Extremophiles (2014) 18:515–523
DOI 10.1007/s00792-014-0636-y

ORIGINAL PAPER

Cloning and expression of three *ladA*-type alkane monooxygenase genes from an extremely thermophilic alkane-degrading bacterium *Geobacillus thermoleovorans* B23

Chanita Boonmak · Yasunori Takahashi · Masaaki Morikawa

Received: 22 October 2013 / Accepted: 19 February 2014 / Published online: 30 March 2014

Abstract An extremely thermophilic bacterium, *Geobacillus thermoleovorans* B23, is capable of degrading a broad range of alkanes (with carbon chain lengths ranging between C11 and C32) at 70 °C. Whole-genome sequence analysis revealed that unlike most alkane-degrading bacteria, strain B23 does not possess an *alkB*-type alkane monooxygenase gene. Instead, it possesses a cluster of three *ladA*-type genes, $ladA\alpha_{B23}$, $ladA\beta_{B23}$, and $ladB_{B23}$, on its chromosome, whose protein products share significant amino acid sequence identities, 49.8, 34.4, and 22.7 %, respectively, with that of *ladA* alkane monooxygenase gene found on a plasmid of *Geobacillus thermodetrificans* NG 80-2. Each of the three genes, $ladA\alpha_{B23}$, $ladA\beta_{B23}$, and $ladB_{B23}$, was heterologously expressed individually in an *alkB1* deletion mutant strain, *Pseudomonas fluorescens* KOB2Δ1. It was found that all three genes were functional in *P. fluorescens* KOB2Δ1, and partially restored alkane degradation activity. In this study, we suggest that *G. thermoleovorans* B23 utilizes multiple LadA-type alkane monooxygenases for the degradation of a broad range of alkanes.

Keywords LadA alkane monooxygenase · *Geobacillus thermoleovorans* B23 · Alkane degradation · Genome

Abbreviations
C12 Dodecane
C16 Hexadecane

Communicated by L. Huang.

C. Boonmak · Y. Takahashi · M. Morikawa (✉)
Division of Biosphere Science, Graduate School of Environmental Science,
Hokkaido University, Sapporo 060-0810, Japan
e-mail: morikawa@ees.hokudai.ac.jp

Introduction

Petroleum hydrocarbons are toxic to animals and plants; however, more than a small number of microorganisms are capable of degrading and using them as carbon and energy sources (Rojo 2009; Das and Chandran 2011). The first step of alkane degradation by aerobic microorganisms is the hydroxylation reaction catalyzed by alkane monooxygenases. One of the best studied enzymes is an AlkB-type alkane monooxygenase, which is widely distributed in bacteria such as Gram-negative *Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Pseudomonas*, and *Stenotrophomonas* and Gram-positive *Mycobacterium*, *Nocardia*, *Prauserella*, *Rhodococcus*, and *Geobacillus* (van Beilen and Funhoff 2007; Takei et al. 2008; Rojo 2009). Tourova et al. (2008) reported that eight different *alkB* gene homologs, *alkB*-geo1 to *alkB*-geo8, are distributed among the members of the genus *Geobacillus*. These homologs occur in each strain in various combinations of four to six *alkB* gene homologs. Analysis of the codon usage in the various *alkB* homologs suggested that the pool of these genes is common to most Gram-positive and certain Gram-negative bacteria (Tourova et al. 2008).

An extremely thermophilic bacterium, *Geobacillus thermoleovorans* strain B23, was previously isolated from the production waters of a subterranean petroleum reservoir at a depth of 2,150 m and a temperature of 105 °C, in Niigata, Japan. Strain B23 degrades a broad range of alkanes (C11–32) at 70 °C by a terminal oxidation pathway, followed by a β-oxidation pathway (Kato et al. 2001a). To understand the metabolism of alkane degradation in this strain, alcohol dehydrogenase and aldehyde dehydrogenase have been previously characterized and demonstrated their high thermostability

and preference for long-chain substrates (Kato et al. 2001b, 2010). Moreover, an investigation of alkane-inducible proteins in this strain revealed that the synthesis of two unique membrane proteins (P16 and P21), together with catalase and superoxide dismutase, was significantly increased during alkane degradation. An acyl-CoA oxidase activity of the type found in eukaryotes was also suggested in strain B23 (Kato et al. 2009). However, the identity of the alkane monooxygenase, which catalyzes the initial step of the alkane degradation pathway, has remained unknown. To obtain further knowledge of the unique alkane degradation pathway in *G. thermoleovorans* B23, whole genome sequence analysis was conducted, and the candidate alkane monooxygenase genes were characterized, as detailed below.

Materials and methods

Strains, plasmids, and media

Geobacillus thermoleovorans B23, was grown in Luria broth (LB) medium for maintenance at 60 °C. *Escherichia coli* DH5α was grown in LB medium at 37 °C and used as the host strain for gene cloning using the cloning vector pUC19 (Yanisch-Perron et al. 1985). The transformants harboring pUC19 and its derivatives were grown in LB medium supplemented with 100 μg/ml ampicillin. *E. coli* MegaX DH10B^{TM} T1^{R} Electrocomp^{TM} cells [Δ(*mrr-hsd*RMS-*mcr*BC); Invitrogen, Carlsbad, CA)] were used for cloning the gene expression plasmids, pCom8:*ladA*α_{B23}, pCom8:*ladA*β_{B23}, and pCom8:*ladB*$_{B23}$. *Pseudomonas fluorescens* KOB2Δ1 is an *alkB1* deletion mutant of *P. fluorescens* CHA0, with dramatically reduced C12–C16 alkane degradation activity (Smits et al. 2001). This strain can thus be used for alkane degradation and growth complementation experiments using alkane monooxygenase genes from other bacteria (Smits et al. 2001). pCom8 is an *E. coli–Pseudomonas* shuttle expression vector based on pUCP25, with a *P. putida* GPo1 *alkB* promoter and a positive regulator, *alkS* (Smits et al. 2001). The transformants harboring pCom8 and its derivatives were grown on LB medium supplemented with the appropriate antibiotics (10 μg/ml gentamicin for *E. coli* and 100 μg/ml gentamicin for *P. fluorescens*; Smits et al. 2002). The recombinant *E. coli* strains and *P. fluorescens* KOB2Δ1 were grown in LB medium at 37 and 30 °C, respectively, unless otherwise noted.

Draft genome sequencing and assembly

Chromosomal DNA from *G. thermoleovorans* B23 was prepared using Marmur method (Marmur 1961). Paired-end libraries were generated from the chromosomal DNA, and sequencing was carried out using Roche 454 GS FLX (Basel, Switzerland) at Hokkaido System Science Co., Ltd. (Sapporo, Japan). The raw sequence data were assembled using GS De Novo Assembler v2.8. The sequence reads were also compared with *Geobacillus kaustophilus* HTA426 reference genome using the multiple genome alignment program Mauve (Darling et al. 2010). Genes were annotated by xBASE bacterial genome annotation service (http://www.xbase.ac.uk/annotation/). The draft genome sequence of *G. thermoleovorans* B23 is available in DDBJ/EMBL/GenBank under the accession numbers BATY01000001–BATY01000209 (Boonmak et al. 2013). The nucleotide sequence between ORF2 and ORF11 (11,986 bp), namely the "*ladAB* gene island," has also been deposited in DDBJ/EMBL/GenBank under the accession number AB727923. The nucleotide sequence for alkanesulfonate monooxygenase gene cluster *ssuCBAED* has been also deposited under AB898656.

Phylogenetic tree analysis

Phylogenetic trees were constructed by Kimura's two-parameter correction (Kimura 1980) using the neighbor-joining method (Saitou and Nei 1987) of *MEGA* version 5 (Tamura et al. 2011). Confidences for the phylogenetic tree were estimated by bootstrap analysis (100 replicates; Felsenstein 1985). Homology search against the protein database was performed using protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1997).

Cloning of *ladA* alkane monooxygenase gene homologs and construction of expression plasmids

ladAα_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ genes were amplified by PCR using KOD-Plus-Neo DNA polymerase (Toyobo, Kyoto, Japan) in a C1000 Touch^{TM} Thermal Cycler (Bio-Rad, Hercules, CA), according to the standard protocol from the supplier. The primers used for PCR amplification of these genes were designed based on the genome sequence of the strain B23, and the restriction sites for *Nde*I (CATATG) and *Sma*I (CCCGGG) were introduced in the primers to facilitate cloning. The primer sequences are as follows: αF (AATTCCATATGGATCAACCCCTATTATTC) and αR (TAGGGCCCGGGCTAGCCATCTGTTCGTGAGC) for *ladA*α_{B23}, βF (AATGGCATATGAGTGTGCGGCAAATG) and βR (GATTCCCGGGTCACACGCGGATCGACTT) for *ladA*β_{B23}, and BF (GGAAACATATGGTTGAATTTATTACGATGG) and BR (CGACCCGGGTTAGATCCATACTTCCGTTTGT) for *ladB*$_{B23}$. The sequences of the genes were confirmed by first cloning the PCR products obtained for the target genes *ladA*α_{B23},

ladAβ_{B23}, and *ladB*$_{B23}$, into the *Sma*I site of pUC19, followed by transformation of *E. coli* DH5α using the heat shock method (Inoue et al. 1990). The nucleotide sequence of the clones obtained was then determined using the BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA), and the sequences were confirmed to be correct. *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ were then separately subcloned as *Nde*I-*Sma*I fragments into the corresponding sites of pCom8 downstream of the *alkB* promoter and used for the transformation of *E. coli* MegaX DH10B^{TM} T1^{R} Electrocomp^{TM} cells according to the standard protocol. The resultant clones of pCom8:*ladA*α_{B23}, pCom8:*ladA*β_{B23}, or pCom8:*ladB*$_{B23}$ were then used for the transformation of *P. fluorescens* KOB2Δ1 (Højberg et al. 1999).

Heterologous gene expression of *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ in *P. fluorescens* KOB2Δ1

In vivo growth complementation test of *P. fluorescens* KOB2Δ1 recombinants containing only pCom8 (as negative control), pCom8:*ladA*α_{B23}, pCom8:*ladA*β_{B23}, and pCom8:*ladB*$_{B23}$ was performed in 5 ml of E2 minimal medium (Lageveen et al. 1988) supplemented with 1 % (v/v) dodecane (C12) or hexadecane (C16), 0.01 % Tween 80, and 2 % (v/v) pre-culture in E2 medium. The cultures were incubated under shaking conditions (120 rpm) at either 30 or 35 °C for 8 days. The experiments were performed in triplicate. Turbidimetry was not useful for measuring cell growth due to high background values because of the formation of oil–water emulsion in the cultures. The growth of *P. fluorescens* KOB2Δ1 recombinants was therefore determined by total protein measurement using the Pierce®BCA Protein Assay Kit (Thermo Scientific, Waltham, MA).

Alkane degradation test was performed in triplicate in 5 ml E2 minimal medium supplemented with 1 % (v/v) standard gas oil (Tokyo Kasei, Tokyo, Japan) and 0.01 % Tween 80 in 50 ml screw cap centrifuge tubes (Catalog no. 18422CTF50; IWAKI, Tokyo, Japan). The cultures were incubated under shaking conditions (120 rpm) at 35 °C for 8 days. The entire culture volume (5 ml) was extracted with 5 ml of acetone:hexane (1:1). After centrifugation at 30,000×*g* for 20 min, the amount of alkanes remaining in the solvent layer was directly analyzed by gas chromatography. A portion (1 µl, split ratio 50:1) of the sample was analyzed by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 µm × 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan). Helium was used as carrier gas at a flow rate of 25 ml/min.

Results and discussion

Whole genome sequence analysis of *G. thermoleovorans* B23

We had tried all the best to amplify *alkB*-type genes by PCR utilizing template genomic DNA from *G. thermoleovorans* B23 with several combinations of degenerate and single sequence primers for *alkB* in *Geobacillus* bacteria (Tourova et al. 2008). However, no *alkB* gene homolog was obtained. In order to identify the genes responsible for the initial oxidation of alkanes in *G. thermoleovorans* B23, whole genome sequence analysis was conducted. The genome sequencing generated 210,521 raw reads covering a total of 87,444,156 bp, achieving approximately >25× genome coverage. Q40 plus bases were 99.94 % reliable. The total G + C mol % was 52.3 %, which is the same in *G. thermoleovorans* CCB_US3_UF5 (NC_016593), and is equivalent to the value of 52.09 % in *G. kaustophilus* HTA426 (NC_006510). The raw sequence data was assembled into 209 contigs (total size 3,353,053 bp). An analysis of 16S rRNA and recN gene sequences of the strain B23 confirmed that this strain belonged to *G. thermoleovorans*, with the two genes showing 99.7 and 100 % identity, respectively, with *G. thermoleovorans* DSM 5366^{T}.

ladA alkane monooxygenase homologs in *G. thermoleovorans* B23 and related *Geobacillus* strains

G. kaustophilus HTA426 does not degrade alkanes and does not contain genes such as *alkB* that encode alkane monooxygenases. To identify candidate genes encoding alkane monooxygenases in the strain B23, we first subtracted the commonly shared orthologous gene regions from the genome sequence of strain B23 by utilizing *G. kaustophilus* HTA426 genome data. Surprisingly, the resulting DNA regions, which were unique to the strain B23, did not have any *alkB*-like alkane monooxygenases, unlike the strain *G. thermoleovorans* DSM 5366^{T} and almost all the other species in the genus *Geobacillus*. We then carefully analyzed the DNA regions unique to strain B23 by Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and found that it carries three possible *ladA*-type alkane monooxygenase genes, namely *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$, in the 11,834 bp region named the "*ladAB* gene island." The three genes encode proteins of 463 (LadAα_{B23} [BAM76377]), 437 (LadAβ_{B23} [BAM76372]), and 372 (LadB$_{B23}$ [BAM76371]) amino acids, respectively. The amino acid sequences of the three proteins showed significant similarity (49.8, 34.4, and 22.7 % identical, respectively; Fig. 1), with the FMN-dependent alkane monooxygenase, LadA [YP_001127577, 440 amino acids],

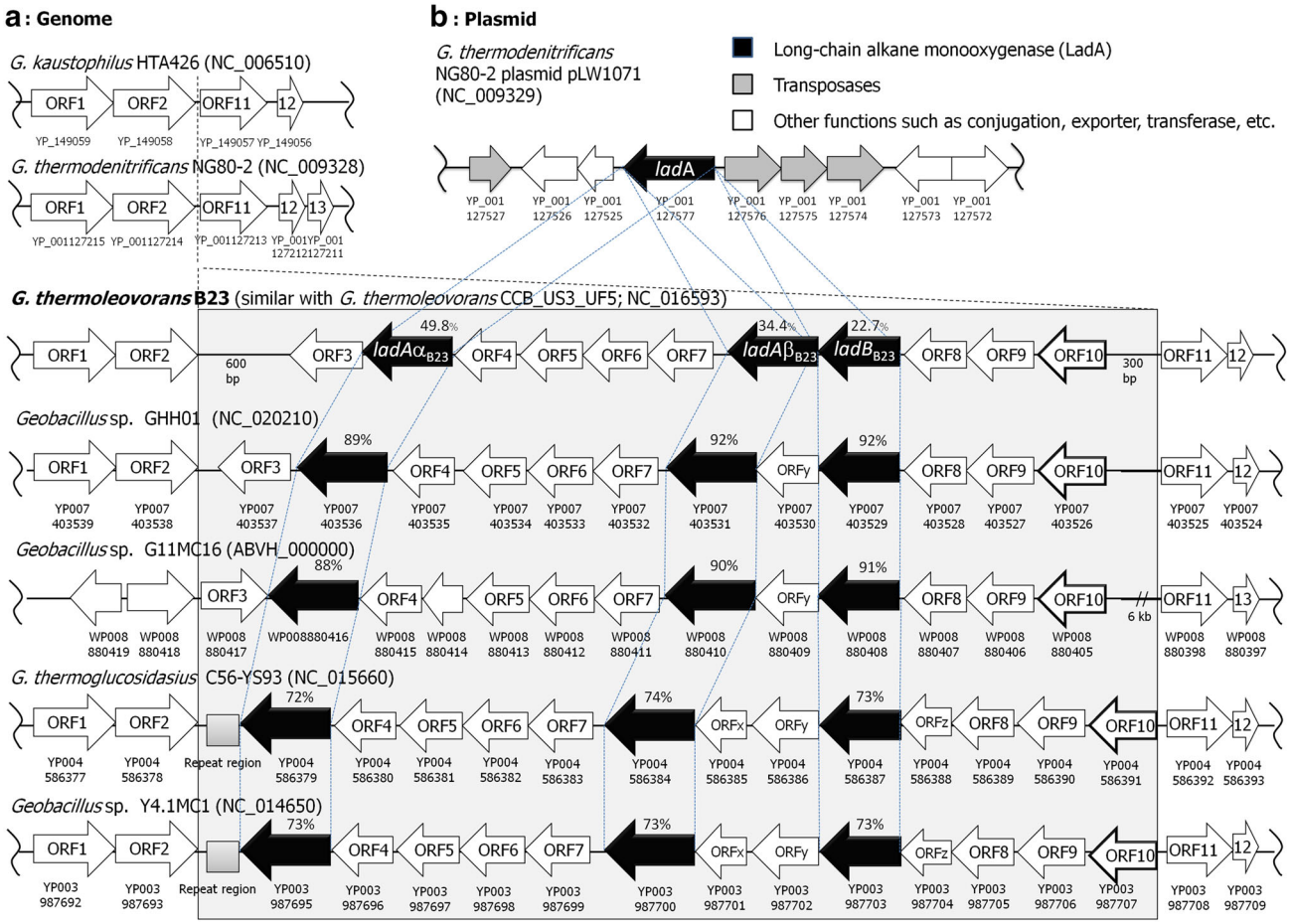

Fig. 1 **a** Alignment of the gene organization of *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ in the chromosome of *G. thermoleovorans* B23 with closely related species from GenBank; and **b** *ladA* on plasmid pLW1071. ORF1, 2, 5, 6, ABC transporter (permease); ORF3, Lysophospholipase-like protein; ORF4, NLPA lipoprotein; ORF7, Acyl-CoA dehydrogenase type 2 domain; ORF8, 12 hypothetical protein; ORF9, LysR family transcriptional regulator; ORF10, NADPH-dependent FMN reductase; ORF11, fructokinases; ORF13, TnpR resolvase; ORFx/ORFy/ORFz, hypothetical protein. The region of "*ladAB* gene island" is shown in *shaded box* in (**b**). Amino acid sequence similarity scores between the *ladA* products of *G. thermodenitrificans* and *G. thermoleovorans* B23 are shown on *arrows*. Other similarity scores on *arrows* are between the respective gene products of B23 and other species

whose gene was located in the plasmid pLW1071 of *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007; Li et al. 2008).

It is generally accepted that the proteins whose mutual sequence identity over 30 % belong to the same enzyme group. Above a cut-off roughly corresponding to 30 % sequence identity, 90 % of the pairs are homologous; below 25 < 10 % are homologous (Rost 1999). Two proteins share 50 % or higher sequence identity, their backbones differ by less than 1 Å RMS deviation. We thus propose the name LadAα_{B23} and LadAβ_{B23} for apparent LadA homologues. While LadB$_{B23}$ shares less identity (22.7 %) with LadA and even higher similarity with putative alkanesulfonate monooxygenases (SsuD) of *G. thermoleovorans* CCB_US3_UF5 [YP_004983996] and well-studied SsuD of *Escherichia coli* K-12 [P80645], 100 and 39.9 % respectively. SsuD is a member of bacterial luciferase family same as LadA-alkane monooxygenase based on structural properties. It differs from LadA in catalytic activity that involves in C–S bond cleavage, not the hydroxylation of a long-chain alkane (Ellis 2010). Highly conserved His12 in SsuD, which is suggested to stabilize negatively charged sulfo group of substrate alkanesulfonate binding, is replaced by neutral Ser11 in all LadB alkane monooxygenases. Interestingly, at another locus on chromosome of B23, there is *ssuCBAED* gene cluster [AB898656] similar to *ssuEADCB* gene cluster of *E. coli* which is required for utilization of aliphatic sulfonates (van der Ploeg et al. 1999). The SsuD encoded in this gene cluster shares 63.8 % identity with *E. coli* SsuD [P80645] and completely conserves all the active site residues (Eichhorn et al. 2002). A phylogenetic tree based on amino acid sequences clearly showed the position of LadB$_{B23}$ in a unique evolutionary lineage distinct from

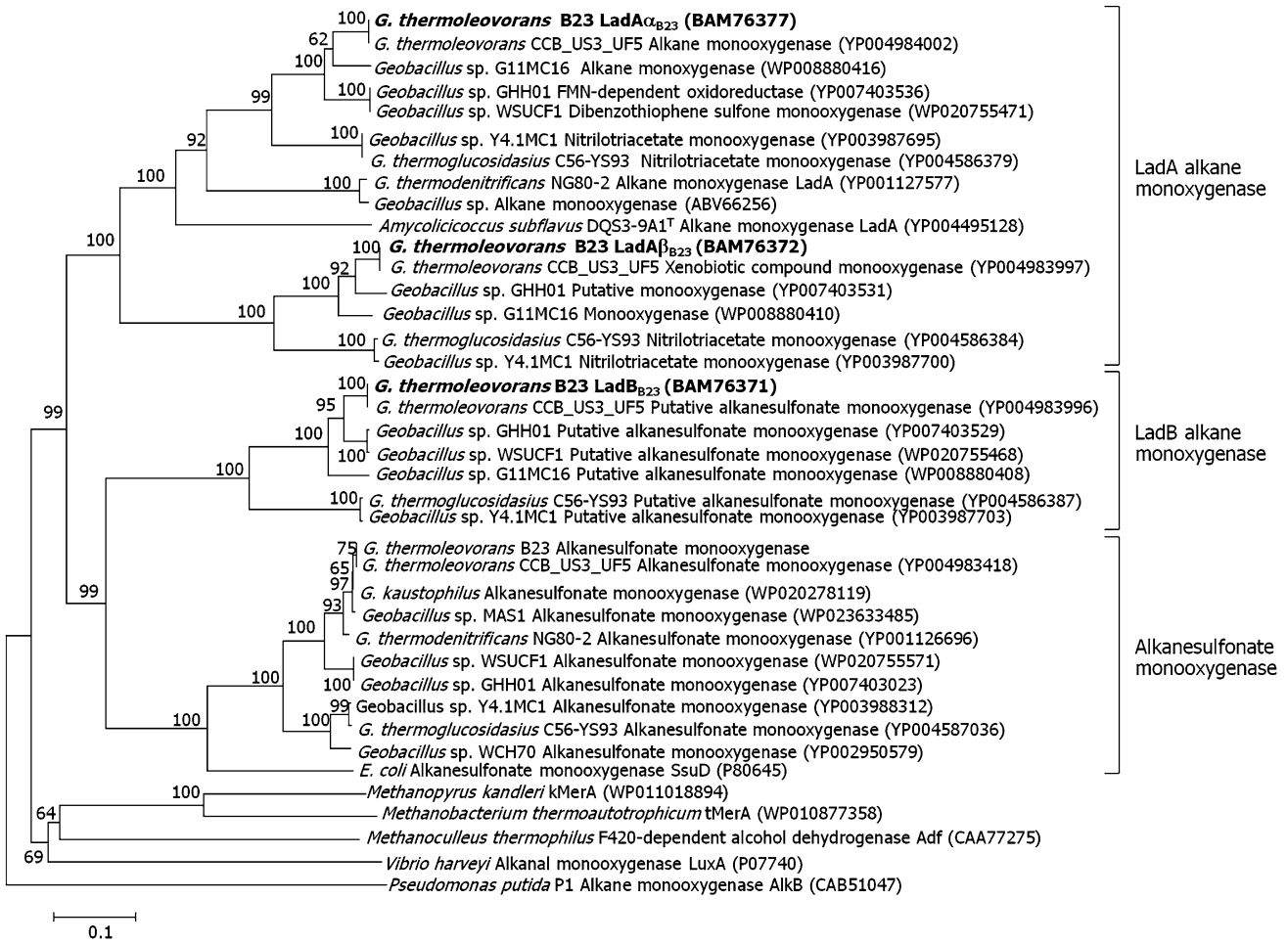

Fig. 2 A phylogenetic tree showing positions of LadAα$_{B23}$, LadAβ$_{B23}$, and LadB$_{B23}$ alkane monooxygenases relative to closely related monooxygenases and bacterial luciferase family enzymes. The phylogenetic tree was constructed from evolutionary distance data corrected by two-parameter transformation, using the neighbor-joining method. Numbers indicate percentages of bootstrap sampling derived from 100 samples. Adf, F420-dependent secondary alcohol dehydrogenase; LuxA, alkanal monooxygenase; kMerA, F420-dependent tetrahydromethanopterin reductase; tMerA, F420-dependent tetrahydromethanopterin reductase chain A; SsuD, F420-dependent alkanesulfonate monooxygenase. *Geobacillus* sp. WSUCF1 also contains a *ladAβ* homolog, but the sequence data (WP_020755469) is insufficient to be included in the tree

SsuD clade (Fig. 2). The above knowledge allowed us to propose LadB$_{B23}$ representing a novel alkane monooxygenase domain group that is independent of LadA and SsuD clade.

Genome alignment analysis of the relevant *Geobacillus* strains revealed that the gene organization in the "*ladAB* gene island" of strain B23 was identical to that of *G. thermoleovorans* CCB_US3_UF5 and quite similar to *Geobacillus* sp. G11MC16, *Geobacillus* sp. Y4.1MC1, and *Geobacillus thermoglucosidarius* C56-YS93, whereas the "*ladAB* gene island" region was completely missing in *Geobacillus kaustophilus* HTA426 and *G. thermodenitrificans* NG80-2 (Fig. 1a). LadA-type alkane monooxygenase is a member of the luciferase family of enzymes, which is classified as a two-component enzyme system. It comprises of (1) a monooxygenase enzyme that catalyzes the oxidation of various substrates such as luciferin, aromatic and polycyclic compounds, long-chain alkanes, and sulfonated compounds and (2) an NADPH-dependent FMN reductase. The FMN reductase provides reduced riboflavin molecule as a prosthetic group of the monooxygenase enzyme to activate an oxygen molecule for the oxidation reaction (Li et al. 2008; Ellis 2010; Morikawa 2010). We found an FMN reductase (ORF10) gene located upstream of the "*ladAB* gene island," as shown in Fig. 1.

Distribution of *alkB* and *ladA* gene homologs among strains belonging to the genus *Geobacillus*

Figure 3 shows a phylogenetic tree of the alkane-degrading *Geobacillus* strains based on their 16S rRNA gene

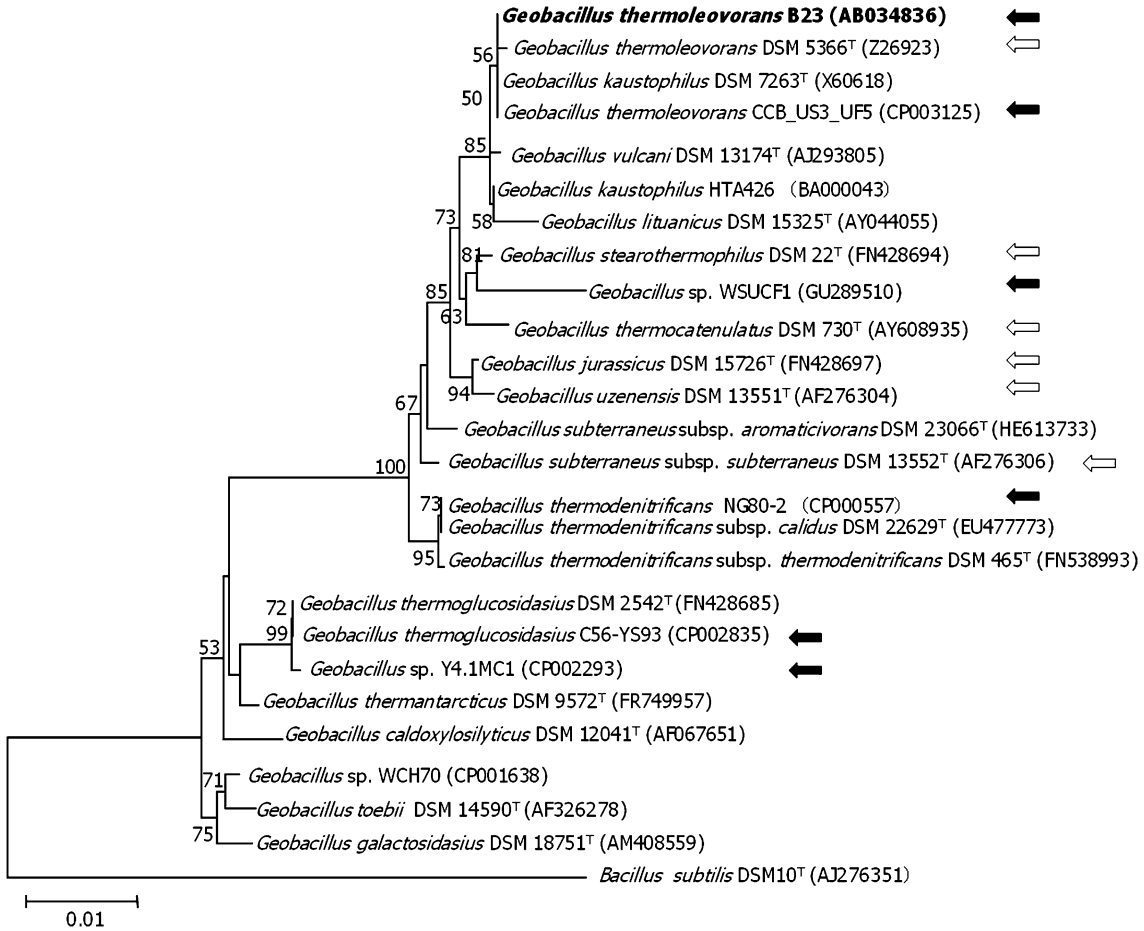

Fig. 3 A phylogenetic tree of *Geobacillus* species based on 16S rRNA gene sequences. The phylogenetic tree was constructed from evolutionary distance data corrected by two-parameter transformation using the neighbour-joining method. *Numbers* indicate percentages of bootstrap sampling derived from 100 samples. *Solid* and *open arrows* indicate the strains harboring *ladA*-type and *alkB*-type genes, respectively. *Geobacillus* sp. [ABV66256] harboring *ladA* was not included in the tree because of the unavailability of its 16S rRNA gene sequence

sequences. It is interesting to note that there is no clear relationship between the species harboring *alkB* and *ladA*. For example, *G. thermoleovorans* strains DSM 5366^T and vw3-1n harbor six *alkB* homologs (Tourova et al. 2008), whereas the *G. thermoleovorans* strains B23 and CCB_US3_UF5 harbor three *ladA* and no *alkB* homologs, suggesting that alkane monooxygenase genes are not species-specific but strain-specific. No strain in the genus *Geobacillus* has ever been reported to harbor both *alkB* and *ladA* alkane monooxygenase genes. There are variations in the copy number of *alkB* homologs among various *Geobacillus* strains and other bacteria, but the copy number of chromosome-encoded *ladA* homologs is three, as reported till date (Fig. 1). It is proposed that the multiple *alkB* homologs found in *Geobacillus* are derived from *Rhodococcus* bacteria, based on their significantly high sequence similarities and similar G + C mol % (Tourova et al. 2008).

G. thermodenitrificans NG80-2 maintains a single copy of *ladA* on the plasmid pLW1071; the presence of transposase genes on the plasmid pLW1071 suggests that *ladA* is an alien gene (Fig. 1b; Feng et al. 2007). It should be worth to note that the G + C mol % of the *ladA* is unusually low 34.8 % when compared with that of the NG80-2 whole genome 49.0 %. In contrast, G + C mol % of $ladA\alpha_{B23}$, $ladA\beta_{B23}$, $ladB_{B23}$, and the whole genome of B23 are 52.9, 53.0, 53.3, and 52.3 %, respectively. It is probable that the '*ladAB* gene island' is originated and evolved in *Geobacillus* bacteria. Recently, a LadA protein homologue [YP_004495128] has been reported from a mesophilic halotolerant bacterium, *Amycolicicoccus subflavus* DQS-9A1 (Nie et al. 2013). This enzyme shares significant amino acid sequence similarity with the LadA in *G. thermodenitrificans* NG80-2 (51.5 % identity), suggesting an evolutionary close relationship.

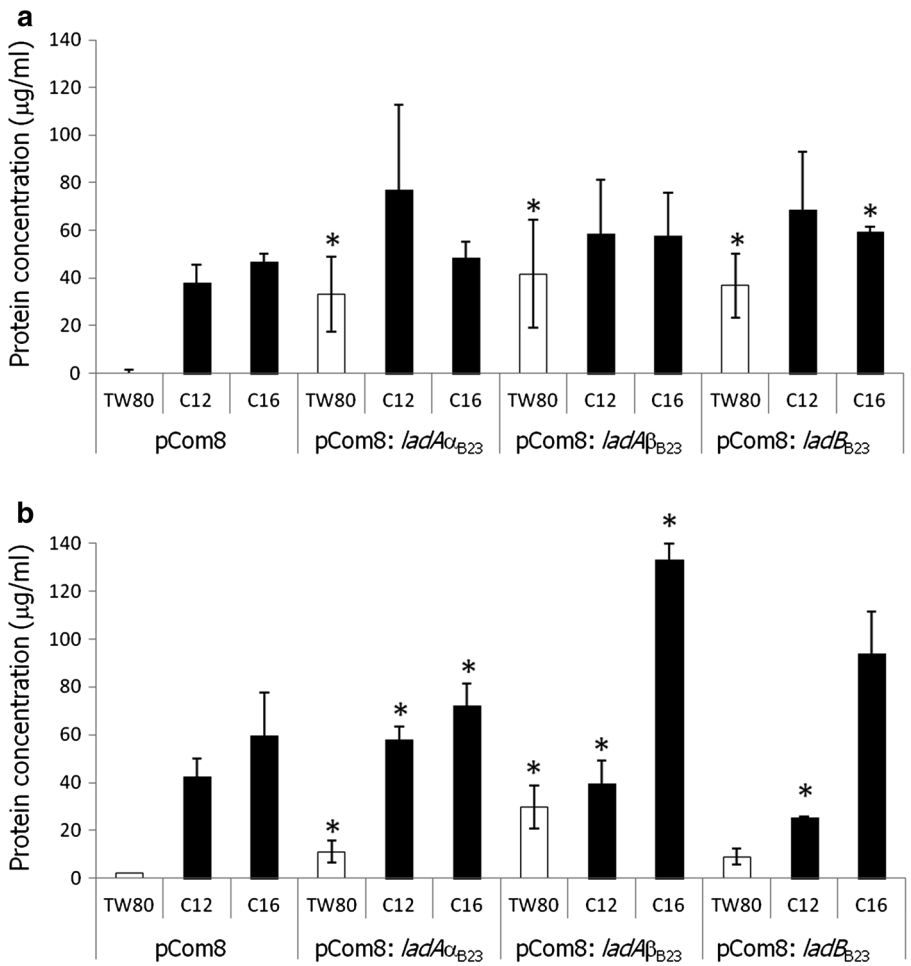

Fig. 4 Complementary growth of *P. fluorescens* KOB2Δ1 recombinant strains harboring pCom8 and its derivatives containing *ladA*α_{B23}, *ladA*βB23, or *ladB*$_{B23}$. Cells were grown on E2 minimal medium supplemented with 1 % (v/v) of *n*-alkane (C12 or C16) and 0.01 % Tween 80 at 30 °C (**a**) and 35 °C (**b**) for 8 days. Cell growth was measured by measuring total protein concentrations. *Single asterisk* $P < 0.05$ compared with control pCom8 only recombinant

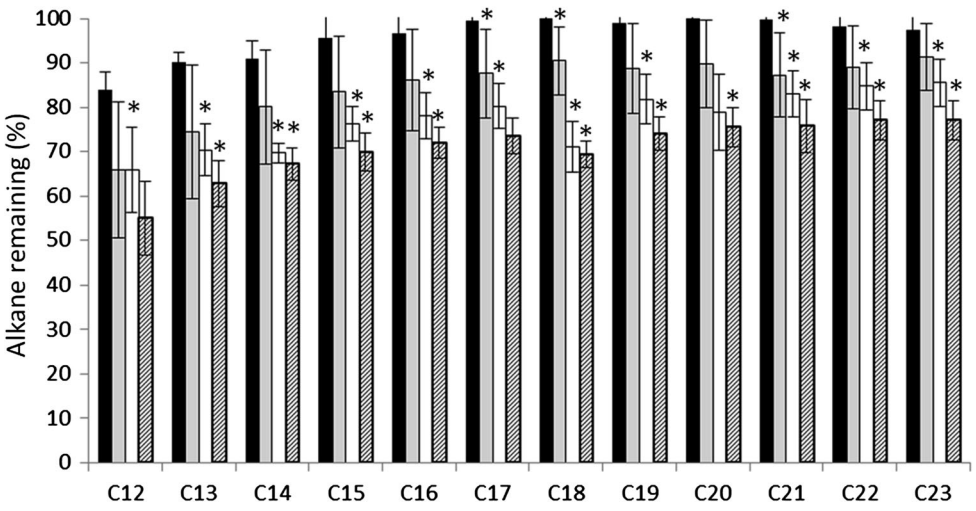

Fig. 5 Alkane degradation activity of *P. fluorescens* KOB2Δ1 recombinant strains harboring pCom8 and its derivatives containing *ladA*α_{B23}, *ladA*β_{B23}, or *ladB*$_{B23}$. Culture conditions were the same as those in Fig. 4. The amount of each alkane remaining in the culture was measured by GC-FID. *Closed bar*, pCom8 only; *gray bar*, pCom8:*ladA*α_{B23}; *open bar*, pCom8:*ladA*β_{B23}; *shaded bar*, pCom8:*ladB*$_{B23}$. *Single asterisk* $P < 0.05$ compared with control pCom8 only recombinant

Heterologous expression of *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ in *P. fluorescens* KOB2Δ1

Because targeted gene disruption was not successful in *G. thermoleovorans* B23, we tested the alkane monooxygenase activities by heterologous gene expression method. The growth of *P. fluorescens* KOB2Δ1 recombinants containing either the vector pCom8 alone (negative control) or pCom8:*ladA*α_{B23}, pCom8:*ladA*β_{B23}, and pCom8:*ladB*$_{B23}$ was examined on E2 minimal medium supplemented with 1 % (v/v) of dodecane

(C12) or hexadecane (C16) and 0.01 % Tween 80 (Polyoxyethylene sorbitan monooleate) at 30 and 35 °C. At 30 °C, which is the optimal temperature for the growth of KOB2Δ1, the growth of the strains harboring pCom8:*ladA*α_{B23}, pCom8:*ladA*β_{B23}, or pCom8:*ladB*$_{B23}$ was slightly better on a medium containing C12 or C16 when compared to the strain harboring pCom8, though the difference was not significant (Fig. 4a). The growth of the control strain (harboring the vector alone) on dodecane and hexadecane was probably a result of the degradation of the C5–C16 alkanes by the putative P450-type oxygenase (YP_007999854) in the KOB2Δ1 (CHA0) strain (van Beilen and Funhoff 2007). Since *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ were obtained from *G. thermoleovorans* B23, a thermophilic bacterium with an optimal alkane degradation temperature of 70 °C (Kato et al. 2001a), the low assay temperature of 30 °C may have caused a structural change or misfolding of the recombinant LadA proteins, resulting in their malfunction. Therefore, we assessed the growth of the cells at 35 °C, which is the highest growth temperature for KOB2Δ1. We found that the recombinant strains harboring pCom8:*ladA*β_{B23} or pCom8:*ladB*$_{B23}$ showed 2.23- or 1.57-fold better growth, respectively, than the negative control (harboring pCom8) on C16 (Fig. 4b). It was also not surprising that Tween 80 supported the growth of *ladA*-homolog-harboring strains because Tween 80 contains oleyl chains in its fatty acid moiety, which could also be a substrate of LadA-type enzymes.

Alkane degradation activity

Alkane degradation experiments at 35 °C revealed that the recombinants harboring the *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ degraded more C12–C23 alkanes than those harboring vector only (Fig. 5), with the following alkane degradation activity: pCom8:*ladA*α_{B23} < pCom8:*ladA*β_{B23} < pCom8:*ladB*$_{B23}$. No significant differences in the substrate specificity (carbon chain length of the alkane) were observed between LadAα_{B23}, LadAβ_{B23}, and LadB$_{B23}$ in mesophilic *P. fluorescens* KOB2Δ1 recombinants at 35 °C. Moreover, there was no lag time for the alkane degradation activity (data not shown), suggesting different gene expression regulation and/or different substrate alkane uptake systems between *G. thermoleovorans* B23 and *P. fluorescens* KOB2Δ1. It is interesting to note that the previously identified LadA of *G. thermodenitrificans* NG80-2 shares the highest sequence identity 49.8 % with the least active enzyme, LadAα_{B23} (Figs. 1, 2).

Conclusions

Unlike the FMN-dependent *lad*A alkane monooxygenase gene which exists in *G. thermodenitrificans* NG80-2 as a single copy on the plasmid pLW1071, whole genome analysis of *G. thermoleovorans* B23 revealed three *ladA* gene homologs *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$, which formed a cluster the "*ladAB* gene island" on the chromosome. A gene encoding FMN reductase, which reduces FMN at the active site of each LadA alkane monooxygenase, was also found in the "*ladAB* gene island." Genome sequence alignment between closely related *Geobacillus* species indicated that the "*ladAB* gene island" was also found on the chromosome of phylogenetically distant *Geobacillus* strains, suggesting that it is present universally. Heterologous gene expression experiments in *P. fluorescens* KOB2Δ1 indicated that *ladA*α_{B23}, *ladA*β_{B23}, and *ladB*$_{B23}$ are the functional alkane monooxygenase genes of *G. thermoleovorans* B23 for degrading a broad range of alkanes.

Acknowledgments *Pseudomonas fluorescens* KOB2Δ1 and pCom8 were kind gifts from Dr. Theo H. M. Smits (Agroscope, Wädenswil, Switzerland). This work was supported by KAKENHI (19380189) to M.M.

References

Altschul SF, Madden TL, Schäffer JZ, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Boonmak C, Takahashi Y, Morikawa M (2013) Draft genome sequence of *Geobacillus thermoleovorans* strain B23. Genome A 1(6):e00944-13

Darling AE, Mau B, Perna NT (2010) Progressive mauve: multiple genome alignment with gene gain, loss, and rearrangement. PLoS One 5:e11147

Das N, Chandran P (2011) Microbial degradation of hydrocarbon contaminants: an overview. Biotechnol Res Int, Article ID 941810

Eichhorn E, Davey CA, Sargent DF, Leisinger T, Richmond J (2002) Crystal structure of *Escherichia coli* alkanesulfonate monooxygenase SsuD. J Mol Biol 324:457–468

Ellis HR (2010) The FMN-dependent two-component monooxygenase systems. Arch Biochem Biophys 497:1–12

Felsenstein J (1985) Confidence limits on phylogenesis: an approach using the bootstrap. Evolution 39:783–791

Feng L, Wang W, Cheng J, Ren Y, Zhao G, Gao C, Tang Y, Liu X, Han W, Peng X, Liu R, Wang L (2007) Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. Proc Natl Acad Sci USA 104:5602–5607

Højberg O, Schnider U, Winteler HV, Sørensen J, Haas D (1999) Oxygen-sensing reporter strain of *Pseudomonas fluorescens* for monitoring the distribution of low-oxygen habitats in soil. Appl Environ Microbiol 65:4085–4093

Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23–28

Kato T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2001a) Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs. J Biosci Bioeng 91:64–70

Kato T, Miyanaga A, Haruki M, Imanaka T, Morikawa M, Kanaya S (2001b) Gene cloning of an alcohol dehydrogenase from thermophilic alkane degrading *Bacillus thermoleovorans* B23. J Biosci Bioeng 91:100–102

Kato T, Miyanaga A, Kanaya S, Morikawa M (2009) Alkane inducible proteins in *Geobacillus thermoleovorans* B23. BMC Microbiol 9:60

Kato T, Miyanaga A, Kanaya S, Morikawa M (2010) Gene cloning and characterization of an aldehyde dehydrogenase from long-chain alkane-degrading *Geobacillus thermoleovorans* B23. Extremophiles 14:33–39

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl Environ Microbiol 54:2924–2932

Li L, Liu X, Yang W, Xu F, Wang W, Feng L, Bartlam M, Wang L, Rao Z (2008) Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the long-chain alkane hydroxylase. J Mol Biol 376:453–465

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218

Morikawa M (2010) Dioxygen activation responsible for oxidation of aliphatic and aromatic hydrocarbon compounds: current state and variants. Appl Microbiol Biotechnol 87:1595–1603

Nie Y, Fang H, Li Y, Chi CQ, Tang YQ, Wu XL (2013) The genome of the moderate halophile *Amycolicicoccus subflavus* DQS3-9A1T reveals four alkane hydroxylation systems and provides some clues on the genetic basis for its adaptation to a petroleum environment. PLoS One 8:e70986

Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 11:2477–2490

Rost B (1999) Twilight zone of protein sequence alignments. Prot Eng 12:85–94

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Smits THM, Seeger MA, Witholt B, van Beilen JB (2001) New alkane-responsive expression vectors for *Escherichia coli* and *Pseudomonas*. Plasmid 46:16–24

Smits THM, Balada SB, Witholt B, van Beilen JB (2002) Functional analysis of alkane hydroxylase from Gram-negative and Gram-positive bacteria. J Bacteriol 184:1733–1742

Takei D, Washio K, Morikawa M (2008) Identification of alkane hydroxylase genes in *Rhodococcus* sp. strain TMP2 that degrades a branched alkane. Biotechnol Lett 30:1447–1452

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tourova TP, Nazina TZ, Mikhailova EM, Rodionova TA, Ekimov AN, Mashukova AV, Poltaraus AB (2008) *alk*B homologs in thermophilic bacteria of the genus *Geobacillus*. Mol Biol 42:217–226

van Beilen JB, Funhoff E (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21

van der Ploeg JR, Iwanicka-Nowickka R, Bykowski T, Hryniewicz MM, Leisinger T (1999) The *Escherichia coli ssuEADCB* gene cluster is required for the utilization of sulfur from aliphatic sulfonates and is regulated by the transcriptional activator CbI. J Biol Chem 274(41):29358–29365

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–109