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EXPERIMENTAL ARTICLES

Taxonomic Diversity of Aerobic Organotrophic Bacteria from Clean Vietnamese Soils and Their Capacity for Oxidation of Petroleum Hydrocarbons

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Abstract—The dominant species and abundance of the cultured aerobic organotrophic bacteria were determined in the clean soils of the Republic of Vietnam. The total number of organotrophs varied from 2.0×10^5 to 5.8×10^8 CFU/g soil. A considerable fraction of the bacterial population (1.1×10^5–9.5×10^6 CFU/ g soil) was able to utilize petroleum hydrocarbons as the sole carbon and energy source. Most of the organisms obtained in pure cultures were gram-positive bacteria; over 70% were hydrocarbon-oxidizing organisms. Analysis of 16S rRNA gene sequences resulted in tentative determination of the taxonomic position of 22 strains, with 12 belonging to the *Firmicutes*, 4, to the *Proteobacteria*, and 6 to the *Actinobacteria*. The most common bacteria capable of hydrocarbon oxidation belonged to the genera *Acinetobacter, Bacillus, Brevibacillus, Chromobacterium, Cupriavidus, Gordonia, Microbacterium, Mycobacterium*, and *Rhodococcus*. Some of the isolated *Bacillus* and *Staphylococcus* strains, as well as one *Pseudomonas* and one *Sinomonas* strain, did not utilize hydrocarbons. Gram-positive degraders, especially members of the order *Actinomycetales*, which exhibited high hydrocarbon-oxidizing activity, gained competitive advantage in the presence of hydrocarbons. This microbial group probably plays an important role in hydrocarbon degradation in tropical soils. Thus, Vietnamese soils, which had no history of petroleum contamination, support numerically significant and taxonomically diverse populations of hydrocarbon-oxidizing bacteria.

Keywords: tropical soils, aerobic hydrocarbon-oxidizing bacteria, petroleum hydrocarbons, 16S rRNA, taxonomic diversity.

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Self-purification is the major mechanism supporting the normal functioning of soil ecosystems under ever-increasing anthropogenic contamination. Petroleum hydrocarbons and their toxic derivatives are common xenobiotics in terrestrial environments [1, 2]. Hydrocarbon-oxidizing bacteria play the key role in their degradation by soil microbial communities. The efficiency of this process in a given environment is to a large degree determined by the presence and adaptive potential of the biological agents for hydrocarbon decomposition.

Soil hydrocarbon-oxidizing bacteria have been isolated mainly from moderate and cold climatic zones, while the information concerning the tropical members of this group is limited [3] and their ecology is poorly studied. Bacteria isolated from petroleum-contaminated tropical soils belonged to the taxa for which capacity for hydrocarbon utilization was already known (*Arthrobacter*, *Pseudomonas*, *Mycobacterium*, *Rhodococcus, Gordonia, Microbacterium, Nocardia, Bacillus,* and *Stenotrophomonas*) [4, 5].

Special attention was paid to actinobacteria. Until recently, this group has been considered a minor component of microbial communities of oil-contaminated sites, while predominance of gram-negative bacteria was noted [6]. Recent research, however, convincingly demonstrated the key role and dominance of the *Actinobacteria* in self-purification and bioremediation of various tropical environments [4, 5, 7, 8]. This is the result of their metabolic versatility, high resistance to the limiting environmental factors, and occurrence both in clean and hydrocarbon-contaminated ecotopes [2, 4, 7].

The Republic of Vietnam is located in the tropical zone. The Vietnamese soils exhibit significant diversity due to the specific geographic location of this country, natural and climatic conditions, and large-scale anthropogenic impact [9]. Their bacterial population is, however, poorly known. The known data are mostly restricted to detection of some bacterial degraders of

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Table 1. Natural and climatic characteristics of the sampling sites

Characteristics	Test stations	
	Hoa Lak	Dom Bay
Geographical position and site characterization	20°9′N, 105°37′E Rural area, 150 km from the sea shore, 30 km SW from Hanoi	12°19′N, 109°29′E Coast of the South China Sea (Che island, 50 m from the water edge), 12 km from Nha Trang
Climate	Tropical monsoon	Humid tropical marine
Seasonality	Rain season (April–September) and dry season (October–March)	Dry (January–August) and moist (September–December) seasons
Annual environmental parameters*:		
Air temperature, °C	+24	+27.5
Air relative humidity, %	78	82
Precipitation, mm	1600	1400
Total solar irradiation, J/m^2	4.5×10^3	6.4×10^3
Rate of chloride precipitation, mg/m^2 per day	–	30–60
Physicochemical parameters of soils**:		
Humidity, %	6.9–39.1	28.0–29.3
Temperature, °C	23–24	24–26
pH	4.7–6.8	6.2–7.7
Total carbon, %	3.1–4.1	1.9–3.2
Total nitrogen, %	0.2–0.4	0.1–0.2
Al_2O_3, %	19.7–25.7	16.8–16.9
Fe_2O_3, %	3.3–17.9	2.0–2.3
C : N	10.7–14.3	10.5–24.0

Notes: * Data of many years of observation of CTS are presented.
** Ranges of the parameters in the upper soil horizon determined in the present work.

such priority pollutants as petroleum products [10] and dioxins [11]. Until recently, several *Acinetobacter* and *Pseudomonas* isolates from the soils strongly contaminated with petroleum were the only hydrocarbon-oxidizing bacteria known from the Vietnamese soils [10]. Natural soils and cultivated soils without pronounced technogenic contamination are especially poorly studied [9].

The goal of the present work was assessment of the taxonomic diversity of the cultured aerobic organotrophic bacteria in clean Vietnamese soils, and of the occurrence of capacity for petroleum hydrocarbon oxidation within this microbial community.

MATERIALS AND METHODS

Characteristics of investigated sites and soils. Typical tropical soils of different regions of the Republic of Vietnam were collected at two sites—Hoa Lak climatic test station (CTS) and Dom Bay marine research and test station (MRTS). Both stations were organized by the Joint Russian-Vietnamese Tropical Research and Technological Center for environmental tests of materials, coatings, and products in humid tropical climate. The distance between the stations is 1300 km. Geographically, they represent Northern and Central Vietnam, respectively. The sites and soils investigated are briefly characterized in Table 1.

Soil samples were collected in November–December from the surface horizon (5–10 cm) in different parts of the test station area and in the vicinity (7 samples for each site). The vegetation was removed from the soil surface immediately before the sampling, and soil was collected into sterile plastic Falcon vials with a broad metal spatula. The samples were treated at the day of collection under aseptic conditions.

Moisture content was determined by drying to constant weight for 20 h at 105°C. The pH was determined in soil extracts (soil and distilled water 1 : 2.5) using a pH meter. Organic carbon and total nitrogen were determined on an Elementar Vario EL III automatic element analyzer (Elementar Analysensysteme GmbH, Germany). The content of Al_2O_3 and Fe_2O_3 was determined on a MAKS-GV roentgen fluorescent scanning spectrometer (Burevestnik, Russia).

The investigated red soils, which were formed under conditions of humid climate and high annual temperatures, are characterized by scrubbing water

regime, loamy and clayey granulometric composition, high content of aluminum and iron oxides, pH_{water} 4.7–7.7, organic matter content from 1.9 to 4.1%, and moisture content from 6.9 to 39.1% (Table 1). The table demonstrates significant variation in the properties of soils of the experimental sites.

Cultivation media and quantification of bacteria. To obtain soil suspensions, soil (10 g) was mixed with 90 mL of pyrophosphate solution ($Na_2P_2O_7$, 2.8 g/L) in a sterile 250-mL flask and homogenized on a shaker (130 rpm) for 2 h. After sedimentation of the soil particles for 30 min, the supernatant was used as inoculum for tenfold dilutions.

Total number of microorganisms was determined by epifluorescence microscopy on 0.2 μm Nucleopore polycarbonate filters stained with DAPI (4,6-diamidino-2-phenylindole).

Cultured bacteria were enumerated by surface plating of 0.1 mL aliquots of tenfold dilutions of solid media. Each sample was plated in three repeats. The results presented are the average values.

Aerobic organotrophic bacteria were detected using the Brain Heart Infusion Agar commercial medium (BHIA, Sigma, B7279, United States). The plates were incubated for 5 days at 24°C.

The plates inoculated with high dilutions (10^4–10^7) of soil suspension and containing 20 to 200 bacterial colonies were used for the isolation of pure cultures of organotrophic microorganisms. The initial selection was based on the differences in colony morphology. The strains were designated HL or DB, depending on the source of isolation.

The number of aerobic hydrocarbon-oxidizing bacteria was determined on Raymond medium with hydrocarbons [12] as the sole carbon source. The plates were incubated in a sealed plastic container (2 L) at 24°C until visually discernible colonies emerged (3 to 6 weeks). Abundance of degrader bacteria was determined from the number of colony-forming units (CFU) or (in the case of higher polycyclic hydrocarbons) from the number of colonies with transparent zones around them [13].

Selective enrichment and isolation of pure cultures of hydrocarbon-oxidizing bacteria. Representative samples of tropical soils reflecting the overall microbiological picture of each station were obtained by mixing the series of seven previously collected samples. The aliquots (1 g) of combined samples were used to inoculate 100 mL of Raymond medium with 100 mg/L of cycloheximide (for prevention of fungal growth) in 500-mL conical flasks. During the next several days, the growth occurred due to consumption of soil organic matter.

After consumption of available substrates (assessed as cessation of increase in the optical density), aliquots of the culture liquid (1 mL) were used to inoculate the flasks with the medium of the same composition. Petroleum from the Moscow oil-processing plant with high content of sulfur and paraffins was used as the sole carbon source (500 μL, 0 days from the beginning of the experiment). The cultures were grown at 24°C under stationary conditions. An inoculated flask without additional carbon sources was used as the control for every experimental series. During the experiment (63 days), two transfers of the enrichments were made, on days 21 and 42, with the content of petroleum increasing to 20 and 80 mL/L, respectively.

At various stages of enrichment, the following parameters were determined: total microbial number (by direct count of DAPI-stained cells), number of organotrophic bacteria (by plating on BHIA), number of hydrocarbon-oxidizing bacteria (by plating on solid Raymond medium with naphthalene, phenanthrene, or C_{12}–C_{16} *n*-alkanes), and the physiological status of the bacterial community (by inoculating liquid Raymond medium with various petroleum derivatives).

At the end of the experiment, the dominant bacteria forming colonies of different morphology were isolated in pure cultures. The strains obtained two-letter designations, with the letter "e" indicating the method of isolation (enrichment with hydrocarbons). At different stages of the experiment, the identity of bacteria forming colonies of the same morphotype was confirmed by sequencing 16S rRNA gene fragments (~500 bp) from several randomly selected colonies, as well as by DNA–DNA hybridization.

Investigation of biodegradation of saturated petroleum hydrocarbons by gas–liquid chromatography (GLC). In order to determine the catabolic potential for hydrocarbons, bacteria were grown at 28–30°C under stationary conditions in liquid Raymond medium with petroleum. The latter was highly sulfurous (sulfur content 2.0–2.2 vol %), paraffin-rich (4.0–4.1 vol %), with specific density of 0.871–0.876 g/cm^3. Uninoculated medium with petroleum was used as the control. The hydrocarbon phase was analyzed in hexane extracts of the culture liquid with bacterial cells and residual oil.

Hydrocarbon-oxidizing activity was determined by decomposition of the *n*-alkanes of the aliphatic fraction on a Kristall 5000.1 gas–liquid chromatograph (Khromatek, Russia) with a flame ionization detector and a 25-m capillary column with Apiezon as the stationary phase. Hydrogen was used as a carrier gas. The column temperature was increased from 100 to 320°C at 5°C/min.

Summary height of phytane and pristane (*iso*C_{19} + *iso*C_{20}) was used as an internal standard. The calculation was considered correct when the *iso*C_{19}/*iso*C_{20} ratio was preserved during the experiment. When this ratio changed by more than 10%, the summary height of C_{28+30} peaks was used as the internal standard. Thus, the calculation enabled us to determine the relative change in the content of an individual *n*-alkane expressed as percentage of its initial content in the petroleum.

Table 2. Quantitative characteristics of the major microbiological parameters of the soils

Soil sample	Parameter			
	Total number of microorganisms*, $\times 10^8$ cells/g soil	Number of organotrophic bacteria,** $\times 10^6$ CFU/g soil	Share of cultured bacteria***, %	Number of hydrocarbon-oxidizing bacteria****, $\times 10^5$ cells/g soil
Hoa Lak station and vicinity				
1	1.0	3.8	3.8	1.4
2	1.2	2.8	2.3	4.0
3	2.1	2.6	1.2	1.5
4	1.3	1.8	1.4	75
5	1.0	0.2	0.2	1.1
6	2.4	6.6	2.8	2.5
7	1.8	42	23.3	65
Dom Bay station and vicinity				
1	54	360	6.7	20
2	32	420	13.1	30
3	28	160	5.7	16.5
4	49	580	11.8	65
5	22	240	10.9	40
6	35	290	8.3	95
7	36	200	5.6	30

Notes: * Results of direct count on DAPI-stained filters.
** Number of aerobic organotrophic bacteria on BHIA medium.
*** The share of cultured bacteria (%) was determined as the ratio between CFU number on BHIA medium to the total number (DAPI) multiplied by 100.
**** Number of hydrocarbon-oxidizing bacteria on Raymond medium with C_{12}–C_{16} *n*-alkanes .

Characterization and identification of bacterial strains. Morphology of bacterial cells and colonies was determined visually under a binocular microscope and phase contrast light microscope. Gram reaction of the cells was determined using a specialized kit (Merck).

Determination of genomic characteristics of pure cultures (DNA G+C content and DNA–DNA hybridization) and amplification and sequencing of 16S rRNA genes were carried out as described previously [14]. The newly obtained sequences were compared to GenBank sequences using the BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST).

The 16S rRNA gene fragments isolated from bacteria from tropical soils were deposited in GenBank under accession nos. JF734312–JF734334.

RESULTS

Microbiological parameters of tropical soils. Both direct count of DAPI-stained cells and the number of cultured aerobic organotrophic bacteria were somewhat higher at the Dom Bay MRTS site (Table 2). While aerobic organotrophic bacteria grown on BHIA medium constituted a relatively small fraction of the total microbial number in Hoa Lak samples (0.2–3.8%), their share in Dom Bay samples was as high as 13.1%. Soil sample no. 7 collected in the vicinity of Hoa Lak in a eucalyptus plantation was exceptional, with cultured bacteria constituting 23.3% of the total microbial number. The CFU values for organotrophic bacteria in the upper soil horizon for the Dom Bay station were stably high (1.6×10^8–5.8×10^8 CFU/g soil), while they varied significantly in the Hoa Lak samples (2.5×10^5–4.2×10^7 CFU/g soil).

Enumeration of hydrocarbon-oxidizing bacteria revealed significant populations of these microorganisms (from 1.1×10^5 to 9.5×10^6 CFU/g soil) in all soil samples (Table 2).

The bacterial community of Hoa Lak soils exhibited significant diversity in colony morphology, while 5–7 morphotypes predominated in Dom Bay soils. Most of them were isolated and maintained in pure culture (Table 3).

Adaptation of soil bacterial communities to hydrocarbons. The spectrum of substrates used by enrichment cultures from tropical soils was investigated under laboratory conditions of exposure to increasing concentrations of petroleum.

Addition of petroleum to enrichment cultures resulted in a significant rearrangement of microbial communities and affected their metabolic potential (Table 4). In the beginning of the experiment, only a narrow spectrum of complex hydrocarbon mixtures,

Table 3. Differentiating phenotypic and genotypic characteristics of the representative bacterial strains isolated from the Vietnamese tropical soils

Strain (no.*)	Gram reaction/Cell shape**	G+C, mol %	Growth on hydrocarbons***	Phylogenetic group	Closest related species according to BLAST (GenBank) (similarity % and no.)*
DB-1 (JF734330)	+/R	34.0	H; N	*Firmicutes*	*Bacillus mycoides* (99 and EU221418.1)
DB-2 (JF734331)	+/R	39.5	H	*Firmicutes*	*Bacillus subtilis* (100 and FJ527656.1)
DB-6 (JF734332)	+/R	34.8	H	*Firmicutes*	*Bacillus cereus* (100 and FJ390480.1)
DB-8 (JF734333)	+/R	34.6	H; N	*Firmicutes*	*Bacillus cereus* (100 and CP000227.1)
HL-5 (JF734317)	+/R	41.0	H; N	*Firmicutes*	*Bacillus aerophilus* (99 and AJ831844.2)
HL-18 (JF734325)	+/R	37.0	H; N	*Firmicutes*	*Bacillus megaterium* (99 and GU252114.1)
DB-3 (JF734329)	+/R	ND	H	*Firmicutes*	*Brevibacillus agri* (99 and AY319301.1)
HL-2 (JF734313)	+/R	ND	H; N	*Firmicutes*	*Brevibacillus agri* (99 and AB039334.1)
HL-21 (JF734326)	+/R	70.4	–	*Firmicutes*	*Brevibacillus invocatus* (99 and AF378232.1)
HL-6 (JF734318)	+/C	33.0	–	*Firmicutes*	*Staphylococcus pasteuri* (99 and AJ717376.1)
HL-11 (JF734321)	+/C	35.5	–	*Firmicutes*	*Staphylococcus arlettae* (100 and EU660331.1)
HL-13 (JF734323)	+/C	32.6	–	*Firmicutes*	*Staphylococcus haemolyticus* (100 and AP006716.1)
HL-1 (JF734312)	–/R	ND	N	β-*Proteobacteria*	*Cupriavidus gilardii* (99 and EF114428.1)
HL-20 (JF734316)	–/R	65.0	H; N	β-*Proteobacteria*	*Chromobacterium violaceum* (99 and AE016825.1)
HL-17 (JF734324)	–/R	ND	H	γ-*Proteobacteria*	*Acinetobacter calcoaceticus* (100 and EF432578.2.1)
HL-10 (JF734320)	–/R	ND	–	γ-*Proteobacteria*	*Pseudomonas acephalitica* (99 and AM407893.1)
HL-4 (JF734315)	+/R-C	ND	–	*Actinobacteria*	*Sinomonas atrocyaneus* (99 and AY605543.1)
DB-10 (JF734334)	+/R-C	67.0	–	*Actinobacteria*	*Microbacteriaceae bacterium* (99 and DQ490451.1)
DB-Ae (JF734328)	+/R-C	68.9	H; N	*Actinobacteria*	*Gordonia terrae* (99 and AB355992.1.1)
HL-Be (JF734327)	+/R-C	66.7	H	*Actinobacteria*	*Mycobacterium fortuitum* (100 and DQ973806.1)
HL-8 (JF734319)	+/R-C	56.4	H; N	*Actinobacteria*	*Rhodococcus equi* (100 and FJ46344.1.1)
HL-3 (JF734314)	+/R-C	66.0	H; N	*Actinobacteria*	*Rhodococcus equi* (100 and AJ741716.1)

Notes: * Accession number of the 16S rRNA gene sequence in the GenBank database.

** Bacterial shape: rods (R) and cocci (S).

*** Designations: H stands for hexadecane, N, for naphthalene, "–" indicates no growth on hexadecane and naphthalene. ND stands for no data.

Table 4. Major microbiological parameters of bacterial enrichment cultures from combined samples of tropical soils in the presence of petroleum

Parameter	Culture from Hoa Lak soil			Culture from Dom Bay soil		
	Incubation time, days					
	0	21	63	0	21	63
Aerobic organotrophic bacteria, cells/mL	5.2×10^5	6.4×10^8	7.6×10^9	3.6×10^6	8.4×10^9	9.2×10^9
n-Alkane-oxidizing bacteria, cells/mL	4.0×10^3	2.8×10^8	7.5×10^9	6.0×10^4	7.2×10^9	9.2×10^9
Naphthalene-oxidizing bacteria, cells/mL	<10*	ND	2.8×10^5	5.4×10^2	ND	7.6×10^6
Phenanthrene-oxidizing bacteria, cells/mL	<10	2.4×10^3	6.2×10^6	<10	3.8×10^3	8.9×10^6
Grow on:						
Diesel fuel	–	+	+	+	+	+
Paraffin oil	+	+	+	+	+	+
Hexane	–	–	+	–	–	–
Octane	–	+	+	–	+	+
Dodecane	+	+	+	+	+	+
Benzoate	+	+	+	+	+	+
Phenol	–	+	+	+	+	+
Benzene	+	+	+	–	–	+
Toluene	+	+	+	+	+	+
Anthracene	–	–	+	–	+	+
Fluorene	–	+	+	–	+	+
Fluoranthene	–	–	+	+	+	+
Pyrene	–	–	+	–	+	+
Acenaphthene	–	–	+	–	–	–
Ethylcyclohexane	–	–	+	–	–	–

Note: Designation <10 is below detection limit. ND stands for no data.

individual hydrocarbons, and their derivatives (a total of 5 to 7 compounds) was used as a source of carbon and energy for the primary enrichment cultures. By the end of the experiment, the growth of enrichment cultures was supported by most of the 15 substrates tested.

On the 21st day of cultivation, the number of organotrophic bacteria increased by more than three to five orders of magnitude, mostly due to an increase in the relative content of *n*-alkane oxidizers (Table 4). By the end of the experiment, the latter constituted 98–100% of the microbial community. A similar increase was observed in the case of naphthalene and phenanthrene degraders. No significant changes in the microbiological parameters were observed in the controls (data not shown).

The structure of the community of degrading bacteria also changed significantly. The total number of morphologically different strains decreased, so that several dominant morphotypes remained. Over 20 dominant members of the hydrocarbon-oxidizing microflora were isolated at different stages of the experiment. Since the results of sequencing of 16S rRNA gene fragments (~500 nucleotides) and DNA–DNA hybridization (homology exceeding 80%) indicated close relations within each morphotype [15] (data not shown), one strain of each colony morphotype was used in subsequent studies. Six pure cultures of hydrocarbon-oxidizing bacteria (HL-Ae, HL-Be, HL-Ce, HL-De, DB-Ae, and DB-Ce) isolated from enrichment cultures were the major subjects of investigation.

Limited bacterial diversity and unique colony morphology made it possible to monitor the dynamics of abundance of individual strains in the course of the experiment by direct CFU counts on solid medium with *n*-alkanes (Fig. 1). In the original enrichment cultures from Hoa Lak on media with *n*-alkanes, bacterial colonies of the HL-Be type (flat dry matted buff) were not found, while the morphotypes HL-Ae (convex transparent glossy), HL-Ce (light pink slimy), and HL-De (dark violet) constituted 7.0, 5, and 2%, respectively. By the end of the first transfer (21 days from the onset of the experiment), the share of the first two morphotypes increased significantly, to 28 and 10%, respectively. On the 63rd day of the experiment,

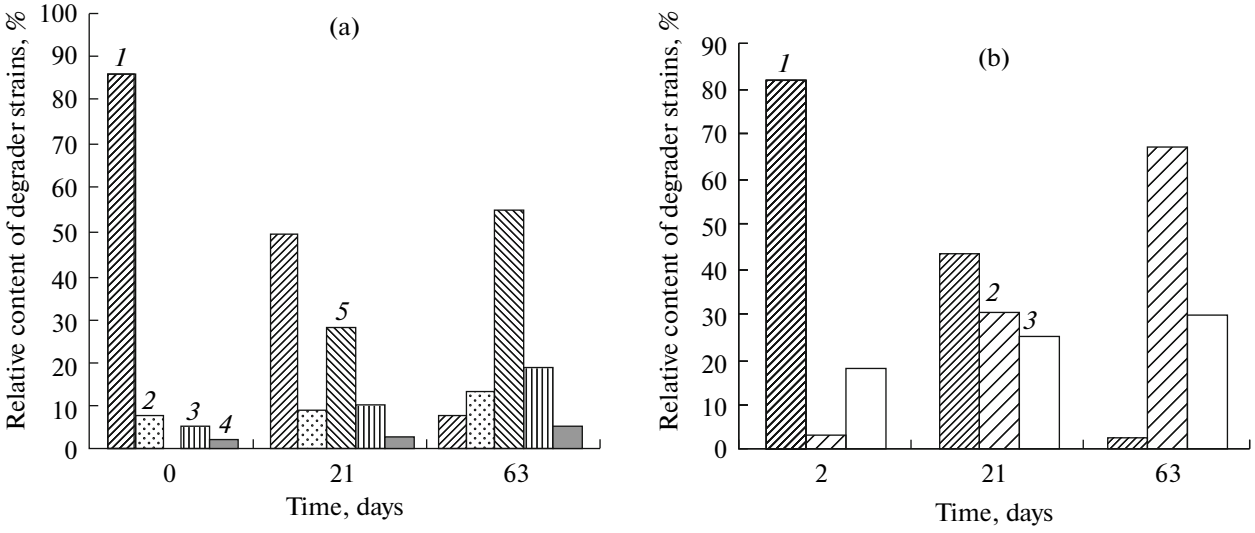

Fig. 1. Relative content of degrader strains (% of the total bacterial number on media with C_{12}–C_{16} *n*-alkanes) in the hydrocarbon-oxidizing community of Hoa Lak (a): other strains (*1*), strain HL-Ae (*2*), strain HL-Be (*3*), strain HL-Ce (*4*), strain HL-De (*5*) and Dom Bay soils (b): other strains (*1*), strain DB-Ae (*2*), and strain DB-Ce (*3*).

these four types constituted 92% of the colonies obtained on Raymond medium with *n*-alkanes (HL-Ae, HL-Be, HL-Ce, and HL-De constituted 13, 55, 19, and 5% of the CFU number, respectively).

In the original enrichment cultures from Dom Bay soils (Raymond medium with paraffins), bacterial colonies of the DB-Ae morphotype (small bright orange convex) were not observed. However, characteristic orange colonies appeared after short exposure to petroleum (on the second day), and their number increased in the course of cultivation and transfers with petroleum (Fig. 1). Bacteria of the second type, DB-Ce (large flat whitish slimy colonies easily merging together), were revealed both in the original soil bacterial community and in enrichments at different hydrocarbon concentrations. Their CFU increased from 18% (day 2) to 25% on the 21st day of the experiment. By the end of the experiment, these two types constituted 97% of the population of hydrocarbon-oxidizing bacteria in the enrichments: 67 and 30% of the colonies were morphologically similar to types DB-Ae and DB-Ce, respectively.

Characterization and identification of pure bacterial cultures isolated from tropical soils. DNA–DNA hybridization was carried out for some bacterial cultures with similar phenotypes and DNA G+C content. The values of DNA homology for strains DB-Ce and DB-2, HL-Be and HL-8, HL-De and HL-20, and HL-Ce and HL-17 were 90, 95, 86, and 79%, respectively. According to existing criteria [15], they correspond to intraspecific differences. One strain of each group (DB-2, HL-8, HL-20, and HL-17) was used in the subsequent work.

Phylogenetic analysis of the 16S rRNA gene sequences from tropical soil strains revealed diversity of heterotrophic bacteria belonging to three major phylogenetic groups: 12 strains belonged to the *Firmicutes*, 4 strains, to the *Proteobacteria*, and 6 strains belonged to the phylum *Actinobacteria* (Table 3). The taxonomic diversity of cultured organotrophic bacteria was much lower at the Dom Bay station than at Hoa Lak (4 and 10 genera, respectively). Although the experimental procedures did not give selective advantage to either gram-positive or gram-negative bacteria, most of the new isolates (17 out of 22), including all Dom Bay strains, belonged to the gram-positive type (Table 3).

Biodegradation of hydrocarbons by bacterial isolates. Out of the 22 identified strains belonging to 12 genera, 15 strains grew on C_{12}–C_{16} alkanes and naphthalene (the simplest polycyclic hydrocarbon) (Table 3). Bacteria of the genera *Acinetobacter, Bacillus, Brevibacillus, Chromobacterium, Cupriavidus, Gordonia, Microbacterium, Mycobacterium*, and *Rhodococcus* were found to be potentially capable of hydrocarbon oxidation. Some members of the phylum *Actinobacteria* were probably the minor components of the natural bacteriocenoses, as can be seen from the absence of their morphotypes in the platings of original soil suspensions (*Gordonia terrae* DB-Ae) or from low CFU numbers (*Mycobacterium fortuitum* HL-Be). They, however, rapidly increased in numbers and were easily revealed under selective enrichment with hydrocarbons (Fig. 1).

Further screening for hydrocarbon-oxidizing activity revealed that gram-positive bacteria (*Mycobacterium fortuitum* HL-Be, *Gordonia terrae* DB-Ae,

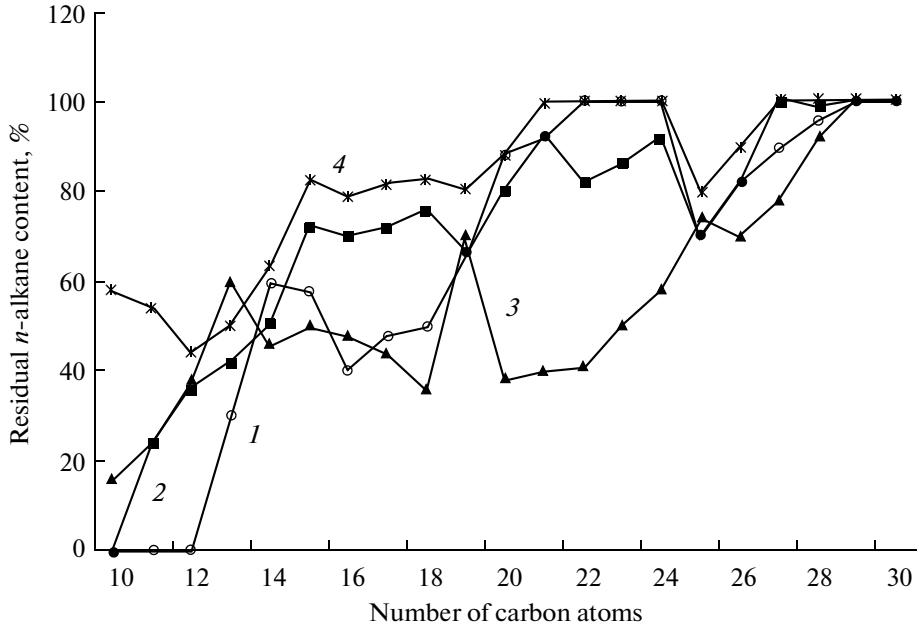

Fig. 2. Utilization of individual *n*-alkanes of petroleum by the isolated microorganisms (cultivation for 7 days): *Mycobacterium fortuitum* HL-Be (*1*), *Gordonia terrae* DB-Ae (*2*), *Acinetobacter calcoaceticus* HL-17 (*3*), and *Rhodococcus equi* HL-8 (*4*).

Rhodococcus equi HL-17, and *Bacillus subtilis* DB-2) and some gram-negative bacteria (*Acinetobacter calcoaceticus* HL-8 and *Chromobacterium violaceum* HL-20) were the most active petroleum degraders. Chromatographic analysis of biodegraded and control petroleum samples revealed preferred utilization of C_{10}–C_{19} *n*-alkanes and the possibility for the degradation of long-chain hydrocarbons up to C_{28}, as well as the differences in the degree and rate of hydrocarbon utilization by specific bacterial strains (Figs. 2 and 3).

DISCUSSION

Size of Microbial Communities and Numbers of Bacterial Populations in Tropical Soils

The results of our quantitative determination of microorganisms were not exactly predictable, since unusually high numbers of hydrocarbon-degrading bacteria (up to 9.5×10^6 CFU/g soil) were sound in the soils under study (Table 2). Hydrocarbon-oxidizing microorganisms have been previously revealed both in the sites contaminated by petroleum hydrocarbons and in clean environments [2, 16]. Expected number of hydrocarbon-oxidizing bacteria per 1 g of clean soil did not exceed 10^2–10^3 cells, while in petroleum-contaminated soils their number increased to 10^6–10^7 cells/g soil, especially in the case of prolonged or repeated contamination [17]. The tropical soils investigated in the present work should be, however, classified as "conditionally clean", since they had never encountered xenobiotics of petroleum nature.

We suggest that this phenomenon could be explained by both the biological characteristics of hydrocarbon-oxidizing bacteria (broad substrate spectrum, ability to utilize low concentrations of organic matter, and high ecological flexibility) and the presence of a biogenic pool of hydrocarbons of biogenic (plant waxes and essential oils) and pyrogenic (mostly fires) origin in slightly disturbed Vietnamese soils. In particular, under conditions of humid climate (Table 1), emission of terpenes [18], widespread hydrocarbons of plant origin susceptible to microbial decomposition [16, 19], was shown to increase significantly. These factors may promote selection of hydrocarbon-oxidizing bacteria and maintain their abundance in the investigated soils at a high level.

High numbers of hydrocarbon-oxidizing bacteria in clean soil environments have been previously reported for Arctic tundra and cold alpine soils [2, 16]. The authors found no correlation between the level of hydrocarbon contamination and the number of soil microbial hydrocarbon degraders. In the present work, numerically significant populations of hydrocarbon-oxidizing bacteria were revealed in clean soils of Vietnam, belonging to the tropical climatic zone.

Adaptation of Bacteria from Tropical Soils to Hydrocarbon Contamination at the Community Level

Rapid adaptation of the autochthonous bacteriocenose of tropical soil to petroleum hydrocarbons (laboratory experiment with petroleum) is an indirect indication of the high catabolic potential and ecologi-

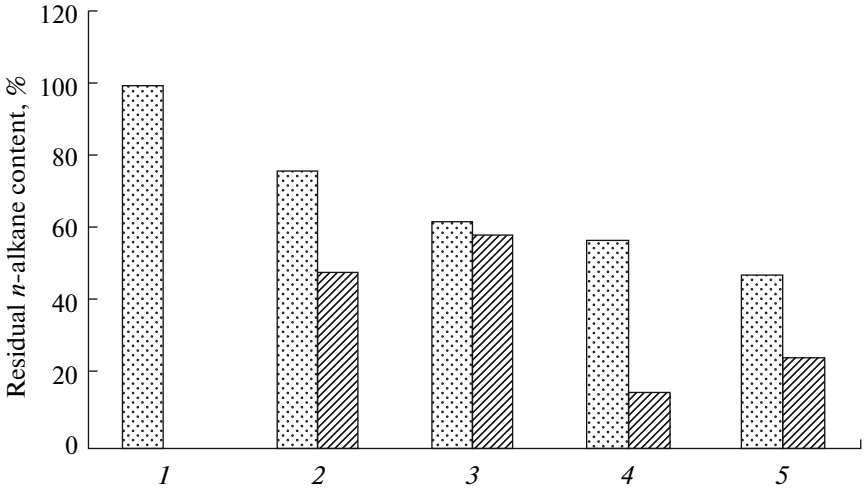

Fig. 3. Residual content of *n*-alkanes in the control petroleum (*1*) and in the petroleum degraded by *Rhodococcus equi* HL-8 (5 and 11 days of cultivation) (*2*), *Mycobacterium fortuitum* HL-Be (5 and 11 days of cultivation) (*3*), *Gordonia terrae* DB-Ae (7 and 31 days of cultivation) (*4*), and *Acinetobacter calcoaceticus* HL-17 (7 and 31 days of cultivation) (*5*).

cal flexibility of soil microbiota. This was evident from a significant increase in the numbers of both nonspecific organotrophic and hydrocarbon-oxidizing bacteria, as well as from widening of the degrading capacity of the community in the course of the experiment (Table 4, Fig. 1).

The original structure of a soil microbial community is a function of geographical location, soil properties, and environmental conditions. In spite of significant differences between the bacterial populations in the soils of the Hoa Lak and Dom Bay test stations, microbial communities reacted to the introduction of petroleum in a similar way: less diverse microbial communities with more versatile and flexible catabolism were selected, with the population enriched with few specific groups of degrading bacteria, primarily, the *Actinobacteria*. These results are in agreement with the earlier data [20] on the greater effect of hydrocarbons, rather than of the geographical origin of the sample, on the functional and species diversity of bacterial communities.

Among the strains isolated from the hydrocarbon-oxidizing communities of both soils after prolonged enrichment with petroleum, gram-positive microorganisms prevailed (actinobacteria of the genera *Gordonia, Mycobacterium*, and *Rhodococcus* and bacteria of the genus *Bacillus*). Some members of gram-negative genera *Acinetobacter* and *Chromobacterium* were also isolated. Predominance of gram-positive bacteria may result from production of antibiotics inhibiting growth of other bacteria, their mire rigid cell wall, and/or capacity for endospore formation, which facilitates their survival in highly unstable, sometimes unfavorable conditions of terrestrial tropical environments, which are prone to significant changes in temperature, humidity, and the nutrient status.

Diversity of Aerobic Organotrophic Bacteria from Tropical Soils and Their Capacity for Growth of Hydrocarbon Substrates

The narrow range of nutrient media, cultivation conditions, and methodic approaches used in the present work is certainly insufficient for complete characterization of the bacterial population under study. However, taxonomically diverse communities of aerobic organotrophic bacteria possessing a high catabolic potential, including capacity for utilization of a broad spectrum of hydrocarbons of petroleum origin, were revealed in the tropical soils of Vietnam.

The cultured bacteria belonged to three large phyla: *Firmicutes, Actinobacteria*, and *Proteobacteria*, which are widespread in soils [21]. Importantly, closely related microorganisms were retrieved from soil of geographically remote regions, e.g., strains HL-6 and DB-1 (*Brevibacillus agri*).

Although typical tropical soils were the sources of bacteria, the cosmopolite species predominated in the cultured bacterial community, which have been revealed worldwide in clean and hydrocarbon-contaminated objects, including soils of the humid tropical, arid, cold Arctic, and moderate climates [2, 4, 22], marine environments [23], and the phyllosphere of higher plants [24]. *Chromobacterium violaceum*, a typical inhabitant of the soil and aquatic ecotopes of the tropical and subtropical zone, was an exception. Thus, these microorganisms are widespread and may survive under quite different environmental conditions.

Many of the isolated bacteria belonged to the genera well-known for their capacity for xenobiotic degradation (*Acinetobacter, Bacillus, Brevibacillus, Gordonia, Microbacterium, Mycobacterium*, and *Rhodococcus*) [2, 7, 25]. Our results are in agreement with the

data of other recent investigations, which showed an important role of the *Actinobacteria* in hydrocarbon degradation in the tropics [4, 7, 8]. The potential of other taxa isolated in the present work (*Chromobacterium* and *Cupriavidus*) is less studied. According to [25], such microorganisms are also involved in hydrocarbon degradation under tropical conditions.

The results of the present work improve our knowledge of the hydrocarbon-oxidizing bacteria from the tropical soils of Vietnam. The high natural background (number and diversity) of bacteria capable of growth on hydrocarbon substrates and the presence of strains with significant hydrocarbon-oxidizing activity indicate the high self-purification potential of tropical soil which have not encountered petroleum contamination.

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