract agar (Carl Roth GhbH). Inoculum for the subsequent transfers was 5% (vol/vol); the cultivation was carried out under static conditions or on a shaker (150 rpm).

The most stable microbial association designated as AG_{S17} was used for further investigation.

Physiological properties of the enrichment AG_{S17}. Control of growth and hydrocarbon degradation. The growth-supporting oxygen concentration was determined by cultivation of the enrichment in the medium with *n*-alkanes under the following aeration modes: (1) under static conditions without forced aeration, at the air partial oxygen pressure (21%) in cotton-plugged 500-mL serum vials with 100 mL of the medium; (2) under specified O_2 concentrations in the gas phase (1–15%) in hermetically sealed vials filled to 2/3 of the volume with the oxygen–argon mixture; and (3) under free aeration in cotton-plugged flasks filled with the medium to 1/5 of the volume on a shaker (150 rpm).

Effect of pH and temperature was studied in the same mineral medium with *n*-pentadecane (*n*-$C_{15}H_{32}$) as a substrate. The optimal growth temperature was determined by incubation of the enrichment at the temperatures from 10 to 55°C with a step of 5°C. The pH optimum for growth was determined by cultivation at different initial pH values within the range from 1.2 to 6.0. The values were adjusted with 10 N H_2SO_4. Since pH did not change significantly during cultivation, no buffers were used to maintain pH in the course of microbial growth.

Capacity for growth under specific cultivation conditions was determined after 2–8 weeks of incubation by measuring OD_{600} in a 10-mm cuvette using a Ultrospec 2100 pro UV/visible spectrophotometer. The cells grown for three days in the medium with *n*-pentadecane (0.05% vol/vol) were harvested by centrifugation, washed with the substrate-free mineral medium, resuspended in 2 mL of the medium, and used to inoculate 125-mL vials with 20 mL of the medium with 0.5% (vol/vol) *n*-pentadecane. The initial optical density (OD_{600}) was 0.08. Each variant was inoculated in five to ten repeats. During the experiment, one vial at a time was used to assess the growth. Prior to OD measurement, the biomass was separated by centrifugation from the culture liquid and the hydrocarbon, washed with the ethanol–chloroform–butanol mixture (10 : 1 : 10), and resuspended in the same volume of the mineral medium.

In some experiments, the total number of microorganisms was determined by the epifluorescence microscopy of diluted suspensions filtered through 0.2 μm Nucleopore polycarbonate membranes and stained with 4,6-diamino-2-phenylindole (DAPI).

Capacity of the bacteria for hydrocarbon oxidation was determined in liquid cultures with the mixture of C_{12}–C_{19} *n*-alkanes and 0.5% (vol/vol) 2,2,4,4,6,8,8-heptamethylnonane ($C_{16}H_{34}$) under static conditions at optimal pH for 20 days. Hexane extracts of the culture liquid with bacterial cells and residual *n*-alkanes were used for analysis of the hydrocarbon phase. For inoculum in these experiments, *n*-pentadecane-grown biomass was used after removal of the residual hydrocarbon by centrifugation under aseptic conditions.

The degradation of *n*-alkanes was studied by GLC using a Shimazdzu GC-2010 Plus gas chromatograph

(Japan) with a flame ionization detector and an SPB™ nonpolar capillary column (1.60 m × 0.25 mm × 0.25 μm) with helium as the carrier gas. The initial temperature was 50°C (1 min), the final temperature was 250°C, and the heating rate was 5°C/min. The peak area for 2,2,4,4,6,8,8-heptamethylnonane ($C_{16}H_{34}$), which was not biodegraded, was used as the internal standard. The experimental results made it possible to calculate the relative change (%) in the content of an individual *n*-alkane in the course of biodegradation compared to the control without microorganisms.

All experiments were carried out in at least three repeats. The results of typical experiments are presented in this work.

Molecular biological analysis of the AG_{S17} enrichment culture. The biomass of the AG_{S17} enrichment grown in liquid medium with C_{12}–C_{19} hydrocarbons for 12 days at 30°C and pH 2.5 was used for the isolation of total DNA. The PowerSoil DNA Kit (MO BIO, United States) was used according to the manufacturer's recommendations. PCR of the 16S rRNA gene fragment was carried out in two stages using the universal bacterial primers 338F-907R and 518FGC-907R for the first and second stage, respectively [10]. PCR products were analyzed in 1.2% agarose gel stained with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) was carried out on a DCode Universal Mutation Detection System (BioRad, United States). PCR fragments were separated in 6% polyacrylamide gel (acrylamide to methylenebis-acrylamide ratio 37.5 : 1) in the 35–65% denaturing gradient (the 100% denaturant contained 7 M urea and 40% formamide), with the denaturant concentration increasing from top to bottom. Electrophoresis was carried out for 17 h in twice diluted TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 7.4) at 60°C and 100 V. The gel was stained for 30 min with ethidium bromide (0.5 μg/mL) and washed for 30 min. The results were recorded using the GelDoc imaging system (BioRad, United States). The major visible bands were excised, and DNA was eluted with sterile deionized water. The product was then reamplified and sequenced using the Big Dye Terminator v. 3 kit on an ABI Prism 3100 automatic sequencer (Applied Biosystems, United States) in the Syntol service laboratory [www.syntol.ru]. Analysis of the sequences was carried out using the BLAST software package [http://www.ncbi.nlm.nih.gov/BLAST].

The sequences of the 16S rRNA gene fragments obtained in the present work (~370 nucleotides) were deposited in the GenBank database under accession nos. JX982826–JX982833.

RESULTS AND DISCUSSION

Isolation, some physiological features, and hydrocarbon-oxidizing activity of the AG_{S17} bacterial association. After three months of incubation of enrichments from a sulfur block sample from the AGC sulfur block (60% humidity, pH 2.2) in the mineral medium with cycloheximide and an *n*-alkane mixture, indications of growth was observed in some cases. These included emulsification of the hydrocarbon phase, visually noticeable turbidity, and, at later stages, pale yellow precipitate. Such cultures were supplemented with fresh medium with cycloheximide and the same paraffin mixture, and the cultivation was continued under the same conditions. The procedure was repeated several times in order to obtain a visually dense microbial suspension (OD_{600} > 1.0). By the time of our experiments, stable enrichment cultures of hydrocarbon-oxidizing bacteria were obtained by sequential transfers. These cultures were characterized by retention of activity for ~20 transfers during two years of batch cultivation, stable growth in liquid medium with *n*-alkanes, and the absence of growth on commercial media (TSA, 1/10 TSA, BHIA, or R2A) (Carl Roth GmbH + Co. KG), as well as on solid media with *n*-alkanes. Further investigation was carried out on one microbial association designated AG_{S17}.

The AG_{S17} enrichment exhibited aerobic metabolism. The best growth in the medium with hydrocarbons was achieved in mixed (shaken) cultures with free air supply (mode 3). Under these conditions, the average cell yield by the end of day 17 was 7.3 × 10^8 cells/mL. Under static cultivation conditions (mode 1), growth was slower, although the cell yield did not change significantly. Limited oxygen supply with a fixed oxygen concentration lower than that of the air (mode 2) resulted in significantly decreased growth rates and cell yields not exceeding 25% of the maximum achieved at good aeration.

Effect of the cultivation parameters (pH and temperature) on the growth of the association is shown on Figs. 1 and 2. Acidic medium with pH from 2.0 to 3.0 was optimal, although growth was possible within the pH range from 1.6 to 5.5 (Fig. 1). The association was thermotolerant; while the optimal growth temperature was 30–35°C, growth occurred within the 20–50°C range (Fig. 2).

The association was characterized by its ability to grow at low pH in liquid medium with hydrocarbons as the sole carbon and energy source. Chromatographic analysis (Table 1) showed that during 20 days of cultivation at 30°C and pH 2.5 in the medium with *n*-pentadecane, it was efficiently oxidized, with the residual concentration not exceeding 7.5%. Under the same conditions, 99.96% of the mixture of C_{12}–C_{19} *n*-alkanes was degraded. All hydrocarbons were consumed, albeit to a different degree, as can be seen from their ratios in the profiles of *n*-alkane degra-

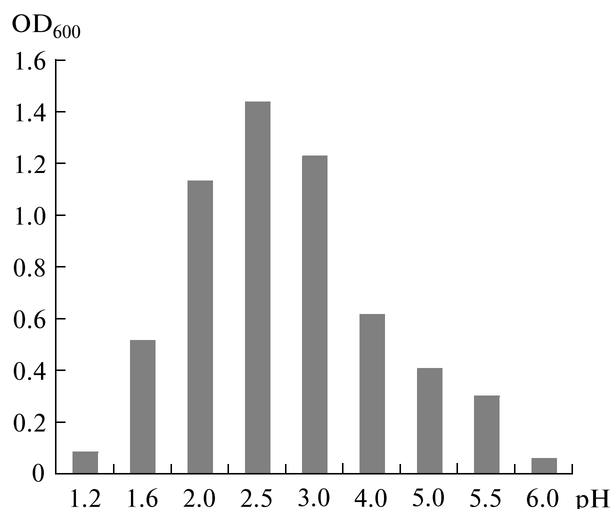

Fig. 1. Growth of the AG_{S17} bacterial association depending on pH. Bacteria were grown for 20 days at 30°C in the medium with *n*-pentadecane.

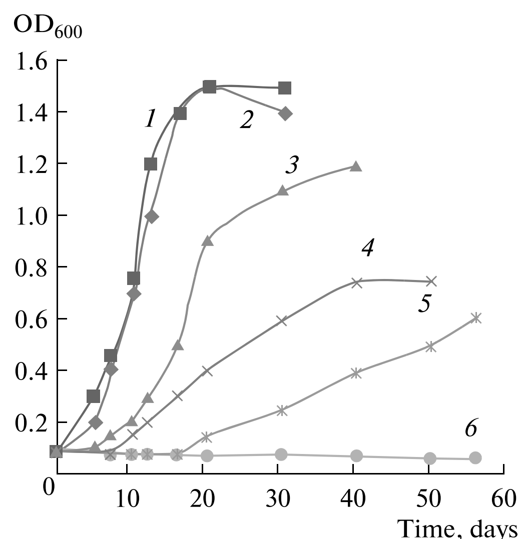

Fig. 2. Growth of the AG_{S17} bacterial association in the medium with *n*-pentadecane at pH 2.5 and at the cultivation temperatures of 35 (*1*), 30 (*2*), 20 (*3*), 40 (*4*), 50 (*5*), and 55°C (*6*).

dation. In the sterile control the ratios of *n*-alkanes did not change.

PCR–DGGE analysis of the 16S rRNA gene fragments from the acidophilic hydrocarbon-oxidizing enrichment AG_{S17}. Our attempts to isolate pure cultures of the microorganisms responsible for hydrocarbon oxidation at low pH proved unsuccessful. Molecular genetic approaches to investigation of microbial communities make it possible to obtain information on the diversity and abundance of their components [10] without isolation of pure cultures. PCR–DGGE analysis was therefore used to investigate the composition of the AG_{S17} enrichment.

Considering the nature of the sample used for enrichment and the duration of cultivation under selective conditions, it was reasonable to expect all the microorganisms in the culture to be acid-tolerant and to participate (directly or indirectly, by creating favorable conditions for the degraders) in hydrocarbon oxidation at extremely low pH. It was therefore not surprising that all bacteria detected in the AG_{S17} enrichment were typical inhabitants of strongly acidic environments.

The results of separation of the amplicons of the ribosomal gene fragments are presented on Fig. 3. The results of sequence analysis of the characteristic profiles (fingerprints) are summarized in Table 2. The number of characteristic DGGE bands was eight,

Table 1. Concentrations of *n*-alkanes in the course of development of the AG_{S17} bacterial association

Compound	Relative content of an *n*-alkane, %		Total content of residual *n*-alkanes, %
	uninoculated control	AG_{S17} culture*	
n-Pentadecane	100.0	7.5	7.5
Mixture of C_{12}–C_{19} hydrocarbons:			0.04
n-Dodecane ($C_{12}H_{26}$)	0.6	0.0	
n-Tridecane ($C_{13}H_{28}$)	8.0	1.4	
n-Tetradecane ($C_{14}H_{30}$)	34.7	2.0	
n-Pentadecane ($C_{15}H_{32}$)	34.4	9.9	
n-Hexadecane ($C_{16}H_{34}$)	15.6	4.5	
n-Heptadecane ($C_{17}H_{36}$)	4.8	0.0	
n-Octadecane ($C_{18}H_{38}$)	1.5	0.0	
n-Nonadecane ($C_{19}H_{40}$)	0.4	0.0	

* Bacterial association AG_{S17} was cultured for 20 days under static conditions at 30°C and pH 2.5 in the mineral medium with C_{12}–C_{19} paraffins.

Fig. 3. DGGE separation of the 16S rRNA gene fragments from the AG_{S17} bacterial association. The bands used for further sequence analyses are marked by arrows and numerals.

including two dominant ones, 3 and 4, which were far remote. This was probably an indication of low diversity within the enrichment.

Sequencing of the major band 3 and of the minor bands 1, 2, 5, and 6 revealed the presence of sulfur-oxidizing bacteria most closely related to *Acidithiobacillus*. This is a genus of aerobic chemolithoautotrophic bacteria which often act as primary producers and important components of the sulfur and iron cycles in natural and anthropogenic extremely acidophilic ecosystems [5, 11, 12]. All the nucleotide sequences (bands 1, 2, 3, 5, and 6) were close to the 16S rRNA gene sequence of the same strain *Ac. thiooxidans* ABRM2011. Since, according to some sources [13], members of this genus (*Ac. ferrooxidans* have two copies of the 16S rRNA gene, we suggest the presence of more than one copy of this gene in the genome of *Ac. thiooxidans*, which could explain the presence of several bands yielding almost identical sequencing results. Primer degeneracy, which in some cases results in emergence of several bands with identical nucleotide sequences [14], is another possible explanation.

Sequence analysis of the 16S rRNA gene fragments from the weakly pronounced bands 7 and 8 revealed the presence of bacteria closely related to *Sulfobacillus* sp. isolate FeSoO-N_4–3-CH (98% similarity). Bacteria of the genus *Sulfobacillus* (family *Alicyclobacillaceae*) are aerobic acidophilic chemolithotrophs, facultative thermophiles, or thermotolerant, capable of using mineral and organic compounds as energy sources and electron donors [15]. While the number of the ribosomal RNA gene copies for sulfobacilli is unknown, the *Firmicutes*, e.g., *Clostridium paradoxum*, were shown to contain up to 15 copies of the rRNA operon [16]. Two bands in the gel probably originated from the fragments of two copies of the 16S rRNA gene.

Sequencing of the PCR fragment of the dominant visible band 4 revealed the presence of bacteria most closely related to *Mycobacterium europaeum* [17] and *M. parascrofulaceum* [18] (98% similarity, Table 2). *M. parascrofulaceum* was described as a slow-growing pathogenic scotochromogenic mesophilic organism, which was isolated from the samples of clinical material from patients with respiratory infections [18]. Free-living strains of this species were, however, subsequently isolated from the water of acidic hot springs in the Yellowstone National Park [19] and were shown to survive and grow within a broad range of temperatures (22–56°C). Although the second bacterium related to the investigated microorganism was very similar to *M. parascrofulaceum* in its cultural and biochemical characteristics, as well as genetically (99.6% 16S rDNA similarity), detailed genetic investigation supported its description as an independent species, *M. europaeum* [17]. No data are available concerning the ability of these mycobacteria to grow on hydrocarbons.

Mycobacteria are common inhabitants of various environments, including soils, silts, plants, and water (natural and municipal) [20, 21]. Some species cause infections in animals and humans. Mycobacteria are characterized by extraordinary high survival under starvation conditions [22], as well as resistance to high temperatures [23] and biocides [20, 24]. Resistance of mycobacteria to unfavorable environmental factors may be due, among other reasons, to their pronounced capacity for formation of biofilms [20] and dormant cells [25]. In spite of wide occurrence in nature, only three publications report detection of uncultured forms [6, 26] or isolation of pure cultures of mycobacteria from extreme environments [19].

Table 2. Results of BLAST analysis of the amplified nucleotide sequences corresponding to the bands in PAG

Band	Most closely related organism (GenBank accession no.)	Similarity, %
1	Uncultured *Acidithiobacillus* sp. clone U2V-bac_f10 (JN982093)	99
	Uncultured bacterium clone kua172 (HM150338)	99
	Acidithiobacillus thiooxidans strain ABRM2011 (JQ034367)	99
2	Uncultured bacterium clone DSJA60 (DQ499268)	99
	Acidithiobacillus thiooxidans strain ABRM2011 (JQ034367)	99
3	Uncultured bacterium clone DSJA60 (DQ499268)	99
	Acidithiobacillus thiooxidans strain ABRM2011 (JQ034367)	99
4	*Mycobacterium europaeum* (FR686462)	98
	Mycobacterium parascrofulaceum (JF271815)	98
	Mycobacterium sp. MOTT-01 (JF271822)	98
5	*Acidithiobacillus thiooxidans* strain ABRM2011 (JQ034367)	99
6	Uncultured bacterium clone DSJA60 (DQ499268)	99
	Acidithiobacillus thiooxidans strain ABRM2011 (JQ034367)	99
7	*Sulfobacillus* sp. isolate FeSo-N4-3-CH (FN870332)	98
	Uncultured bacterium clone RI_Dun_a11 (EU376020)	98
8	*Sulfobacillus* sp. isolate FeSo-N4-3-CH (FN870332)	98
	Uncultured bacterium clone RI_Dun_a11 (EU376020)	98

Organotrophic mycobacteria are common in xenobiotic-contaminated ecotopes. Capacity for hydrocarbon degradation was established for some members of the genus [27]. All known hydrocarbon-oxidizing mycobacteria are able to grow on conventional laboratory media at neutral pH and belong to the phylogenetic branch of fast-growing mycobacteria [28]. Two exceptions have been found. *M. paraffiinicum* is a species described in 1956 [29], but validated quite recently, with its taxonomic status of an independent species confirmed [30]. These mesophilic bacteria isolated from hydrocarbon-contaminated soils have low growth rates (at least 7 days before formation of macroscopic colonies) and are able to oxidize ethane and other *n*-alkanes [25]. The amended description of this taxon provides no information on the pH range for its growth. Another slow-growing hydrocarbon-oxidizing mycobacterium is the already mentioned bacterium related to *M. montefiorense* [6].

An intriguing analogy may be traced between the enrichment described in [6] and the bacterial association studied in the present work. In both cases, the tentative hydrocarbon degraders belonged to mycobacteria, the organisms were not obtained in pure culture, they originated from extreme environments with low pH values, and hydrocarbons were used as sole sources of carbon and energy. In the present work, however, aliphatic hydrocarbons were degraded, while in [6] degradation of such polycyclic hydrocarbons as phenanthrene and pyrene was reported.

Although DGGE is known to provide for rapid and efficient identification of the dominant microorganisms in microbial communities [10], the minor components may be overlooked. While we cannot rule out the possibility that the mycobacteria revealed in the present work grow on the intermediate products of hydrocarbon oxidation produced by other bacteria, which are present in the enrichment in significant numbers, but are not detected by PCR–DGGE, it does not seem highly probable.

Thus, the combination of two selective factors (low pH and the presence of hydrocarbons) resulted in successful enrichment of a unique hydrocarbon-oxidizing bacterial association, including acidophilic sulfur-oxidizing bacteria and a member of slow-growing mycobacteria. Molecular approaches revealed numerous nucleotide sequences related to *Ac. thiooxidans* in the total DNA of the microbial community. Attempts to detect bacteria related to *M. parascrofulaceum* in the sulfur block samples prior to enrichment were, however, unsuccessful (Ivanova et al., unpublished data). The latter could probably be due to the low numbers of such bacteria under nonselective environmental conditions. Interestingly, other authors [5, 6] stressed the importance of the choice of the relevant conditions (e.g., low pH of the original samples and necessity for preliminary enrichment) for obtaining and mainte-

nance of the cultures of acidophilic hydrocarbon-degrading microorganisms. The AG_{S17} enrichment is the first and presently the only acidophilic bacterial thermotolerant association isolated from sulfur blocks and capable of aerobic oxidation of petroleum hydrocarbons. Its optimal growth occurs under strongly acidic conditions, which are generally uncharacteristic of the chemical parameters of sulfur blocks. We suggest that other components of the microbial community, which interact with the "body" of sulfur blocks, provide for survival of the biodegrading bacteria at the storage site. Longer terms of open storage of sulfur, the presence of such acid-producing microorganisms of the oxidative branch of the sulfur cycle as *Acidithiobacillus* and *Sulfobacillus* species, and elevated humidity (resulting from atmospheric precipitation, leaks of condensed steam from the technological equipment, etc.) may result in developing the favorable conditions for persistence and development of acidophilic hydrocarbon-degrading microorganisms.

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