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**Gene cloning and functional analysis of triple alkane
monooxygenases from *Geobacillus thermoleovorans* B23**
Geobacillus thermoleovorans B23 の三連型アルカン酸素添加酵素
に関する遺伝子クローニングと機能解析

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ABSTRACT

An extremely thermophilic bacterium, *Geobacillus thermoleovorans* B23 which was isolated from a deep subterranean oil reservoir at Niigata, Japan, is capable of degrading broad range alkanes (C11-C32) at 70°C by terminal oxidation pathway, followed by β -oxidation pathway. Whole genome sequence analysis revealed that B23 did not have *alkB*-type alkane monooxygenases genes like most alkane degrading bacteria but it carried three gene homologs namely *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} on its chromosome in a unique region, namely “*ladAB* gene island”. The amino acid sequences deduced from these three gene homologs were significantly similar to FMN- dependent alkane monooxygenase, LadA, encoded in a plasmid of *Geobacillus thermodetrificans* NG 80-2 with 50%, 33% and 21% identity, respectively. I also found a gene encoding FMN reductase in the upstream of *ladAB* gene island, which provides reduced flavin for the monooxygenase enzyme to activate oxygen molecule in alkane oxidation reaction.

In order to confirm that the three gene products are active alkane monooxygenases, heterologous expression of *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} have been performed in *Pseudomonas fluorescens* KOB2 Δ 1. The strain KOB2 Δ 1 is a mutant of alkane degrading *P. fluorescens* CHA0 with *alkB1* deletion which no longer grows in medium containing C12 - C16 of *n*-alkanes as sole carbon sources. It was found that all these gene homologs functioned in *P. fluorescens* KOB2 Δ 1 and partially restored alkane degradation activity. Alkane degradation test at 35°C for 8 days revealed that all the recombinants harboring either *ladA* α_{B23} , *ladA* β_{B23} or *ladA* γ_{B23} degraded more C12-C23 alkanes than vector only. Alkane degradation activity of the three recombinant strains was *ladA* α_{B23} < *ladA* β_{B23} < *ladB* β_{B23} . It was also found that the *ladAB* gene structure is widely shared in *Geobacillus* strains. Here, I report that a group of *G. thermoleovorans* utilizes multiple LadA-type alkane monooxygenases for degradation of wide range alkanes.

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INTRODUCTION

Due to rising of petroleum consumption, crude oil extraction has continuously increased worldwide to support the bulk of energy requirement. However, petroleum hydrocarbons are also one of the most prevalent pollutants which has great impact to ecosystems if they are accidentally contaminated to natural environments. Hydrocarbons are highly toxic to plants and animals, but not to several microorganisms. They affect not only to microbial activities and communities but also the physical and the chemical properties of soil. (Samanta *et al.*, 2002; Labud *et al.*, 2007; John *et al.*, 2011). Several researches of microbial hydrocarbon degradation have come to light for over half past century

Alkanes are saturated hydrocarbons which can be aliphatic (*n*-alkanes), cyclic (cycloalkanes) or branched (isoalkanes). Carbon numbers range from C1 up to C60. It can constitute up to 50% of crude oil, depending on the oil source. Many microorganisms are capable to degrade alkanes, both in aerobic and anaerobic condition. The composition of the main groups of alkane monooxygenases which are responsible to alkane oxidation is shown in Table 1.

Table 1 Enzyme classes involved in the oxidation of alkanes. (van Beilen & Funhoff, 2007; Throne-Holst *et al.*, 2007)

Enzyme class	Composition and cofactors	Substrate range	Presence shown in
Soluble methane monooxygenase (sMMO)	$\alpha 2\beta 2\gamma 2$ hydroxylase; dinuclear iron reductase, [2Fe–2S], FAD, NADH regulatory subunit	C1–C8 (halogenated)-alkanes, alkenes, cycloalkanes	<i>Methylococcus</i> , <i>Methylosinus</i> , <i>Methylocystis</i> , <i>Methylomonas</i> , <i>Methylocella</i>
Particulate methane monooxygenase (pMMO)	$\alpha 3\beta 3\gamma 3$ hydroxylase trimer composed of PmoA, PmoB, PmoC; mononuclear copper and dinuclear copper in PmoB	C1–C8 (halogenated)-alkanes, alkenes, cycloalkanes	<i>Methylococcus</i> , <i>Methylosinus</i> , <i>Methylocystis</i> , <i>Methylobacter</i> , <i>Methylomonas</i> , <i>Methyломicrobium</i> , etc.
AlkB-type alkane monooxygenase	Membrane monooxygenase; dinuclear iron rubredoxin; mononuclear iron rubredoxin reductase, FAD, NADH	C5–C16 alkanes, fatty acids, alkylbenzenes, cycloalkanes, etc.	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Amycolicococcus subflavus</i> , <i>Burkholderia</i> , <i>Geobacillus</i> , <i>Mycobacterium</i> , <i>Pseudomonas</i> , <i>Rhodococcus</i> , etc.
AlmA-type alkane monooxygenase	Soluble AlmA alkane monooxygenase, NADH/flavin reductase	C10–C40 alkanes	<i>Acinetobacter</i> sp. DSM 17874
LadA-type alkane monooxygenase	Soluble LadA alkane monooxygenase, (homo-dimer), FMNH ₂	C15–C36 alkanes	<i>Geobacillus denitrificans</i> NG80-2 and <i>Amycolicococcus subflavus</i> DQS3-9A1 ^T
Bacterial P450 oxygenase systems (CYP153, class I)	P450 oxygenase; P450 heme ferredoxin; iron–sulfur ferredoxin reductase, FAD, NADH	C5–C16 alkanes, (cyclo)-alkanes, alkylbenzenes, etc.	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Amycolicococcus subflavus</i> , <i>Caulobacter</i> , <i>Mycobacterium</i> , <i>Rhodococcus</i> , <i>Sphingomonas</i> , etc.
Dioxygenase	Homodimer; copper, FAD	C10–C30 alkanes	<i>Acinetobacter</i> sp. M-1, <i>Alcanivorax borkumensis</i> SK2

Alkanes are hydrophobic compounds, insoluble in water, and chemically inert, thus alkane degradation by microorganism depends on substrate accessibility and specialized enzymes systems to utilize *n*-alkanes as a carbon and energy source (Wentzel *et al.*, 2007). At moderate temperature, biodegradation of alkane is limited by low water solubility of substrates. It is generally recognized that the solubility of crude oil and the rate of biodegradation processes could be improved at high temperature conditions. Therefore, thermophilic bacteria offer crucial advantages over mesophilic or psychrophilic bacteria when they are applied to ex-situ bioremediation processes (Kato *et al.*, 2009; Liu *et al.*, 2009).

Geothermally heated oilfield with a temperature of 60-85°C or higher, in which liquid hydrocarbons are presence, are a unique ecological niche for thermophilic, hydrocarbon-oxidizing bacteria. Even though, petroleum reservoirs are anaerobic environments dominated by anaerobic microorganisms, aerobic bacteria can also be found due to injection water often contains dissolved oxygen. *Geobacillus* is an aerobic, thermophilic, spore forming bacteria. It is obligately thermophilic, growth temperature range is 37-75 °C, with an optimum growth at 55-65°C. Many of *Geobacillus* strains were isolated from subterranean high temperature petroleum reservoirs in various geographic regions around the world and reported to be able to degrade alkanes (Liu *et al.*, 2009; Nazina *et al.*, 2001; Nazina *et al.*, 2005; Tourova *et al.*, 2008; Wang *et al.*, 2006). There're two alkane monooxygenase classes which have been reported in the genus *Geobacillus*. First is the most popular AlkB-type alkane monooxygenase which functions to C5-C16 *n*-alkanes. It is a three-component enzyme system comprising with (1) an integral-membrane diiron monooxygenase (AlkB), (2) a soluble rubredoxin (AlkG) and (3) a NADH/rubredoxin reductase (AlkT) as shown in Fig. 1 (Morikawa, 2010). AlkB alkane monooxygenase is widely distributed in other bacteria, such as Gram-negative bacteria of genera *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Alcanivorax*, and *Burkholderia* and Gram-positive bacteria of genera *Rhodococcus*, *Mycobacterium*, *Nocardia*, and *Prauserella* (Rojo, 2009; van Beilen and Funhoff, 2007). Tourova *et al.* (2008) reported eight different homologues of AlkB-type alkane monooxygenase, AlkB-geo1 to AlkB-geo8, in genus *Geobacillus*. The *alkB-geo* homologues have highly diverse, 59.7–96.7% similarity to each others, and occur in each strain in various combinations, 4-6 homologues (Table 2). Analysis of the codon usage in the *alkB* homologs suggested that the pool of these genes was common in most Gram-positive and certain Gram-negative bacteria (Fig.2).

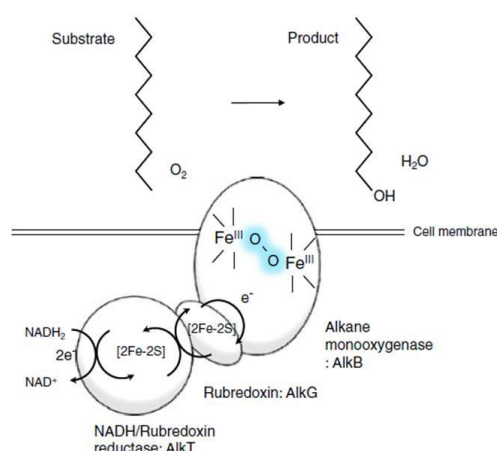


Fig. 1 Electron transfer system of AlkB-type alkane monooxygenases. (Morikawa, 2010)

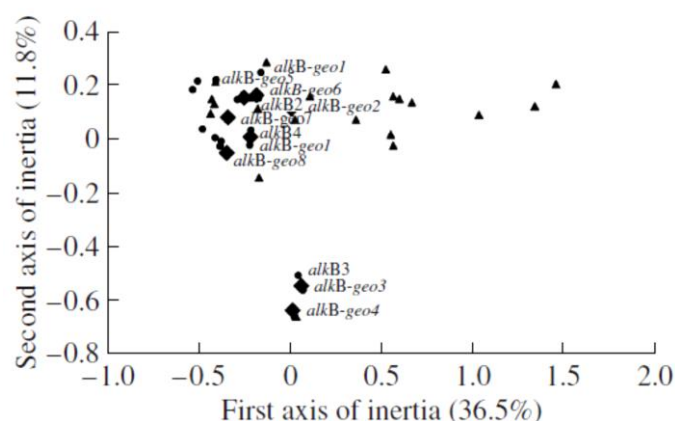


Fig. 2 Correspondence analysis of relative synonymous codon usage in *alkB* of (◆) *Geobacillus* and other (●) Gram-positive (*alkB1*, *alkB2*, *alkB3*, and *alkB4*: *alkB* homologs of *Rhodococcus* sp. Q15) and (▲) Gram-negative bacteria. (Tourova *et al.*, 2008)

Table 2 Diversity of *alkB* sequences in the genus *Geobacillus*. (Tourova *et al.*, 2008)

alkB homolog	Nearest cultivable relative according to BLAST analysis	Nucleotide sequence similarity ¹ , %	Amino acid sequence similarity ² , %	Total clones	Number of clones of particular strains										
					<i>G. stearothermophilus</i>	<i>G. thermocatalytus</i>	<i>G. thermoglucosidarius</i>	<i>G. thermoleovorans</i>		<i>G. garagensis</i>	<i>G. uzenensis</i>		<i>G. subterraneus</i>	<i>G. jurrassicus</i>	<i>G. toebii</i>
					DSM 22 ^T	VKM B-1259 ^T	3Feng	DSM 5366 ^T	vw3-In	DSM 15378 ^T	DSM 13551 ^T	VKM B-2228	DSM 13552 ^T	DSM 15726 ^T	B1024
alkB-geo1	<i>Rhodococcus erythropolis</i> NRRL B-16531 alkane 1-monooxygenase (alkB4)	99.2	100	47	3	4	3	3	2	4	3	5	12	7	1
alkB-geo2	<i>R. erythropolis</i> NRRL B-16531 alkane 1-monooxygenase (alkB4)	90.0	98.6	75	11	15	13	9	5	3	7	3	2		7
alkB-geo3	<i>Nocardia</i> sp. H17-1 alkane 1-monooxygenase (alkB3)	87.7	89.7	31	2	1	1	4	3	7	7			4	2
alkB-geo4	<i>R. erythropolis</i> NRRL B-16531 alkane 1-monooxygenase (alkB3)	96.7	96.6	55	3	1	1	3	7	6	1	13	11	7	2
alkB-geo5	<i>R. erythropolis</i> 50-V alkane 1-monooxygenase (alkB2)	95.4	100	12			2	1	1		1			1	6
alkB-geo6	<i>R. erythropolis</i> NRRL B-16531 alkane 1-monooxygenase (alkB2)	99.0	100	3				1				1		1	
alkB-geo7	Natural clone alkG4-35k, alkane 1-monooxygenase (alkB)	69.8	71.2	2					1		1				
alkB-geo8	<i>Rhodococcus</i> sp. Q15 alkane 1-monooxygenase (alkB1)	70.0	67.4	1	1										
Total clones				226	20	21	20	21	19	20	20	22	25	20	18

¹ Between the nearest *alkB* from GenBank and *Geobacillus alkB* fragments.

² Between the sequences inferred from the nearest *alkB* from GenBank and *Geobacillus alkB* fragments.

Another enzyme class which has been reported in the genus *Geobacillus* is LadA-type alkane monooxygenase which has only been reported in plasmid pLW1071 of *Geobacillus thermodenitrificans* NG80-2 (Fig. 3a). NG80-2 was isolated from a deep-subsurface oil reservoir in Dagang oilfield, Northern China. It grows between 45°C - 73°C (optimum 65°C)

and can utilize long-chain (C15-C36) alkanes as a sole carbon source (Fig. 3b). NG80-2 doesn't contain any *alkB* homologs but carry plasmid-borne *ladA* gene (Feng *et al.*, 2007). The crystal structure of LadA monooxygenase suggested that LadA was a flavoprotein monooxygenase that utilized dioxygen to introduce an oxygen atom into the substrate. It's two components enzyme system comprising with (1) **an NAD(P)Hdependent flavin reductase** for reduction of flavin and (2) **a LadA monooxygenase** that uses reduced flavin as a co-factor for oxidation reaction (Fig. 4) (Li *et al.*, 2008).

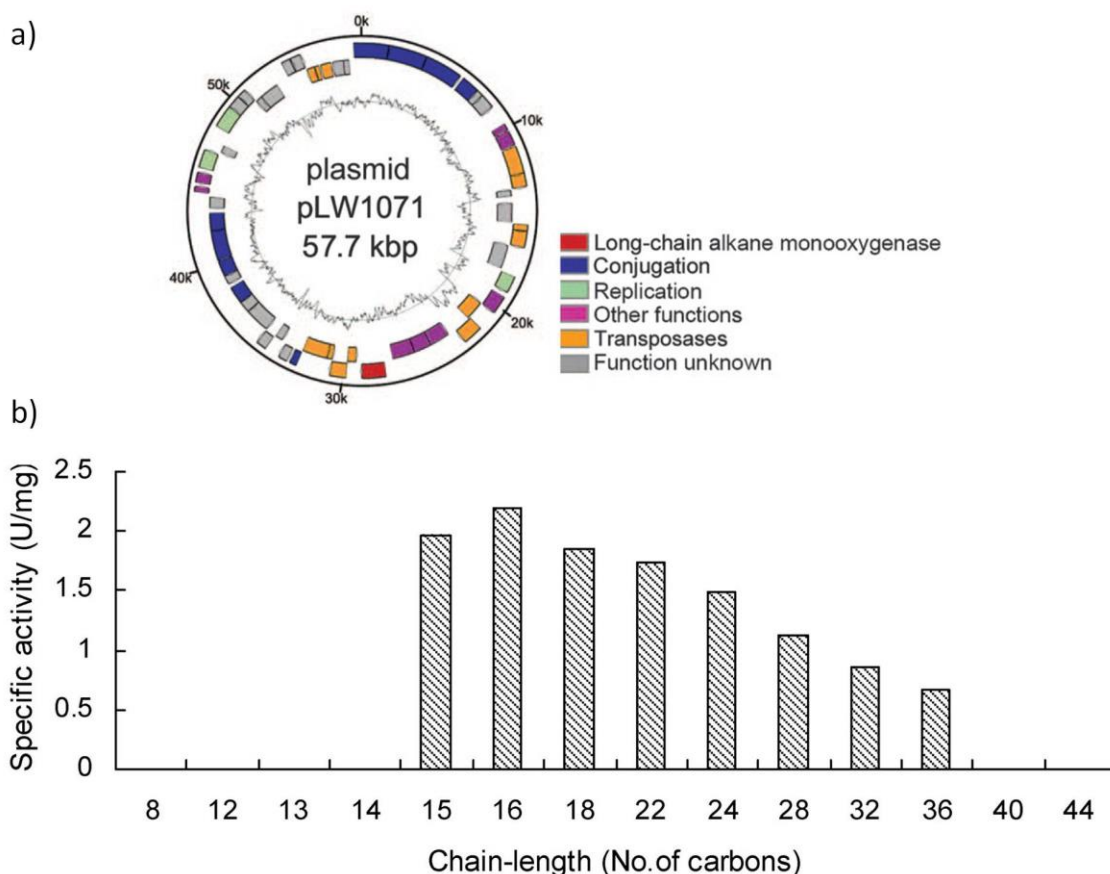


Fig. 3 LadA-type alkane monooxygenase. a) Circular maps of the *G. thermodenitrificans* NG80-2 plasmid pWL1071 shows ORFs color-coded according to their assigned functions. b) Specific activity of purified LadA on alkanes with different chain lengths. (Feng *et al.*, 2007)

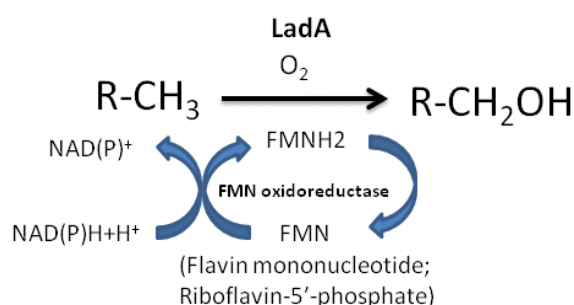


Fig. 4 Reaction catalyzed by the FMN-dependent alkane monooxygenase enzyme, LadA (modified from Ellis, 2010).

An extremely thermophilic bacterium, *Geobacillus thermoleovorans* strain B23, was previously isolated from production water (crude oil-water emulsion) at 2,150 meter-depth (105°C) of subterranean petroleum reservoir in Niigata, Japan 2001. It was found to be able to degrade broad range aliphatic alkanes (C11-C32) at 70°C by terminal oxidation pathway, followed by β -oxidation pathway (Kato *et al.*, 2001a). Cell morphology of B23 shown in Fig.5, the cells became longer and thicker after 14-day growth on alkanes.

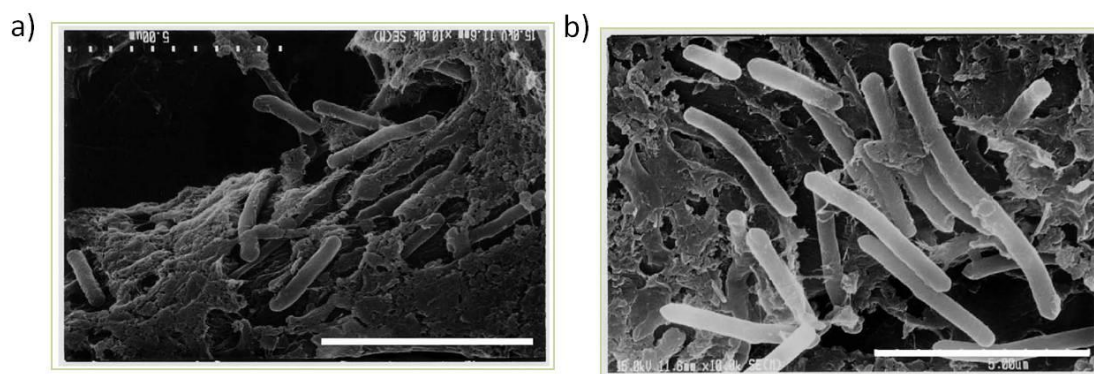


Fig. 5 Vegetative cells of *G. thermoleovorans* B23. Cells became longer and thicker compared with before (a) cultivation in 0.1% (v/v) alkane mixture after (b) grown at 70°C 14days. Scale bar = 5 μ m. (Kato *et al.*, 2009)

An investigation of alkane inducible proteins in B23 revealed that two membrane proteins (P16 and P21), catalase and superoxide dismutase (P24) productions were significantly increased during alkane degrading activity while acyl-CoA dehydrogenase activity was found to be replaced by eukaryotic-type acyl-CoA oxidase in β -oxidation pathway (Kato *et al.*, 2009). In order to understand the unique metabolisms of alkane degrading pathway, genes which are involved in alkane degradation of B23 such as alcohol dehydrogenase and aldehyde dehydrogenase also have been studied (Kato *et al.*, 2001b; Kato *et al.*, 2010). Schematic of alkane degrading pathway of B23 was shown in Fig. 6. After initial oxidation of the *n*-alkane, the corresponding alcohol is subsequently oxidized further by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding aldehyde and carboxylic acid, respectively. The carboxylic acid then serves as a substrate for acyl-CoA synthetase, and the resulting acyl-CoA enters the β -oxidation pathway. However, alkane monooxygenase which catalyzes oxidation of alkanes to primary alcohol at the initial reaction of the terminal oxidation pathway, has still remained unknown.

The type strain of *G. thermoleovorans*, LEH-1^T (= DSM 5366^T), could degrade alkane immediately in basal salt medium (LBM) supplement with 0.1% standard gas oil after incubation and effectively degraded alkanes with carbon chain length of 10 to 16 (Fig. 7 a, b). The result was supported by Tourova *et al.*, 2008 who reported the presence of *alkB* homologues in *Geobacillus*. LEH-1^T carried 6 homologues, *alkB-geo1* to *alkB-geo8*, therefore it could degrade well only medium chain alkanes. On the other hand, *G. thermoleovorans* B23 was found to have 7 day-lag in alkane degradation, suggested that the enzyme which was responsible to alkane degradation was inducible enzyme, and could degrade C11-C23 alkanes in standard gas oil efficiently (Fig. 7 a, b). These results implied that alkane monooxygenase in B23 might be of a family different from AlkB-type alkane monooxygenase.

In order to complete the knowledge of alkane degrading pathway of *G. thermoleovorans* B23, alkane monooxygenase genes were explored and analyzed in this work.

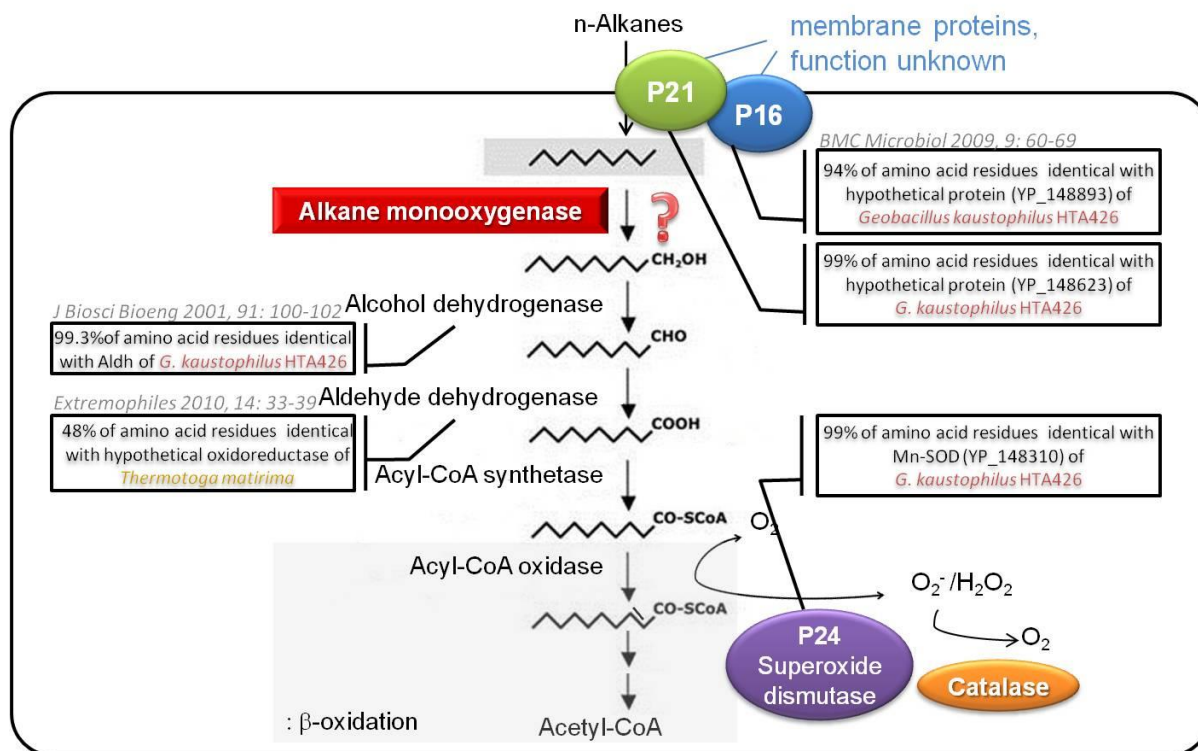


Fig. 6 Schematic of alkane degrading pathway of *G. thermoleovorans* B23.

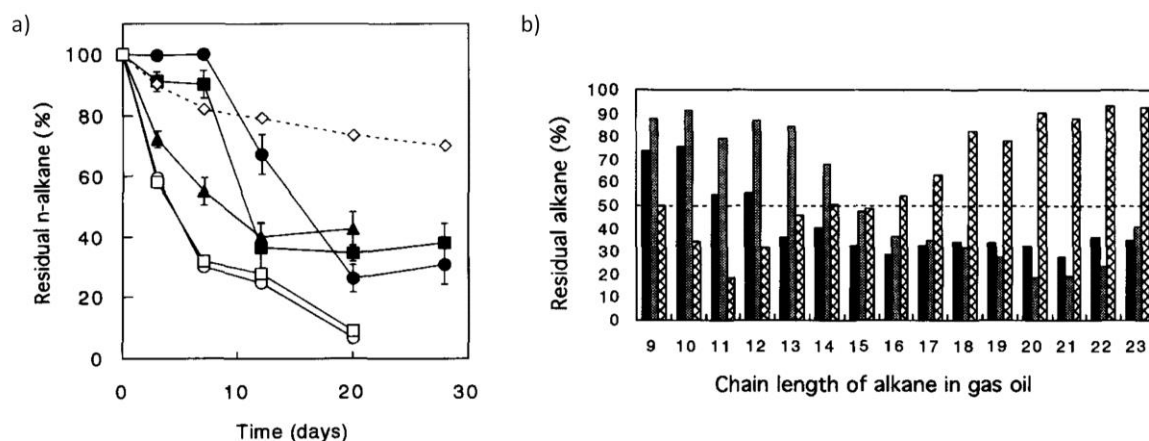


Fig. 7 Biodegradation of standard gas oil by *G. thermoleovorans* strain B23, H41 and LEH-1^T at 70°C. a) Degradation activity in LBM supplement with 0.1% standard gas oil. Residual alkanes represent the amount of alkanes (C9-C23) extracted. ●, B23; ■, H41; ▲, LEH-1^T; ◇, no cell. Culture of B23 and H41 were grown in LBM medium supplement with 0.1% standard gas oil for 13 days, then harvested and re-inoculated into the same medium or another 20 days. The degradation results were presented by ○, B23 and □, H41. b) Remaining residual alkanes (C9-C23) after cultivated in LBM medium supplement with 0.1% standard gas oil for 20 days. ■, B23; ●, H41; □, LEH-1^T. (Kato *et al.*, 2001a)

CHAPTER I

Genome analysis of *Geobacillus thermoleovorans* B23

1.1 Materials and methods

1.1.1 Draft genome sequencing and assembly

Chromosomal DNA from *G. thermoleovorans* B23 was prepared using Marmur's method (Marmur 1961). Paired-end libraries were generated from the chromosomal DNA and sequenced using the Roche 454 GS FLX (Basel, Switzerland) at Hokkaido System Science Co., Ltd. (Sapporo, Japan). Raw sequence data were assembled into 209 contigs (3,353,053 bp) using GS De Novo Assembler v2.8. The sequence reads were also compared with the *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 accession numbers BATY01000001-BATY01000209 (Boonmak *et al.*, 2013).

1.1.2 Phylogenetic tree analysis

The sequences were compared pairwise by BLASTN search (Altschul *et al.*, 1997) and were aligned with genome data of related *Geobacillus* species retrieved from GenBank database. Phylogenetic trees were constructed by Kimura's two-parameter correction (Kimura, 1980) using neighbor-joining method (Saitou & Nei, 1987) of MEGA version 5 (Tamura *et al.*, 2011). Confidences for the phylogenetic tree were estimated by bootstrap analysis (1000 replicates) (Felsenstein, 1985).

1.2 Results and discussion

1.2.1 Whole genome analysis of *G. thermoleovorans* B23

I had tried all the best to amplify *alkB*-type genes by PCR utilizing several combinations of degenerate and single sequence primers for *alkB* in *Geobacillus* bacteria (Tourova *et al.*, 2008). However, no *alkB* gene homologue was obtained. In order to identify genes responsible for initial oxidation of alkane, whole genome analysis was conducted. The genome sequencing generated 210,521 raw reads covering a total of 87,444,156 bp, achieving approx. >x25 genome coverage. Q40 plus bases were 99.94% reliability. Total mol%G+C content was 52.3%, which was the same value of *G. thermoleovorans* CCB_US3_UF5 (NC_016593) and equivalent to 52.09% of *G. kaustophilus* HTA426 (NC_006510). There were total of 209 contigs (over 100 bp) with 3,353,053 bp. The sequence reads were also aligned with the *G. kaustophilus* HTA426 reference genome using the multiple genome alignment program Mauve (Darling *et al.*, 2010). Analysis of 16S rRNA and *recN* gene sequences of B23 confirmed that the strain indeed belonged to *G. thermoleovorans* with 99.7% and 100% identity with *G. thermoleovorans* DSM 5366^T, respectively.

1.2.2 *ladA* alkane monooxygenase-like gene homologs in *G. thermoleovorans* B23 and related *Geobacillus* strains

G. kaustophilus HTA426 is a non-alkane degrading strain and does not have genes encoding alkane monooxygenase such as *alkB* (Takami *et al.*, 2004). I first subtracted the commonly shared orthologous gene regions from the genome sequence of B23 by utilizing *G. kaustophilus* HTA426 genome data with Mauve. B23 did not have any *alkB*-type alkane monooxygenase like its type strain, *G. thermoleovorans* LEH-1^T, and almost species in the genus *Geobacillus*. Next, I carefully analyzed the DNA regions of B23 which were absence in HTA426 by BlastX (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and found that B23 carried three genes, namely *ladA*_{αB23}, *ladA*_{βB23} and *ladB*_{B23}, in the 11,834 bp unique region, namely “*ladAB* gene island”. The three genes encode proteins of 463 (*LadA*_{αB23} [BAM76377]), 437 (*LadA*_{βB23} [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; Table 3), with the FMN-dependent alkane monooxygenase, *LadA* [YP_001127577, 440 amino acids], whose gene was located in the plasmid pLW1071 of *Geobacillus thermodenitrificans* NG80-2 (Feng *et al.*, 2007; Li *et al.*, 2008).

Table 3 Deduced amino acid sequence similarities of the three homologs with respect to *LadA* alkane monooxygenase from *G. thermodenitrificans* NG80-2.

Deduced amino acid sequence	<i>LadA</i> _{αB23}		<i>LadA</i> _{βB23}		<i>LadB</i> _{B23}	
	Identities	Positives	Identities	Positives	Identities	Positives
<i>LadA</i> _{αB23}	-	-	-	-	-	-
<i>LadA</i> _{βB23}	35%	49%	-	-	-	-
<i>LadB</i> _{B23}	25%	42%	25%	41%	-	-
<i>LadA</i> -NG80-2	50%	67%	34%	51%	23%	40%

BlastP search of *LadAB* against *Geobacillus* genomes revealed that there were several strains that have highly similar enzymes. The closest relative proteins of *LadA*_{αB23}, *LadA*_{βB23} and *LadB*_{B23} were alkane monooxygenase (YP004984002), a xenobiotic compound monooxygenase (YP004983997) and alkanesulfonate monooxygenase (YP004983996) of *G. thermoleovorans* CCB_US3_UF5 with 100% similarity. *LadA*_{αB23}, *LadA*_{βB23} and *LadB*_{B23} formed a phylogenetic cluster with *LadA* alkane monooxygenase of *G. thermodenitrificans* NG80-2 and other enzymes in luciferase family, which is distinctly separated from alkanesulfonate monooxygenase (SsuD) and well-studied *AlkB* alkane monooxygenases with high bootstrap values (Fig. 8).

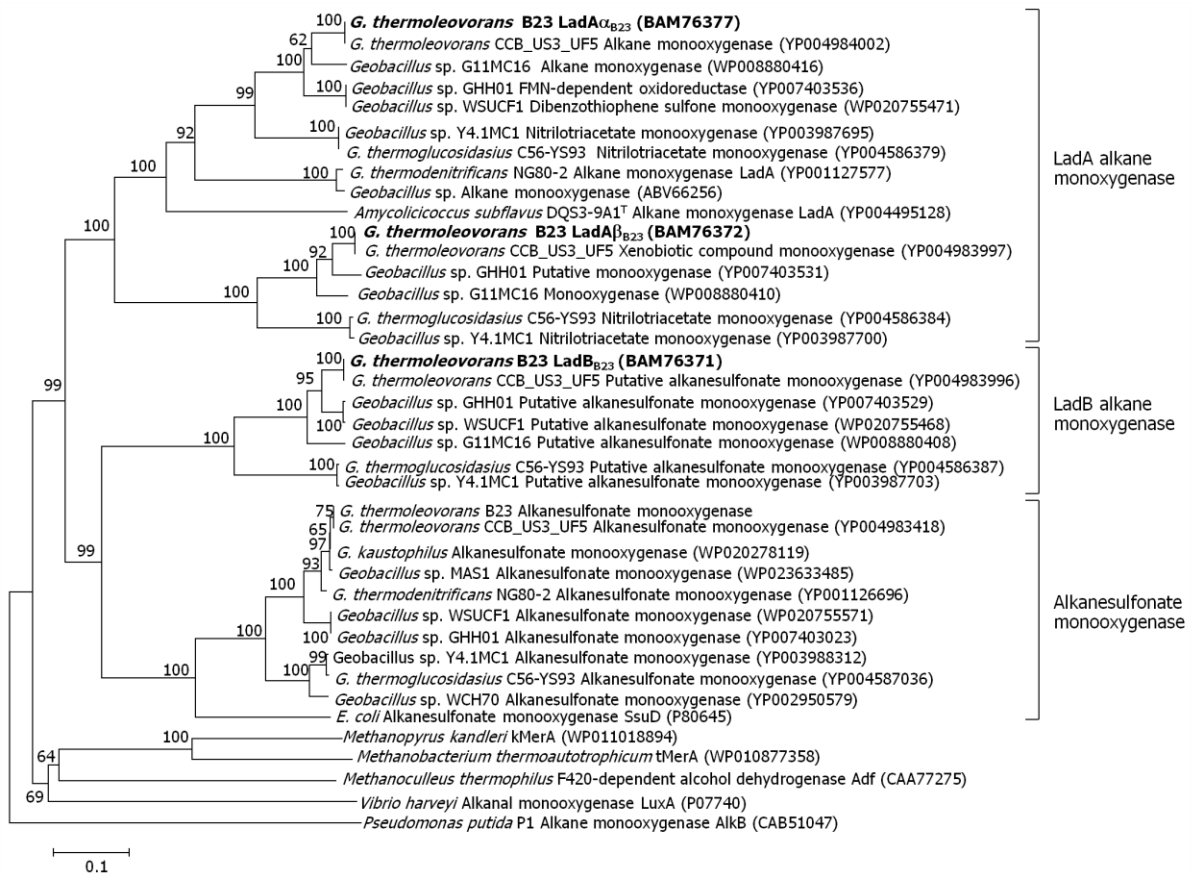


Fig. 8 Phylogenetic tree based on the deduced amino acid sequences, showing positions of $LadA\alpha_{B23}$, $LadA\beta_{B23}$ and $LadB\beta_{B23}$ alkane monooxygenases, with respect to closely related sequences from BLAST search and other bacterial luciferase family members. The phylogenetic tree was constructed from evolutionary distance data corrected by two-parameter transformation, using the neighbor-joining method. Numbers indicate percentages of bootstrap sampling, derived from 1000 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.

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% less than 10% are homologous (Rost, 1999). Two proteins share 50% or higher sequence identity, their backbones differ by less than 1 Å RMS deviation. I thus propose the name $LadA\alpha_{B23}$ and $LadA\beta_{B23}$ for apparent LadA homologues. While $LadB\beta_{B23}$ shares less identity with LadA (22.7%) and even higher similarity with putative 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 with neutral Ser11 in all LadB alkane monooxygenases. Interestingly, at another locus on chromosome of B23, there is a *ssuCBAED* gene cluster

(AB898656) same as *ssuEADCB* gene cluster of *E. coli* which is required for utilization of aliphatic sulfonates (van der Ploeg *et al.*, 1999). The SsuD of the cluster shares 63.8% identity with *E. coli* SsuD [P80645] and completely conserves all the active site residues (Eichhorn *et al.*, 2002). Phylogenetic tree based on amino acid sequences showed position of LadB_{B23} on an unique cluster, distinct from SsuD clade (Fig. 8). Above knowledge allowed us to propose LadB_{B23} representing a new alkane monooxygenase group that is neither LadA nor SsuD group.

LadA-type alkane monooxygenase is classified as a two component system enzyme with other enzymes in luciferase family. It composes 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) a FMN reductase. FMN reductase provides reduced riboflavin molecule as a prosthetic group of the monooxygenase enzyme to activate oxygen molecule for oxidation reaction (Li *et al.*, 2008; Ellis, 2010; Morikawa, 2010). I also found a FMN reductase (ORF10) gene located in the upstream of *ladAB* gene island as shown in Fig. 9. Genome alignment of relevant *Geobacillus* strains revealed that the gene organization in *ladAB* gene island of B23 was identical with *G. thermoleovorans* CCB_US3_UF5, and quite similar to that of *Geobacillus* sp. G11MC16, *Geobacillus* sp. Y4.1MC1 and *Geobacillus thermoglucosidarius* C56-YS93, while the *ladAB* gene island region was completely missing in *Geobacillus kaustophilus* HTA426 and *G. thermodenitrificans* NG80-2 (Fig. 9). The nucleotide sequence between ORF2 and ORF11 (11,986 bp), namely “*ladAB* gene island”, has been also deposited to DDBJ/EMBL/GenBank under accession number AB727923.

1.2.3 Distribution of *alkB* and *ladA* gene homologs in *Geobacillus* strains

Even though gene organization and regulation of *alk* clusters has been reported and well-studied in several bacteria such as *Acinetobacter*, *Alcanivorax borkumensis*, *Mycobacterium tuberculosis*, *P. aeruginosa*, *P. fluorescens*, *P. putida* and *Rhodococcus* (Smits *et al.*, 2002; van Beilen *et al.*, 2001; Whyte *et al.*, 2002), knowledge of *alkB* genes in *Geobacillus* still limited. So far, studying of *alkB* homologs in *Geobacillus* has been done only by PCR amplification by degenerate primers and DNA sequencing (Tourova *et al.*, 2008; Liu *et al.*, 2009). At the time of this study, eleven whole genomic and plasmid sequences of *Geobacillus* strains were available in GenBank database (<ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>), *G. kaustophilus* HTA426 (uid58227), *G. thermodenitrificans* NG80-2 (uid58829), *G. thermoglucosidasius* C56_YS93 (uid48129), *G. thermoleovorans* CCB_US3_UF5 (uid82949), *Geobacillus* sp. C56_T3 (uid49467), *Geobacillus* sp. HH01 (uid188479), *Geobacillus* sp. JF8 (uid215234), *Geobacillus* sp. WCH70 (uid59045), *Geobacillus* sp. Y412MC52 (uid55381), *Geobacillus* sp. Y412MC61 (uid41171) and *Geobacillus* sp. Y4_1MC1 (uid55779). In order to searching for localization of *alkB* homologs in the genus *Geobacillus*, all whole genome sequences of *Geobacillus* which were mentioned above were analyzed by multiple genome alignment program Mauve (Darling *et al.*, 2010) and carefully searched by BlastX (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using *alkB-geo1* to *alkB-geo8* sequences as queries (Tourova *et al.*, 2008). Surprisingly, all of them including strains which carry *ladAB* gene island, do not harbor any *alkB* homologs. Therefore, no *Geobacillus* strain has ever been reported that harbors both *alkB*-type and *ladA*-type alkane monooxygenase genes.

Although many thermophilic bacteria strains which are capable of degrading alkane have been isolated, knowledge of alkane monooxygenases in thermophilic bacteria is still limited. According to Fig. 10, showing a phylogenetic tree analysis of alkane degrading *Geobacillus* strains based on the 16S rRNA gene sequences. Based on evolutionary, there is no clear relationship between the species harboring *alkB*-type and *ladA*-type genes. *G. thermoleovorans* LEH-1^T and vw3-1n harbor six *alkB* homologs (Tourova *et al.*, 2008) while *G. thermoleovorans* B23 harbors three *ladA* and no *alkB* homologues, suggesting that alkane monooxygenase genes are not species specific but strain specific.

The presence of multiple *alkB* alkane monooxygenase genes is a general feature of the genus *Geobacillus*, total eight homologs. Each *Geobacillus* strains may carry three to six homologs with various combinations (Tourova *et al.*, 2008) but the copy number of genome encoding *ladA* is three or one as far as ever reported (Fig. 10). It is proposed that multiple *alkB* in *Geobacillus* are derived from *Rhodococcus* bacteria based on their significant high sequence similarity, similar mol%G+C contents and codon usage (Tourova *et al.*, 2008). The various combinations of *alkB* homologs in genome of particular strains also occurred in *Rhodococcus*, *alkB1* to *alkB4* (Whyte *et al.*, 2002). It might occur because of location of the *alkB* genes. van Beilen *et al.* (2001) reported that *alkB* cluster of *Pseudomonas putida* GPo1 and *P. putida* P1 located between IS elements, therefore these genes were part of a transposon.

The *ladAB* gene island has been identified only in thermophilic *Geobacillus* strains, suggesting that it had been originally appeared and evolved in this genus. It generally occurs in chromosome of *Geobacillus* as three homologs, *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} , except *Geobacillus* sp. WSUCF1 which doesn't contain *ladA* β_{B23} . In the case of *G. thermodenitrificans* NG80-2 which harbors a single copy of *ladA* in plasmid pLW1071, there were transposase genes located in the plasmid pLW1071 suggesting that the *ladA* of NG80-2 is an alien gene (Fig. 3a, Feng *et al.*, 2007). It should be worth to note that the G+C mol% of the *ladA* of *G. thermodenitrificans* NG80-2 is unusually low 34.8% when compared with that of the whole genome 49.0%. In contrast, G+C mol% of *ladA* α_{B23} , *ladA* β_{B23} , *ladB* β_{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 while *alkB* homologs is appeared as a result of horizontal gene transfer.

No *ladA* gene homologue is yet found in the genome of *Rhodococcus* bacteria, suggesting its distribution and evolution mechanism is different from *alkB*. This speculation is supported by the fact that (i) there is no mobile element around *ladAB* gene island location and (ii) there are quite different habitats of obligate thermophilic *Geobacillus* and other mesophilic bacteria, including *Rhodococcus*. It is possible to isolate *Geobacillus* strains from moderate temperature environments but it should exist as bacterial spores which are impossible of genetic transfer between species. Recently, a LadA homologue (YP_004495128) has been also 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 relationship. It still remains unknown how *A. subflavus* DQS-9A1 obtained or created this enzyme gene in their evolution.

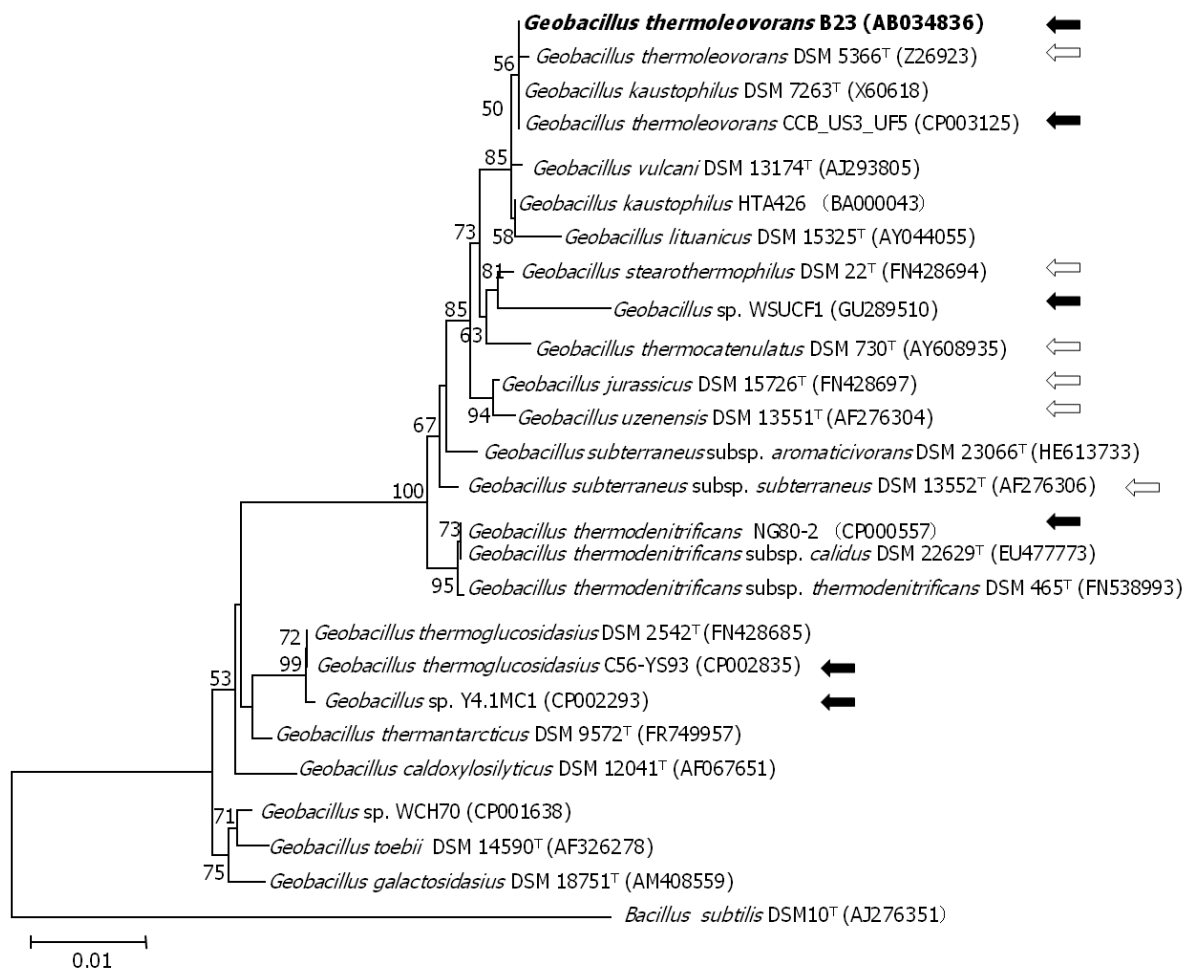


Fig. 10 Phylogenetic tree based on 16S rRNA gene sequences with respect to type strains of the genus *Geobacillus*. The phylogenetic tree was constructed from evolutionary distance data corrected by two-parameter transformation, using the neighbor-joining method. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. Black arrow indicated the strains which harboring *ladA*-type alkane monooxygenase genes. White arrow indicated the strains which have been reported to harbor *alkB*-type alkane monooxygenase genes (Tourova *et al.*, 2008).

CHAPTER II

Heterologous expression of $LadA_{B23}$ in *Pseudomonas fluorescens* KOB2Δ1

2.1 Materials and methods

2.1.1 Strains and plasmid

Heterologous expression of the three candidates genes, $ladA\alpha_{B23}$, $ladA\beta_{B23}$ and $ladA\gamma_{B23}$, has been done in *Pseudomonas fluorescens* KOB2Δ1, a mutant of *P. fluorescens* CHA0 with *alkB1* deletion. KOB2Δ1 can no longer grow in C₁₂-C₁₆ of *n*-alkanes but can oxidize longer alkanes (C₁₈ to C₂₈) because of unidentified second alkane oxidation system (Smits *et al.*, 2002). Plasmid pCom8 is an *E. coli*-*Pseudomonas* expression vector which based on pUCP25 and *P. putida* GPo1 *alkBp* promoter (Smits *et al.*, 2001). The bacterial strains and plasmids used or constructed in this study are listed in Table 4.

Table 4 Strains and plasmids used in heterologous gene expression of $LadA_{B23}$ in *P. fluorescens* KOB2Δ1.

Strain or plasmid	Relevant characteristics (genotype)	Reference
Strains		
<i>E. coli</i> DH5α	Cloning strain for pUC19	-
<i>E. coli</i> DH10B	Cloning strain for pCom8	Invitrogen
<i>P. fluorescens</i> KOB2Δ1	Mutant of <i>P. fluorescens</i> CHA0 with <i>alkB1</i> deletion; C ₁₂ -C ₁₆ Alk ⁻ , C ₁₈ -C ₂₈ Alk ⁺	Smits <i>et al.</i> , 2002
Cloning and expression vectors		
pUC19	Cloning vector, Ap ^r	Yanisch-Perron <i>et al.</i> , 1985
pCom8	Broad-host range expression vector; P _{<i>alkB</i>} , Gm ^r <i>oriT</i> <i>alkS</i> *	Smits <i>et al.</i> , 2001
pCom8: $ladA\alpha_{B23}$	pCom8 with $ladA\alpha_{B23}$	This study
pCom8: $ladA\beta_{B23}$	pCom8 with $ladA\beta_{B23}$	This study
pCom8: $ladB_{B23}$	pCom8 with $ladB_{B23}$	This study

* P_{*alk*}, *alkB* promoter; Gm^r, aminoglycoside acetyltransferase (*aacC1*); *alkS*, transcriptional regulator

Table 5 Primers used in heterologous gene expression of $LadA_{B23}$ in *P. fluorescens* KOB2Δ1

Genes	Length (bp)	Primer name	Sequences	Restriction site
$ladA\alpha_{B23}$	1392	p8αF	5' AATTCCATATGGATCAACCCCTATTATTC 3'	5'NdeI-SmaI3'
		p8αR	5' TAGGGCCCCGGGCTAGCCATCTGTTCTGTGAGC 3'	
$ladA\beta_{B23}$	1314	p8βF	5' AATGGCATATGAGTGTGCGGCAAATG 3'	5'NdeI-SmaI3'
		p8βR	5' GATTCCCGGGTCACACGCGGATCGACTT 3'	
$ladB_{B23}$	1119	p8γF	5' GGAAACATATGGTTGAATTTATTACGATGG 3'	5'NdeI-SmaI3'
		p8γR	5' CGACCCGGGTTAGATCCATACTTCCGTTTGT 3'	

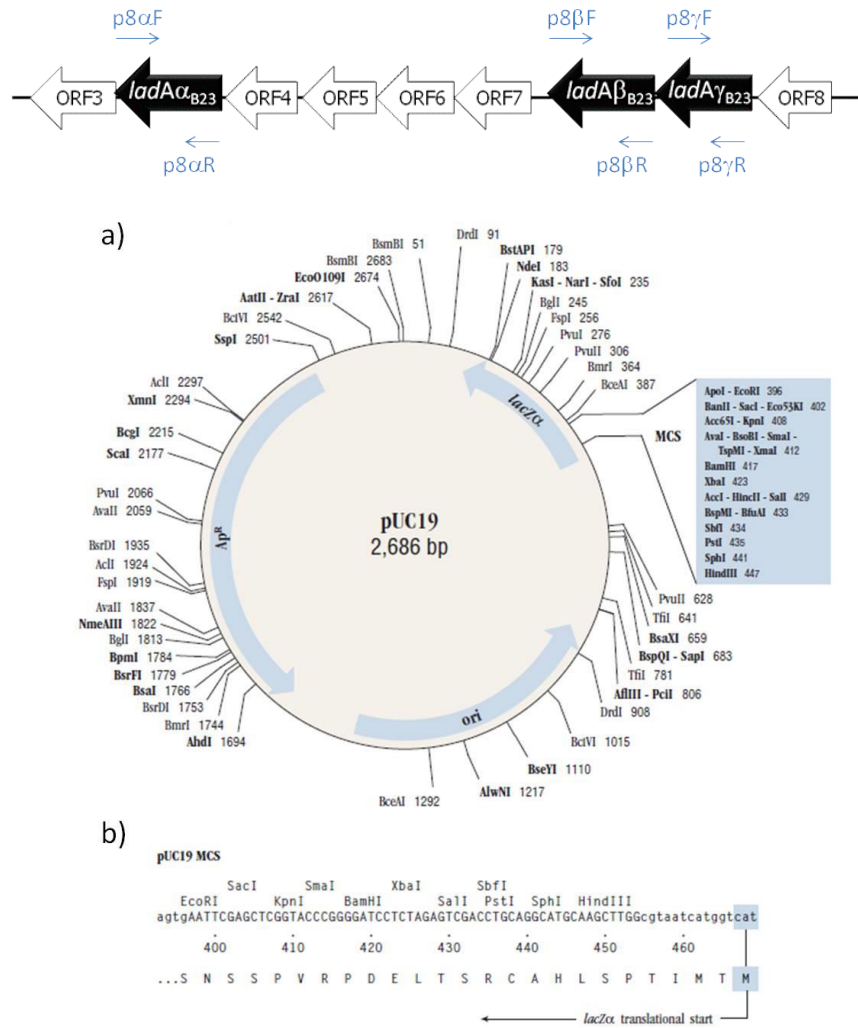


Fig. 11 Physical map of pUC19 (a) and its multiple cloning sites (b).

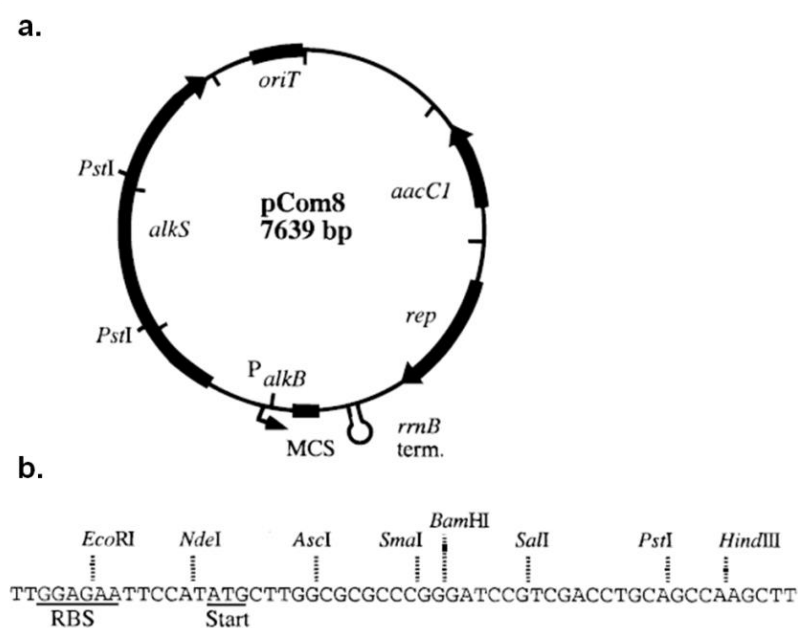


Fig. 12 Physical map of pCom8 (a) and its multiple cloning sites (b).

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

Genomic DNA of B23 was prepared by Marmur's method (Marmur 1961). The *ladA* α_{B23} , *ladA* β_{B23} and *ladB* $_{B23}$ genes were amplified by C1000 TouchTM Thermal Cycler (Bio-Rad, Hercules, CA) with KOD-Plus-Neo DNA polymerase (Toyobo, Kyoto, Japan) according to standard protocol from supplier. PCR cycles were shown in Appendix C.1. The primers used for PCR amplification of these genes were designed from genome sequences with modification of *NdeI* and *SmaI* restriction sites (Table. 5). In order to confirm genes sequences, the PCR fragments of *ladA* α_{B23} , *ladA* β_{B23} and *ladA* γ_{B23} genes were cloned into *SmaI* site of pUC19 and transformed *E. coli* DH5 α by heat shock method (Inoue *et al.*, 1990). The nucleotide sequence was determined by BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3100 DNAsequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA) and confirmed no error was contained in the PCR products. The *ladA* α_{B23} , *ladA* β_{B23} and *ladA* γ_{B23} fragments were separately subcloned into pCom8 between the gap of *NdeI* and *SmaI* site at downstream of *alkB* promoter. Each pCom8 containing *ladA* α_{B23} , *ladA* β_{B23} and *ladA* γ_{B23} genes were transferred into *E. coli* MegaX DH10BTM T1^R ElectrocompTM cells according to the standard protocol and then transferred into *P. fluorescens* KOB2 Δ 1 (Højberg *et al.*, 1999). *P. fluorescens* KOB2 Δ 1 is a mutant of *P. fluorescens* CHA0 with *alkB1* deletion, which dramatically reduced C12-C16 alkane degradation activity (Smits *et al.*, 2001). Protocols are available in Appendix C.1.

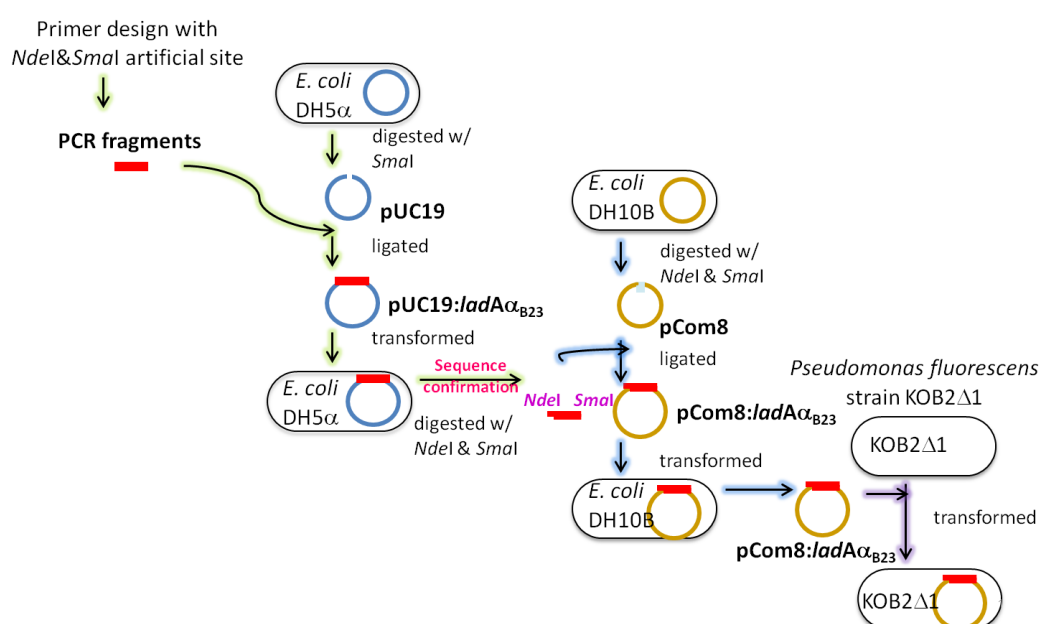


Fig. 13 Scheme of cloning of *ladA* alkane monooxygenase-like homologs and construction of expression plasmids in *P. fluorescens* KOB2 Δ 1.

2.1.3 Heterologous gene expression of *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} in *P. fluorescens* KOB2 Δ 1

2.1.3.1 Growth complementation test

In vivo growth complementation test of *P. fluorescens* KOB2 Δ 1 recombinants containing pCom8 only (as negative control), pCom8:*ladA* α_{B23} , pCom8:*ladA* β_{B23} and pCom8:*ladB* β_{B23} were performed on 5 ml of E₂ minimal salts medium (Lageveen *et al.*, 1988; Appendix A) supplemented with 1% (vol/vol) of *n*-alkane (C12 or C16) and 0.01% tween80 (Polyoxyethylene sorbitan monooleate). Turbidometry was not useful for measuring cell growth due to high background values by forming oil-water emulsion in the culture. Therefore, growth of *P. fluorescens* KOB2 Δ 1 recombinants was determined by total protein measurement using Pierce[®] BCA Protein Assay Kit (Thermo Scientific, USA). Protocols are available in Appendix C.2.

2.1.3.2 Alkane degradation test

Alkane degradation test was performed in triplicate in 5 ml of E₂ minimal salts medium supplemented with 1% (vol/vol) of standard gas oil and 0.01% tween80 surfactant in 50 ml screw cap-centrifuge tube 18422CTF50 (IWAKI, Tokyo, Japan). The cultures were incubated at 120 rpm and 35°C for 8 days. Five ml of whole culture supernatant was extracted by 5 ml acetone: hexane (1:1). After centrifuge at 30,000xg 20 min, the amount of remaining alkanes in the samples was directly analyzed by gas chromatography. A portion (1 μ l, split ratio 50:1) of the sample was analyzed by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 μ m x 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan, GC/MS). Helium was used as carrier gas at a flow rate 25 ml/min. Protocols are available in Appendix C.3.

2.2 Results and discussion

2.2.1 Growth complementation of *P. fluorescens* KOB2 Δ 1 recombinants

At 30°C, which is the optimal temperature for growth of KOB2 Δ 1, the recombinant strains harboring *ladA* α_{B23} , *ladA* β_{B23} or *ladA* γ_{B23} could grow on a medium containing dodecane (C12) or hexadecane (C16) slightly better than vector only (pCom8) but the difference was not significant (Fig. 14a). Growth of vector only strain on dodecane and hexadecane was still observed probably because that putative P450-type oxygenase (YP_007999854) in KOB2 Δ 1 (CHA0) degraded C5-C16 alkane (van Beilen & Funhoff, 2007). Since *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} were originated from *G. thermoleovorans* B23, a thermophilic bacterium whose optimal temperature for alkane degradation was 70°C (Kato *et al.*, 2001a), protein expression at low temperature condition such as 30°C might have caused structure change or misfolding of LadA-type proteins and malfunctions. Therefore, we next tested the cell growth at 35°C which is the highest growth temperature for KOB2 Δ 1. It was found that the recombinant strains harboring *ladA* β_{B23} or *ladB* β_{B23} clearly showed better growth than vector only on hexadecane (C16) by 2.23 and 1.57-folds, respectively (Fig.14b). It is not surprising that tween 80 also supported growth of *ladA* harboring strains because

tween 80 contains oleyl-chain (C16) in the fatty acid moiety which could be also a substrate of LadA-type proteins.

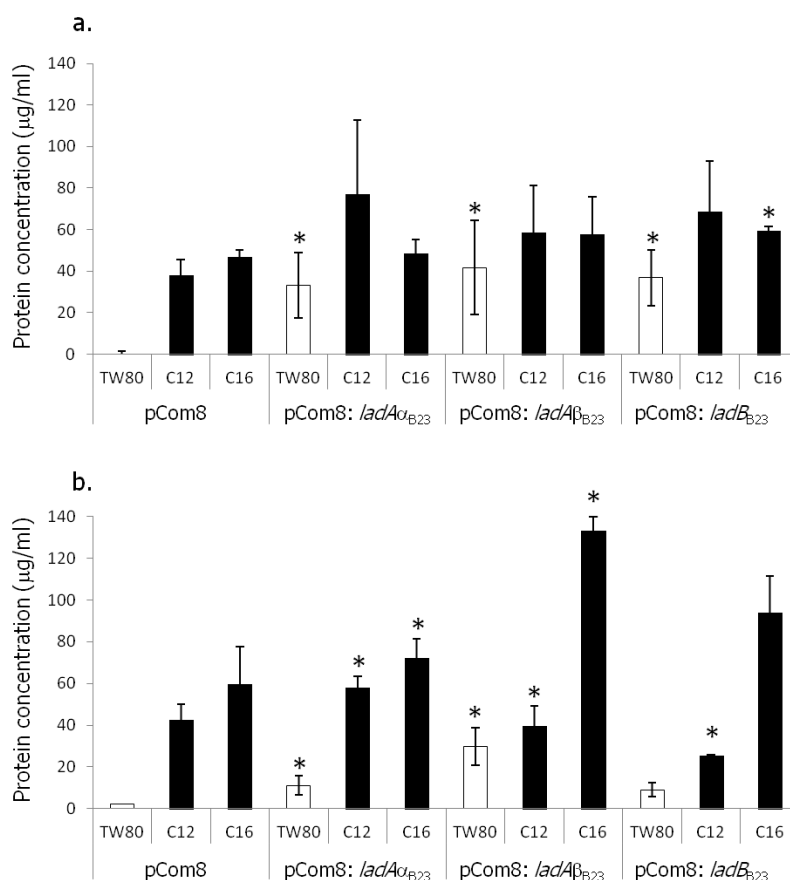


Fig. 14 Complementary growth of *P. fluorescens* KOB2Δ1 recombinants containing pCom8 and pCom8 derivatives of *ladA*_{αB23}, *ladA*_{βB23} and *ladB*_{B23} on E₂ minimal salts medium supplemented with 1% (vol/vol) of *n*-alkane (C12 or C16) and 0.01% tween80 (surfactant) at 30°C (a) and 35°C (b), for 8 days. **P* < 0.05 compared with control pCom8 only recombinant.

2.2.2 Alkane degradation test

Alkane degradation test at 35°C for 8 days revealed that all the recombinants harboring either *ladA*_{αB23}, *ladA*_{βB23} or *ladB*_{B23} indeed degraded more C12-C23 alkanes than vector only (Fig. 15). The amount of each alkane in the bottle after 8 days at 35°C incubation was used for 100% alkane degradation activity among the three recombinants showed quite similar degradation patterns for each alkanes (Fig. 15, 16). Even though all of three genes used same *alkB* promoter on pCom8 vector, the recombinants containing *ladA*_{αB23} and *ladB*_{B23} gradually degraded C12-C23 alkanes after incubation while the recombinants which harboring *ladA*_{βB23} had 6-day lag before started degradation. Although, there was 6-day lag of degradation in the *ladA*_{βB23} recombinant cells, the remaining amount of alkane (C16, C18, and C20) decreased to similar amount in culture of *ladB*_{B23} recombinant 2 days later.

Alkane degradation activity of the three recombinant strains was in the order of $ladA\alpha_{B23} < ladA\beta_{B23} < ladB_{B23}$. *P. fluorescens* KOB2 Δ 1 is a mutant of *P. fluorescens* CHA0 with *alkB1* deletion which can no longer grow in C12-C16 of *n*-alkanes but grow in C18-C28 (Smits *et al.*, 2002). From our study, the recombinant *P. fluorescens* KOB2 Δ 1: pCom8 could not degrade long chain alkane at 35°C but degraded C12-C16 alkanes even it doesn't contain *alkB1* gene. It might be occurred because p450 oxygenase of host cells, which responses for C5-C16 alkane (van Beilen & Funhoff, 2007) worked during incubation while larger enzyme such the original AlkB could not work at 35°C.

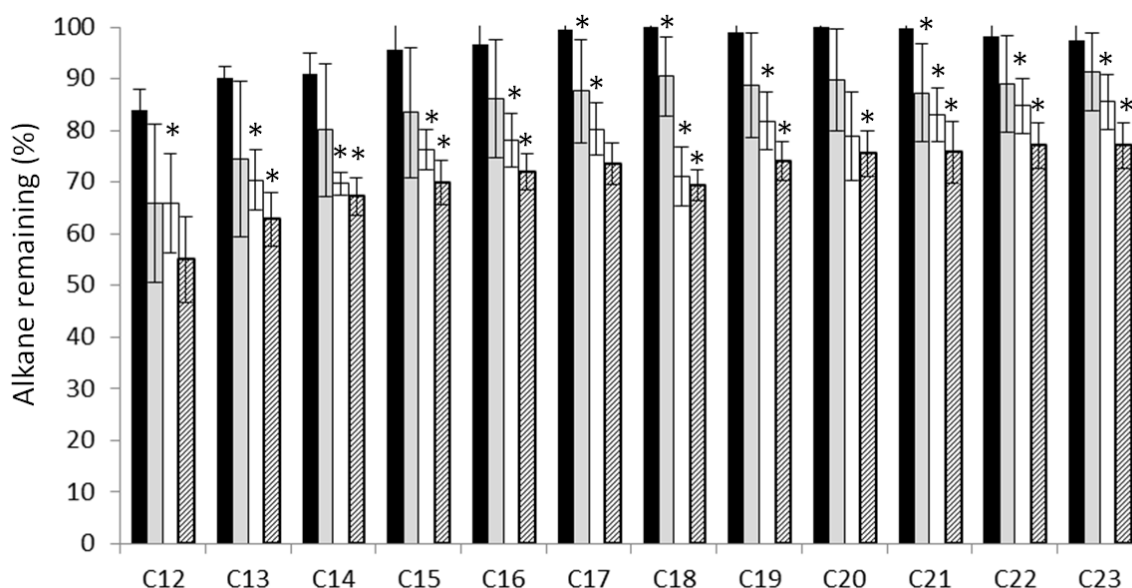


Fig. 15 Remaining alkane after cultivated *P. fluorescens* KOB2 Δ 1 recombinants harboring pCom8 and pCom8 derivatives of *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} on E₂ minimal salts medium supplemented with 1% (vol/vol) of standard gas oil and 0.01% tween80 surfactant at 35°C for 8 days. ■, pCom8; ■, pCom8: *ladA* α_{B23} , □, *ladA* β_{B23} , shade bar, *ladB* β_{B23} . **P* < 0.05 compared with control pCom8 only recombinant.

It is interesting that *ladB* β_{B23} has been misidentified to alkanesulfonate monooxygenase gene (Fig. 7) and showed the highest alkane degradation among the three genes. Moreover, *LadA* in *G. thermodenitrificans* NG80-2 had least identity with *LadA* γ_{B23} (22.7%) among these three genes (Fig. 8).

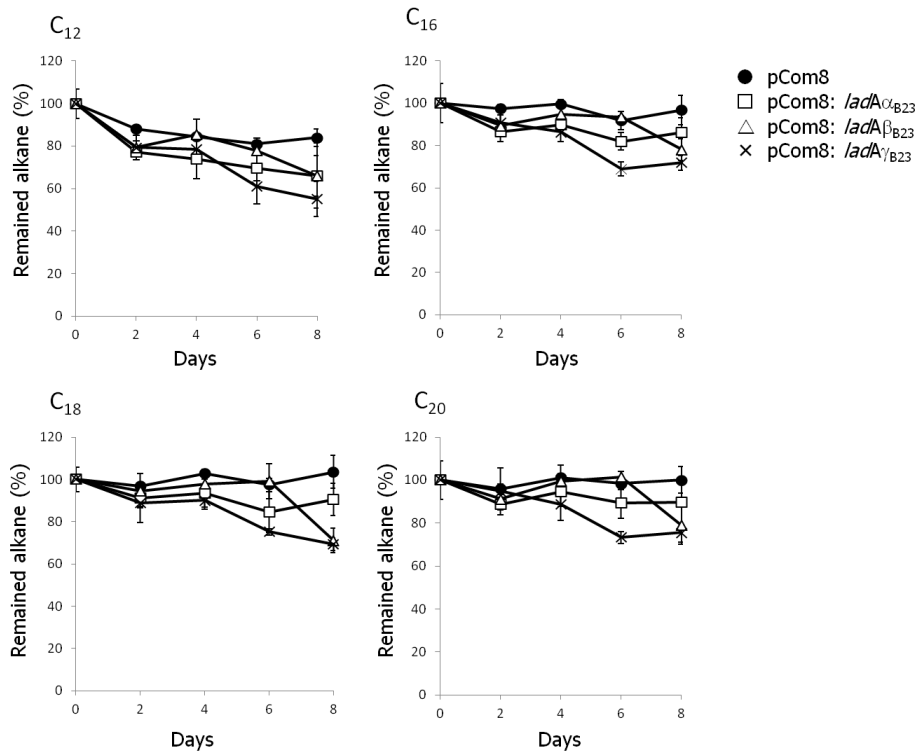


Fig. 16 Alkane degradation activity of *P. fluorescens* KOB2Δ1 recombinants containing pCom8 and pCom8 derivatives of *ladA* α _{B23}, *ladA* β _{B23} and *ladB*_{B23} on E₂ minimal salts medium supplemented with 1% (vol/vol) of standard gas oil and 0.01% tween80 surfactant at 35°C, showing degradation pattern on 0-8 days.

In conclusion, my experimental results from whole genome analysis, growth complementation test and alkane degradation test strongly suggested that *ladA* α _{B23}, *ladA* β _{B23} and *ladB*_{B23} are the most probable alkane monooxygenase genes in *G. thermoleovorans* B23. However, due to the presence of unknown secondary alkane degradation system and unsuitable condition for gene expression of thermostable enzymes in *P. fluorescens* KOB2Δ1, I next tried heterologous gene expression in *Geobacillus kaustophilus* MK93 which is a thermophilic bacterium and doesn't carry *alkB* and *ladA*-type genes in the genome of original strain HTA426.

CHAPTER III

Heterologous expression of $LadA_{\alpha B23}$ in *Geobacillus kaustophilus* MK93

3.1 Materials and methods

3.1.1 Strains and plasmids

Table 6 Strains and plasmids used in heterologous gene expression of $LadA_{\alpha B23}$ in *G. kaustophilus* MK93.

Strain or plasmid	Relevant characteristics (genotype)	Reference
Strain		
<i>E. coli</i> DH5 α	Cloning strain for pUC19	-
<i>E. coli</i> DH10B	Cloning strain for pGAM48	-
<i>E. coli</i> BR408	Cloning strain for methylation of <i>Geobacillus kaustophilus</i> HTA426 contained; 1) dam methylation system of <i>E. coli</i> ER1793, Δ dcm ; 2) pIR408 (<i>hsdMS</i> , p15A ori, Cm ^r) ; 3) conjugative promoting vector, pUB307 (RP1 ori, Km ^r , Tet ^r)	Suzuki <i>et al.</i> , 2012b
<i>Geobacillus kaustophilus</i> MK93	Mutant of <i>G. kaustophilus</i> HTA426 (Δ pyrF Δ pyrR GK707::P _{gk704} <i>bgaB</i>)	Suzuki <i>et al.</i> , 2013
Cloning and expression vectors		
pUC19	Cloning vector, Amp ^r	-
pGAM48	Integrative vector of <i>G. kaustophilus</i> HTA426 contained P _{gk704} , maltose promoter, GK707 and <i>pyr</i> of HTA426, and ori ^T , pUC ori and Ap ^r of <i>E. coli</i>	Suzuki <i>et al.</i> , 2012a
pGAM48: <i>ladA</i> α_{B23}	pGAM48 with <i>frd</i> and <i>ladA</i> α_{B23}	This study

3.1.1.1 *Geobacillus kaustophilus* MK93

G. kaustophilus MK93 has been constructed based on *G. kaustophilus* HTA426, which α -amylase gene (GK707) is inserted by β -galactosidase gene (*bgaB*) and maltose promoter of HTA426 (P_{gk704}), Δ pyrF Δ pyrR GK707::P_{gk704} *bgaB*) (Suzuki *et al.*, 2013). *G. kaustophilus* HTA426 is a thermophilic bacillus which was isolated from deep-sea sediment of the Mariana Trench. It grows between 42°C and 74°C with the optimum at 60°C (Takami *et al.*, 2004). The β -galactosidase gene (*bgaB*) is a marker for counter selection with X-gal. Orotidine-5'-phosphate decarboxylase genes (*pyrF* and *pyrR*) which are involved in uracil synthesis, were knockout for counter selection with 5-fluoroorotic acid (5-FOA). 5-FOA is non-toxic substance but after being digested with orotidine-5'-phosphate decarboxylase, product of the reaction, 5-fluorouracil, is high toxic to bacteria. Therefore, MK93 requires uracil for growth and is resistant to 5-FOA. Maltose promoter (P_{gk704}) was inserted in the upstream of *bgaB*, therefore the expression is inducible by maltose (Suzuki *et al.*, 2012a).



Fig. 17 Integrative site for integrative vector on *G. kaustophilus* MK93 chromosome with insertion of P_{gk704} and *bgaB* on α -amylase gene, *GK707*.

3.1.1.2 Integrative expression vector pGAM48

Vector pGAM48 is integrative expression vector for *G. kaustophilus* HTA426, originated from pRK2013. It contained P_{gk704} , maltose promoter, *GK707* and *pyr* of HTA426, and ori^T , pUC ori and ampicillin resistant gene (Amp^r) of *E. coli*. Multiple cloning site (MCS) of pUC19, *Hind*III to *Sph*I, and P_{gk704} , were inserted at *Hind*III-*Sph*I on *GK707*. The *pyrF* gene maker was inserted; therefore the recombinant of pGAM48 can grow in uracil-free medium and sensitive with 5-FOA (Suzuki *et al.*, 2012b).

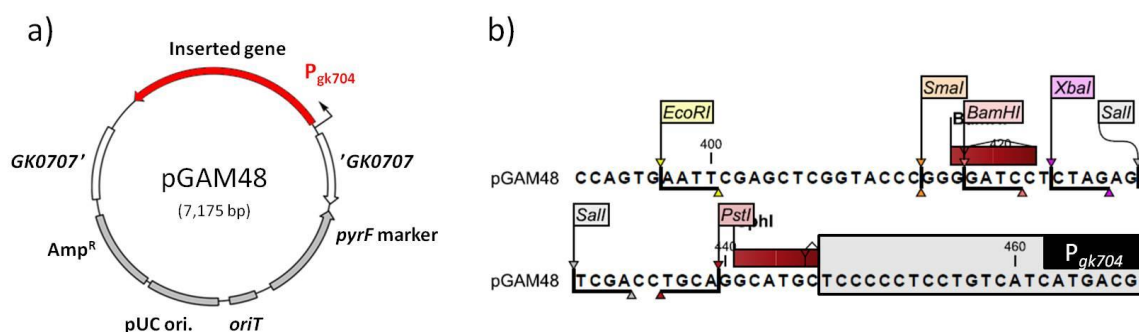


Fig. 18 Integrative expression vector pGAM48 (7,175 bp). a) Structure and genetic map of pGAM48. b) Multiple cloning site (MCS) in pGAM48 for the cloning of target genes.

3.1.1.3 *E. coli* BR408, strain for DNA methylation and conjugative plasmid transfer

E. coli BR408 is a cloning strain for methylating DNA specific for *G. kaustophilus* HTA426. It contains (1) DNA methylation system for HTA426, *dam*, Δdcm and plasmid pIR408 (*hsdMS*, p15A ori , Cm^r) (2) Conjugative promoting vector, pUB307 (RP1 ori , Km^r , Tet^r). *E. coli* BR408 was originated from *E. coli* K12 with Dcm (DNA cytosine methyltransferase) modification system deletion. Plasmid DNA which is introduced to *E. coli* BR408 will be methylated by modification system and cannot be degraded by restriction systems type IV, McrA, McrBC and Mrr and type II, M.AlwI and AlwI. *E. coli* BR408 harbors plasmid pIR408 which carries *hsdRMS* genes, type I restriction and modification system, of HTA426, allowing its DNA can be maintained in HTA426 cells (Suzuki *et al.*, 2012b).

(1) DNA methylation system for HTA426

DNA methylation is the mechanism which specific DNA sequences are methylated by addition of a methyl group to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. It consists of Restriction-Modification (R-M) systems. Foreign DNA with different modification or no methylation will be degraded by sequence-specific restriction enzymes and cleaved. Therefore, the methylation of own DNA can act as a part of cell immune system to protect bacteria from infection by alien DNA and bacteriophage. DNA methylation system of HTA426 is shown in Fig. 20.

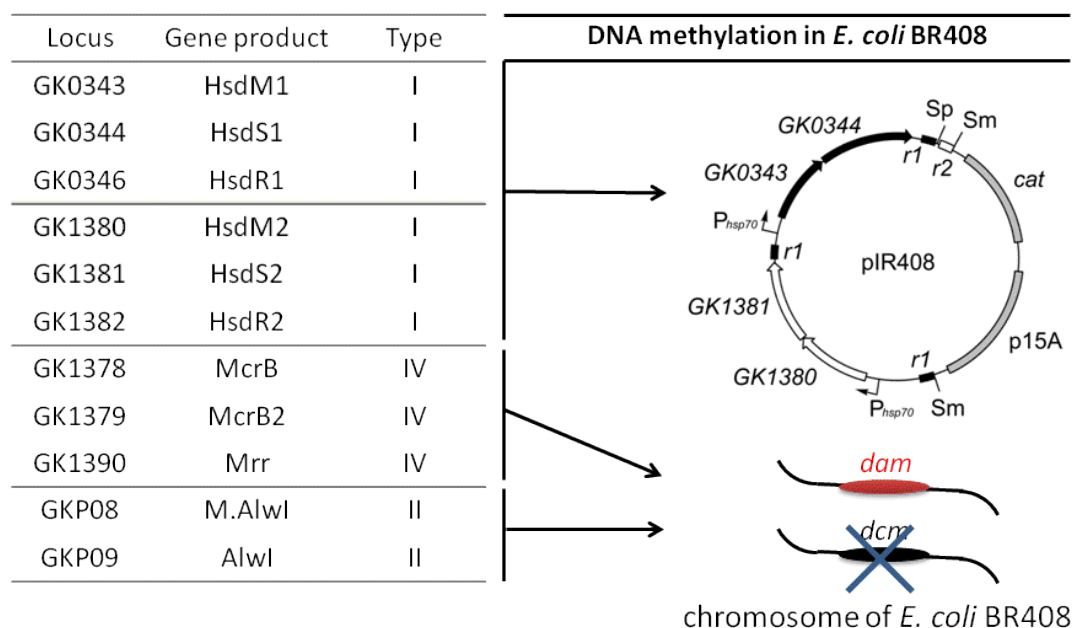


Fig. 19 DNA methylation system of *G. kaustophilus* HTA426 and *E. coli* BR408. R-M system type I HsdMSR (Host specificity for DNA), Hsd subunit for modification, specificity and restriction; R-M system type II AlwI, Dam (DNA adenine methyltransferase), Dcm (DNA cytosine methyltransferase); R-M system type IV McrABC (methyl-cytosine restricting system) and Mrr (methylated adenine recognition and restriction system)

(2) Conjugative promoting vector, pUB307

The helper plasmid pUB307 is a conjugative, broad-host-range plasmid which was originated from plasmid RP1. The plasmid pUB307 has been reported to be capable of promoting the transfer of gonococcal resistance plasmids from *E. coli* to *N. gonorrhoeae*. It can promote mobilization of large plasmid efficiently but pUB307 cannot be introduced or maintained in the host, even it contains *OriT*, because of its replication deficiencies (Piffaretti *et al.*, 1988).

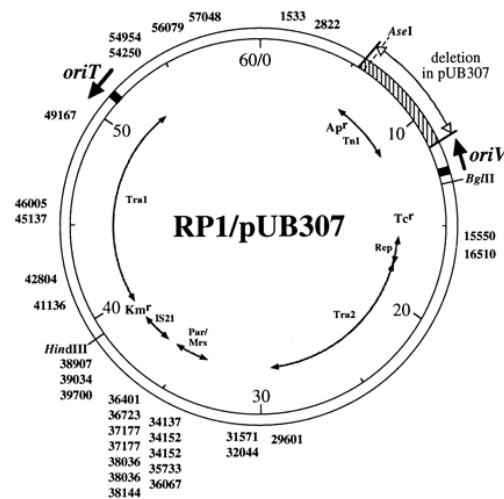


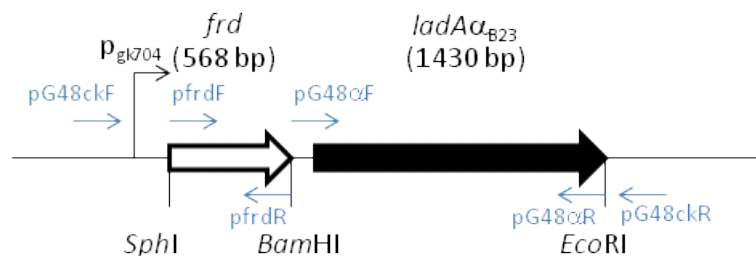
Fig. 20 Physical map of pUB307. (Hu & Derbyshire, 1998)

3.1.1.4 Primer design

LadA alkane monooxygenase is two component enzymes which needs FMN reductase for reducing flavin after reaction (Fig. 4). I found that HTA426 carried only flavin reductase (GK1652, substrate is flavin), but not FMN reductase (uses flavin mononucleotide as substrate). Therefore, the genes to be introduced in *G. kaustophilus* MK93 were *frd* (FMN reductase, ORF10) and *ladA* α_{B23} genes.

Table 7 Primers use in heterologous gene expression of *LadA* α_{B23} in *G. kaustophilus* MK93.

Genes	PCR length (bp)	Primer name	Sequence	Restriction site
<i>frd</i>	568	pfrdF	5' GTTGCATGCCTGATATCGTTATTCTCAACGGAAA 3'	<i>Sph</i> I
		pfrdR	5' GGGGATCCCTTAGTTTGTGAAACTTGGGAATAGCA 3'	<i>Bam</i> HI
<i>ladA</i> α_{B23} (include SD sequence)	1,430	pG48 α F	5' GGGGATCCGTCTTGAAGGGAGGGCAAAT 3'	<i>Bam</i> HI
		pG48 α R	5' GGGAATTCCTAGCCATCTGTTCTGTGAGC 3'	<i>Eco</i> RI
<i>frd</i> , <i>ladA</i> α_{B23} (for check crossover in MK93 chromosome)	2,129	pG48ckF	5' TGGAGATGAGTGGTGCAATC 3'	-
		pG48ckR	5' GTTTTCCCAGTCACGACGTT 3'	-



3.1.2 Cloning of *frd* and *ladA* α_{B23} alkane monooxygenase genes and construction of expression plasmid

Genomic DNA of B23 was prepared by Marmur's method (Marmur, 1961). The *frd* and *ladA* α_{B23} genes were amplified by C1000 TouchTM Thermal Cyclers (Bio-Rad, Hercules, CA) with KOD-Plus-Neo DNA polymerase (Toyobo, Kyoto, Japan) according to standard protocol from supplier. The primers used for PCR amplification of these genes were designed from genome sequences with modification of *Sph*I, *Bam*HI and *Eco*RI restriction sites (Table 7). PCR cycles were shown in Appendix C.4. In order to confirm genes sequences, the PCR fragments of *frd* and *ladA* α_{B23} genes were separately subcloned into *Hinc*II site of pUC19 and transformed to *E. coli* DH5 α by heat shock method (Inoue *et al.*, 1990). The nucleotide sequence was determined by BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA) and confirmed no error was contained in the PCR products. The recombinant pUC19: *frd* plasmid was extracted and digested by restriction enzymes, *Sph*I and *Bam*HI while the recombinant pUC19: *ladA* α_{B23} plasmid was digested by *Bam*HI and *Eco*RI. The *frd* and *ladA* α_{B23} fragments were purified by gel electrophoresis. DNA was extracted from gel and kept at -20°C. The *frd* fragment was ligated to pGAM48 and cloned into *E. coli* DH10B by electroporation. The recombinant pGAM48: *frd* plasmid was extracted and digested by restriction enzymes, *Bam*HI and *Eco*RI, then ligated with *ladA* α_{B23} fragment (Fig. 22). The recombinant plasmid was cloned into *E. coli* DH10B for amplification. Due to different DNA R-M system of *E. coli* and *G. kaustophilus* HTA426, the recombinant of plasmid pGAM48 from *E. coli* DH10B needed to be cloned into *E. coli* BR408 for DNA methylation (Fig. 20) before transconjugated to *G. kaustophilus* MK93. The recombinant vector pGAM48: *frd/ladA* α_{B23} was cloned into *E. coli* DH10B by electroporation according to standard protocol of Bio-Rad manual (USA). The recombinant plasmid was introduced into *G. kaustophilus* MK93 by conjugative DNA transfer and gene integration (Suzuki *et al.*, 2013). Protocols are available in Appendix C.4.

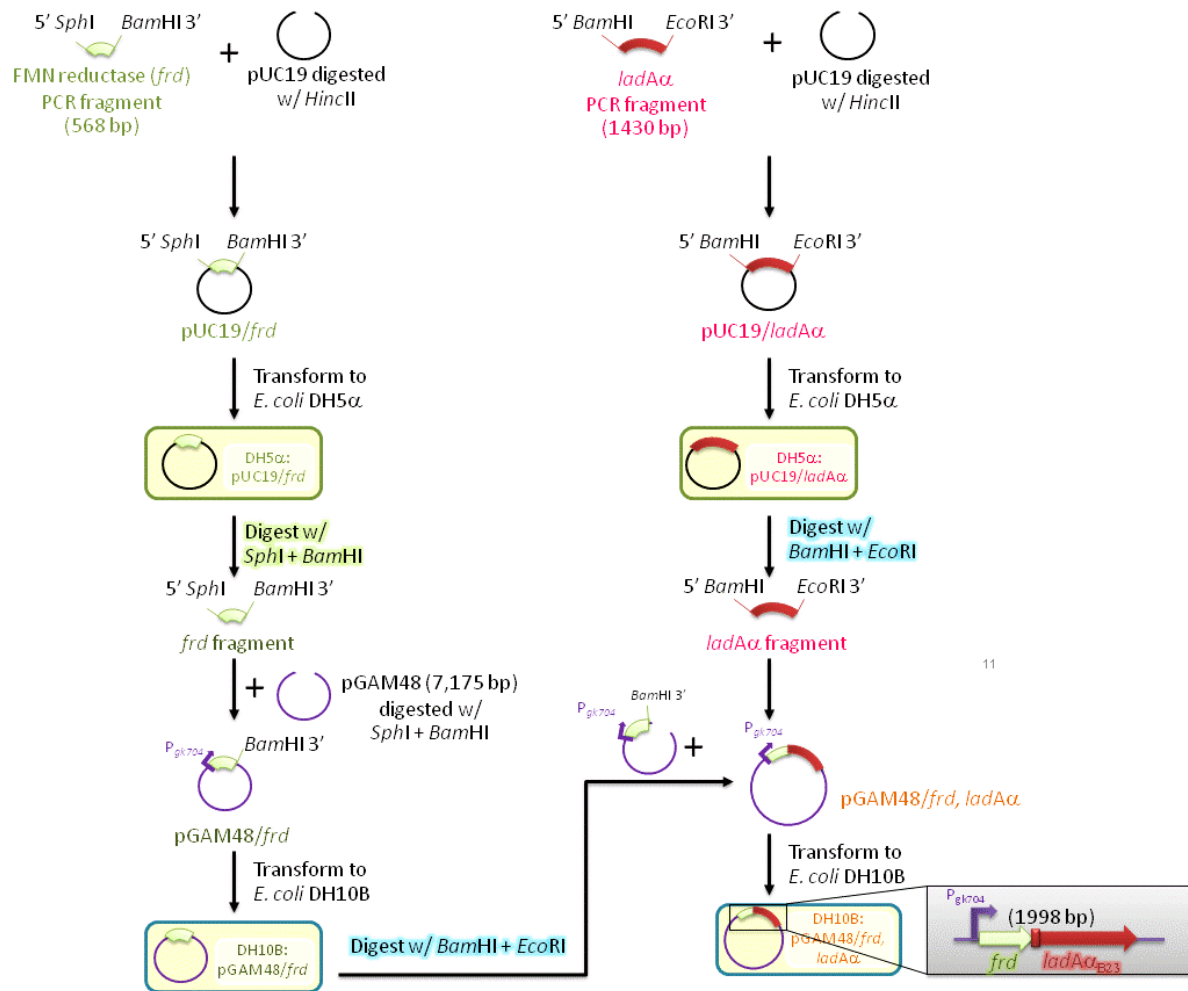


Fig. 21 Construction of pGAM48 recombinant with *frd* and *ladAα_{B23}* genes.

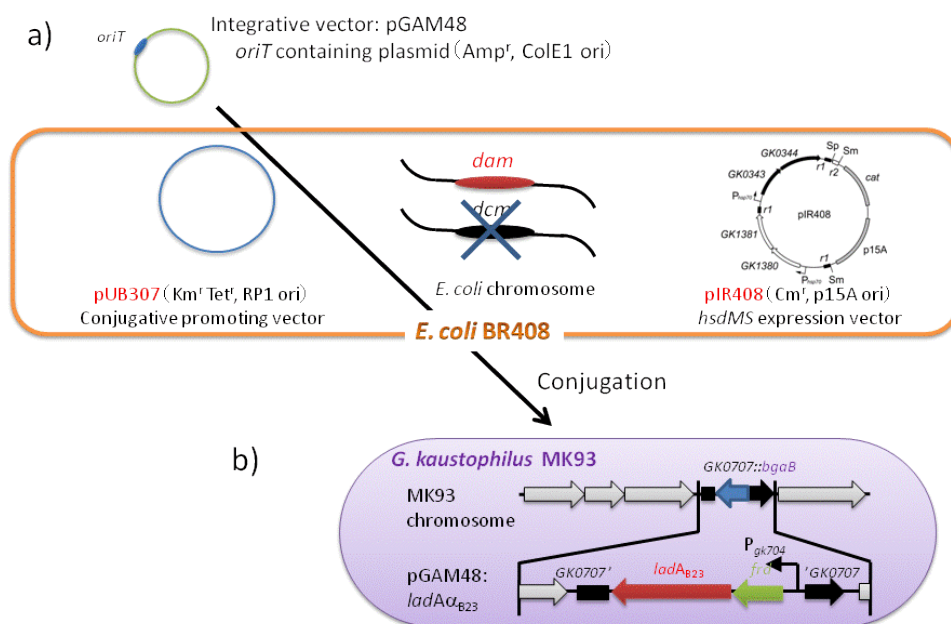


Fig. 22 a) DNA methylation system *E. coli* BR408, b) single crossover of *frd* and *ladAα_{B23}* genes to chromosome of *G. kaustophilus* MK93.

3.1.3 Alkane degradation test

Objective of this experiment is to determine suitable condition of alkane degradation for future use. Alkane degradation test was performed on 10 ml of Minimal salts medium for MK93 (Suzuki *et al.*, 2013) supplemented with 1% (vol/vol) of *n*-eicosane (C20 alkane) in 100 ml glass bottle in triplicate. Maltose was added to final concentration of 1% (vol/vol) to induce maltose promoter (P_{gk704}). 1% (vol/vol) of glucose was used as negative control. 0.01% (vol/vol) of tween80 surfactant was added to test whether surfactant affect on enzyme activity. The cultures were incubated at 70°C for 3 days. Ten ml of whole culture supernatant was extracted by 10 ml acetone: hexane (1:1). After centrifuge at 30,000xg 20 min, the amount of remaining alkanes in the samples was directly analyzed by gas chromatography. A portion (1 μ l, split ratio 50:1) of the sample was analyzed by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 μ m x 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan, GC/MS). Helium was used as carrier gas at a flow rate 25 ml/min. Protocols are available in Appendix C.4.

3.2 Results and discussion

G. kaustophilus MK93 recombinants which harboring *frd/ladA* α_{B23} genes were successfully constructed. The four strains, M1, M3, M10 and L5, were resistant to 5-FOA and could not grow on uracil free medium, suggested that they lost pGAM48 plasmid. All recombinants formed white colonies on MMS containing x-gal indicated that they lost the β -galactosidase gene maker. Colony PCR also confirmed that *frd/ladA* α_{B23} genes were successfully crossover into chromosome of MK93 tandemly (Fig. 22).

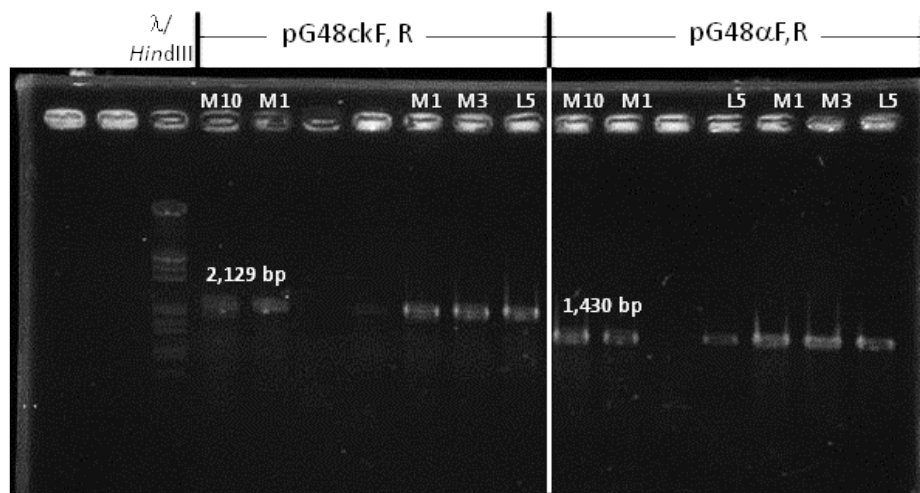


Fig. 23 Colony PCR of *G. kaustophilus* MK93 recombinants M1, M3, M10 and L5 showing *frd/ladA* α_{B23} fragment (2,129 bp) amplified by primer pGK48ckF, R (left) and *ladA* α_{B23} gene (1,430 bp) amplified by primer pG48αF, R (right).

Alkane degradation activity of recombinant strain M1, harboring *frd* and *ladA*_{B23} genes on Minimal salts medium supplemented with 1% (vol/vol) of *n*-eicosane (C20 alkane) at 70°C for 3 days shown in Fig. 24. Surprisingly, the host cell, *G. kaustophilus* MK93, could degrade *n*-eicosane (26.01% and 34.92% in presence of glucose and maltose, respectively, without addition of tween80), despite without both *alkB* and *ladA*-type genes on the chromosome. The result suggested that there is other unknown long-chain alkane degradation system in *G. kaustophilus* MK93. Tween80 surfactant clearly showed repression effect on alkane degradation especially in medium containing glucose. It might occur because the unknown long-chain alkane degradation system doesn't have substrate specificity and prefer to utilize oleyl-chain in the fatty acid moiety of tween80. However, *ladA*_{B23} gene from B23 was successfully expressed and actively functioned in recombinant M1. In absence of tween 80, it degraded 58.57% of *n*-eicosane in medium containing 1% (vol/vol) of maltose, better than MK in same condition by 1.68 fold.

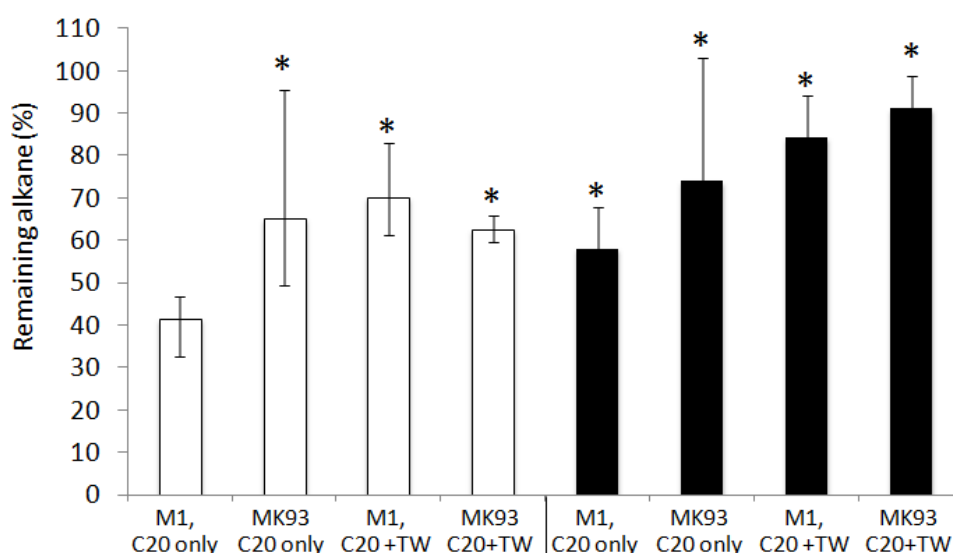


Fig. 24 Remaining alkane after cultivated host *G. kaustophilus* MK93 and recombinant M1, harboring *frd* and *ladA*_{B23} genes on Minimal salts medium (Suzuki *et al.*, 2013) supplemented with 1% (vol/vol) of *n*-eicosane (C20 alkane) at 70°C for 3 days. □, medium containing 1% (vol/vol) of maltose; ■, medium containing 1% (vol/vol) of glucose. **P* < 0.05 compared with the positive recombinant M1 on medium containing 1% (vol/vol) maltose without tween80.

CHAPTER IV

Conclusion

This is first report of multiple *ladA* alkane monooxygenase homologs in chromosome of *Geobacillus* bacteria. While *ladA* alkane monooxygenase gene exists in *G. thermodenitrificans* NG80-2 as a single copy on plasmid, whole genome analysis of *G. thermoleovorans* B23 revealed three *ladA*-type gene homologs, *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} , formed a cluster called '*ladAB* gene island' on B23 chromosome. Moreover, a FMN reductase gene (ORF10) which provides reduced riboflavin molecule as a prosthetic group of the monooxygenase enzyme, also found in the upstream of *ladA*-type genes (Fig. 9). The *ladA* gene of NG80-2 is flanked by two transposases, IS21 family, which indicated that it's an alien gene and moved into the current position on the plasmid by the two IS21 elements. The two IS21 elements were still intact, implied that incident was recent event (Feng *et al.*, 2007). Unlike NG80-2, there's no transposase located at upstream and downstream region of the *ladAB* gene island and the cluster was found on the chromosome of phylogenetically distant *Geobacillus* strains, suggested its universality and common in the genus *Geobacillus*. *LadA* α_{B23} and *LadA* β_{B23} alkane monooxygenase are definitely formed a distinct clade in phylogenetic tree based on deduced amino acid sequences; however, *LadB* β_{B23} alkane monooxygenase is clearly shared evolutionary lineage with alkanesulonate monooxygenase (SsuD). Evolutionary trait of *alkB*-type and *ladA*-type alkane monooxygenase genes in *Geobacillus* still unclear but it is possible that the *LadA/B* family had evolved from the SsuD family. Even though, SsuD is a common enzyme in bacteria, there is no report for the *ladAB* gene island except in *Geobacillus* bacteria. It suggests the *LadA/B* family has unique evolution mechanism which might be provided by extremely habitats of the genus while *alkB*-type genes were originated from mesophilic bacteria. Genome sequence alignment between closely related *Geobacillus* indicated that the *ladAB* gene island was found on the chromosome of several *Geobacillus* strains, suggested its common and wide distribution in the genus.

While the G+C mol% of the *ladA* of *G. thermodenitrificans* NG80-2 is unusually low 34.8% (whole genome 49.0%), and G+C mol% of *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} of B23 are 52.9%, 53.0% and 53.3%, respectively (whole genome 52.3%), the G+C mol% of *ladA* homolog of *Amycolicococcus subflavus* DQS-9A1 is 60.0% (whole genome 62.24%) (Nie *et al.*, 2013). These data suggests that there is high variety of *LadA/B* family in extremophilic bacteria.

Alkane degradation activities of *LadA* α_{B23} , *LadA* β_{B23} and *LadB* β_{B23} were successfully detected from gene expression in *P. fluorescens* KOB2 Δ 1. Only heterologous expression of *ladA* α_{B23} gene has been performed in *G. kaustophilus* MK93, however, the alkane degradation activity of *LadA* α_{B23} was clearly detected. In the conclusion, the results from whole genome analysis, heterologous gene expression in *P. fluorescens* KOB2 Δ 1 and *G. kaustophilus* MK93 indicated that *ladA* α_{B23} , *ladA* β_{B23} and *ladB* β_{B23} genes are functional alkane monooxygenase genes for *G. thermoleovorans* B23 to degrade broad range alkanes.

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To all of my labmates and friends in Japan and Thailand, thank you so much for their encouragements and supports. Last, I would like to thank to my family who always inspirits my life.

APPENDICES

A. REAGENTS AND BUFFERS

TAE buffer (40mM Tris acetate, 1mM EDTA)

50x stock solution

Tris base	242	g/l
Glacial acetic acid	57.1	ml/l
0.5M EDTA pH 8.0	100	ml

Working solution: 1x TAE buffer; 20 ml 50x TAE, 980 ml MilliQ

TBE buffer (45mM Tris borate, 1mM EDTA)

5x stock solution

Tris base	54	g/l
Boric acid	27.5	g/l
0.5M EDTA pH 8.0	20	ml

Working solution: 0.5x TBE buffer; 100 ml 5x TBE, 900 ml MilliQ

1M Tris Cl

Dissolve 121.1 g Tris base in 800 ml MilliQ, adjust pH 7.4 or 7.6 or 8.0 by conc. HCl. Add MilliQ to 1 liter.

0.5M EDTA pH 8.0

Dissolve 186.1 g di-Sodium EDTA in 800 ml MilliQ, adjust pH 8.0 with NaOH (~20g of NaOH pellet). Add MilliQ to 1 liter and autoclave.

Tris EDTA (TE), autoclave

TE pH 7.4; 100 mM Tris Cl pH 7.4, 10 mM EDTA pH 8.0

TE pH 7.6; 100 mM Tris Cl pH 7.6, 10 mM EDTA pH 8.0

TE pH 8.0; 100 mM Tris Cl pH 8.0, 10 mM EDTA pH 8.0

3M Sodium acetate

Dissolve 408.3 g sodium acetate.3H₂O in 800 ml MilliQ, adjust pH 5.2 or 7.0 with glacial acetic. Add MilliQ to 1 liter and autoclave.

TB buffer

MnCl ₂ .4H ₂ O	10.88	g/l
CaCl ₂ .2H ₂ O	2.2	g/l
KCl	18.65	g/l
PIPES (0.5 M, pH 6.7)	20	ml/l

Add MilliQ to 1 liter and autoclave.

B. MEDIA

LB medium

Tryptone	10	g/l
Yeast extract	5	g/l
NaCl	10	g/l

SOB medium (Inoue *et al.*, 1990)

Tryptone	20	g/l
Yeast extract	5	g/l
NaCl	0.5	g/l
2M KCl	1.25	ml/l

Adjust pH 7.0 by 5N NaOH (~0.2 ml), autoclave and then add 10 ml of 1M MgCl₂ to 1 liter medium.

SOC medium (Inoue *et al.*, 1990)

Add 1M sterile glucose solution into SOB medium, final concentration of 20 mM glucose.

E2 minimal salts medium (Lageveen *et al.*, 1988)

NaNH ₄ HPO ₄ ·4H ₂ O	3.5	g/l
K ₂ HPO ₄ ·3H ₂ O	7.5	g/l
KH ₂ PO ₄	3.7	g/l
0.1 M MgSO ₄ ·7H ₂ O	10	ml/l
Microelement stock solution	1	ml/l

Microelement stock solution

FeSO ₄ ·7H ₂ O	2.78	g/l
MnCl ₂ ·4H ₂ O	1.98	g/l
CoSO ₄ ·7H ₂ O	2.81	g/l
CaCl ₂ ·2H ₂ O	1.47	g/l
CuCl ₂ ·2H ₂ O	0.17	g/l
ZnSO ₄ ·7H ₂ O	0.29	g/l

Minimal salt medium for MK93 (Suzuki *et al.*, 2013)

D-glucose	10	g/l
Casamino acids (Difco)	1	g/l
Na ₂ HPO ₄ ·12H ₂ O	2.5	g/l
NH ₄ Cl	1	g/l
K ₂ SO ₄	0.3	g/l
MgSO ₄ ·7H ₂ O	0.4	g/l
1 M Tris-HCl	10	ml/l
Trace element stock solution	1	ml/l

Trace element stock solution (100 ml)

MnCl ₂ ·4H ₂ O	0.3	g
CaCl ₂ ·2H ₂ O	0.5	g
FeCl ₃ ·6H ₂ O	0.7	g
CuSO ₄ ·5H ₂ O	0.02	g
ZnSO ₄ ·7H ₂ O	0.04	g
H ₃ BO ₃	0.001	g
CoCl ₂ ·6H ₂ O	0.005	g
NiCl ₂ ·6H ₂ O	0.001	g
EDTA	0.025	g

C. PROTOCOLS

1. Cloning of *ladA* alkane monooxygenase-type gene homologs and construction of expression plasmids

Protocol outline

- 1.1) PCR amplification
- 1.2) Transformation of *E. coli* DH5 α by pUC19 using heat shock method
 - 1.2.1) Preparation of competent *E. coli* (modified from QIAGEN protocol)
 - 1.2.2) Preparation of ligation mixture
 - 1.2.3) Transformation by heat shock method
- 1.3) DNA sequencing
- 1.4) Transformation of *E. coli* DH10B by pUC19 using electroporation
 - 1.4.1) Preparation of pCom8 and derivatives
 - 1.4.2) Preparation of electrocompetent cells of *E. coli* DH10B
 - 1.4.3) Transformation of *E. coli* DH10B by electroporation
- 1.5) Transformation of *P. fluorescens* KOB2 Δ 1 by pCom8 using electroporation
 - 1.5.1) Preparation of pCom8 vector and derivatives
 - 1.5.2) Preparation of electrocompetent cells of *P. fluorescens* KOB2 Δ 1
 - 1.5.3) Transformation of *P. fluorescens* KOB2 Δ 1 by electroporation

1.1) PCR amplification

Genomic DNA of B23 was prepared using Marmur's method (Marmur 1961). The *ladA* α _{B23}, *ladA* β _{B23} and *ladB*_{B23} genes were amplified by C1000 TouchTM Thermal Cycler (Bio-Rad, USA) with KOD-Plus-Neo DNA polymerase (Toyobo, Japan) according to standard protocol of KOD-Plus-Neo DNA polymerase. The primers used for PCR amplification of these genes were designed from genome sequences with modification of *Nde*I and *Sma*I restriction sites (Table 4). PCR mixture was purified by gel electrophoresis. The DNA bands which were expected size were cut and extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan). DNA concentration was determined by spectrophotometer at wavelength 260 nm for double strand DNA. Thus, an absorbance (A_{260}) of 1 corresponds to a concentration of 50 μ g/ml of double strand DNA.

PCR cycle

PCR step	p8 α (1.4 kb)	p8 β (1.3 kb)	p8 γ (1.1 kb)
Pre-denature	-----94°C, 2 min-----		
Denature	-----98°C, 10 sec-----		
Annealing	53°C, 30 sec	59°C, 30 sec	55°C, 30 sec
Extension	68°C, 45 sec	68°C, 45 sec	68°C, 45 sec

1.2) Transformation of *E. coli* DH5 α by pUC19 using heat shock method

In order to confirm genes sequences, the PCR fragments of *ladA* α _{B23}, *ladA* β _{B23} and *ladA* γ _{B23} genes were cloned at *Sma*I site of pUC19 and transformed to *E. coli* DH5 α by heat shock method (Inoue *et al.*, 1990).

1.2.1) Preparation of competent *E. coli* (modified from QIAGEN protocol)

1. *E. coli* DH5 α cells was streaked on LB agar plate and incubated at 37°C overnight.
2. Single bacterial colony was picked and inoculated to 10 ml LB broth, incubate at 37°C overnight.
3. Seed culture 1 ml was added to 100 ml pre-warmed 37°C SOB medium (appendix 2), incubate at 37°C until OD₆₀₀ reach 0.7 – 0.8 (1-2h).
4. Competence culture was kept on ice for 5-10 min.
5. The culture was transferred to sterile-centrifuge tube and centrifuged at 4°C 7200 rpm (4000xg) for 5 min.
6. Supernatant was discarded. Remained pellet was gently resuspended in 30 ml of cold 4°C TB buffer (appendix 1). Cell suspension was kept on ice for 5-10 min then centrifuged at 4°C 7200 rpm (4000xg) 5 min.
7. Supernatant was discarded. Remained pellet was gently resuspended in 4 ml of cold 4°C TB buffer. Cell suspension was kept on ice for 5-10 min then centrifuged at 4°C 7200 rpm (4000xg) 5 min. Repeat step 7 twice.
8. In order to prepare frozen stocks of competent cells (Molecular Cloning, 3rd ed Protocol 23 p. 1.109), 140 μ l of DMSO was added to 4 ml of resuspend cells, mixed and kept on ice for 15 min. Additional 140 μ l of DMSO was added, mixed and kept on ice 15 min.
9. Cell suspension was aliquot to chilled sterile tubes then immediately snap-freeze by store tubes at -70°C until needed. Final concentration of DMSO was 7%.

1.2.2) Preparation of ligation mixture

Plasmid pUC19 was extracted from *E.coli* host by QIAprep Spin Miniprep Kit (QIAGEN, USA) and completely digested by *Sma*I and purified by gel electrophoresis. The DNA bands which were expected sizes of pUC19 (2,686 bp), *ladA* α _{B23} fragment (1,392 bp), *ladA* β _{B23} fragment (1,314 bp) and *ladA* γ _{B23} fragment (1,119 bp) were cut and kept in 1.5 ml Eppendorf tube. DNA was extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan). DNA ligation was performed by DNA Ligation Kit Ver.2.1 (Takara, Japan). DNA mixture and ligation has been done according to standard protocol from supplier. Concentration of insert DNA and plasmid and their size affects on ligation step, therefore calculation of concentration and size of each DNA species is necessary. For most standard cloning, vector: insert ratio is 1:3 molar. Total DNA in ligation reaction should be around 100 ng in 10 μ l reaction.

$$\text{Concentration of insert (ng/ml)} = \frac{\text{Concentration of vector (ng/ml)} \times \text{Size of insert (kb)} \times 3}{\text{Size of vector (kb)}}$$

Restriction enzyme digestion

*Sma*I (TOYOBO, Japan): Total reaction 50 μ l

Plasmid DNA	30	μ l
10x <i>Sma</i> I buffer	5	μ l
<i>Sma</i> I	2.5	μ l
MilliQ	12.5	μ l
Incubate at 30°C for overnight		

1.2.3) Transformation by heat shock method (Inoue *et al.*, 1990)

1. 100 μ l of cold competent cells was mixed with 40-50 ng DNA mixture at 4°C and kept on ice for 5-10 min, gently mixed every 2-3 min.

2. Heat shock was performed by put cell-DNA mixture tube in water bath 42°C for 45 sec then immediately added 1 ml SOC medium to the mixture and transferred whole culture to 15 ml culture tube.
3. The culture was grown on SOC medium at 37°C for 45 min. Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 100 µg/ml ampicillin, 0.01 M isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.2% x-gal, incubate at 37°C overnight. White colonies were selected and restreak on the same selective medium.
4. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and PCR fragment insertion was confirmed by colony PCR and restriction enzymes digestion, *EcoRI* and *HindIII*, then run gel electrophoresis. The sequence fidelity was checked by DNA sequencing method.

Restriction enzyme digestion

EcoRI and *HindIII* (TOYOBO, Japan) : Total reaction 10 µl

Plasmid DNA	2	µl
H buffer	1	µl
<i>EcoRI</i>	0.25	µl
<i>HindIII</i>	0.25	µl
MilliQ	6.5	µl

Incubate at 37°C for 1-1.5 h.

1.3) DNA sequencing

The nucleotide sequence was determined by BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3100 DNAsequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA), according to protocol from supplier. Anyway, DNA purification and precipitation are necessary after PCR step.

Purification of extension products for sequencing

1. Reaction was prepared as follow,

Big Dye product	10 µl
3M Sodium acetate	2 µl
20 mg/ml glycogen	1 µl
MilliQ	10 µl

2. The mixture was mixed well. Fifty microliter of 100% ethanol was added, mixed and kept in dark at room temperature for 5-10 min.
3. Samples were centrifuged at 15,000 rpm; room temperature for 10 min. Supernatant was completely discarded.
4. One hundred microliter of 70% ethanol was added, and then centrifuged at 15,000 rpm; room temperature for 10 min. Supernatant was completely discarded.
5. Samples were wrapped by aluminum foil to keep in dark and dried in vacuum chamber for 20-30 min/ or kept in 37°C 15 min. Samples can keep in -20°C for a while after this step.
6. Twelve microliter of Hi-Di-formamide was added before applied to DNA sequencer.

1.4) Transformation of *E. coli* DH10B by pCom8 using electroporation

1.4.1) Preparation of pCom8 and derivatives

Plasmid pCom8 and pUC19 which harboring the three genes, *ladA* α_{B23} , *ladA* β_{B23} and *ladA* γ_{B23} genes, were extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and completely digested by restriction enzymes *Nde*I and *Sma*I. The mixture after digestion was purified by gel electrophoresis. The DNA bands which were expected sizes of pCom8 (7,639 bp), *ladA* α_{B23} fragment (1,392 bp), *ladA* β_{B23} fragment (1,314 bp) and *ladA* γ_{B23} fragment (1,119 bp) were cut and kept in 1.5 ml Eppendorf tube. DNA was extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan) and ligated by DNA Ligation Kit Ver.2.1 (Takara, Japan). Vector: insert ratio was 1:3 molar.

Double restriction enzyme digestion

*Nde*I and *Sma*I (TOYOBO, Japan): Total reaction 200 μ l

Plasmid DNA	10	μ l
10x BSA	20	μ l
10x T buffer	20	μ l
<i>Nde</i> I	10	μ l
MilliQ	140	μ l

Incubate at 37°C for 2 h. Then add *Sma*I 10 μ l and incubate overnight (for complete digestion)

1.4.2) Preparation of electrocompetent cells of *E. coli* DH10B (Bio-Rad manual, USA)

1. *E. coli* DH10B cells was streaked on LB agar plate and incubated at 37°C overnight.
2. Single bacterial colony was picked and inoculated to 5 ml LB broth, incubate at 37°C overnight.
3. Seed culture 5 ml was added to 500 ml pre-warmed 37°C LB broth, incubate at 37°C until OD₆₀₀ reach 0.5-0.7.
4. Competence culture was chilled on ice for 20 min.
5. The culture was transferred to sterile-centrifuge tube and centrifuged at 4°C 7200 rpm (4000xg) for 15 min.
6. Supernatant was discarded. Remained pellet was gently resuspended in 500 ml of iced-cold 10% glycerol. Cell suspension was centrifuged at 4°C 7200 rpm (4000xg) 15 min.
7. Supernatant was discarded. Remained pellet was gently resuspended in 250 ml of iced-cold 10% glycerol. Cell suspension was centrifuged at 4°C 7200 rpm (4000xg) 15 min.
8. Supernatant was discarded. Remained pellet was gently resuspended in 20 ml of iced-cold 10% glycerol. Cell suspension was centrifuged at 4°C 7200 rpm (4000xg) 15 min.
9. Supernatant was discarded. Remained pellet was gently resuspended in 1-2 ml of iced-cold 10% glycerol.
10. Forty microliter of cell suspension was aliquot to chilled sterile tubes then immediately snap-freeze by store tubes at -80°C until needed.

1.4.3) Transformation of *E. coli* DH10B by electroporation (Bio-Rad manual, USA)

1. Forty microliter of competent cell and 100-500 ng DNA were mixed in chilled microcentrifuge tube and transferred to cold 0.1-cm cuvette.
2. After kept on ice 1 min, the cuvette was put into chamber. Set MicroPulser™ (BIORAD, USA) to EC1 mode (2.0kV, 200 Ω , 25 μ F) and electroporated. Then immediately added 1 ml SOC medium to the mixture and transferred whole culture to 15 ml culture tube.
3. The culture was grown on SOC medium at 37°C for 45 min. Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 10 μ g/ml gentamicin overnight. Transformants were selected and restreak on the same selective medium.

4. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and target genes were confirmed by colony PCR and restriction enzymes digestion, *EcoRI* and *HindIII*, then run gel electrophoresis.

1.5) Transformation of *P. fluorescens* KOB2Δ1 by pCom8 using electroporation

1.5.1) Preparation of pCom8 vector and derivatives

Plasmid pCom8 (empty vector) and derivatives which harboring *ladA*_{αB23}, *ladA*_{βB23} and *ladA*_{γB23} genes were extracted from *E. coli* DH10B by using QIAprep Spin Miniprep Kit (QIAGEN, USA)

1.5.2) Preparation of electrocompetent cells of *P. fluorescens* KOB2Δ1 (Højberg *et al.*, 1999)

1. *P. fluorescens* KOB2Δ1 was streaked on LB agar plate and incubated at 30°C overnight.
2. Single bacterial colony was picked and inoculated to 5 ml LB broth, incubate at 30°C overnight.
3. Seed culture 0.25 ml was added to 25 ml pre-warmed 30°C LB broth, incubate at 35°C until OD₆₀₀ reach 0.5-1.0.
4. The culture was transferred to sterile-centrifuge tube and centrifuged at 4°C 7200 rpm (4000xg) for 5 min.
5. Supernatant was discarded. Remained pellet was gently resuspended in 10 ml ice-cold glycerol-MOPS solution (15% (w/v) glycerol and 1mM 3-morpholinopropanesulfonic acid). Cell suspension was centrifuged at 4°C 7200 rpm (4000xg) 5 min. Repeat this step twice.
6. Remained pellet was gently resuspended in 200 μl ice-cold glycerol-MOPS solution. The electrocompetent cells was keep on ice and used within 2-3 h.

1.5.3) Transformation of *P. fluorescens* KOB2Δ1 by electroporation (Højberg *et al.*, 1999)

1. Forty microliter of competent cell and 100-500 ng DNA were mixed in chilled microcentrifuge tube and transferred to cold 0.2-cm cuvette.
2. After kept on ice 1 min, the cuvette was put into chamber. Set MicroPulser™ (BIORAD, USA) to EC2 mode (2.5kV/cm, 200Ω, 25 μF) and electroporated. Then immediately added 1 ml pre-warm SOC medium to the mixture and transferred whole culture to 15 ml culture tube.
3. The culture was grown on SOC medium at 35°C for 3 h. Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 100 μg/ml gentamicin, incubated at 35°C overnight. The culture plate was transferred to 30°C and incubated for 48 h. Transformants were selected and restreak on the same selective medium.
4. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and target genes were confirmed by colony PCR and restriction enzymes digestion, *EcoRI* and *HindIII*, then run gel electrophoresis.
5. Positives clones were maintained in LB broth containing 10% glycerol, keep at -80°C.

2. Growth complementation test of *P. fluorescens* KOB2Δ1 recombinants

1. *P. fluorescens* KOB2Δ1 recombinants containing pCom8 only (as negative control), pCom8:*ladA*_{B23}, pCom8:*ladA*_{B23} and pCom8:*ladB*_{B23} were streaked on LB agar plate and incubated at 30°C overnight.
2. Bacterial cells were collected from LB agar plate and suspended in E₂ minimal salts medium (Lageveen *et al.*, 1988; appendix 2) without carbon source, final concentration at OD₆₀₀ = 0.6.
3. The cells were inoculated to test medium, 5 ml E₂ minimal salts medium supplemented with 1% (vol/vol) of *n*-alkane (C12 or C16) and 0.01% tween80 at 2% (vol/vol) of test medium. Every experiment was done in triplicate. The cultures were incubated at 120 rpm, 30°C or 35°C for 8 days.
4. Growth of cultures was determined by total protein measurement using Pierce® BCA Protein Assay Kit (Thermo Scientific, USA), according to the standard protocol from supplier.

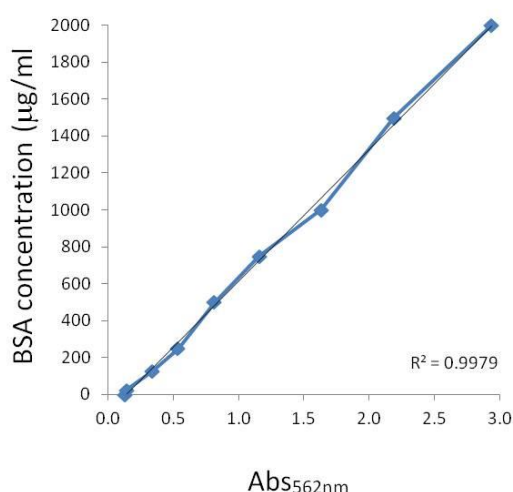


Fig. 25 Typical color response curves for bovine serum albumin (BSA) using the standard protocol.

3. Alkane degradation test of *P. fluorescens* KOB2Δ1 recombinants

1. *P. fluorescens* KOB2Δ1 recombinants containing pCom8 only (as negative control), pCom8:*ladA*_{B23}, pCom8:*ladA*_{B23} and pCom8:*ladB*_{B23} were streaked on LB agar plate and incubated at 30°C overnight.
2. Bacterial cells were collected from LB agar plate and suspended in 5 ml of E₂ minimal salts medium supplemented with 1% (vol/vol) of standard gas oil and 0.01% tween80 surfactant in 50 ml screw cap-centrifuge tube 18422CTF50 (IWAKI, Tokyo, Japan) in triplicate, final concentration at OD₆₀₀ = 0.6. The cultures were incubated at 120 rpm and 35°C for 8 days.
3. Five ml of whole culture supernatant was extracted by 5 ml acetone: hexane (1:1) and centrifuged at 30,000xg 20 min. The amount of remaining alkanes in the samples 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 x 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass

spectrometer (JEOL, Tokyo, Japan, GC/MS). Helium was used as carrier gas at a flow rate 25 ml/min.

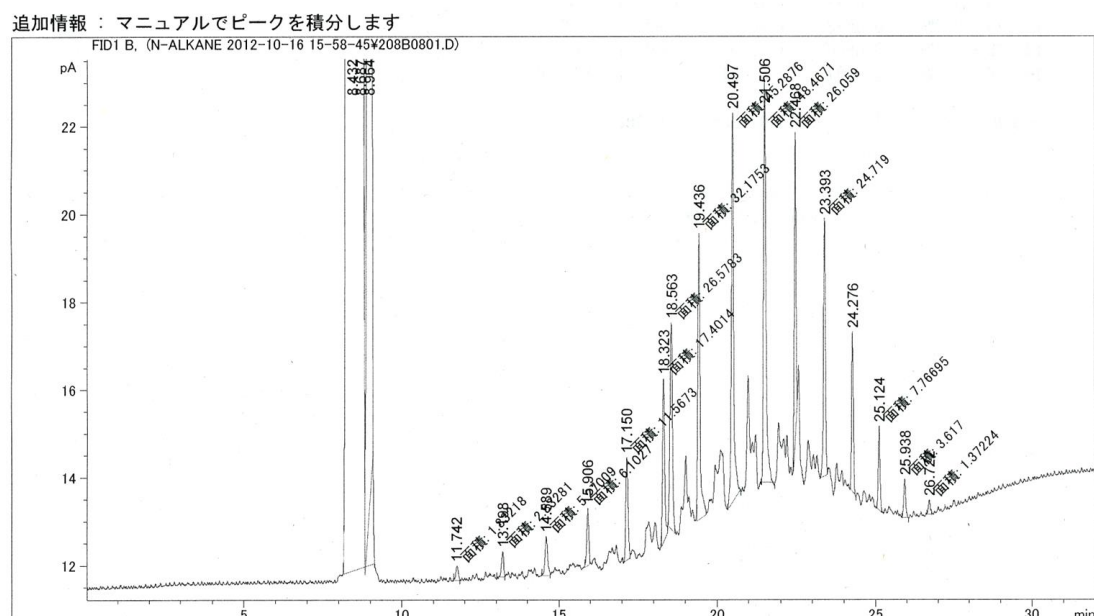


Fig. 26 Chromatogram of 1% standard gas oil by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 μ m x 30 m non-polar capillary HP-1 column and FID detector (GC/FID).

Table 8 Retention time and composition of 1% standard gas oil by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 μ m x 30 m non-polar capillary HP-1 column and FID detector (GC/FID).

Retention time	Alkane	Peak area	Conc. (ppm)	Composition (%)
11.742	C9	1.83218	1.38	0.7
13.198	C10	2.83281	2.13	1.1
14.589	C11	5.27009	3.97	2.1
15.906	C12	6.10270	4.59	2.5
17.150	C13	11.56726	8.70	4.6
18.323	C14	17.40138	13.09	7.0
19.436	C15	32.17530	24.21	12.9
20.497	C16	45.28764	34.08	18.2
21.506	C17	48.46705	36.47	19.5
22.468	C18	26.05902	19.61	10.5
23.393	C19	24.71899	18.60	9.9
24.276	C20	14.42159	10.85	5.8
25.124	C21	7.766950	5.84	3.1
25.938	C22	3.617000	2.72	1.5
26.722	C23	1.372240	1.03	0.6

4. Cloning of *frd* and *ladA* α_{B23} alkane monooxygenase genes and construction of expression plasmids

Protocol outline

- 2.1) Construction of pGAM48 recombinant with *frd* and *ladA* α_{B23} genes
 - 2.1.1) PCR amplification
 - 2.1.2) Construction of pUC19 recombinant with *frd* and *ladA* α_{B23} genes
 - 2.1.3) DNA sequencing
 - 2.1.4) Construction of pGAM48 recombinant with *frd* and *ladA* α_{B23} genes
 - 2.1.4.1) Transferring pGAM48: *frd* from *E. coli* DH5 α to *E. coli* DH10B by electroporation
 - 2.1.4.2) Transferring pGAM48: *frd*, *ladA* α_{B23} to *E. coli* DH10B by electroporation
- 2.2) Cloning of pGAM48: *frd*, *ladA* α_{B23} in *E. coli* BR408 for DNA methylation
- 2.3) Transconjugation to *G. kaustophilus* MK93
- 2.4) Crossover of *frd* and *ladA* α_{B23} to MK93 chromosome

4.1) Construction of pGAM48 recombinant with *frd* and *ladA* α_{B23} genes

4.1.1) PCR amplification

Genomic DNA of B23 was prepared using Marmur's method (Marmur 1961). The *frd* and *ladA* α_{B23} genes were amplified by C1000 TouchTM Thermal Cycler (Bio-Rad, USA) with KOD-Plus-Neo DNA polymerase (Toyobo, Japan) according to standard protocol of KOD-Plus-Neo DNA polymerase. The primers used for PCR amplification of these genes were designed from genome sequences with modification of *Sph*I, *Bam*HI and *Eco*RI restriction sites (Table 7). PCR mixture was purified by gel electrophoresis. The DNA bands which were expected size were cut and kept in 1.5 ml Eppendorf tube. DNA was extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan).

PCR cycle for *frd* and *ladA* α_{B23} genes

PCR step	pfrd (567 bp)	pG48 α (1430 bp)
Pre-denature	----- 94°C, 2 min -----	-----
Denature	----- 98°C, 10 sec -----	-----
Annealing	59°C, 30 sec	59°C, 30 sec
Extension	68°C, 30 sec	68°C, 45 sec

4.1.2) Construction of pUC19 recombinant with *frd* and *ladA* α_{B23} genes

Competent cells of *E. coli* DH5 α were prepared according to Inoue *et al.*, 1990 (p. 18). Plasmid pUC19 was extracted from *E. coli* host by QIAprep Spin Miniprep Kit (QIAGEN, USA) and completely digested by *Hinc*II and purified by gel electrophoresis. The DNA bands which were expected sizes of pUC19 (2,686 bp), *frd* fragment (568 bp) and *ladA* α_{B23} fragment (1,430 bp) were cut and kept in 1.5 ml Eppendorf tube. DNA was extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan). DNA ligation of

pUC19-*frd* and pUC19- *ladA* α_{B23} gene was performed by DNA Ligation Kit Ver.2.1 (Takara, Japan), according to standard protocol from supplier. Vector: insert ratio was 1:3 molar.

Restriction enzyme digestion

HincII (TOYOBO, Japan): Total reaction 50 μ l

Plasmid DNA	30	μ l
M buffer	5	μ l
<i>HincII</i>	2.5	μ l
MilliQ	12.5	μ l

Incubate at 30°C for overnight (for complete digestion)

DNA transformation has been done by heat shock method (Inoue *et al.*, 1990, p 19). Transformants were properly diluted with appropriated dilution and spread on LB agar supplemented with 100 μ g/ml ampicillin, 0.01 M isopropyl β -D-1-thiogalactopyranoside (IPTG) and 0.2% x-gal, incubate at 37°C overnight. White colonies were selected and restreak on the same selective medium. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and PCR fragment insertion was confirmed by colony PCR and restriction enzymes digestion, *EcoRI* and *HindIII*, then run gel electrophoresis. The sequence with no error was checked by DNA sequencing method.

4.1.3) DNA sequencing

The nucleotide sequence was determined by BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3100 DNAsequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA) and confirmed no error was contained in the PCR products (p. 20).

4.1.4) Construction of pGAM48 recombinant with *frd* and *ladA* α_{B23} genes

4.1.4.1) Transferring pGAM48: *frd* from *E. coli* DH5 α to *E. coli* DH10B by electroporation

The recombinant pUC19: *frd* vector and integrative vector pGAM48 were extracted from *E. coli* hosts and completely digested by *SphI* and *BamHI*. Purification has been done by gel electrophoresis. The DNA bands which were expected sizes of pGAM48 (7,175 bp) and *frd* fragment (568 bp) were cut and extracted from gel by QIAquick Gel Extraction Kit (QIAgen, Japan). DNA ligation of pGAM48-*frd* gene was performed by DNA Ligation Kit Ver.2.1 (Takara, Japan), according to standard protocol from supplier. Vector: insert ratio was 1:3 molar.

Double restriction enzyme digestion

SphI and *BamHI* (Takara, Japan): Total reaction 100 μ l

Plasmid DNA	10	μ l
10x K buffer	10	μ l
<i>SphI</i>	5	μ l
<i>BamHI</i>	5	μ l
MilliQ	70	μ l

Incubate at 37°C for overnight (for complete digestion)

The recombinant vector pGAM48: *frd* was cloned into *E. coli* DH10B by electroporation (Bio-Rad manual, USA; p. 21). Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 100 μ g/ml ampicillin, incubated at 37°C overnight. Transformant colonies were selected and restreak on the same

selective medium. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and PCR fragment insertion was confirmed by colony PCR and restriction enzymes digestion, *SphI* and *BamHI*, then run gel electrophoresis.

4.1.4.2) Transferring pGAM48: *frd*, *ladA* α_{B23} to *E. coli* DH10B by electroporation

The recombinant pGAM48: *frd* vector and the recombinant pUC19: *ladA* α_{B23} vector were extracted from *E. coli* hosts and completely digested by restriction enzymes, *BamHI* and *EcoRI*. Purification has been done by gel electrophoresis. The DNA bands which were expected sizes of pGAM48: *frd* (7,743 bp) and *ladA* α_{B23} fragment (1,430 bp) were cut and extracted from gel by QIAquick Gel Extraction Kit (QIAGEN, Japan). DNA ligation of pGAM48: *frd* and *ladA* α_{B23} gene was performed by DNA Ligation Kit Ver.2.1 (Takara, Japan), according to standard protocol from supplier. Vector: insert ratio was 1:3 molar.

Double restriction enzyme digestion

BamHI and *EcoRI* (Takara, Japan): Total reaction 100 μ l

Plasmid DNA	10	μ l
10x K buffer	10	μ l
<i>BamHI</i>	5	μ l
<i>EcoRI</i>	5	μ l
MilliQ	70	μ l

Incubate at 37°C for overnight (for complete digestion)

The recombinant vector pGAM48: *frd/ladA* α_{B23} was cloned into *E. coli* DH10B by electroporation (Bio-Rad manual, USA; p. 21). Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 100 μ g/ml ampicillin, incubated at 37°C overnight. Transformant colonies were selected and restreak on the same selective medium. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and PCR fragment insertion was confirmed by colony PCR and restriction enzymes digestion, *BamHI* and *EcoRI*, or *SphI* and *EcoRI* then run gel electrophoresis.

4.2) Cloning of pGAM48: *frd*, *ladA* α_{B23} to *E. coli* BR408 for DNA methylation

Electrocompetent cells of *E. coli* BR408 were prepared according to Bio-Rad manual (USA, p. 20). The recombinant vector pGAM48: *frd/ladA* α_{B23} was extracted from *E. coli* hosts by QIAprep Spin Miniprep Kit (QIAGEN, USA). Transformation has been performed according to standard protocol of Bio-Rad manual (USA, p. 21). Transformants were diluted with appropriated dilution and spread on LB agar supplemented with 12.5 μ g/ml chloramphenicol, 25 μ g/ml kanamycin and 50 μ g/ml ampicillin overnight. Transformants were selected and restreak on the same selective medium. Recombinant plasmid was extracted by QIAprep Spin Miniprep Kit (QIAGEN, USA) and target genes were confirmed by colony PCR.

4.3) Transconjugation to *G. kaustophilus* MK93

4.3.1) Preparation of *E. coli* BR408 culture (Donor)

E. coli BR408 recombinant which harboring pGAM48: *frd/ladA* α_{B23} was cultured on 5 ml LB broth supplemented with 12.5 μ g/ml chloramphenicol, 25 μ g/ml kanamycin and

50 µg/ml ampicillin and incubated at 37°C overnight. Cell was harvested and washed by 10 ml LB broth to remove antibiotics. Final cell concentration was OD₆₀₀ = 0.1.

4.3.2) Preparation of *G. kaustophilus* MK93 culture (Recipient)

G. kaustophilus MK93 seed culture was grown in 20 ml LB broth at 55°C overnight. Seed culture was added to 20 ml fresh LB broth at 0.1% (v/v) and incubated at 60°C until OD₆₀₀ reached 0.5. The culture was kept standing at room temperature until cool down around 35-37°C.

4.3.3) Transconjugation and screening

1. *E. coli* BR408 recombinant which harboring pGAM48: *frd/ladAα_{B23}* and *G. kaustophilus* MK93 cultures were mixed in ratio 1: 9, *E. coli* BR408 1 ml: *G. kaustophilus* MK93 9 ml.
2. The mix culture was filtrated through sterile 0.22 mm Ø membrane filter. Supernatant was discarded and the filter was pasted on LB agar plate without antibiotic (to keep MK93 survive). The plate was kept at 37°C overnight, *E. coli* BR408 should generate conjugation tube and transconjugation occurs in this step. Therefore, vibration during incubation should be avoided.
3. The membrane was washed in basal minimal salt medium (MMS; appendix 2) and then spread on uracil free-MMS agar containing 50 µg/ml x-gal, incubated at 60°C overnight. Blue colonies were collected and purified. The transconjugants which were grown on the medium were expected to contain pGAM48: *frd/ladAα_{B23}* as free plasmid in cells (*ura* prototroph and 5-FOA sensitive).
4. The transconjugants were confirmed presence of pGAM48: *frd/ladAα_{B23}* by testing on uracil free-MMS agar containing 50 µg/ml 5-FOA and 50 µg/ml x-gal, incubated at 60°C overnight. Positive clones which could not grow on the medium were selected and checked again by colony PCR.

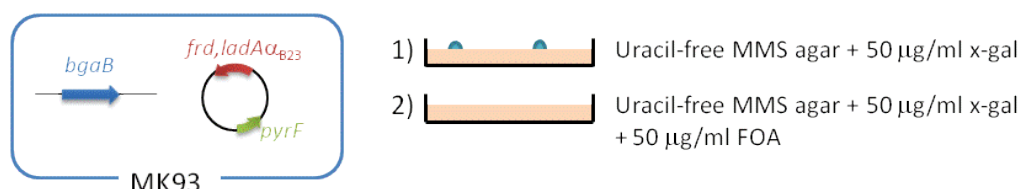


Fig. 27 pGAM48: *frd/ladAα_{B23}* presences as free plasmid in MK93. The transconjugants which contained *pyrF* marker on vector pGAM48 can grow on uracil-free medium (1) but sensitive to 5-FOA (2). Colonies were blue on medium containing x-gal because of β-galactosidase gene on chromosome of MK93.

4.4) Crossover of *frd* and *ladAα_{B23}* to MK93 chromosome

1. The transconjugants were cultured on 5 ml LB broth or 5 ml MMS broth supplemented with 10 µg/ml uracil and incubated at 60°C overnight for 2 generations. The integrative vector pGAM48 were expected to be lost and *frd/ladAα_{B23}* fragment should crossover to chromosome of MK93 at integrative site (*GK707::P_{gk704} bgaB*) in this step.
2. The 2 generations-culture was spread on MMS agar plate supplemented with 10 µg/ml uracil, 50 µg/ml 5-FOA and 50 µg/ml x-gal, incubated at 60°C overnight. White colonies were collected and purified. Positive clones need uracil for growth (*ura* auxotroph because

of loss of *pyrF* marker on pGAM48 vector) and presence as white colony (loss of β -galactosidase gene).

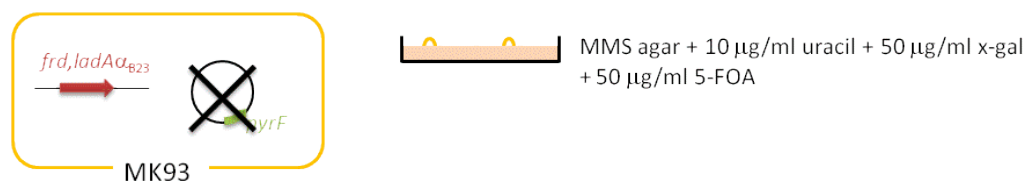


Fig. 28 pGAM48 loss and *frd/ladA α _{B23}* fragment integrated to MK93 chromosome. The transconjugants required uracil for growth and resistance to 5-FOA. Colonies were white on medium containing x-gal because β -galactosidase gene on chromosome of MK93 loses.

3. Positive clones were purified and confirmed insertion of *frd/ladA α _{B23}* fragment by colony PCR using primer pG48 α F, R and pG48ckF, R.

PCR cycle for pG48ckF, R

Pre-denature	94°C, 2 min
Denature	98°C, 10 sec
Annealing	60°C, 30 sec
Extension	68°C, 1 min 5 sec

5. Alkane degradation test of *G. kaustophilus* MK93 recombinant

1. *G. kaustophilus* MK93 (as negative control) and MK93 recombinant of *frd/ladA α _{B23}* genes were cultured on 250 ml Minimal salts medium for MK93 (Suzuki *et al.*, 2013) supplemented with 1% glucose and 10 μ g/ml uracil and incubated at 60°C overnight.
2. Bacterial cells were collected by centrifuge at 7200 rpm (4000xg) 10 min, washed with 400 ml sugar-free MMS broth supplemented with and 10 μ g/ml uracil twice and then resuspend with sugar-free MMS broth supplemented with and 10 μ g/ml uracil. Final cell concentration was adjusted to OD₆₀₀ = 0.6. Tween80 surfactant was added to final concentration of 0.01% in order to test whether it can increase alkane solubility.
3. 10% (vol/vol) glucose or 10% (vol/vol) maltose was added to final concentration of 1% (vol/vol) carbon source. Ten milliliter of cell cultures were aliquot to 100 ml glass bottle in triplicate, 10% of *n*-eicosane was added to final concentration of 1% (vol/vol) of alkane in medium. The cultures were incubated at 70°C for 3 days.
4. Ten ml of whole culture supernatant was extracted by 10 ml acetone: hexane (1:1) and centrifuged at 30,000xg 20 min. The amount of remaining alkanes in the samples was directly analyzed by gas chromatography. A portion (1 μ l, split ratio 50:1) of the sample was analyzed by GC system HP6890 (Agilent, Palo Alto, CA) with a 340 μ m x 30 m non-polar capillary HP-1 column and FID detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, Japan, GC/MS). Helium was used as carrier gas at a flow rate 25 ml/min.

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- Boonmak, C., Y. Takahashi, and M. Morikawa. 2013. Draft Genome Sequence of *Geobacillus thermoleovorans* B23. *Genome Announc.* 1(6): e00944-13.
- Boonmak, C., Y. Takahashi, and M. Morikawa. 2014. Gene cloning and expression of three LadA-type alkane monooxygenase homologs from an extremely thermophilic alkane-degrading *Geobacillus thermoleovorans* B23. *Extremophiles* (submitted).

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