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EXPERIMENTAL ARTICLES

Detection and Transcription of *n*-Alkane Biodegradation Genes (*alk*B) in the Genome of a Hydrocarbon-Oxidizing Bacterium *Geobacillus subterraneus* K

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Abstract—The diversity and localization of *alkB* genes in the genome of the hydrocarbon-oxidizing bacterium *Geobacillus subterraneus* K, as well as the transcription of *alkB* genes, were studied as functions of culture growth phase and the hydrocarbon substrates utilized. Analysis of 96 clones containing inserted *alkB* genes revealed six *alkB* homologs in the strain under study: *alkB-geo1, alkB-geo2, alkB-geo3, alkB-geo4, alkB-geo5*, and *alkB-geo6*. In addition, real-time PCR of the total DNA of strain K revealed one more homolog, *alkB-geo7*. Chromosomal localization of *alkB* genes was demonstrated in strain K. During exponential growth on *n*-alkanes of various chain length (*n*-$C_{16}H_{34}$ and *n*-$C_{22}H_{46}$), formation of mRNA of highly homologous *alkB-geo5* and *alkB-geo6* genes was observed; in the beginning of the stationary phase, *alkB-geo4* mRNA was formed. The functional role and conditions of induction of the enzymes encoded by the rest of the homologs detected in strain K, that is, *alkB-geo1, alkB-geo2,* and *alkB-geo7*, remain unknown, which calls for further investigations of *alkB* genes in thermophilic bacteria under various growth conditions and with the use of various oil hydrocarbons as substrates.

Keywords: alkB genes, homologs, RNA, cDNA, mRNA, *Geobacillus*, thermophilic bacteria, oil biodegradation.

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Along with the genes providing for the vitally important functions, prokaryotic genomes contain considerable amount of genes associated with the ability to adapt to the changing environment (genes of antibiotic resistance, biodegradation, pathogenicity, etc). Adaptational genes are not compulsory for the population representatives; they are often located on plasmids, and this promotes their variability and horizontal transfer between the population members. Therefore, implementing adaptation genes as phylogenetic markers is limited, if not impossible. However, their phylogenetic analysis may be useful for understanding of the evolution of prokaryotes and may be of practical importance.

This group includes genes that provide for the ability to degrade hydrocarbons, *n*-alkanes in particular. This ability is widespread among bacteria, but, despite the great ecological and practical importance of the *n*-alkane biodegradation process, its characteristics, as well as the phylogeny and diversity of hydrocarbon-oxidizing bacteria in natural thermophilic communities, remain poorly studied.

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The process of *n*-alkane oxidation and organization of *alk*-operon were studied in mesophilic gram-negative bacteria (the genera *Pseudomonas, Acinetobacter, Stenotrophomonas, Alcanivorax*, and *Burkholderia*) and gram-positive bacteria (the genera *Rhodococcus, Mycobacterium, Nocardia*, and *Praserella*) [1–4]. A wide variety of *n*-alkane biodegradation genes was revealed even within single strains [5]. Alkane-hydroxylase of *Pseudomonas putida* consists of three enzymes: alkane monooxygenase, rubredoxin, and rubredoxin reductase [6, 7]. The key enzyme is the alkane monooxygenase encoded by the *alkB* gene. Location of *alk*-operon may be either chromosomal or plasmid. For example, the *alk*-operon of *P. putida* GPo1 is located on the plasmid OCT and that of strain *P. putida* P1 on the chromosome. In both cases these genes are flanked by IS elements [6, 7].

Data published on genes responsible for hydrocarbon biodegradation by thermophilic bacteria are scarce. *Geobacillus thermoleovorans* 70 and *G. thermoglucosidasius* TR2 each were found to contain a single sequence of *alkB* gene [8, 9]. The *alkB* gene of strain 70 had 96% homology with *alkB* genes of *Rhodococcus erythropolis* [10]. The *alkB* gene expression in *G. ther-*

moleovorans 70 was demonstrated both during the course of cultivation on *n*-hexadecane-containing medium and in soil samples [9]. Nucleotide sequences of the complete genomes of *Geobacillus kaustophilus* HTA426 and *Geobacillus thermodenitrificans* NG80-2 have been determined [10, 11]. In *G. thermodenitrificans* the *ladA* gene was revealed, encoding a protein that may cleave long-chain alkanes. The gene is contained in plasmid pLW1071 and is 1323 bp long [12]. Meanwhile, no *alkB* genes were detected in these genomes.

The genes *alkB* were also studied in 11 strains of the genus *Geobacillus* (*G. uzenensis* U^T and X, *G. subterraneus* 34^T, *G. gargensis* Ga^T, *G. jurassicus* $DS1^T$, *G. toebii* B1024, *G. thermoglucosidasius* 3Feng, *G. stearothermophilus* DSM 22^T, *G. thermoleovorans* DSM 5366^T and vw3-ln, and *G thermocatenulatus* VKM $B\text{-}1259^T$) [13]. Analysis of 226 clones containing *alk*B insertion (18–25 clones for each of 11 strains) revealed eight different homologs, *alk*B-*geo1*–*alk*B-*geo8* (59.7–96.7% homology). Six *alk*B homologs of geobacilli were closely related to the *alk*B*4*, *alk*B*3*, and *alk*B*2* genes found earlier in *R. erythropolis* NRRL B-16531 and Q15 [14]. Two variants of *alk*B homolog sequences of geobacilli turned out to be unique. The most widely occurring were the *alk*B-*geo1* and *alk*B-*geo4* genes: they were revealed in the genomes of all studied geobacilli strains. The least frequent homologs were revealed in the genomes of only two strains (*alk*B-*geo7*) or even of a single strain (*alk*B-*geo8*). The number of *alk*B homologs in a single geobacillus strain reached six. It was supposed that all homologs could be found in each of the strains upon studying a larger number of clones.

Chinese researchers [15] revealed three *alkB* gene homologs (*alkB-geo1, alkB-geo4*, and *alkB-geo6*) in the genome of *Geobacillus stearothermophilus* MH-1. Analysis of nucleotide and translated amino acid sequences of the *alkB-geo6* cluster was performed. Five open reading frames were detected, coding for AlkB2, two rubredoxins, and two regulatory proteins close to those of *Rhodococcus* strains NRRL B-16531 and Q15.

The aim of the present work was detailed analysis of the diversity and localization of the *alkB* genes present in the genome of the hydrocarbon-oxidizing bacterium *Geobacillus subterraneus* K, as well as a study of their transcription as a function of the culture growth phase and of the hydrocarbon substrates used.

MATERIALS AND METHODS

Medium composition and cultivation conditions. In this work, the bacterium *Geobacillus subterraneus* K (VKM B-2225) isolated from the Uzen' oil field (Kazakhstan) was studied [16]. To isolate total DNA, the strain was grown in rich LB medium (10.0 g/l Bacto tryptone, 5.0 g/l yeast extract, 5.0 g/l NaCl) for 12 h at 60°C or in mineral salt medium (MSM) with oil (0.4 vol %) as a substrate [17].

In order to isolate RNA, cells were grown with a single carbon and energy source, which was 2 g/l glucose or sodium acetate or one of the hydrocarbons *n*-$C_{16}H_{34}$ or *n*-$C_{22}H_{46}$ (0.4 vol %).

Strain K growth curves on hydrocarbons under batch cultivation conditions were plotted using the values of optical density at 600 nm (OD_{600}). The culture was grown overnight on MSM with glucose as a single source of carbon and energy [17]. The cells were harvested by centrifugation and washed once with fresh medium without substrates. The washed cells were resuspended in 1 ml of MSM and introduced into flasks containing 20 ml MSM supplemented with 0.4 vol % *n*-hexadecane ($C_{16}H_{34}$) or *n*-docosane ($C_{22}H_{46}$); OD_{600} after inoculation was 0.04. Then cells were grown at 60°C to the OD_{600} of 0.5 (two culture passages on hydrocarbon-containing medium). To measure the optical density of the culture in a medium supplemented with a hydrocarbon, the biomass was separated by centrifugation and resuspended in an equal volume of sterile MSM. Growth on oil-containing medium was monitored by chromatographic analysis of the content of *n*-alkanes (% of that in uninoculated control) in the degraded oil as described earlier [18].

DNA and RNA isolation. Total DNA preparations were obtained by means of a technique described earlier [19]. To isolate total RNA, a 1.5-ml aliquot of an exponential or early stationary phase culture was taken and cooled on ice; then biomass was harvested by centrifugation at 5000 *g* for 5 min at 4°C. The supernatant was discarded, and cells were immediately resuspended in 100 μl of TE buffer (10 mM *Tris*, 1 mM EDTA) with lysozyme (300 μg). Cell suspension was allowed to stand at room temperature for 5 min, cells were sedimented by centrifugation (5000 *g*, 4°C, 5 min), and the lysozyme buffer was removed. Then the cells were lysed and RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. The isolated RNA was dissolved in 20 μl of mQ water. Plasmid DNA was isolated from the strain K biomass grown in 100 ml of the medium to OD_{600} 0.5–0.6 using a QIAGEN Plasmid Midi Kit according to the protocol of low-copy plasmid or cosmid isolation. The molecular weight of plasmid DNA was determined after electrophoresis in 0.7% agarose gel (35 V, 6 h) by comparison with the DNA preparations of known molecular weights.

DNA amplification. To amplify *alk*B gene fragments, degenerate oligonucleotide primers were used according to the previously established protocol [13]: the forward Alk-BFB primer (5'-GGT ACG GSC AYT TCT ACR TCG A-3') and the reverse Alk-BRB primer (5'-CGG RTT CGC GTG RTG RT-3').

Cloning and sequencing of PCR products. Amplified *alk*B gene fragments approximately 500 nucle-

Primers designed to target various *alk*B genes of geobacilli

*alk*B homolog	Primer name	Sequence, 5'–3'	Product length, bp
*alk*B-*geo1*	ST1F	TCGCGGTACAAGGAAAACTT	173
	ST1R	AGACTGCCGAACAGAACCAC	
*alk*B-*geo2*	ST2F	GCTATTGCCGGTTTCACATT	157
	ST2R	AGAGGAAAAGGTTGCTGACG	
*alk*B-*geo3*	ST3F	CAGATATTGCCCTGGCTGTT	204
	ST3R	GCTGTGTCGTTGCAATTGAT	
*alk*B-*geo4*	ST4F	GCAGATACTGCCCTGGCTAC	214
	ST4R	GTGGTGGTCACTGTGTCGTT	
*alk*B-*geo5*	ST5/6F	CTGCATTCGTGGTTGATGTC	221
	ST5R	GTGCAGATGTGATCGCTGTT	
*alk*B-*geo6*	ST6R	CAGGAAGATGTTGGTGCAGA	243
*alk*B-*geo7*	ST7F	ATTTGCTGGAGGTGGTGAAC	154
	ST7R	CGCTGCAGGTGATACAAGAA	
*alk*B-*geo8*	ST8F	ATCCTCCCGTACCTGTTCCT	194
	ST8R	CGCTGCAGGTGATACAGAAA	

otides long were cloned in the plasmid vector pTZ57RT (Fermentas, Lithuania). Clones containing DNA inserts of the expected size (~500 bp) were sequenced using plasmid primers M13D and M13R on a 3730 DNA Analyzer using BigDye^R Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, United States).

The obtained sequences were analyzed using the NCBI BLAST software (www.ncbi.nlm.nih.gov/blast/).

Primer design. Primers specific to the eight *alk*B homologs revealed earlier [13] were designed using the BioEdit v. 5.06 software, Lasergene, and Oligo v. 6 (see table). The specificity of amplification of different *alk*B homologs using the designed primers was verified by PCR using serial dilutions of plasmids with the inserts of *alk*B gene fragments.

Reverse transcription. The obtained RNA preparations were purified from residual DNA with DNase (Sileks) used in the amount of 0.5 activity units per reaction. Procedures of cDNA isolation were described previously [19]. The obtained cDNA was immediately used as a PCR template. The successful reverse transcription reaction and cDNA synthesis on total RNA was verified by amplification of *16S rRNA* genes using universal primers [13]. The presence and quality of cDNA of mRNA were evaluated by amplification of gene fragments of topoisomerase II, gyrase (*gyr*B), and topoisomerase IV (*par*E) using primers specific to these genes of geobacilli [20]. For amplification of the *alk*B genes, we used only cDNA preparations that allowed amplification of *gyr*B and *par*E genes, which testified that a sufficient amount of cDNA had been synthesized.

Real-time PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction was performed in 25 μl of a reaction mixture containing 1x PCR buffer, 0.2 mM dNTP, ROX and SYBR Green as dyes (ZAO Sintol, Russia) and 0.3 U of *Taq* polymerase. Chromosomal and plasmid DNA preparations were diluted to the required number of copies according to the manufacturer's recommendations and used as templates in PCR. Amplification was performed in the following mode: DNA denaturation (95°C, 10 min) and then 40 cycles of denaturation (95°C, 15 s) and primer annealing and extension (63°C, 1 min). After the amplification, the product dissociation curve was constructed. The length of the amplified *alk*B fragments was approximately 200 bp.

The total PCR product fluorescence intensity was analyzed as a function of the amplicon melting temperature. The basal level of the threshold number of cycles was determined by the negative control, which was PCR in the absence of a template. A positive signal was determined by the specific melting temperature and fluorescence intensity of the product upon each amplification cycle. A parabolic dependence of fluorescence intensity on the number of cycles above the threshold level evidenced the specific PCR product synthesis.

The nucleotide sequences of *alk*B-*geo2*, *alk*B-*geo3*, *alk*B-*geo5*, and *alk*B-*geo6* genes of *G. subterraneus* K revealed by cloning were deposited in the GenBank under accession numbers JF508466–JF508469.

RESULTS AND DISCUSSION

***G. subterraneus* K growth on oil and individual *n*-alkanes.** Representatives of *Geobacillus subterraneus* widely occur in natural habitats, including high-temperature oil fields in various geographical regions. Studies of the processes of oil biodegradation and of

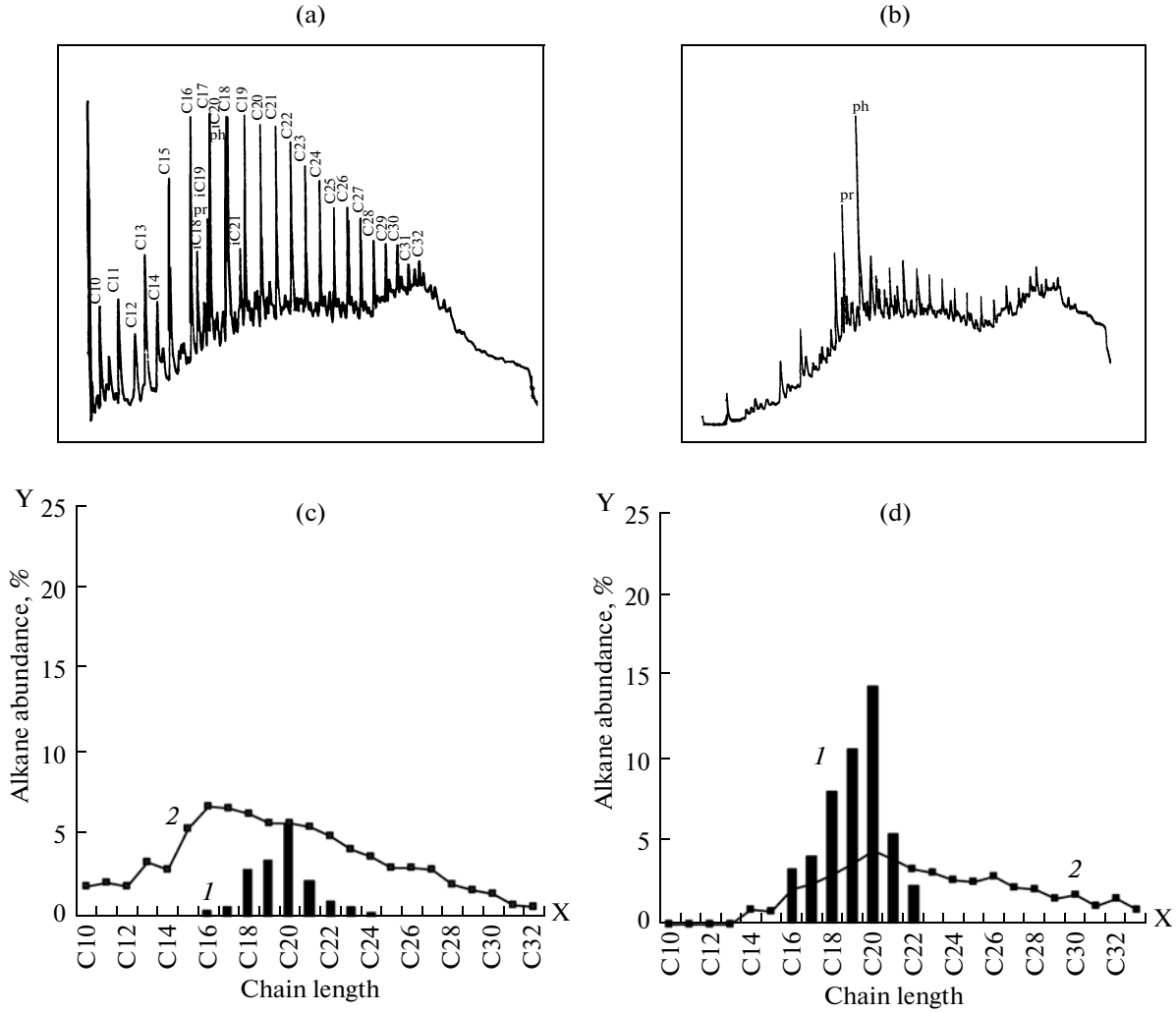

Fig. 1. Results of chromatography of the saturated hydrocarbons of oil in sterile control (a) and in a *G. subterraneus* K culture (b) and *n*-alkane degradation profiles in control (c) and after *G. subterraneus* K incubation over 14 days at 60°C (d). The alkane content, %, is plotted against the ordinate axis, and the number of carbon atoms is plotted against the abscissa axis. Designations: *1*, branched alkanes; *2*, straight alkanes.

the distribution and expression of *alk*B genes in a large number of various strains are of considerable scientific interest. Strain K isolated from the Uzen' field (Kazakhstan) [16] has not previously been implemented in *alk*B gene search. In the present work, strain K growth on Devon oil from the Romashkinskoe oil field was studied under batch culture conditions. Oil hydrocarbon consumption was monitored by changes in the chromatographic profile of saturated hydrocarbons in degraded oil (Figs. 1b and 1d) in comparison with *n*-alkane profile in sterile medium (Figs. 1a and 1c). As follows from these figures, the strain grew on oil-consuming components of the saturated hydrocarbon fraction, including normal and branched alkanes and discarding pristan (i-$C_{19}H_{40}$) and phytane (i-$C_{20}H_{42}$) (Figs. 1b and 1d). The strain K growth curves presented in Fig. 2 evidence that exponential growth phase started earlier and larger biomass was produced by the stationary phase in the medium containing *n*-hexadecane (n-$C_{16}H_{34}$) than in the medium containing *n*-docosane (n-$C_{22}H_{46}$). During the second culture passage on medium with *n*-hexadecane or *n*-docosane, the biomass was sampled to study transcription of *alk*B homologs (Fig. 2).

Diversity of *alkB* genes in *G. subterraneus* strain K. Total DNA of strain K grown on oil-containing medium was used to amplify *alk*B gene fragments with degenerate primers targeting the most conserved region of the geobacillus *alk*B gene. 96 clones containing 500-bp-long inserts were obtained. The clones were analyzed using specific primers designed to target the eight *alk*B homologs revealed earlier in bacteria of the genus *Geobacillus* (*alk*B-*geo1*–*alk*B-*geo8*) [13]. Since *alk*B-*geo1* and *alk*B-*geo4* homologs were previously detected in all of the 11 studied geobacillus strains [13], primary analysis of clones by real-time

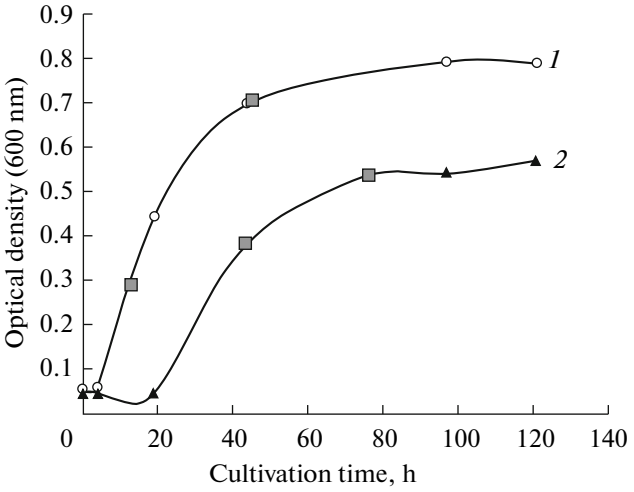

Fig. 2. Growth curves of *G. subterraneus* K in media with *n*-hexadecane (*1*) or *n*-docosane (*2*) at 60°C. Squares mark the moments of sampling.

PCR was performed using primers to *alk*B-*geo1* and *alk*B-*geo4*. Other clones were analyzed by DNA sequencing using plasmid primers. The obtained nucleotide sequences were compared to the known sequences of *alk*B-*geo* homologs, as well as with *alk*B genes of various bacteria deposited in GenBank. Six homologs of *alk*B gene were revealed in *G. subterraneus* strain K—namely, *alk*B-*geo1* (7 clones), *alk*B-*geo2* (27 clones), *alk*B-*geo3* (15 clones), *alk*B-*geo4* (40 clones), *alk*B-*geo5* (4 clones), and *alk*B-*geo6* (3 clones).

We also performed real-time PCR on the total DNA of strain K using specific primers targeting the eight homologs of *alk*B gene. This allowed us to reveal one more homolog, *alk*B-*geo7*, in addition to the six homologs mentioned above. Earlier, *alk*B-*geo7* was detected in only two geobacillus strains, *G. uzenensis* U^T and *G. toebii* vw3-1n [20], which was formerly referred to *G. thermoleovorans* [13]. The *alk*B-*geo7* and *alk*B-*geo8* homologs are the rarest in geobacilli and considerably differ from other homologs in the gene primary structure [13]. The *alk*B-*geo8* homolog could not be revealed in strain K by any of the methods used.

Thus, increasing the number of analyzed clones containing the insert of *alk*B gene fragments and application of specific primers allowed us to detect seven of the eight gene homologs known for geobacilli [13]. Apparently, analysis of a sufficient number of clones may reveal more homologous genes in other geobacillus strains as well. Lack of species specificity of *alk*B genes in geobacilli precludes their use as molecular markers in studies of natural diversity of geobacilli. At the same time, when used in complex molecular biology investigations, the *alk*B genes may provide additional information on functioning of geobacilli in natural thermophilic communities.

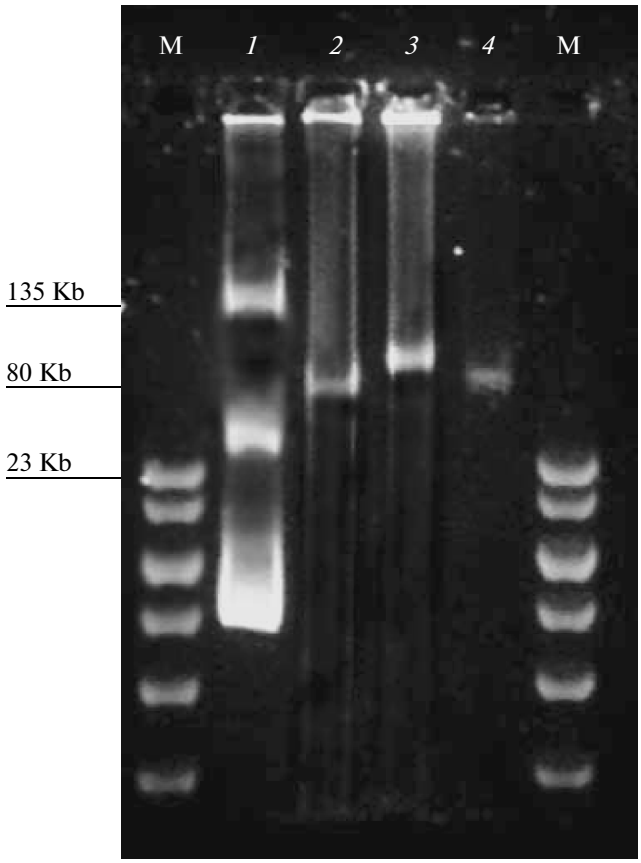

Fig. 3. DNA electrophoresis. *1*, bacmid DNA bMON 14272 preparation (136 Kb, Invitrogen); *2*, plasmid fraction of DNA preparation from *Pseudomonas* sp. Bi12n (~80 Kb); *3*, plasmid fraction of DNA preparation from *G. subterraneus* K; *4*, chromosomal fraction of DNA preparation from *G. subterraneus* K; M, DNA molecular weight marker (Lambda *Hind*III, 23 Kb, Invitrogen).

Localization of *alk*B genes in the genome of *G. subterraneus* K. The close affinity of most *alk*B genes of geobacilli to those of rhodococci revealed previously allowed us to assume involvement of these genes in the processes of horizontal transfer [13]. It is known that the most commonly involved in the transfer processes are adaptation genes located on plasmids. To determine the localization of *alk*B genes in the genome of *G. subterraneus* K, we performed separate isolation of chromosomal and plasmid DNA from the biomass. The plasmid found in strain K had a size of approximately 100 kb (Fig. 3).

To verify the separation efficiency of chromosomal and plasmid DNA, amplification was performed with the *gyr*B-specific primers known to be present in the genome in a single copy and to be localized on the chromosome [20]. In this experiment PCR product of *gyr*B gene was synthesized efficiently only with the chromosomal DNA preparation as the template (Fig. 4). A weak band in lane 4 indicate trace admixture of chromosomal DNA in the plasmid prepara-

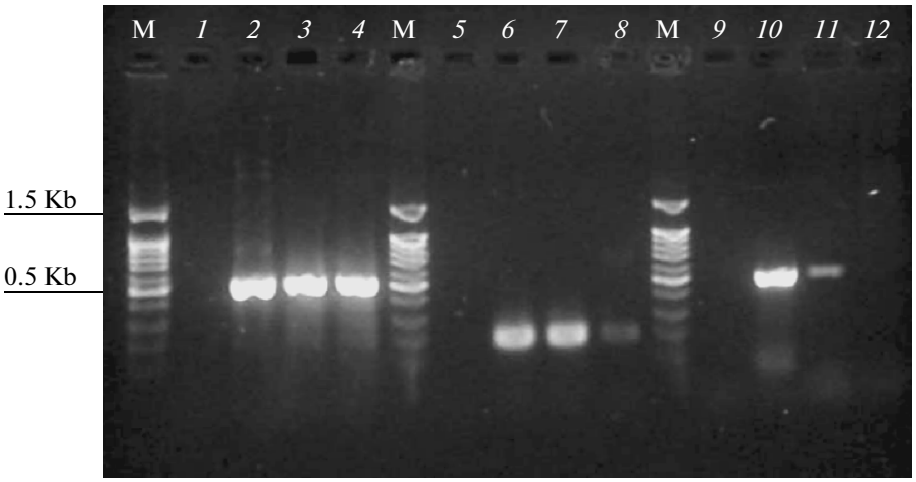

Fig. 4. Amplification of the 16S rRNA (*1–4*), *gyr*B (*5–8*), and *alk*B (*9–12*) genes using genome DNA (*3*, *7, 11*) and plasmid DNA (*4*, *8*, and *12*) of *G. subterraneus* K as the template. Plasmid DNA of clones containing DNA insert of the relevant genes were used as positive controls (*2*, *6*). M, DNA molecular weight marker (100-bp DNA ladder, Fermentas). *1, 5, 9*, negative controls without template.

tion. Amplification of *alk*B gene fragment using the degenerate primers (AlkBFB and Alk-BRB) also proceeded only on the chromosomal DNA template.

Using real-time PCR, the formation of *alk*B gene was studied as a function of the amount of template DNA (50–500 ng per reaction). In the case of plasmid DNA, no specific PCR product was observed in the range of concentrations studied, while in the case of chromosomal DNA *alk*B gene amplification started already at 50 ng of DNA per reaction.

These results evidence chromosomal localization of *alk*B genes in the genome of *G. subterraneus* K.

Transcription of *alk*B gene homologs in *G. subterraneus* K cells. The presence of several *alk*B gene copies in the genome of a single organism is not a peculiar characteristic of geobacilli. Two genes, *alk*B1 and *alk*B2, were detected in the gram-negative bacteria *Pseudomonas aeruginosa* PAO1 and PR1 [21], *Acinetobacter* sp. M1 [22], and *Alcanivorax borkumensis* SK2 [23]. Four homologs were detected in the genomes of gram-positive bacteria *R. erythropolis* NRRL B-16531 and *Rhodococcus* sp. Q15 [14]. Apparently, multiple copying of alkane hydroxylase is a mechanism of adaptation of hydrocarbon-utilizing bacteria to the environmental conditions. Two adaptation mechanisms were demonstrated experimentally: (1) two homologous genes are expressed in different phases of culture growth, as in *P. aeruginosa* [21], providing adaptation for the changing growth conditions (pH, temperature, aeration, etc.), and (2) expression of different *alk*B gene homologs occurs as a function of the chain length of the *n*-alkane substrate, as in *Acinetobacter* sp. M1 and *A. borkumensis* SK2 [21, 22].

To determine the functional role of the gene homologs in strain K, experimental evaluation of gene transcription (*alk*B gene mRNA synthesis) during the exponential and stationary phases of growth at 60°C was performed with employment of *n*-alkanes of differing chain length (*n*-$C_{16}H_{34}$ and *n*-$C_{22}H_{46}$). Culture growth curves and time points of sample collection are presented in Fig. 2. Cultures grown on glucose and acetate under the same conditions were used as negative controls.

The number of *alk*B gene copies in the cDNA preparation obtained with degenerate primers was very low (Ct 35–37), which did not allow us to analyze transcription of the homologs using cDNA directly. Thus, to obtain enough copies of *alk*B genes and, thus, to reliably detect mRNA of the *alk*B homologs, preliminary amplification using degenerate primers targeting *alk*B gene cDNA was performed to amplify the number of copies of cDNA of all *alk*B homologs present in the genome of strain K. Upon amplification, the PCR product was diluted 100 times and used as a template for PCR employing primers specific to each of the eight homologs. In order to verify that the reamplification signal is produced by cDNA and not by residual DNA, a control was set reamplifying the PCR product obtained on the RNA template; the dilution of the template was the same in the experiment and in the control.

The reaction was expected to proceed only on cDNA preparations derived from cultures grown on *n*-hexadecane and *n*-docosane. Indeed, the culture grown on glucose produced a negative result. However, an unexpected positive result was observed with the culture grown on acetate. To determine which of the gene homologs are transcribed on this substrate, a second amplification round was performed with primers specific to each of the *alk*B gene homologs. The positive signal in PCR performed on the cDNA template was shown to be generated by cDNA

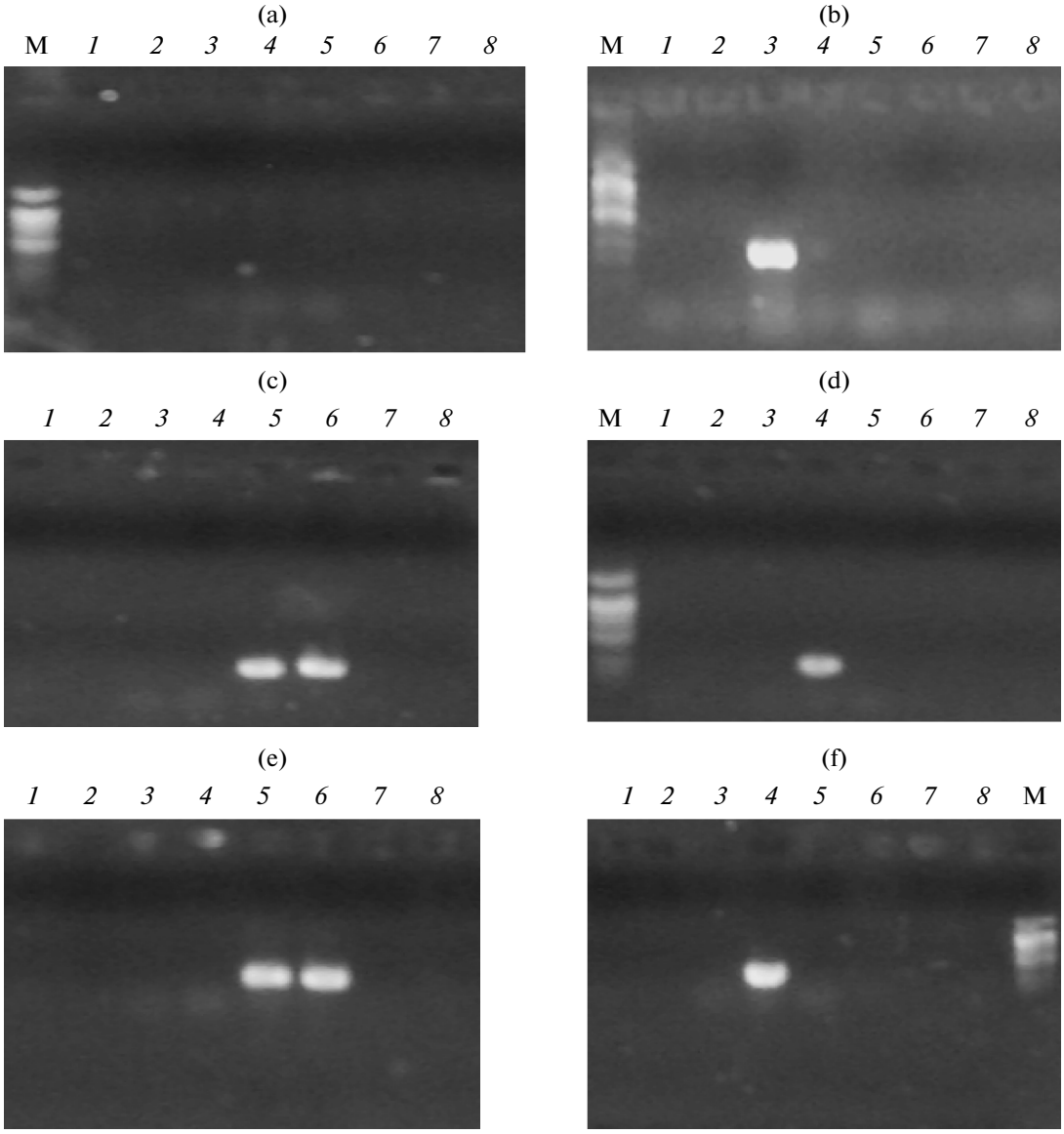

Fig. 5. Reamplification of *alk*B genes with primers specific to cDNA obtained form *G. subterraneus* K culture in the exponential phase of growth on media with glucose (a), acetate (b), *n*-hexadecane (c), or *n*-docosane (d) and in the stationary phase of growth on media containing *n*-hexadecane (e) or *n*-docosane (f). M, DNA molecular weight marker; *1–8*, PCR with specific primers targeting *alk*B-*geo1*, *alk*B-*geo2*, *alk*B-*geo3*, *alk*B-*geo4*, *alk*B-*geo5*, *alk*B-*geo6*, *alk*B-*geo7*, *alk*B-*geo8* respectively.

of *alk*B-*geo3* homolog (Fig. 5b). Further experiments are needed to explain the induction of transcription of an alkane monooxygenase gene homolog during growth on acetate.

In the exponential-phase culture (OD_{600} of 0.28) grown on *n*-hexadecane, the presence of *alk*B-*geo5* and *alk*B-*geo6* homologs in cDNA was detected by PCR (Fig. 5c) and real-time PCR (Fig. 6a) reamplification with the use of specific primers. According to the Ct values (22.41 and 23.35, respectively), the numbers of mRNAs of the two homologs were practically equal. In the exponential-phase culture (OD_{600} of 0.38) grown on *n*-docosane, transcription of *alk*B-*geo5* and *alk*B-*geo6* (Figs. 5e and 6c) was also found to occur in practically equal amounts (Ct 18.25 and 19.27, respectively).

Thus, data on mRNAs of *alk*B homologs synthesized in the exponential phase of growth on *n*-alkanes with different chain lengths (C_{16} and C_{22}) coincide. In both cases synthesis of *alk*B-*geo5* and *alk*B-*geo6* mRNA was observed, while mRNAs of other homologs were not detected. Notably, sequences of *alk*B-*geo5* and *alk*B-*geo6* are highly homologous to each other and to the *alk*B2 gene of *R. erythropolis* NRRL B-16531 (up to 99% identity at the nucleotide and up to 100% at the amino-acid level) [13]. Our studies provide evidence that the nearly identical

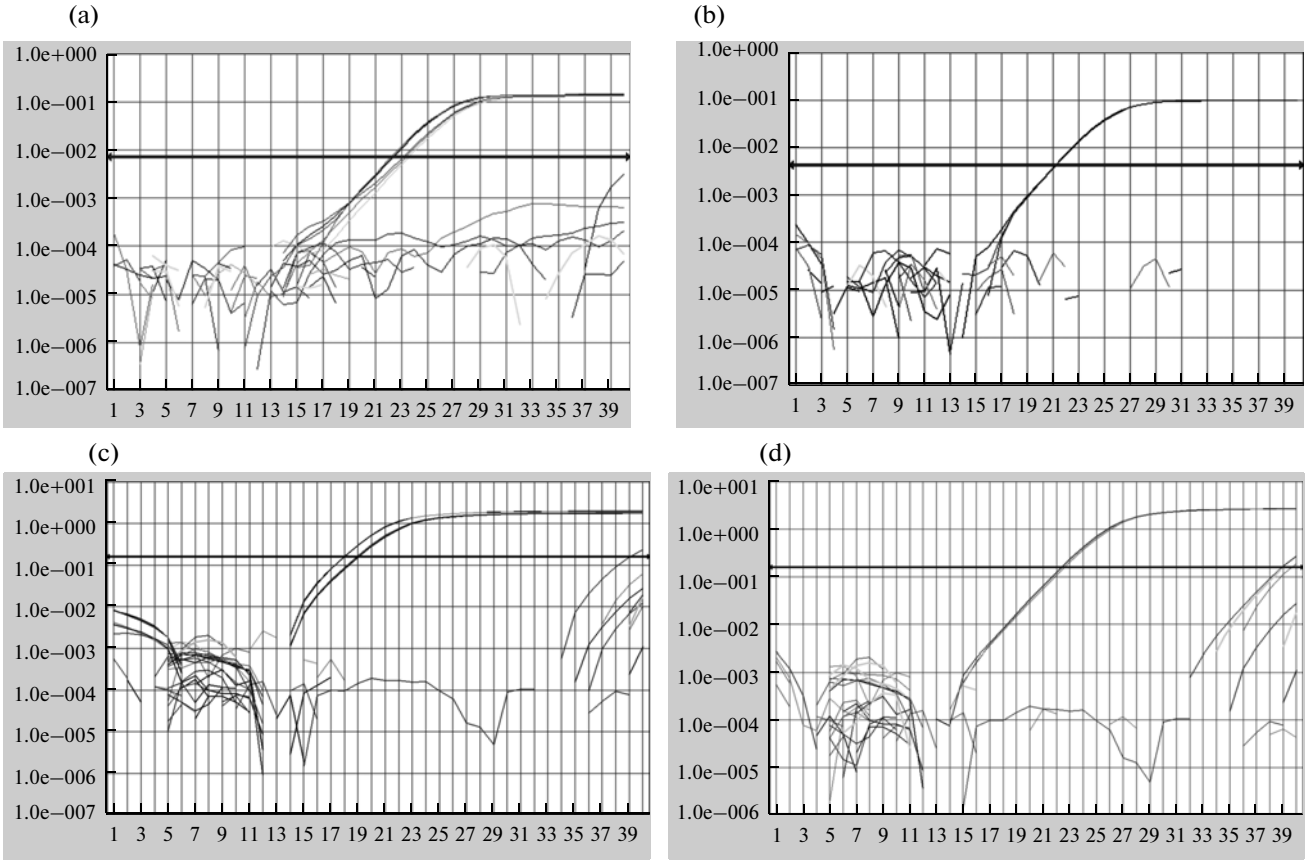

Fig. 6. Reamplification by real-time PCR of the *alk*B genes with primers specific to cDNA obtained from *G. subterraneus* K culture in the exponential phase of growth on media with *n*-hexadecane (a) or *n*-docosane (c) and in the stationary phase of growth on media with *n*-hexadecane (b) and *n*-docosane (d).

structures of these homologs result in the same physiological function.

Further study of mRNA synthesis at the beginning of the stationary phase of growth of strain K on the *n*-alkanes C_{16} and C_{22} (culture OD_{600} of 0.71 and 0.54, respectively) also revealed matching results: cells grown on both substrates in the beginning of the stationary phase yielded mRNA of a single homolog, *alk*B-*geo4* (Figs. 5d, 5f, 6b, and 6d).

Thus, the chain length of the studied *n*-alkane substrates *n*-$C_{16}H_{34}$ and *n*-$C_{22}H_{46}$ did not influence the differential transcription of *alk*B gene homologs in *G. subterraneus* K. Selective transcription of various *alk*B gene homologs depended on the culture growth conditions. In the exponential growth phase, that is, under optimal growth conditions, *alk*B-*geo5* and *alk*B-*geo6* homologs are transcribed, while in the stationary phase, under conditions unfavorable for growth, *alk*B-*geo4* is transcribed. It should be noted that, similarly to the *alk*B-*geo5* and *alk*B-*geo6*, *alk*B-*geo4* pair, *alk*B-*geo3* forms a pair of close homologs with *alk*B-*geo4* [13]. However, the level of their homology is lower, 89% at nucleotide and 92% at amino acid level, which favored reliable selective amplification with specific primers. Despite the similarity of the primary structures, the functions of these homologous genes turned out to be different. Notably, sequences close to that of the *alkB-geo4* homolog were found among the analogous genes of the database, including *alkB3* gene of *R. erythropolis* NRRL B-16531, while the *alkB-geo3* homolog has been detected only in geobacilli [13], its role needing further investigations.

It was found [9] that the growth of *G. thermoleovorans* T70 on *n*-hexadecane over 48 h at 55°C proceeded with formation of mRNA of the *alk*B gene. According to our data, such growth conditions correspond to the early stationary phase of the growth of geobacilli on hexadecane. In the absence of hexadecane, no mRNA of *alk*B gene was formed. The analysis of *alk*B gene homologs in 12 geobacillus strains performed by Tourova et al. [13] allows the *alk*B gene of *G. thermoleovorans* T79 [9] to be referred to *alk*B-*geo4* homologs. Thus, under conditions of early stationary phase of growth on hexadecane, *alk*B-*geo4* homologs are transcribed in *G. thermoleovorans* T79 and *G. subterraneus* K. The *alk*B gene revealed in the genome of *G. thermoglucosidasius* TR2 is close to the *alk*B-*geo6* homolog of geobacilli [8, 13].

The results obtained in the present work allow us to assume functional roles of three of the eight known *alk*B gene homologs of geobacilli. The conditions of induction of enzymes determined by the rare homologs *alk*B-*geo7* and *alk*B-*geo8*, as well as by the *alk*B-*geo1* and *alk*B-*geo2* homologs common for all geobacilli, remain unknown. Supposedly, these variants of the enzyme are induced by other hydrocarbon components of oil or under other growth conditions; therefore, further studies of structural organization and functioning of *alk*B genes in thermophilic bacteria are required.

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