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Analysis of the Alkane Hydroxylase Gene and Long-Chain Cyclic Alkane Degradation in *Rhodococcus*

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Abstract

To characterize the long-chain cyclic alkane (*c*-alkane) degradation of bacteria in *Rhodococcus*, we analyzed the relationship between the alkane hydroxylase gene (*alkB*) and long-chain *c*-alkane degradation in 19 species. Eleven strains which were isolated from nature using long-chain *c*-alkane as a substrate were identified as *R. erythropolis*, and all were shown to carry the *alkB* [*alkB* R2 type]. This gene type was also carried by two other species, *R. rhodochrous* and *R. baikonurensis*. In total, 17 species of the genus *Rhodococcus* carried *alkB*, but the gene types differed from each other. The two species *R. rhodnii* and *R. coprophilus* did not carry *alkB*, and their long-chain *c*-alkane degradation levels were low.

Keywords

Alkane Hydroxylase, *alkB*, Bioremediation, Long-Chain *c*-Alkane, *Rhodococcus*

1. Introduction

In recent years, environmental pollution by petroleum hydrocarbons has been a serious problem worldwide. To clean up hydrocarbon-contaminated sites, a range of treatments can be performed, including incineration, chemical oxidation (Fenton reaction), washing, and evaporation. However, a lot of energy is required for these treatments [1] [2] [3] [4]. Biological treatments such as bioremediation have been investigated as an effective system of hydrocarbon degradation [5] [6] [7] [8] [9], and many microorganisms with the ability to degrade petroleum hydrocarbons have been isolated from their natural habitat and characterized [10] [11] [12] [13] [14]. However, it is difficult for bacteria to degrade long-chain hydrocarbons, especially long-chain cyclic alkanes (*c*-alkanes) [15] [16]. Therefore,

the isolation of bacteria capable of degrading long-chain hydrocarbons is important for the bioremediation of hydrocarbons [17] [18] [19].

Kubota *et al.* previously isolated hydrocarbon-degrading bacteria (HDB) from their natural habitats, and determined their phylogenetic relationships based on partial sequences of the 16S rRNA gene [20]. This identified many isolated species as belonging to the genera *Pseudomonas*, *Rhodococcus*, *Gordonia*, and *Acinetobacter* [20]. The strains belonging to the genera *Rhodococcus* and *Gordonia* degraded not only long-chain normal alkanes (*n*-alkanes) but also *c*-alkanes as the sole carbon and energy source [20] [21] [22] [23]. This indicated that the strains degrading long-chain *c*-alkanes have potential for cleaning petroleum hydrocarbon pollutants, because long-chain *n*-alkanes and *c*-alkanes remain in the soil for long periods.

The first step of degrading long-chain *c*-alkanes, the oxidation of hydrocarbons, is catalyzed by alkane hydroxylases (Alk) [24] [25]. For many HDB, *alkB* genes encoding Alk have been well analyzed [26] [27] [28] [29]. *alkB* genes are categorized as seven types (*alkB*1 to *alkB*7) [30] [31], and many strains of the genus *Rhodococcus* are known to carry *alkB*2. However, the relationship between different types of *alk* genes and the *c*-alkane degradation ability has not been characterized for each species. Moreover, *alkB* varies among species of the genus *Rhodococcus*, and oxidizable substrates differ depending on the *alkB* gene type [32]. For example, Fukuhara *et al.* [33] revealed that the *R. erythropolis* strain NDKK6, carrying the *alkB* R2 type, showed a high *c*-alkane degradation ability. Furthermore, the carbon number of the alkyl side chain seemed to influence *c*-alkane degradation [34] [35] [36] [37]. A previous study isolated 11 strains of HDB from the genus *Rhodococcus* [20], but they have not been characterized yet.

In the present study, we investigated the relationship between *alkB* genes and long-chain *c*-alkane degradation for 11 isolated strains of *Rhodococcus* and 19 strains obtained from the NITE Biological Resource Center (NBRC), Japan.

2. Materials and Methods

2.1. Bacterial Strains

Thirty bacterial strains were used as long-chain *c*-alkane-degrading bacteria. Eleven strains (*R. erythropolis* NDKK1, *R. erythropolis* NDKK2, *R. erythropolis* NDKK5, *R. erythropolis* NDKK6, *R.* e*rythropolis* NDKK7, *R. erythropolis* NDKK48, *R. erythropolis* ODNM1C, *R. erythropolis* NDKY82A, *R. erythropolis* ODMI54, *R. erythropolis* ODNM2B, and *R. erythropolis* NDMI144) were isolated in our previous study [20], and 19 species (*R. erythropolis* NBRC15567, *R. rhodochrous* NBRC15564, *R. baikonurensis* NBRC100611, *R. wratislaviensis* NBRC100605, *R. opacus* NBRC100624, *R. ruber* NBRC15591, *R. equi* NBRC101255, *R. percoletus* NBRC100626, *R. jostii* NBRC16295, *R. triatomae* NBRC103116, *R. koreensis* NBRC100607, *R. corynebacterioides* NBRC14404, *R. zopfii* NBRC100606, *R. tukisamuensis* NBRC100609, *R. maanshanensis* NBRC100610, *R. pyridinivorans*

NBRC100608, *R. kroppenstedtii* NBRC103113, *R. rhodnii* NBRC100604, and *R. coprophilus* NBRC100603) were obtained from the NBRC.

2.2. Identification of Bacterial Strains

The 11 strains from our earlier study were identified based on the full length 16S rRNA gene sequence. These strains were cultivated in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 0.5% NaCl) at 30˚C with shaking at 200 rpm [36]. Total DNA was extracted from the culture, and the 16S rRNA gene sequence was determined [38] [39]. Sequence data were deposited in the DNA Data Bank of Japan (DDBJ) database (https://www.ddbj.nig.ac.jp/index-e.html) under accession numbers LC107434 to LC107444. The sequences were also compared with data in the GenBank database using BLAST+ 2.2.31 which is available at https://www.ncbi.nlm.nih.gov/blast/. Phylogenetic analyses were carried out for the 11 strains and the additional 19 species whose sequence data were obtained from the GenBank database using ClustalW (version 2.1). The phylogenetic tree was constructed by the neighbor-joining method and bootstrap values were calculated by 1000 replications.

2.3. Sequences of *alkB* and *alkB* [*alkB* R2 Type] Genes

Sequences of *alkB* were also determined for 30 strains. PCR was conducted using primers *alkB*-F (5'-AACTAYMTCGARCAYTAYGG-3')/*alkB*-R (5'-TGRTCKSTCGYTGVARGTG-3') and *alkB* R2-F (5'-CGGTTGTGTCGCAGGA-TC-3)/ *alkB* R2-R (5'-AACGACTGCGCCAGAGTGAT-3') [31] to confirm the presence of *alkB* and *alkB* genes [*alkB* R2 type], respectively. *alkB* and *alkB* gene [*alkB* R2 type] PCR products were 140 bp and 100 bp, respectively. *alkB* primers were designed for the consensus region of HDB. *alkB* sequences were determined by the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and sequence data were deposited in the DDBJ database under accession numbers LC107445 to LC107470. Phylogenetic analysis was performed for *alkB* sequences as described above.

2.4. Analysis of *c*-Alkane Degradation by *Rhodococcus* Strains

To evaluate bacterial long-chain *c*-alkane degrading abilities, 30 strains were cultivated in modified SW medium with undecylcyclohexane (UDC), dodecylcyclohexane (DDC), and tridecylcyclohexane (TDC) substrates. Pre-culture of each strain was prepared in LB medium. Substrate hydrocarbon (0.10 g) and pre-culture (1 ml) were inoculated in 100 ml of modified SW medium (1% v/w; per liter: 1.21 g NH_4NO_3, 14.3 g $Na_2HPO_4{\cdot}12H_2O$, 5.44 g KH_2PO_4, 0.5 g NaCl, 0.247 g $MgSO_4$, 2.78 mg $FeSO_4{\cdot}7H_2O$, 14.7 mg $CaCl_2{\cdot}2H_2O$, 2.01 mg $ZnSO_4{\cdot}7H_2O$, 0.15 mg $[NH_4]_6Mo_7O_{24}{\cdot}4H_2O$, 2 mg $CuSO_4{\cdot}5H_2O$, 0.4 mg $CoCl_2{\cdot}6H_2O$, 1.49 mg $MnSO_4{\cdot}5H_2O$, 0.5 g polypeptone, and 0.25 g yeast extract) in a baffle flask before incubating at 30˚C with shaking at 120 rpm.

After 3 days of cultivation, 30 ml of chloroform-methanol (3:1) mixture was added to 100 ml medium and mixed well before centrifuging at 4000× g for 30 min. The separated organic layer was taken for gas-chromatographic analysis using a flame ionized detector (GC-FID) (GC-2010, Shimadzu, Kyoto, Japan). The degradation test was carried out in triplicate. The degradation ratio was calculated from the rate of decrease of the peak area of the gas-chromatogram as follows:

$$1 - (\text{Peak area after cultivation}/\text{Peak area before cultivation}) \times 100\ (\%)$$

The concentration of hydrocarbon in the soil was measured using the OCMA-355 oil content analyzer (Horiba, Kyoto, Japan).

3. Results and Discussion

3.1. Identification of Long Chain *c*-Alkane-Degrading Bacteria and Analysis of Alkane Hydroxylase Genes in *Rhodococcus*

To identify *Rhodococcus* strains isolated in our previous study [20], we determined the 16S rRNA gene sequences (1429 - 1472 bp). Phylogenetic analysis showed that all strains belonged to *R. erythropolis*, based on similarity with the reference strain *R. erythropolis* MPU33 (DNA databank accession number: AB334770) (**Figure 1**). These strains were isolated from a culture medium containing long chain *c*-alkane as the sole carbon source [20], indicating that the strains carried *alkB*.

The presence and type of *alkB* genes in the strains were next examined. All strains carried *alkB* genes, with 13 *Rhodococcus* species of the 29 strains used in this study shown to carry *alkB* (**Table 1** and **Table 2**). *alkB* sequences of *R. erythropolis* isolated from nature were 98.1% - 100% homologous with the *alkB* R2 type of *R. erythropolis* NDKK6 that has high *c*-alkane degradation ability (**Table 1**). The *alkB* sequence from *R. erythropolis* NBRC15567 also showed high similarity with *alkB* R2 type (95.7%). Additionally, two other strains (*R. rhodochrous* and *R. baikonurensis*) obtained from NBRC carried *alkB* genes with approximately 100% homology to *alkB* R2 type (**Table 2**).

Ten strains also carried *alkB* genes, but homology analysis showed that their sequences differed from *alkB* R2 type (21.9% to 78.3% homologous) so original *alkB* names were given to each *alkB* gene type (**Table 2**). The similarity of *R. opacus* and *R. percolatus alkB* genes was 99.7% (**Figure 2**), so they were named *alkB* ROP type. The remaining 6 species lacked *alkB* genes. Phylogenetic analysis based on *alkB* revealed separate clusters of *alkB* [*alkB* R2 type] and *alkB* [non-*alkB* R2 type] groups (**Figure 2**). In the *Rhodococcus* genus, 3 groups were categorized as follows: first group included species such as *R. baikonurensis* and *R. rhodochrous* carrying *alkB* R2 type, another group carried other types of *alkB* genes, and the third group lacked any *alkB* genes.

3.2. Degradation of Long Chain *c*-Alkane by *Rhodococcus* Species Carrying *alkB* R2 Type

The degradation rates of long chain *c*-alkanes (UDC, DDC, and TDC) by 14

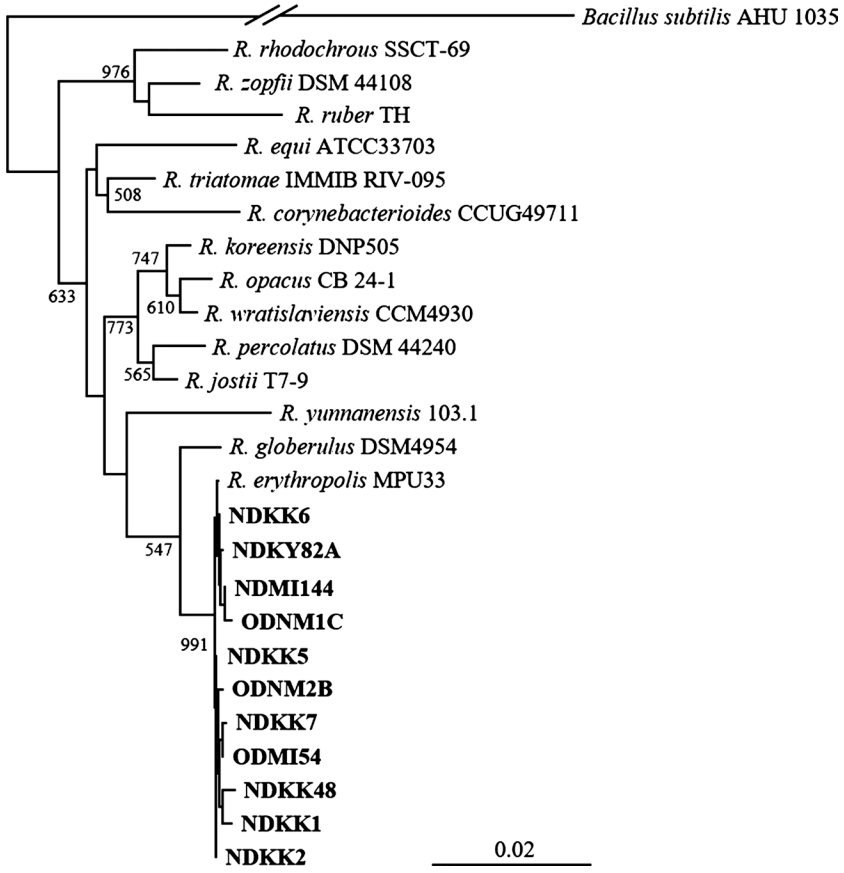


Figure 1. Phylogenetic tree based on 16S rRNA gene sequences of 26 strains of genus *Rhodococcus*. Sequences of the strain shown in bold were determined in this study and the other strains were taken from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The tree was constructed by the neighbor-joining method. The scale bar indicates the number of substitutions per site. Bootstrap values (1000 replicates) greater than 50% are indicated in the corresponding nodes.

Table 1. The presence of *alkB* genes and similarity to *alkB* [*alkB* R2 type] genes.

Strain	*alkB* Gene	Similarity to *alkB* Gene [*alkB* R2 Type] (%)*
R. erythropolis NDKK1	+	99.6
R. erythropolis NDKK2	+	100
R. erythropolis NDKK5	+	99.9
R. erythropolis NDKK7	+	99.8
R. erythropolis NDKK48	+	99.7
R. erythropolis ODNM1C	+	99.8
R. erythropolis NDKY82A	+	98.1
R. erythropolis ODMI54	+	99.7
R. erythropolis ODNM2B	+	99.2
R. erythropolis NDMI144	+	99.7
R. erythropolis NBRC15567	+	95.7

**alkB* gene [*alkB* R2 type] from *Rhodococcus erythropolis* NDKK6 [1].

Table 2. The presence of *alkB* genes similarity to *alkB* genes [*alkB* R2 type], and types of *alkB* gene.

Strain	*alkB* Gene	Similarity to *alkB* Gene [*alkB* R2 Type] (%)*	Type of *alkB* gene
R. rhodochrous NBRC15564	+	100	*alkB* R2
R. baikonurensis NBRC100611	+	99.7	*alkB* R2
R. wratislaviensis NBRC100605	+	22.6	*alkB* RW
R. opacus NBRC100624	+	23.4	*alkB* ROP
R. ruber NBRC15591	+	30.5	*alkB* RR
R. equi NBRC101255	+	78.3	*alkB* RE
R. percolatus NBRC100626	+	21.9	*alkB* ROP
R. jostii NBRC16295	+	30.1	*alkB* RJ
R. triatomae NBRC103116	+	30.1	*alkB* R1
R. koreensis NBRC100607	+	30.5	*alkB* RK
R. corynebacterioides NBRC14404	+	24.1	*alkB* RC
R. zopfii NBRC100606	+	30.7	*alkB* RZ
R. tukisamuensis NBRC100609	−		
R. maanshanensis NBRC100610	−		
R. pyridinivorans NBRC100608	−		
R. kroppenstedtii NBRC103113	−		
R. rhodnii NBRC100604	−		
R. coprophilus NBRC100603	−		

**alkB* gene [*alkB* R2 type] from *Rhodococcus erythropolis* NDKK6 [1].

Rhodococcus strains carrying *alkB* R2 type are shown in Table 3. The average degradation rates of UDC, DDC, and TDC were 55.5%, 31.8%, and 55.2%, respectively. Strain ODNM2B showed the highest degradation rate (98.9%) of UDC. Similarly, strains NDKK2 and ODNM2B showed superior degradation of DDC and TDC (42.9% and 94.6%, respectively). Figure 3 shows the degradation of UDC by *R. erythropolis* NDKK6. These observations of *R. erythropolis* strains including our identified strains and one from NBRC indicate that *alkB* R2 type plays an important role in the degradation of long chain *c*-alkanes, and that *Rhodococcus* carrying *alkB* R2 type can utilize *n*-hexadecane [40].

3.3. Degradation of Long Chain *c*-Alkane by Strains Carrying Non-*alkB* R2 Type

The degradation rates of long chain *c*-alkanes (UDC, DDC, and TDC) by 10 *Rhodococcus* species carrying non-*alkB* R2 type are shown in Table 4. Average degradation rates of UDC, DDC, and TDC were 49.4%, 29.4%, and 53.9%, respectively. Although strains of the genus *Rhodococcus* carrying *alkB* genes can degrade hydrocarbons such as long chain *c*-alkanes, the degradation rates of strains carrying non-*alkB* R2 type were lower than those carrying *alkB* R2 type

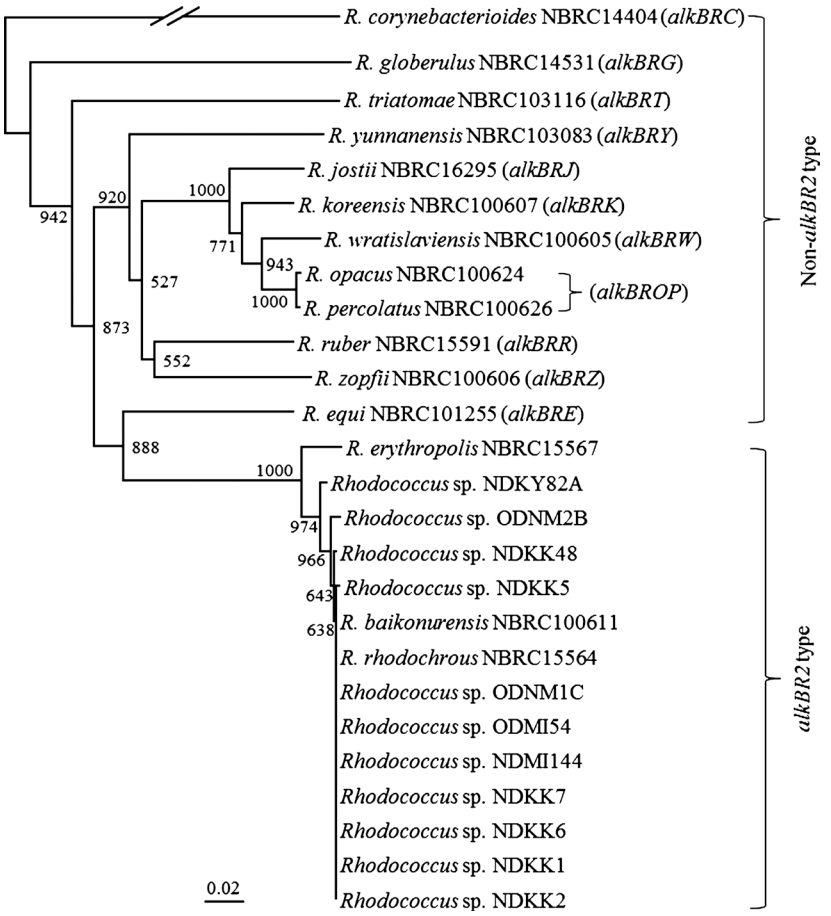


Figure 2. Phylogenetic tree based on alkane hydroxylase (*alkB*) gene sequences of 24 strains of the genus *Rhodococcus*. The scale bar indicates the number of substitutions per site. Corresponding gene names of each strain and group of strains are followed by names in parenthesis and braces, respectively. The tree was constructed by the neighbor-joining method. Bootstrap values (1,000 replicates) greater than 50% are indicated in the corresponding nodes.

(Table 3 and Table 4). This indicates that *alkB* R2 type was more suitable for the degradation of long chain *c*-alkanes. Moreover, the higher degradation rates of UDC and TDC compared with DDC suggest that long chain *c*-alkanes with an odd number of carbon atoms are easier to biodegrade by *Rhodococcus* species [21].

3.4. Degradation of Long Chain *c*-Alkane by Strains Carrying No *alkB* Genes

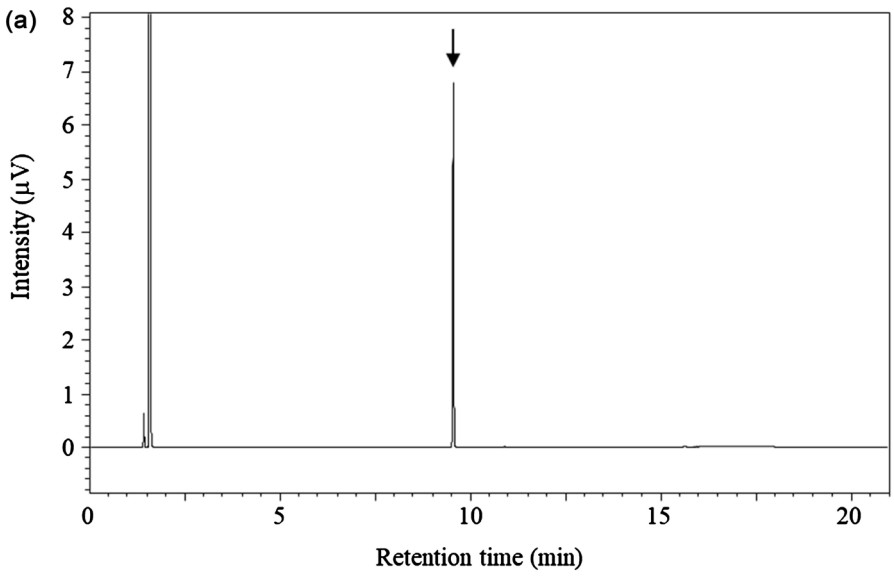
The degradation rates of long chain *c*-alkanes (UDC, DDC, and TDC) by 6 strains carrying no *alkB* genes are shown in Table 5. *R. rhodnii* NBRC100604 had the highest degradation rate of UDC, DDC, and TDC among all 6 strains. The average degradation rates of UDC, DDC, and TDC by these 6 strains were 19.3%, 13.3%, and 19.1%, respectively. The average degradation ratios of UDC, DDC, and TDC by strains carrying no *alkB* genes were lower than in these by strains carrying *alkB* [*alkB* R2 type] and *alkB* [non-*alkB* R2 type]. Strains of genus

Rhodococcus carrying *alkB* genes had higher degradation abilities of long-chain *c*-alkane than those of strains carrying no *alkB* genes (Table 3 and Table 5). However, *R. rhodnii* NBRC100604 carrying no *alkB* gene showed a degradation rate of above 30%. The ability of long-chain *c*-alkane degradation might be regulated

Table 3. The degradation of long-chain *c*-alkanes by stains harboring *alkB* [*alkB* R2 type] in the genus *Rhodococcus*.

Strain	Degradation Ratio (%)*		
	Undecylcyclohexane (UDC)	Dodecylcyclohexane (DDC)	Tridecylcyclohexane (TDC)
R. erythropolis NDKK1	16.0 ± 7.5	31.7 ± 8.7	17.9 ± 7.3
R. erythropolis NDKK2	6.7 ± 10.7	42.9 ± 10.2	23.5 ± 3.2
R. erythropolis NDKK5	66.7 ± 0.2	42.2 ± 1.8	40.6 ± 10.0
R. erythropolis NDKK6	38.8 ± 1.0	18.9 ± 6.2	75.7 ± 6.1
R. erythropolis NDKK7	53.5 ± 3.6	42.1 ± 4.0	46.7 ± 9.1
R. erythropolis NDKK48	40.4 ± 2.5	33.1 ± 12.6	81.4 ± 0.1
R. erythropolis ODNM1C	78.2 ± 2.4	41.8 ± 2.9	91.0 ± 1.1
R. erythropolis NDKY82A	52.7 ± 4.9	39.1 ± 9.2	78.4 ± 1.5
R. erythropolis ODMI54	68.2 ± 5.0	37.8 ± 8.2	46.8 ± 2.6
R. erythropolis ODNM2B	98.9 ± 0.2	30.3 ± 3.5	94.6 ± 6.7
R. erythropolis NDMI144	91.4 ± 3.9	27.7 ± 10.3	39.9 ± 8.3
R. erythropolis NBRC15567	64.5 ± 9.4	0.0 ± 1.5	75.4 ± 1.5
R. erythropolis NBRC15564	50.6 ± 3.9	41.6 ± 9.1	39.6 ± 7.1
R. baikonurensis NBRC100611	50.4 ± 3.8	16.0 ± 3.0	20.6 ± 5.7
Average	55.5	31.8	55.2

*n = 3.



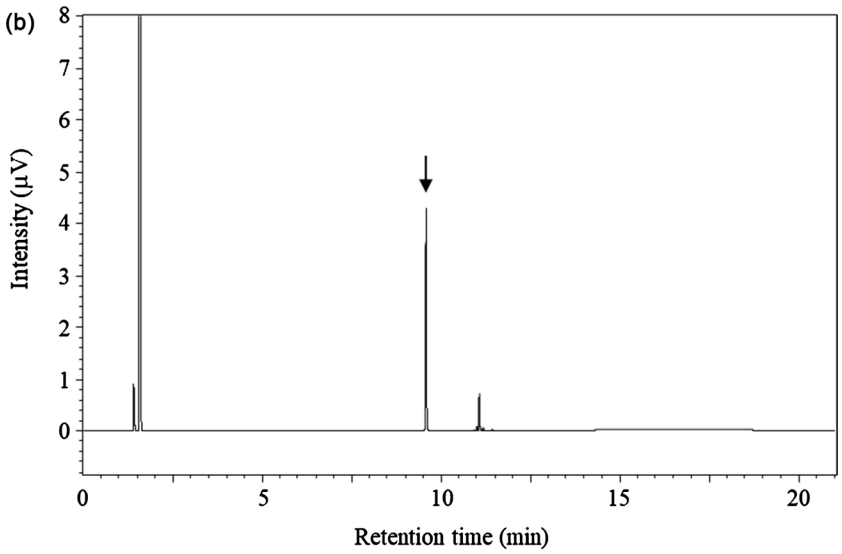


Figure 3. Gas chromatograms of UDC degraded by *R. erythropolis* NDKK6. Arrows indicate the peak of UDC. Images of gas chromatograms of LB medium with UDC substrate cultivated without (a) and with (b) *R. erythropolis* NDKK6.

Table 4. The degradation of long-chain *c*-alkanes by stains harboring *alkB* genes [non-*alkB* R2 type] in the genus *Rhodococcus*.

Strain	Type of *alkB* Gene	Degradation Ratio (%)*		
		Undecylcyclohexane (UDC)	Dodecylcyclohexane (UDC)	Tridecylcyclo-hexane (UDC)
R. wratislaviensis NBRC100605	*alkB* RW	39.7 ± 5.2	58.6 ± 4.0	25.1 ± 2.1
R. opacus NBRC100624	*alkB* ROP	94.9 ± 1.4	56.8 ± 5.8	43.1 ± 0.4
R. percoletus NBRC100626	*alkB* ROP	47.1±13.5	35.7 ± 2.3	60.6 ± 11.1
R. ruber NBRC15591	*alkB* RR	98.2 ± 1.6	55.9 ± 3.6	43.2 ± 3.4
R. equi NBRC101255	*alkB* RE	48.9 ± 2.1	39.3 ± 3.4	98.4 ± 1.6
R. jostii NBRC16295	*alkB* RJ	29.8 ± 4.9	27.8 ± 10.7	65.0 ± 8.2
R. triatomae NBRC103116	*alkB* R1	34.0 ± 14.2	15.9 ± 7.4	75.3 ± 1.7
R. koreensis NBRC100607	*alkB* RK	6.0 ± 10.6	4.2 ± 5.4	5.5 ± 2.3
R. corynebacterioides NBRC14404	*alkB* RC	41.4 ± 6.5	0.0 ± 10.2	62.3 ± 0.6
R. zopfii NBRC100606	*alkB* RZ	53.9 ± 9.1	0.0 ± 9.7	60.3 ± 5.3
Average		49.4	29.4	53.9

*n = 3.

Table 5. The degradation of long-chain *c*-alkanes by stains harboring no *alkB* genes in the genus *Rhodococcus*.

Strain	Degradation Ratio (%)*		
	Undecylcyclohexane (UDC)	Dodecylcyclohexane (UDC)	Tridecylcyclo-hexane (UDC)
R. tukisamuensis NBRC100609	26.0 ± 1.4	4.1 ± 11.0	20.1 ± 8.0
R. maanshanensis NBRC100610	18.3 ± 7.4	13.4 ± 4.2	18.6 ± 3.4
R. pyridinivorans NBRC100608	2.0 ± 1.4	11.2 ± 13.1	19.6 ± 10.2
R. kroppenstedtii NBRC103113	12.3 ± 5.2	1.7 ± 1.0	15.9 ± 4.2
R. rhodnii NBRC100604	34.7 ± 11.5	34.2 ± 3.8	34.8 ± 3.3
R. coprophilus NBRC100603	22.2 ± 3.3	15.4 ± 6.7	5.6 ± 7.1
Average	19.3	13.3	19.1

*n = 3.

by the alkane hydroxylase gene, while genus *Rhodococcus* spp. carrying no *alkB* genes may degrade long-chain *c*-alkanes by other degradation systems such as those involving the cytochrome gene [41].

3.5. Alkane Hydroxylase Gene-Carrying Bacteria and Their Roles in Material Circulation in Nature

c-alkane-degrading bacteria belonging to the genus *Rhodococcus* isolated from their natural habitats in our previous study were closely related to *R. erythropolis* and strains harboring *alkB* R2 type [33]. Although many *c*-alkane-degrading bacteria of the genus *Rhodococcus* are widely distributed, long-chain *c*-alkanes are limited in the natural environment. These bacteria may utilize hydrocarbons such as the wax of leaves and aromatic compounds, which include linear or cyclic hydrocarbon components. Thus, these bacteria harboring alkane hydroxylase genes might contribute to the material circulation of several kinds of hydrocarbon compounds in nature [42].

4. Conclusion

To characterize the long-chain cyclic alkane (*c*-alkane) degradation of bacteria in *Rhodococcus*, 11 strains were isolated from nature using long-chain *c*-alkane as a substrate. These strains were identified as *R. erythropolis*, and all were shown to carry the *alkB* R2 type. It was also confirmed that the degradation ratios of long-chain *c*-alkanes by *Rhodococcus* carrying the *alkB* R2 type of gene (55.5% of UDC, 31.8% of DDC, and 55.2% of TDC) were higher than in those carrying the non-*alkB* R2 type of gene (49.4% of UDC, 29.4% of DDC, and 53.9% of TDC) and no *alkB* gene (19.3% of UDC, 13.3% of DDC, and 19.1% of TDC).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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