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ORIGINAL RESEARCH PAPER

Identification of alkane hydroxylase genes in *Rhodococcus* sp. strain TMP2 that degrades a branched alkane

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Abstract *Rhodococcus* sp. TMP2 is an alkane-degrading strain that can grow with a branched alkane as a sole carbon source. TMP2 degrades considerable amounts of pristane at 20°C but not at 30°C. In order to gain insights into microbial alkane degradation, we characterized one of the key enzymes for alkane degradation. TMP2 contains at least five genes for membrane-bound, non-heme iron, alkane hydroxylase, known as AlkB (*alkB1–5*). Phylogenetical analysis using bacterial *alkB* genes indicates that TMP2 is a close relative of the alkane-degrading bacteria, such as *Rhodococcus erythropolis* NRRL B-16531 and Q15. RT-PCR analysis showed that expressions of the genes for AlkB1 and AlkB2 were apparently induced by the addition of pristane at a low temperature. The results suggest that TMP2 recruits certain alkane hydroxylase systems to utilize a branched alkane under low temperature conditions.

Keywords Alkane · *alkB* · Biodegradation · Pristane · *Rhodococcus* sp. TMP2

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Introduction

Alkanes are highly reduced compounds with various structures, including short and long-chain, branched, cyclic or aromatic forms. These compounds are highly abundant in nature due to their less volatile and insoluble properties. Although many bacteria are known to metabolize various types of alkanes through distinct processes (van Beilen and Funhoff 2005), the key step for alkane degradation is recognized to be the oxygenation at a terminal methyl group (Rehm and Reiff 1981). For example, linear medium- and long-chain alkanes are oxidized by alkane monooxygenase (AlkB) or cytochrome P450s (van Beilen and Funhoff 2007). In general, alkane-degrading bacteria contain multiple genes for alkane hydroxylase and are capable of utilizing versatile alkanes (van Beilen et al. 2002). These observations suggest distinct roles of each member of the enzymes; however, detailed catalyses of alkane hydroxylase have been less well characterized.

We had previously isolated an alkane-degrading bacterium, *Rhodococcus* sp. TMP2, which grew on a minimum-agar plate overlaid with 2, 6, 10, 14-tetramethylpentadecane (pristane) as a sole carbon source (Kunihiro et al. 2005). TMP2 has an optimum growth temperature of 30°C and degrades comparable amounts of *n*-alkanes (C9–C24) at temperatures ranging from 10 to 30°C. The degradation rate of pristane by TMP2 is high at 20°C, but no degradation is observed at 30°C. These observations suggest that

diverse metabolisms for alkane degradation may be taking place in TMP2 cells grown at different temperatures.

We found decreased rates of pristane degradation when equal amounts of *n*-alkane and pristane were added to the incubation of TMP2 (Kunihiro et al. 2005). This observation suggests that degradation pathways for *n*-alkane and pristane may be identical or partially overlapped. In order to shed light on pristane degradation by TMP2, the organization and expression of the genes for alkane hydroxylase in TMP2 were investigated.

Materials and methods

Bacterial strains and culture conditions

Strain TMP2 was previously isolated from the soil of a rice field in Japan (Kunihiro et al. 2005). It was grown in Luria broth (LB; 1% bactotryptone, 0.5% yeast extract, 0.5% NaCl) or minimum salt medium BM2 (Kunihiro et al. 2005). *Escherichia coli* DH5α was used as a host strain for the general cloning of DNA fragments. Cloning vectors pBluescript II SK^+ (Stratagene, La Jolla, CA) and pGEM-T EASY (Promega, Madison, WI) were used in *E. coli* DH5α.

Alkane degradation test

Overnight cultures of TMP2 (LB, 30°C) were collected by centrifugation (12,000 × g, 5 min) and washed twice with BM2. Culture suspension was adjusted at $OD_{600} = 5$ in 10 ml of BM2. After being supplemented with various carbon sources (0.1%), the cells were incubated at 20 or 30°C with shaking at 120 rpm. The remaining hydrocarbons were extracted with 10 ml hexane/acetone (1:1, v/v) at 12 h intervals. Each sample included 20 ppm (w/v) biphenyl as an internal standard. The amount of each hydrocarbon was quantified by the GC equipped with a non-polar capillary column HP-1 (Hewlett-Packard), as described previously (Kunihiro et al. 2005). Each data was the average of triplicate experiments.

Identification of TMP2 *alkB* genes

Chromosomal DNA of TMP2 was isolated by a general phenol/SDS method (Sambrook and Russell 2001). PCRs were carried out in 50 µl with KOD plus DNA polymerase (Toyobo, Osaka, Japan) and a primer set based on bacterial *alkB* gene sequences (see Supplementary Table 1). The PCR programme was 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 60°C, 2 min at 68°C; 5 min at 68°C and 4°C until further use. PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGEM-T EASY. Nucleotide sequences of the PCR fragments were determined by BigDye terminator cycle sequencing on an ABI sequencer according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems, Wellesley, MA). Sequence data have been submitted into the nucleotide database under the accession numbers AB377307 (*alkB1*), AB377308 (*alkB2*), AB377309 (*alkB3*), AB377310 (*alkB4*) and AB377311 (*alkB5*), respectively. Distance trees based on multiple sequence alignments were generated with ClustalX (version 2.0; Thompson et al. 1997), using the neighbor-joining method with 1000 bootstrap trials.

Reverse transcriptase (RT) PCR analysis

Total RNA was isolated from bacterial cultures using RNAeasy Mini Kit (Qiagen). cDNA was synthesized from 1 µg DNase-treated RNA in 20 µl, using random-primed reverse transcription (Reverse Transcription System, Promega, Madison, WI). One µl of RT products was amplified in 30 µl using KOD Plus DNA polymerase (Toyobo) and a primer set for each cDNA (Supplementary Table 1). The PCR condition was 2 min at 94°C; 30 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 68°C; 2 min at 68°C. The PCR products were run on 2% agarose gel in 1 × TBE (89 mM Tris-borate, 2 mM EDTA) and visualized by ethidium bromide staining. As an internal control to normalize the amount of templates, 16S rRNA was used. The linearity between the amount of templates and intensity of signals was verified by comparison at different cycle numbers of PCR.

Scanning electron microscopy (SEM)

TMP2 cells, grown at 20 or 30°C (BM2, 24 h) with hexadecane or pristine, were stepwise fixed with 5% (w/v) OsO_4 and 2% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.0). After fixation, the samples were absolutely dehydrated by increasing

concentrations of ethanol and gold-coated in a deep vacuum. The samples were examined with a Model S-2400 (Hitachi, Japan) scanning electron microscope.

Results and discussion

Alkane consumption by TMP2

Previous work has shown that TMP2 grows better at 30°C than at 10 or 20°C, whereas this strain effectively degrades pristine only at low temperatures (Kunihiro et al. 2005). To assess alkane degradation by TMP2 further, we inoculated equal amounts of overnight culture cells ($OD_{600} = 5$) and evaluated their abilities to degrade different alkanes (Fig. 1). During the first day, TMP2 assimilated up to 80% *n*-alkane (hexadecane) at both temperatures. TMP2 preferentially metabolized pristane at 20°C and also degraded it at 30°C during longer incubation times. Only a small decrease in a cyclic alkane (decylcyclohexane) could be observed with relatively higher rates at 20°C. From these results, it is obvious that TMP2 is capable of degrading three tested alkanes, whereas each extent of degradation is various and influenced by temperatures.

Identification of *alkB* genes

Major intermetabolites of pristane are monoic acids in *Rhodococcus* strains (Nakajima et al. 1985; Sharma and Pant 2000). This implies the involvement of monoterminal oxidation pathways for pristane consumption. One of the bacterial pathways that initiates the aerobic metabolism of alkanes is a three-component alkane hydroxylase system, which contains a membrane-bound alkane hydroxylase AlkB, and two soluble electron transfer components (rubredoxin and rubredoxin reductase). Several alkane-degrading strains contain multiple genes for AlkB, which are proposed to act on overlapping substrate recognitions (van Beilen and Funhoff 2007). We designed highly degenerate primers for the *alkB* genes and used these for PCR amplification (Supplementary Table 1). Sequence analysis of the PCR products showed that the predicted amino acid sequences were classified into five members, all of which possessed signatures specific to AlkB (Hist-1, Hist-2, HYF and Hist-3 motives). We designed five genes as *alkB1* to *alkB5* in TMP2 (Fig. 2).

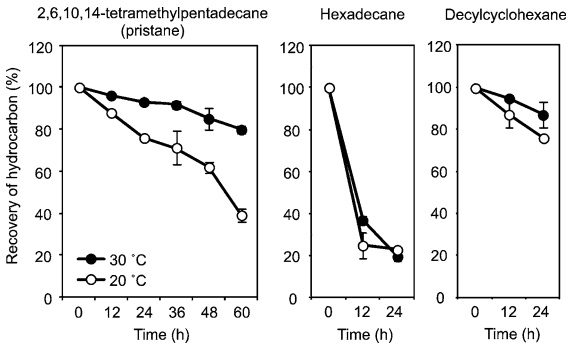

Fig. 1 Alkane consumption rates by *Rhodococcus* sp. TMP2, grown at 20 or 30°C

In a phylogenetical analysis, *alkB1–4* from TMP2 represents the closest homologies to the corresponding member of *R. erythropolis* NRRL B-16531 and Q15 (Whyte et al. 2002) and also exhibited higher similarities to those of *Nocardia* sp. H17-1, which is an oil-degrading bacterium isolated from oil-contaminated soil (Baek et al. 2006). The *alkB5* gene is a novel member that locates at the deep position of the *alkB4* branch. In the past studies, the presence of a fifth member of rhodococcal *alkB* genes has been suspected by the cross-hybridization with the DNA probes for *R. fascians* 115-H *alkB115* and *R. fascians* 154-S *alkB154*, but its gene has not yet been cloned (van Beilen et al. 2002). Database searches predicted higher similarities with two uncultured bacteria and *Brevundimonas* sp. *alkB*-like genes, whereas those with *alkB115* and *alkB154* were relatively low. The survey in our bacterial collections indicated the presence of a homologous *alkB5* gene in another alkane-degrading *Rhodococcus* sp. T12 (Kunihiro et al. 2005). These results indicate that TMP2 has at least five different *alkB* homologues and *alkB5* may be general in *Rhodococcus* spp.

Expression patterns of TMP2 *alkB* genes

To examine further how five *alkB* genes are regulated in TMP2 cells, RT-PCR analysis was used to compare their transcript levels during the incubation with alkanes (Fig. 3). The *alkB3*, *alkB4*, and *alkB5* transcripts were present at constant levels in cells grown in the minimum salt medium containing 0.1% hexadecane at 20 and 30°C. Transcript levels of *alkB1* and *alkB2* rose slightly after the onset of

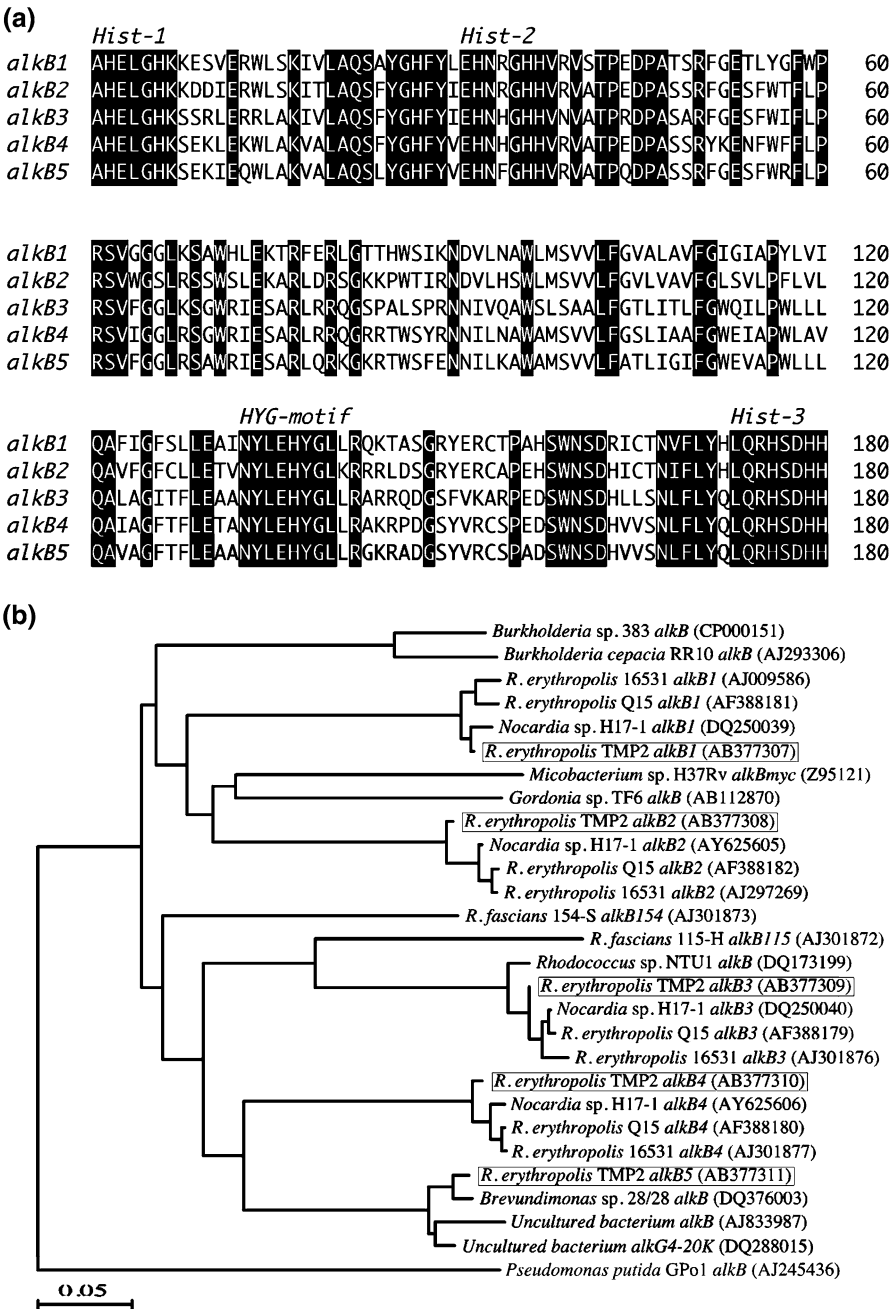

Fig. 2 Comparison of amino acid sequences among alkane hydroxylases in *Rhodococcus* sp. TMP2, and a phylogenetical relationship among bacterial *alkB* genes. (**a**) Amino acid sequence alignment comparing the predicted AlkB proteins of TMP2. Identical residues conserved in all AlkBs are highlighted in black. The three conserved histidine boxes are indicated above. (**b**) Phylogenetical tree among the nucleotide sequences of bacterial *alkB* genes. Each sequence is denoted by the strain, the gene name and the database accession number. Scale bar, 0.05 substitution per nucleotide site

incubation with hexadecane, especially at 20°C, and declined in the later stages of incubation. When cells were grown in the medium containing pristane, transcript levels of *alkB3*, *alkB4*, and *alkB5* were also constitutive at both temperatures, whereas those of *alkB1* and *alkB2* increased more rapidly and significantly at 20°C than they did at 30°C. Taken together, the results of this study demonstrate that the *alkB3–5* genes express constitutively, whereas expressions of *alkB1* and *alkB2* should be controlled by growth conditions. Temporal appearances of *alkB1* and *alkB2* transcripts seem to be linked with higher degradation rates of pristane as seen in Fig. 1, implying that effective alkane degradations are attributed to the induction of *alkB1* and *alkB2* genes in TMP2 cells.

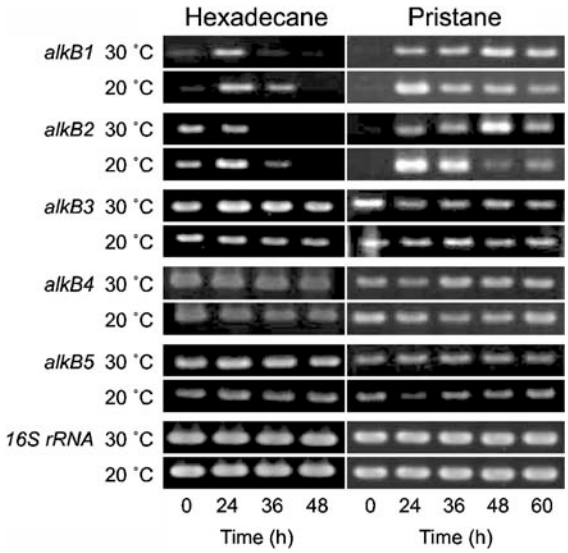

Fig. 3 Transcript levels of five *alkB* genes in *Rhodococcus* sp. TMP2 during the incubation with 0.1% (v/v) alkanes (hexadecane, pristane) at 20 or 30°C

Previous analysis of the alkane hydroxylase system in *Pseudomonas putida* GPo1 revealed that its three components are not present in equal ratios in the cells and alkane hydroxylases might share a limiting number of electron transfer components (Staijien et al. 2000). Whyte et al. (2002) reported that similar organizations of *alkB1 and alkB2* genes in *R. erythropolis* NRRL B-16531 and Q15 constitute an operon-like structure including the genes for electron transfer components, whereas other members are located separately. At this stage, organizations of *alkB* genes in TMP2 are elusive. If *alkB1* and *alkB2* genes of TMP2 are co-transcribed with other component genes, as was the case for NRRL B-16531 and Q15, encoded electron transfer components might be shared by other standalone types of AlkBs (AlkB3–5) and be capable of contributing to overall elevations of the catalysis for alkane degradation. In this context, it is plausible that the transcript levels of *alkB1* and *alkB2* are stimulated in response to the addition of alkanes (Fig. 3).

SEM of TMP2 cells

In this study, we had noticed that TMP2 cells grew into flocs with relatively dispersal and culture turbidities were high at 20°C, whereas cells grown at 30°C were highly aggregated in spite of the presence of hexadecane or pristane (data not shown). SEM observation revealed that the surfaces of cell flocks were smooth at 30°C, but those seen at 20°C were rough and surrounded by many capsular compounds (Fig. 4). These observations indicate that drastic changes of the cell surface structure might be occurring at low temperatures.

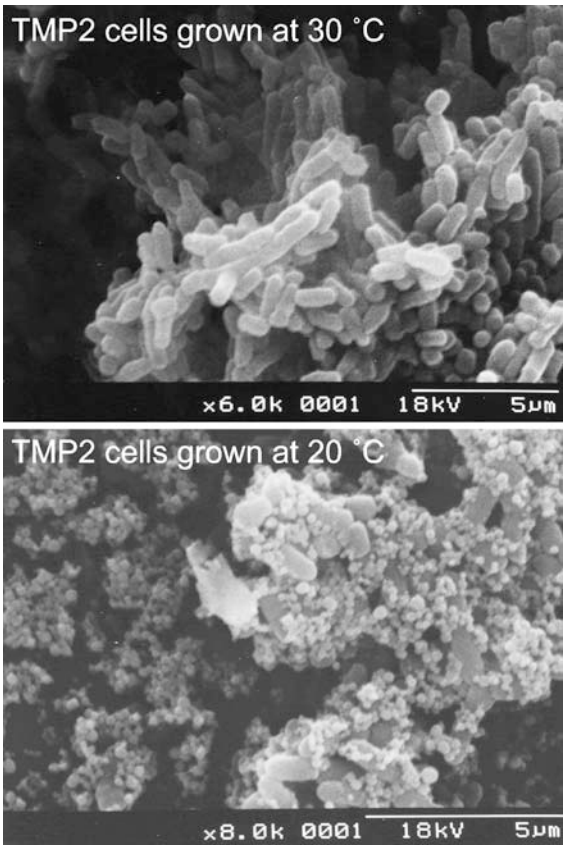

Fig. 4 Scanning electron microscope (SEM) photographs of *Rhodococcus* sp. TMP2 cells grown at 20 or 30°C with 0.1% (v/v) pristane (BM2, 24 h)

It should be noted that *Rhodococcus* strains change cell surface properties in response to growth conditions, aiming to take up and assimilate hydrophobic compounds (Sanin et al. 2003; Whyte et al. 1999). Biosurfactant production is one of the strategies to metabolize hydrophobic compounds (Lang and Philp 1998; Rapp and Gabriel-Jürgens 2003). *Rhodococcus* sp. NCIM 5126 changes the composition of cellular fatty acids in response to the supplied hydrocarbons so that the increased lipophilicity may facilitate the uptake and import of hydrocarbons (Sharma and Pant 2000). SEM reveals that the NCIM 5126 cells are

smooth rod-shaped and grow separately: this is different from TMP2. Because TMP2 is not a biosurfactant producer (Kunihiro et al. 2005) and branched alkane is a more hydrophobic than linear alkane, direct contact with the cell surface and hydrocarbons is important for effective assimilations. The addition of pristane at low temperatures should trigger a major change of surface properties of TMP2 cells where recruitments of certain alkane hydroxylase systems might be anticipated.

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