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EXPERIMENTAL ARTICLES

Diversity of the *alkB* Genes of *n*-Alkane Biodegradation in Thermophilic Hydrocarbon-Oxidizing Bacteria of the Genera *Geobacillus*, *Parageobacillus*, and *Aeribacillus*

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Abstract—Analysis of complete genomes of thermophilic bacteria of the genus *Geobacillus*, oxidizing *n*-alkanes and crude oil revealed the *ladA* gene and its homologues. In the genomes of some strains, the *ladA* gene was not detected, although they were capable of growth on *n*-alkanes. Cloning with degenerate primers has previously revealed eight homologues of the *alkB* gene (*alkB-geo1*–*alkB-geo8*) encoding alkane hydroxylases in *Geobacillus*. In the present work, investigation of the *alkB* genes of several new strains of thermophilic, hydrocarbon-oxidizing bacilli was carried out. In the clone libraries obtained using degenerate primers for the *alkB* genes from five strains of the genera *Geobacillus*, *Parageobacillus*, and *Aeribacillus,* mostly the universal homologous genes *alkB-geo1* and *alkB-geo4* were revealed. Additional PCR amplification with specific primers for each of the eight known *alkB* homologues revealed the universal homologous genes only in some of the studied strains. A correlation was found between the set of the *alkB-geo3*–*alkB-geo6* homologus genes from *Geobacillus subterraneus* strain K amplified with specific primers and the previously revealed a set of these homologous genes transcripted at different cultivation conditions. This correlation may be due to accumulation of the copies of individual homologues under different cultivation conditions, which results in higher sensitivity of specific primers. The least common homologue, *alkB-geo7*, which was not detected in the relevant clone libraries, was found in two strains, indicating the possibility of development of more specific primers for amplification of this homologue in order to reveal hydrocarbon-oxidizing bacteria of related genera *Geobacillus*–*Parageobacillus* in molecular ecological research.

Keywords: alkB genes, homologues, thermophilic bacteria, *Geobacillus*, *Parageobacillus*, *Aeribacillus*

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The currently available information concerning the genes involved in *n*-alkane biodegradation is mainly related to mesophilic microorganisms. Most commonly, *n*-alkane degradation in mesophilic bacteria is mediated by alkane hydroxylase, a three-component enzyme system whose key element is alkane monooxygenase (AlkB) (van Beilen et al., 2003; Nie et al., 2014). Thermophilic bacteria of the genus *Geobacillus* and of the recently described closely related genera *Parageobacillus* and *Aeribacillus* are common inhabitants of oil fields; they can utilize crude oil, individual *n*-alkanes, and aromatic hydrocarbons (Nazina et al., 2005; Marchant and Banat, 2010). However, the genes responsible for biodegradation of *n*-alkanes in these bacteria remain poorly studied.

The first thermophilic bacteria found to possess the *alkB* sequences were *G. thermoleovorans* 70 and *G. thermoglucosidasius* TR2 (Sharkey et al., 2004; Marchant et al., 2006). The *alkB* genes encode alkane-1 monooxygenase, the key enzyme of the three-component alkane hydroxylase system. In subsequent studies, partial *alkB* sequences were determined for 11 strains of geobacilli (Tourova et al., 2008; Korshunova et al., 2011). For the first time, eight *alkB* variants were identified in geobacilli: *alkB-geo1*, *alkB-geo2*, *alkB-geo3*, *alkB-geo4*, *alkB-geo5*, *alkB-geo6*, *alkB-geo7*, and *alkB-geo8* (Tourova et al., 2008). Different strains of geobacilli possessed three to seven alkane monooxygenase gene homologues (*alkB*), and only two of these were universally present in all strains (Tourova et al., 2008; Korshunova et al., 2011). A high level of similarity was observed between certain *alkB* homologs found in *Geobacillus* and *Rhodococcus* strains (Whyte et al., 2002; Marchant et al., 2006; Tourova et al., 2008; Liu et al., 2009). Primers specific to these eight *alkB* homologues were designed to investigate *alkB* expression at different stages of cul-

ture growth on different hydrocarbon substrates (Korshunova et al., 2011). A study of mRNAs levels of *alkB* homologues in exponential- and stationary-phase *G. subterraneus* strain K growing on $C_{16}H_{34}$ and $C_{22}H_{46}$ hydrocarbons showed that the central role in the degradation of medium- and long-chain *n*-alkanes in this strain belonged to *alkB-geo4*, *alkB-geo5*, and *alkB-geo6* homologues. In particular, *alkB-geo5* and *alkB-geo6* homologues were expressed in the cells of a culture growing exponentially under optimal conditions, while *alkB-geo4* was expressed in the stationary phase under conditions of limited substrate availability (Korshunova et al., 2011).

Little information is available on the organization of the *alkB* gene clusters in thermophilic bacilli. As a rule, *alkB* gene studies are based on amplification of their sequence fragments. The complete sequence and structure of an *alk* operon has so far been obtained only for *alkB-geo6* homologue in *Geobacillus* sp. MH-1 (Liu et al., 2009). Moreover, location of the *alkB* genes in the genome of thermophilic geobacilli still remains uncertain. The results of experiments on amplification of chromosomal and plasmid DNA of the hydrocarbon-oxidizing strain *G. subterraneus* K seemed to suggest that *alkB* genes were located in the chromosomal component of its genome (Korshunova et al., 2011). However, by present, none of the completely sequenced chromosomes of different *Geobacillus* strains was found to contain the *alkB* genes.

At the same time, genomes of hydrocarbon-oxidizing geobacilli were found to possess a different gene involved in biodegradation of *n*-alkanes: *ladA* and its homologues (Feng et al., 2007; Boonmak et al., 2014). For the first time, some of the strains analyzed were shown to possess both *alkB* and *ladA* genes (Tourova et al., 2016).

Recently, we isolated several new strains of spore-forming thermophilic hydrocarbon-oxidizing bacteria: *Geobacillus* sp. 1017 and *Aeribacillus pallidus* 8m3 (Tourova et al., 2010; Shestakova et al., 2011). In our previous study, we described the phylogenetic position of these strains, the presence of *alkB* genes in their genomes, as well as their ability to oxidize *n*-alkanes of crude oil (Tourova et al., 2016).

The goal of the present work was to investigate the composition of *alkB* homologues in the genomes of the newly identified strains of the genera *Geobacillus, Parageobacillus,* and *Aeribacillus*, as well as in the type strain *Geobacillus icigianus* G1w1^T (Bryanskaya et al., 2014, 2015).

MATERIALS AND METHODS

Bacterial strains. The study was performed using the strains of spore-forming thermophilic hydrocarbon-oxidizing bacteria *Geobacillus* sp. 1017 (=VKM B-3132) and *Aeribacillus pallidus* 8m3, which were isolated previously from formation water of the high-temperature Kongdian bed of the Dagang oilfield (Hebei province, China) (Tourova et al., 2010; Shestakova et al., 2011), as well as *Geobacillus icigianus* G1w1^T (=VKM B-2853^T=DSM 28325^T) isolated from a hot spring in Kamchatka (courteously provided by the workers of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences; Bryanskaya et al., 2014, 2015). Alkane-1 monooxygenase gene screening was also performed in the previously studied strains *G. subterraneus* K and *Parageobacillus* (previously, *Geobacillus*) *toebii* B-1024 (Tourova et al., 2010).

Growth media and culture conditions. Thermophilic bacteria were cultured in the medium of the following composition (g/L): NH_4Cl, 1.0; K_2HPO_4, 1.5; KH_2PO_4, 0.75; $CaCl_2 \cdot 2H_2O$, 0.02; $MgSO_4 \cdot 7H_2O$, 0.2; KCl, 0.1, NaCl, 2.0; yeast extract, 1.0; mixture of C_{10}–C_{20} *n*-alkanes, 10 mL/L, pH 7.0–7.2. Bacteria were grown for 7–10 days at 60°C as static cultures.

DNA isolation. DNA was isolated from bacterial biomass using the DIAtom^{TM}DNA Prep100 reagent kit (Biokom, Russia) according to the manufacturer's protocol with minor modifications. Purified DNA was dissolved in 50 μL of deionized water (MQ) and used as a PCR template.

Amplification of *alkB* gene sequences. PCR primers and conditions used for amplification of *alkB* alkane monooxygenase genes are listed in Table 1. Amplification was performed in two rounds. In the first round, PCR was performed in 10 μL reaction mixture using 5–25 ng DNA as a template. The product obtained in the first round was used as a template in the second PCR round. The reaction protocol for *alkB* amplification was as follows: initial denaturation, 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; final elongation, 8 min at 72°C.

The PCR products were purified from the primers by precipitation under mild conditions. After ammonium acetate and ethanol were added to the final concentration of 1.125 M and 65%, respectively, the sample was incubated for 20 min at room temperature and centrifuged for 20 min at 13000 *g*. The pellet was washed with 70% ethanol. The purified PCR product was dissolved in 10 μL MQ.

The PCR products were analyzed by electrophoresis in agarose gel (1 or 1.5%, depending on the expected product size) prestained with ethidium bromide. The products were purified by precipitating DNA with ethanol solution containing 0.75 M ammonium acetate at room temperature.

Cloning and sequencing of PCR products. Amplified fragments of bacterial *alkB* genes (~500 bp long) were purified and cloned using the TA cloning vector kit (Promega, United States) as recommended by the manufacturer. The clones carrying an insert within the

Table 1. Primers used for amplification of alkane-1 monooxygenase genes

Gene	Primer name	Primer sequence, 5'–3'	Product size, bp	Reference
*alk*B	Alk-BFB Alk-BRB	GGTACGGSCAYTTCTACRTCGA CGGRTTCGCGTGRTGRT	459	Tourova et al., 2008
*alk*B-*geo1*	ST1F ST1R	TCGCGGTACAAGGAAAACTT AGACTGCCGAACAGAACCAC	173	Korshunova et al., 2011
*alk*B-*geo2*	ST2F ST2R	GCTATTGCCGGTTTCACATT AGAGGAAAAGGTTGCTGACG	157	
*alk*B-*geo3*	ST3F ST3R	CAGATATTGCCCTGGCTGTT GCTGTGTCGTTGCAATTGAT	204	
*alk*B-*geo4*	ST4F ST4R	GCAGATACTGCCCTGGCTAC GTGGTGGTCACTGTGTCGTT	214	
*alk*B-*geo5*	ST5/6F ST5R	CTGCATTCGTGGTTGATGTC GTGCAGATGTGATCGCTGTT	221	
*alk*B-*geo6*	ST6R	CAGGAAGATGTTGGTGCAGA	243	
*alk*B-*geo7*	ST7F ST7R	ATTTGCTGGAGGTGGTGAAC CGCTGCAGGTGATACAAGAA	154	
*alk*B-*geo8*	ST8F ST8R	ATCCTCCCGTACCTGTTCCT CGCTGCAGGTGATACAGAAA	194	

pGEM-T plasmid (pGEM-T easy vector kit, Promega) were detected using the standard plasmid-specific primers M13F or M13R. The incorporated fragments were sequenced using the specific Alk-BFB and Alk-BRB primers.

The obtained *alkB* gene sequences of were deposited in the GenBank under accession numbers MG720135–MG720142.

Sequencing of *alkB* fragments amplified by PCR was performed on an automated 3730 DNA Analyzer unit using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, United States) as recommended by the manufacturer.

Preliminary analysis of the obtained sequences was performed by searching the NCBI GenBank database of reference species' sequences using the BLAST software. The sequences were edited and analyzed using the BioEdit software (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html).

RESULTS

Detection of *alkB* genes by amplification with degenerate oligonucleotide primers. In our previous work, using degenerate primers to the most conserved *alkB* region (Alk-BFB–Alk-BRB), we detected ~500-bp-long fragments corresponding to the target product in *Geobacillus* sp. 1017, *Aeribacillus pallidus* 8m3, and *G. icigianus* G1w1^T, which showed that the genomes of these strains contained the *alkB* genes (Tourova et al., 2016).

To determine the repertoire of *alkB* homologues in the newly found strains, PCR with degenerate *alkB* primers was used to create clone libraries of 31–47 clones per strain carrying the corresponding gene fragment inserts (altogether, 120 clones). BLAST-mediated comparison of the fragment sequences revealed their 75–100% similarity to the corresponding *alkB* fragments of different *Geobacillus* and *Rhodococcus* strains represented in the GenBank database; therefore, all cloned fragments belonged indeed to the same gene family.

Table 2 presents the results of comparative analysis of the *alkB* sequence fragments determined in the new strains and the corresponding sequences of *alkB-geo1–alkB-geo8* homologues described in geobacilli previously (Tourova et al., 2008).

Altogether, the newly studied strains possessed three of the eight *alkB* homologues: *alkB-geo1*, *alkB-geo4,* and *alkB-geo6* in different combinations. Among them, *alkB-geo1* and *alkB-geo4* were universally present in all these genomes, as it was also the case in the *Geobacillus* strains studied previously (Tourova et al., 2008). The lowest diversity of *alkB* genes was observed in the genome of strain 8m3, which included only the two universal homologues.

Detection of *alkB* genes by PCR with the primers specific to different *alkB* homologues described in geobaccili. In our initial study of *G. subterraneus* K, we obtained an *alkB* clone library using PCR with degenerate primers and identified the homologous genes *alkB-geo1*, *alkB-geo2*, *alkB-geo4*, and *alkB-geo6* (Tourova et al., 2008). After expanding the library from 22

Table 2. Diversity of *alkB*-genes identified in thermophilic hydrocarbon-oxidizing bacilli of the genera *Geobacillus*, *Parageobacillus*, and *Aeribacillus* by analysis of clone libraries obtained using degenerate primers

alkB homologue	Closest cultured relative determined by BLAST analysis	Nucleotide sequence identity,* %	Amino acid sequence identity,** %	Total number of clones	Number of clones obtained for individual strains				
					Geobacillus sp. 1017	*G. subterraneus* K	*G. icigianus* G1w1^T	*P. toebii* B-1024	*Aeribacillus pallidus* 8m3
alkB-geo1	*Rhodococcus erythropolis* NRRL B-16531 alkane-1 monooxygenase (*alkB4*)	99.2	100	92	44	12	10	1	25
alkB-geo2	*R. erythropolis* NRRL B-16531 alkane-1 monooxygenase (*alkB4*)	90.0	98.6	37		30		7	
alkB-geo3	*Nocardia* sp. H17-1 alkane-1 monooxygenase (*alkB3*)	87.7	89.7	17		15		2	
alkB-geo4	*R. erythropolis* NRRL B-16531 alkane-1 monooxygenase (*alkB3*)	96.7	96.6	87	1	53	25	2	6
alkB-geo5	*R. erythropolis* 50-V alkane-1 monooxygenase (*alkB2*)	95.4	100	9		3		6	
alkB-geo6	*R. erythropolis* NRRL B-16531 alkane-1 monooxygenase (*alkB2*)	99.0	100	12	2	4	6		
alkB-geo7	Not found								
alkB-geo8	Not found								
Total number of clones				254	47	117	41	18	31

* Between the most closely related *alkB* gene from GenBank and *alkB* gene fragments of the bacilli studied.
** Between translated sequences of the most closely related *alkB* gene from GenBank and *alkB* gene fragments of the bacilli studied.

Table 3. Diversity of the *alkB* genes identified in thermophilic hydrocarbon-oxidizing bacilli of the genera *Geobacillus*, *Parageobacillus*, and *Aeribacillus* using specific primers

Genus, species, strain	*alkB* gene homologues							
	alkB-geo1	*alkB-geo2*	*alkB-geo3*	*alkB-geo4*	*alkB-geo5*	*alkB-geo6*	*alkB-geo7*	*alkB-geo8*
Geobacillus sp. 1017	+	+	+	+	+	+		
G. subterraneus K			+	+	+	+		
G. icigianus G1w1^T		+					+	
P. toebii B-1024		+	+		+	+	+	
Aeribacillus pallidus 8m3				+				

to 94 clones, we were able to identify two more *alkB* gene homologues in the strain K genome: *alkB-geo3* and *alkB-geo5* (Korshunova et al., 2011). However, the least frequently occurring *alkB-geo7* homologue could be detected only by means of specially designed primers specific to each of the eight *alkB* homologues described in geobacilli. Taking this into account, we performed an additional screening of the new strains' genomes by amplifying individual *alkB* genes with primers specific to each of the *alkB* homologues.

The results of PCR with the primers specific to each of the eight *alkB* homologues were only in a partial agreement with the data obtained by clone library screening. In particular, only four of the seven previously identified gene homologues were detected in *G. subterraneus* K: *alkB-geo3* to *alkB-geo6*; moreover, this assortment of *alkB* homologues agreed with the repertoire of mRNAs produced at different stages of culture growth on hydrocarbons (Korshunova et al., 2011). Similarly to strain K, the universal *alkB-geo1* homologue was not detected in most strains studied, except for strain 1017. Another universal homologue, *alkB-geo4* could not be detected in B-1024 and G1w1^T. At the same time, PCR with specific primers showed that these strains possessed the rare *alkB-geo7* homologue, which could not be detected by clone library screening. Thus, five of the 14 currently studied strains of hydrocarbon-oxidizing geobacilli were found to possess this rare genus-specific *alkB* homologue.

DISCUSSION

Most *Geobacillus* strains (including the 14 strains investigated by our group) have been shown to be able to degrade *n*-alkanes (Tourova et al., 2016). Numerous PCR-based experiments showed that their genomes contained the *alkB* genes that encode alkane-1 monooxygenase, the key element of the alkane hydroxylase complex (Sharkey et al., 2004; Marchant et al., 2006; Tourova et al., 2008, 2016; Korshunova et al., 2011). Moreover, by means of self-formed adaptor PCR, a gene cluster that included a complete *alkB-geo6* homologue, two rubredoxin genes, and a putative transcription-regulating protein were identified in the genome of *Geobacillus* sp. MH-1 (Liu et al., 2009). However, none of the completely sequenced bacterial genomes of members of the genera *Geobacillus, Parageobacillus*, and *Aeribacillus* was found to contain these genes. This is also true for the strains G1w1^T, 8m3, K, and 1017 analyzed in the present work, for which genome sequence data are also available (Braynskaya et al., 2014; Poltaraus et al., 2016a, 2016b; Kadnikov et al., 2017).

The results of PCR-based experiments suggest that *Geobacillus* genomes contain multiple copies of *alkB* genes. However, in the absence of data on the structure of complete gene sequences, it is currently impossible to design universal primers for specific amplification of all gene copies present in geobacilli. The degenerate primers that are currently used for these purposes were developed based on the analysis of the *alkB* genes of other hydrocarbon-oxidizing bacteria (mainly rhodococci and pseudomonads) and therefore may have different specificity to individual gene variants. This may probably explain the quantitative proportions observed in *alkB* clone libraries derived from different *Geobacillus* strains. In particular, *alkB* homologues with the highest representation in the clone libraries developed based on pure cultures of *Geobacillus* strains were *alkB-geo1* to *alkB-geo6*, whose sequences exhibit most similarity to the corresponding genes of rhodococci (Tourova et al., 2008). Noteworthy, the results of BLAST analysis of the GenBank database suggested that the *alkB* homologues most widely represented in uncultured hydrocarbon-oxidizing bacilli from different habitats were *alkB-geo5* and *alkB-geo6*.

The fact that hydrocarbon-utilizing bacteria possess multiple copies of alkane monooxygenase genes probably reflects their adaptation to environmental conditions. The functional role of these genes may be determined by the composition of available hydrocarbon substrates or by various biological and physicochemical parameters, including culture growth stage, as well as by a combination of these factors. It was shown previously that, although the clone library derived from *G. subterraneus* K contained *alkB-geo1* to *alkB-geo6* homologues, only *alkB-geo5* and *alkB-geo6* were expressed in a strain K culture exponentially

growing on hydrocarbons with different chain length, while *alkB-geo4* was expressed in the stationary phase (i.e., under unfavorable growth conditions). Moreover, *alkB-geo3* expression was observed in a culture growing on acetate, which has not been explained so far (Korshunova et al., 2011). The agreement between these observations and the results of PCR with homologue-specific primers obtained for strain K in the present work may be explained by accumulation of particular *alkB* homologue copies depending on the conditions of culture growth, which may enhance the sensitivity of the corresponding primers. The same phenomenon may underlie the discrepancy between the results obtained by clone library analysis using degenerate primers and by PCR with the primers specific to individual *alkB* homologues, especially since the functional role of the other *alkB* homologues has not been determined so far.

The results concerning the *alkB-geo7* homologue, which has been detected only in geobacilli, are worth special discussion. In our previous experiments with clone libraries, only two clones representing two different strains were found to carry an insert with an *alkB-geo7* fragment (Tourova et al., 2008). At the same time, the GenBank database contains two closely related (99% similarity) sequence fragments from uncultured bacteria which were revealed as a result of *alkB* gene cloning from the samples collected in oilfields of different geographical locations (AC JF405893 and AC JX171304, Verde et al., 2013). This fact confirms that detection of the rare *alkB-geo7* homologue is not an artifact. Although *alkB-geo7* could not be detected in the *alkB* clone library derived from strain K even after its expansion, it was successfully revealed using the specific primer pair (Korshunova et al., 2011). Similar results were obtained for strains G1w1^T and B-1024: in these strains, *alkB-geo7* also could be detected only using the specific primer. These results inspire hope that specific primers can be developed to use *alkB-geo7* amplification as a marker for detection of thermophilic hydrocarbon-oxidizing bacteria of the closely related genera *Geobacillus*–*Parageobacillus* in molecular ecological studies.

Although PCR with different types of primers was successfully employed to detect *alkB* genes in hydrocarbon-oxidizing bacilli, the fundamental issue concerning genomic localization of these genes remains unresolved. Taking into account that none of the *Geobacillus* chromosomes that have been completely sequenced so far was found to contain any *alkB* genes, it is logical to expect that they must be located in the plasmid component of the bacterial genomes. This notion also agrees with the earlier hypothesis implying that these genes were transferred to geobacilli from rhodococci (or some related microorganisms) (Tourova et al., 2008). Further research of the plasmid component of the genomes of different *Geobacillus* strains may help to resolve this issue.

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