Multiple alkane hydroxylase systems in a marine alkane degrader, *Alcanivorax dieselolei* B-5

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Summary

*Alcanivorax dieselolei* strain B-5 is a marine bacterium that can utilize a broad range of *n*-alkanes (C₅–C₉₆) as sole carbon source. However, the mechanisms responsible for this trait remain to be established. Here we report on the characterization of four alkane hydroxylases from *A. dieselolei*, including two homologues of AlkB (AlkB1 and AlkB2), a CYP153 homologue (P450), as well as an AlmA-like (AlmA) alkane hydroxylase. Heterologous expression of *alkB1*, *alkB2*, *p450* and *almA* in *Pseudomonas putida* GPo12 (pGEc47ΔB) or *P. fluorescens* KOB2Δ1 verified their functions in alkane oxidation. Quantitative real-time RT-PCR analysis showed that these genes could be induced by alkanes ranging from C₈ to C₉₆. Notably, the expression of the *p450* and *almA* genes was only upregulated in the presence of medium-chain (C₁₆–C₂₄) or long-chain (C₂₅–C₉₆) *n*-alkanes, respectively; while *alkB1* and *alkB2* responded to both medium- and long-chain *n*-alkanes (C₁₂–C₂₀). Moreover, branched alkanes (pristane and phytane) significantly elevated *alkB1* and *almA* expression levels. Our findings demonstrate that the multiple alkane hydroxylase systems ensure the utilization of substrates of a broad chain length range.

Introduction

In 2005, we reported the isolation from coastal seawater and deep sea sediment, the classification and identification of *Alcanivorax dieselolei* (Liu and Shao, 2005). In the following years, *A. dieselolei* has been continuously detected in surface water, deep seawater bodies or sediments of many sites worldwide, including: the East Pacific Ocean, South China Sea, Mediterranean Sea, Atlantic Ocean, Persian Gulf, Ago Bay (Japan) and Southwest Indian Ocean (GenBank Accession Numbers FM957534, GQ153648, EU853416, FJ218198, AB453735, FJ685648, EU440990, etc.); demonstrating that it is a cosmopolitan marine bacterium as well as a key petroleum hydrocarbon degrader. *Alcanivorax dieselolei*, a γ-proteobacterium, is a member of the hydrocarbonoclastic bacteria, which are present at low or undetectable levels in unpolluted environments, but bloom dramatically after an oil spill, becoming the predominant microbe in polluted marine waters (Yakimov et al., 2007). Like other hydrocarbonoclastic bacteria, *A. dieselolei* cannot assimilate sugars or amino acids as sources of energy and carbon, but can only utilize some organic acids and alkanes. Notably, the spectrum of alkanes utilized by *A. dieselolei* (at minimum C₅–C₉₆) (Liu and Shao, 2005) is substantially broader than those of most other previously described alkane degraders (van Beilen and Funhoff, 2007). This endows *A. dieselolei* with a tremendous potential for marine oil pollution mitigation.

Alkane degradation is generally initiated by alkane hydroxylases, which convert alkanes to alkanols. There are three categories of alkane hydroxylases that act on short-, medium- and long-chain alkanes respectively (van Beilen and Funhoff, 2007). Methane monooxygenase-like enzymes usually hydroxylate gaseous short-chain alkanes (C₁–C₄), while membrane-bound non-haem iron (AlkB) (van Beilen et al., 1994) or cytochrome P450 monooxygenases oxidize medium-chain alkanes (C₅–C₉₆). The AlkB protein from *Pseudomonas putida* GPo1 is presently the best-characterized alkane hydroxylase (van Beilen et al., 1994). It catalyses the first step of alkane degradation with help of two electron transfer proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) (van Beilen et al., 1994). Over the past decade, *alkB*-like hydroxylase genes have been detected in a wide range alkane-degrading bacteria, including α-, β- and γ-proteobacteria; as well as in some high G+C content Gram-positive bacteria (Smits et al., 2002). Many of these contain more than one *alkB* homologue, such as *Pseudomonas aeruginosa* PA01 (*alkB1* and *alkB2*), *Rhodococcus erythropolis* Q15 (*alkB1*–4) and Acinetobacter sp. M-1 (*alkMa* and *alkMb*). The soluble class
Il-type three-component P450/CYP153 enzyme systems are usually present in the medium-chain alkane degraders that do not possess alkB-like genes (van Beilen et al., 2006). One exception is Alcanivorax borkumensis SK2, the first described and most extensively studied Alcanivorax species (Yakimov et al., 1998). It carries three P450 genes and two alkB genes (van Beilen et al., 2004; 2006; Schneiker et al., 2006), both enzymes systems oxidize medium-chain alkanes; particularly alkB1, which acts on \( C_2-C_{12} \); alkB2, which acts on \( C_9-C_{18} \) (van Beilen et al., 2004); and P450s, which oxidizes \( C_8-C_{10} \) substrates at the very minimum (Schneiker et al., 2006; van Beilen et al., 2006). As A. borkumensis SK2 is capable of degrading at least \( C_5-C_{23} \) chain length alkanes (van Beilen et al., 2004), it has been anticipated that the existence of other unknown enzyme system(s) in A. borkumensis SK2 may be responsible for long-chain alkane (> \( C_{10} \)) hydroxylation. To date, very little is known about the alkane hydroxylases that act on long-chain alkanes. However, two recent publications have revealed that a flavin-binding monooxygenase (AlmA) in Acinetobacter sp. DSM 17874 supports its growth on long-chain alkanes up to \( C_{36} \) (Throne-Holst et al., 2007); and that a soluble monomeric monooxygenase (LaD) in Geobacillus thermodenitrificans NG80-2 can oxidize \( C_{15}-C_{36} \) in vitro (Feng et al., 2007).

In this study, we characterized multiple functional alkane hydroxylase systems that exist in A. dieselolei strain B-5, including two AlkB-, one P450- and one AlmA-like hydroxylases. The expression of these genes was induced by alkanes of different chain length, indicating that complementary expression profiles is one of the mechanisms by which A. dieselolei B-5 ensures its capability to degrade a wide range of alkanes.

### Results

**Cloning and sequence analysis of alkB1 and alkB2 genes**

In our taxonomic analysis of A. dieselolei (Liu and Shao, 2005), we cloned a partial sequence (425 bp) from an alkB-like gene (named alkB1 here) from B-5 chromosomal DNA, using highly degenerate primers (MonF and MonR, Table 1). To obtain the full-length sequence and flanking regions of alkB1, a genomic DNA library was constructed by cloning partially Sau3AI-digested chromosomal DNA fragments into plasmid pUC18. The Escherichia coli DH10B transformants harbouring gel-purified approximately 8 kb DNA fragments were screened on Luria–Bertani (LB) agar plates by colony hybridization, using the partial alkB1 sequence as a probe. A 5.1 kb A. dieselolei B-5 DNA fragment containing the alkane hydroxylase gene was cloned. Inverse PCR was subsequently used to obtain the adjacent DNA fragments. The assembled DNA segment was 9.2 kb in length. Using the same probe, Southern blotting analysis showed that there was only one copy of alkB1 in the B-5 chromosome (data not shown).

In the GenBank, the deduced full-length AlkB1 sequence (394 aa) shared the highest levels of identity with the \( \beta \)-proteobacteria AlkB (for example 63% with Ralstonia pickettii 12J and 61% with Burkholderia cenocepacia J2315) and significantly lower levels of homology with all the other AlkB (for example 43% with Acinetobacter sp. ADP1 AlkM, 43% with A. borkumensis SK2 AlkB1 and only 37% with SK2 AlkB2). The phylogenetic tree was constructed, and A. dieselolei B-5 AlkB1 fell into the \( \beta \)-proteobacteria cluster, whose integrity was supported in 100% of the neighbour-joining trees generated (Fig. 1A), 98% of maximum-parsimony and 100% of maximum-likelihood trees, which indicated that the branching pattern was stable.

### Table 1. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>alkBf</td>
<td>AAY ACW GSN CAY GAR MTN GGN CAY AA</td>
</tr>
<tr>
<td>alkBr</td>
<td>TGR TGR TCB GAR TGN CGY TG</td>
</tr>
<tr>
<td>almAfw</td>
<td>GGGGNACNTGGGAYCTNTT</td>
</tr>
<tr>
<td>almAwr</td>
<td>ATRTNCGYTTNAGNTCC</td>
</tr>
<tr>
<td>alkB1f</td>
<td>ACG AAT ACA TAT GAA GAC CGT AAC C</td>
</tr>
<tr>
<td>alkB1r</td>
<td>TTA GAA GCT TGA GTG GGT CAA TT</td>
</tr>
<tr>
<td>alkB2f</td>
<td>AGC ATA TG A GCA AGG CAG TGT AGT TCA</td>
</tr>
<tr>
<td>alkB2r</td>
<td>TAA AGG TT A GTG TGC TGG TGG CCA CCC C</td>
</tr>
<tr>
<td>p450f</td>
<td>ACT TAA TAT AG CAC TTA TAG ATC AAG</td>
</tr>
<tr>
<td>p450r</td>
<td>GCC AAT TGA GTT AAA AAA CGC CAG TTG</td>
</tr>
<tr>
<td>almApF</td>
<td>TACATGCAACATTGGCGCGCTG</td>
</tr>
<tr>
<td>almApR</td>
<td>ATAAACTGAGCCCATCGGCG</td>
</tr>
<tr>
<td>2inf</td>
<td>TTC CAG TGT ACT TCC GCC A</td>
</tr>
<tr>
<td>2inr</td>
<td>GAA TTT CCA GAT GCT TTC GC</td>
</tr>
<tr>
<td>RT-gyrF</td>
<td>GTG AAG GTG CCT GAT CCC AAG</td>
</tr>
<tr>
<td>RT-gyrR</td>
<td>GGG CTG CAT CGA TAA TCT TCT G</td>
</tr>
<tr>
<td>RT-p450F</td>
<td>GGA GTT GCG GGT GGT GTG ATT</td>
</tr>
<tr>
<td>RT-p450R</td>
<td>ACA AGG ACC CAC GAC GCA A</td>
</tr>
<tr>
<td>RT-alk1F</td>
<td>ACC GTG ATC GGC AGC TT</td>
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<tr>
<td>RT-alk1R</td>
<td>CCA ACC ACA GCA TCA GGA CA</td>
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<tr>
<td>RT-alk2F</td>
<td>GGA AAT TCG TAC TGG GGG AGA</td>
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<tr>
<td>RT-alk2R</td>
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</tr>
<tr>
<td>RT-almAF</td>
<td>ACA GTG GCA AAC GGG TGG</td>
</tr>
<tr>
<td>RT-almAR</td>
<td>CGG TGG CAT AGG TGG</td>
</tr>
</tbody>
</table>

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Fig. 1. Neighbour-joining phylogenetic trees based on the alignment of amino acid sequences of alkane monooxygenases from A. dieselolei B-5 and reference strains.

A. Neighbour-joining phylogenetic tree based on the alignment of amino acid sequences of alkane monooxygenases. The tree was based on almost full-length alkB-like alkane monooxygenases comprising 378–483 amino acids. A. dieselolei B-5 is highlighted.

B. Neighbour-joining phylogenetic tree based on the alignment of amino acid sequences of cytochrome P450 family. The tree was based on almost full length of B-5 p450 and reference sequences. The reference P450 sequences were retrieved from the database constructed by Dr David Nelson (http://drnelson.uthsc.edu/CytochromeP450.html) and the NCBI database.

C. Neighbour-joining phylogenetic tree based on the alignment of amino acid sequences of flavin-binding monooxygenase (almA). The tree was based on almost full length of B-5 almA and reference sequences. The scale bar denotes divergence per percentage between sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

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Fig. 2. Organization of alkane hydroxylase gene segments from *A. dieselolei* B-5 aligned to homologous genes from *A. borkumensis* SK2 or *Marinobacter* sp. ELB17.

A. alkB1 gene cluster, gene designations: alkB1, alkane hydroxygenase; ABO_2696, short-chain dehydrogenase; ABO_2697, hypothetical protein; ABO_2698, phospholipase D; ABO_2699, hypothetical protein.


C. p450 gene cluster, p450, cytochrome P450 monooxygenases; alkJ2, alcohol dehydrogenase; fdx, ferredoxin; araC, AraC family transcription regulator; ABO_0194, monooxygenase; ABO_0195, lipase/esterase; ABO_0196, short-chain dehydrogenase; ABO_0197, metal-dependent hydrolase; ABO_0188, TelR family transcriptional regulator.

D. almA gene cluster, almA, flavin-binding monooxygenase; ABO_0198, metal-dependent hydrolase; ABO_0188, metal-dependent hydrolase; ABO_0189, dehydrogenase; ABO_0190, putative monooxygenase; ABO_0191, hydrolase; ABO_0192, hypothetical protein; ABO_0193, hypothetical protein.

Homologous genes are highlighted by shaded areas. The percentages show amino acid sequence identity between the corresponding ORFs with the same pattern. The asterisk indicates the closest relative in GenBank.
protein of 63% identity to Marinobacter sp. ELB17 AMP-dependent synthetase and ligase. This is followed by another ORF (orf1) orientated in the opposite direction, showing 56% identity to the A. borkumensis SK2 phospholipase D (Fig. 2A). Genes encoding two polypeptides showing 79% (orf6) and 66% (orf7) amino acid identities to A. borkumensis SK2 proteins were found downstream of alkB1. The close relation with A. borkumensis SK2 genes led us to compare this alkB1 region with the recently released genome sequence of A. borkumensis SK2 (Schneiker et al., 2006); as a result, a similar region in the chromosome of strain SK2 was retrieved. As shown in Fig. 2A, the composition of these two gene regions is very similar, except that in strain B-5, orf2 and orf4–alkB1 are located in the intergenic regions between orf1–orf3 and orf3–orf6 respectively. This suggests that the two regions may share the same origin and may have been acquired by a horizontal gene transfer, and evolved separately. However, in the alkB1 region, we failed to find any sequences known to play a role in horizontal gene transfer.

We then sought to clone other alkB genes responsible for the primary oxidation of n-alkanes in A. dieselolei B-5. A partial DNA sequence of alkB2 was first obtained using another degenerated primer set (alkBf and alkBr, Table 1). A 448 bp internal gene fragment of an alkB gene homologue was amplified. The complete sequence of alkB2 was obtained using inverse PCR with primers 2inF and 2inR. The full-length AlkB2 amino acid sequence showed highest similarity (68% identity) with Marinobacter sp. ELB17 alkane-1 monoxygenase; but only shared 45.3% identity with A. dieselolei B-5 AlkB1 and 34% identity with A. borkumensis SK2 AlkB2. As shown in Fig. 1A, A. dieselolei B-5 AlkB2 branched with Marinobacter sp. ELB17 and Moritella sp. PE36 AlkB, and formed a separate group with those of γ-proteobacteria, such as P. putida Gpo1 AlkB and A. borkumensis SK2 AlkB1. Upstream of alkB2 and in the opposite orientation, there is a gene segment (orf1) that encodes a peptide lacking C-terminus, which showed highest identity (58%) to N-terminus of the Marinobacter sp. ELB17 AraC transcriptional regulator (MELB17-10563) (Fig. 2B). Furthermore, within the intergenic region between orf1 and alkB2, a 7 bp direct repeat (GTCAGCTT) is found 90 bp upstream of the start codon of alkB2. This direct repeat has been shown to represent the typical binding sequence of AraC or XylS (Gallegos et al., 1997). As is the case for alkB1, determined by Southern blot, there is only one copy of each of the alkB2 genes in the A. dieselolei B-5 chromosome (data not shown).

**Cloning and sequence analysis of p450 and almA genes**

By using highly degenerated primers CF and CR (Kubota et al., 2005), an 831 bp fragment of a putative p450 gene was amplified. To obtain the full-length sequence of this p450 gene, Tail-PCR (thermal asymmetric interlaced PCR) was applied. The cloned DNA was then subjected to sequencing. After assembly, the sequence of an 8.8 kb section of chromosomal DNA was obtained. The deduced amino acid sequence of p450 gene displayed 84% identity to that of A. borkumensis SK2 p450 (b) or (c) (Fig. 1B). Analysis of genes upstream and downstream of A. dieselolei B-5 p450 revealed that like those of alkB1 they shared highest identities with A. borkumensis SK2 proteins and formed a very similar gene cluster to that of the SK2 p450 (c) operon (Fig. 2C). In B-5, orf3 is located immediately upstream of orf4 and p450, and encoded a peptide that was only homologous to the N-terminus of the AraC regulatory protein, and was 771 bp shorter than the araC homologue in the SK2 p450 (c) gene cluster. However, no direct repeat was found within the intergenic region between orf2 and orf3. A BlastX analysis revealed that the intergenic region between orf2 and orf3 did not share sequence identity higher than 40% with any genes in the GenBank. Additionally, both orf1 and orf2 that were located within this sequenced segment did not share similarity to any genes putatively required for alkane oxidation.

By using highly degenerated primers almAwf and almAwr (Table 1), a 1131 bp fragment of a putative almA gene was amplified. To obtain the full-length sequence and flanking regions of almA, as is the case for alkB1, a genomic DNA library was constructed by cloning partially Sau3Al-digested chromosomal DNA fragments into plasmid pUC18. The E. coli DH10B transformants harbouring gel-purified approximately 9.0 kb DNA fragments were screened on LB agar plates by colony hybridization, using the partial almA sequence as a probe. An 8.7 kb fragment containing the almA gene was cloned. As determined by Southern blot, there is also only one copy of each of the p450 and almA genes in the A. dieselolei B-5 chromosome respectively (data not shown).

The deduced full-length AlmA sequence (501 aa) shared the highest identity (72%) with a putative flavin-containing monoxygenase of A. borkumensis (Fig. 2D), and much more distantly related to other AlmAs, for example only 48.6% with the AlmA of Acinetobacter sp. DSM 17874 (Fig. 1C). Sequence analysis showed that the gene organization of A. dieselolei B-5 almA region is similar to that of A. borkumensis SK2 putative flavin-containing monoxygenase operons. Notably, a putative alcohol dehydrogenase gene (71% identity to A. borkumensis SK2 dehydrogenase) is positioned immediately downstream of almA and is orientated in the same direction (indicated as orf6; Fig. 2D). Downstream of orf5 and oriented in the same direction, an ORF (orf6) was present that encodes a protein of 72% identity to A. borkumensis SK2 putative metal hydrolase ABO_0188. Upstream of
Pseudomonas fluorescens KOB2 recombinant strain harbouring alkB1 n-alkane hydroxylases, which catalyses the hydroxylation of alkB1 indicated that B-5 alkane hydroxylase genes.

To determine whether the isolated p450 gene encodes a functional alkane hydroxylase, B-5 p450 gene was cloned into recently developed expression vector pCom12-PxF200R1500 instead (van Beilen et al., 2006), as the p450 function needs the help of ferredoxin and ferredoxin reductase. The resulting plasmid, pCom12B5-p450, was subsequently transformed into P. putida Gpo12 and P. fluorescens KOB2Δ1. A comparable high growth rate (0.35–0.48 h⁻¹) was observed in P. putida p450 recombinants on C₆–C₁₂ n-alkanes. Although both P. putida recombinants grew on C₆, a reproducible growth rate was difficult to be recorded probably because of the high volatility of this compound or its toxicity as cell membrane lipid solvents. Unlike alkB1, the introduction of p450 allowed P. putida recombinants grow on C₁₃. On the other hand, in P. fluorescens KOB2Δ1, p450 allowed a substantial growth of the host on C₁₀–C₁₆, with the fastest growth rate on C₁₄.

To determine whether the isolated p450 gene encodes a functional alkane monooxygenase, an expression recombinant plasmid pCom8B5-almA was constructed by inserting the B-5 almA into vector pCom8, and transformed into P. fluorescens KOB2Δ1. Subsequently, the growth of the recombinant on n-alkanes shorter than C₁₈ was tested. The results showed that the recombinant strain had a low but substantive growth rate on C₁₂–C₁₆ (Table 2). The shortest doubling time of 5.37 h was observed on C₁₆.

Transcriptional expression of alkB1, alkB2, p450 and almA genes

To test the expression profiles of four genes putatively involved in A. dieselolei B-5 alkane degradation, cells were grown in SM1 medium with selected n-alkanes as their sole carbon source. Branched alkanes, pristane (C₂₀H₄₀) and phytane (C₂₀H₄₂), were also included. When cultures reached mid-exponential phase, the total RNA was isolated and subjected to real-time PCR analysis. Cells grown on sodium acetate as their sole carbon source were used as controls. Of the n-alkanes tested, C₁₄, C₁₆, C₁₈, C₂₀, C₂₂, C₂₄ and C₂₆ significantly induced the expression of alkB1 compared with controls. The largest

<table>
<thead>
<tr>
<th>Table 2. Growth rates (h⁻¹) of Pseudomonas recombinants containing the A. dieselolei B-5 alkane hydroxylase genes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas recombinants</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>P. putida Gpo12 (pGEC47ΔB)</td>
</tr>
<tr>
<td>pCom8B5-alkB1</td>
</tr>
<tr>
<td>pCom12B5-p450</td>
</tr>
<tr>
<td>P. fluorescens KOB2Δ1</td>
</tr>
<tr>
<td>pCom8B5-alkB1</td>
</tr>
<tr>
<td>pCom8B5-alkB2</td>
</tr>
<tr>
<td>pCom8B5-almA</td>
</tr>
<tr>
<td>pCom12B5-p450</td>
</tr>
</tbody>
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increase (13.7-fold) was observed with C24. Interestingly, \textit{alkB1} also strongly responded to branched alkanes, pristane (15.5-fold) and phytane (17.8-fold). All other alkanes produced much smaller or no response (Fig. 3A).

The expression of \textit{alkB2} was induced by C12–C26 \textit{n}-alkanes to similarly high levels (5.7- to 8.0-fold increases). The inclusion of branched alkanes, or \textit{n}-alkanes shorter than C12 or longer than C26 did not produce significant responses in \textit{alkB2} transcription (Fig. 3B). Only \textit{n}-alkanes shorter than C16 significantly increased \textit{p450} mRNA level. The highest increase (12.4-fold) was observed for C12. On the contrary, when grown on longer-chain alkanes (C32, C34 and C36), the expression of \textit{p450} was remarkably depressed, compared with basal expression levels within control cultures (Fig. 3C).

Unlike the results obtained for the \textit{alkB1}, \textit{alkB2} and \textit{p450} genes, \textit{almA} mRNA levels responded to long-chain \textit{n}-alkanes (C56–C38). Notably, longer-chain \textit{n}-alkanes (C28–C36) produced high increases (7.0- to 12.9-fold). Branched alkanes (pristane and phytane) also elicited strong transcriptional increase of \textit{almA} (Fig. 3D).

### Discussion

Being a cosmopolitan marine bacterium, \textit{A. dieselolei} is a promising candidate for oil pollution mitigation due to its wide substrate range hydrocarbon-degrading abilities. Understanding the genetic determinants responsible for its versatile alkane degradation abilities is of importance for the development of effective bioremediation strategies. In this study, we demonstrated that the indigenous marine organism, \textit{A. dieselolei} B-5 possesses at least four functional alkane hydroxylase genes: \textit{alkB1}, \textit{alkB2}, \textit{p450} and \textit{almA} that jointly provide this bacterium with the ability to utilize a wide substrate spectrum of alkanes.

Neighbour-joining phylogenetic analyses of \textit{alkB}-type alkane hydroxylases showed four distinct clusters that separated well from each other (Actinobacteria, \textit{\beta}-, \gamma- and \textit{\alpha}-proteobacteria). In case of \textit{A. dieselolei} B-5, \textit{AlkB2} branched with other \gamma-proteobacteria AlkBs, while \textit{AlkB1} is located in the \textit{\beta}-proteobacteria cluster and is closely related to \textit{Burkholderia} AlkBs. One explanation for this is horizontal gene transfer, which has been suggested to
occur in many alkane-degrading bacteria (Smits et al., 2002). Consistent with this proposition, when we compared the A. dieselolei B-5 alkB1 gene region with that of A. borkumensis SK2 (ABO_2696–2699), an apparent ‘gain or loss of DNA sequences’ was observed (Fig. 2A). Furthermore, the G + C content of the alk gene clusters is generally significantly lower than that of the rest of the genome (Yakimov et al., 2007). A G + C content of 62.1% was determined in A. dieselolei B-5. However, the alkB1 and alkB2 genes in B-5 only have G + C content of 54.1 and 49.7% respectively. It also needs to be noted that the B-5 alkB2 gene region and the orf2 in alkB1 region shared the highest protein identity with homologues genes from Marinobacter sp. ELB17, and that the genome of ELB17 has a similar G + C content (54%) to alkBs (xp01738706). More efforts are needed to depict the origin and spreading of alkB genes in marine bacteria.

Analysis of the ORFs in the A. dieselolei B-5 alkB1 gene locus revealed that they all encoded proteins putatively involved in the process of alkane degradation. Of these, orf1 encoded a polypeptide closely related to phospholipase D and cardiolipin synthase, which are required in the processes of phospholipid biosynthesis (Dowhan, 1997). Cardiolipin was shown to involve in activation of membrane-bound enzymes, like AlkB (Navarro et al., 1984; Jensen and Schutzbach, 1988). Moreover, homologues of zinc-containing alcohol dehydrogenase (orf4), short-chain alcohol dehydrogenase (orf7) and long-chain fatty acid CoA ligase (orf2) are known to play important roles in alkanol oxidation and β-oxidation respectively. In A. borkumensis, these alkane degradation genes were reported to be expressed only in alkane-induced cells (Sabirotova et al., 2006). Orf6, which is located immediately downstream of alkB1, encodes a polypeptide similar to a MerR family transcriptional regulator, which regulates gene expression in response to a variety of stresses (Brown et al., 2003). In this protein, the conserved DNA binding ‘winged HTH’ motif of the MerR regulators was also found. Within the A. dieselolei B-5 alkB1 region, orf4 is located adjacent to alkB1. BlastX analysis of the 324 aa Orf4 polypeptide revealed that it contains the conserved domain of the alcohol dehydrogenase superfamily. Alcohol dehydrogenase (ADH) converts alcohols into aldehydes performing the 2nd reaction in alkane oxidation pathway. To test if orf4 encodes a functional ADH, we cloned and expressed it in E. coli. However, we could not detect any alcohol dehydrogenase or quinone reductase activity in form of either the purified polypeptide or the crude cell extract (data not shown). Although these seven ORFs may be functionally related, they are not predicted to be co-transcribed for their different orientations. Even though orf1, orf3 and alkB1 are oriented in the same direction, an inverted repeat (10 bp stem, 4 bp loop) similar to a Rho-independent terminator was observed 13 bp downstream from the stop codon of orf3.

In the case of A. borkumensis SK2, AlkB1 and AlkB2 acted on C12–C12 and C12–C16 alkanes, respectively; P450s oxidized C22–C10 substrates (van Beilen et al., 2004; 2006; Schneker et al., 2006). In this report, AlkB1 and AlkB2 from A. dieselolei B-5 can be induced by alkanes of a broader range, covering medium- to long-chain n-alkanes (C12–C26). The substrate spectrum of A. dieselolei B-5 almA (C22–C36) neatly complements those of alkB and p450 genes. Similarly to Acinetobacter sp. DSM 17874 (Throne-Holst et al., 2007), almA in A. dieselolei B-5 was not induced by short- or medium-chain alkanes.

Interestingly, although B-5 almA did not response to the induction of C12–C26 alkanes in the transcriptional analysis (Fig. 3D), the Pseudomonas recombinant harbouring almA showed a substantive growth on C12–C16 alkanes (Table 2), suggesting B-5 almA encoded alkane hydroxylase is capable of oxidizing medium-chain n-alkanes. Further mutational analysis revealed that the almA deficient cells failed to grow on long-chain n-alkanes, C22–C36 (data not shown). These results clearly indicated that the ability of B-5 to degrade long-chain n-alkanes is dependent on the expression of almA. To our best knowledge, this is the first report showing that in Alcanivorax, almA encodes a functional alkane hydroxylase and is induced by long-chain n-alkanes of C22–C36.

Pristane and phytane are isoprenoid hydrocarbons that are widely present in the biosphere; introduced during oil spills, or produced during diagenesis (Koopmans et al., 1999). They are often used as a relatively ‘inert’ biomarker in oil degradation studies (Atlas et al., 1992). It has previously been shown that the higher ability of Alcanivorax to degrade branched alkanes enabled this bacterium to predominate in oil-containing seawater (Hara et al., 2003). In the case of A. borkumensis SK2, isoprenoid hydrocarbon (phytane) seems to be a strong inducer of cytochrome P450a and alkB2 gene expression (Schneker et al., 2006; van Beilen et al., 2006). Interestingly, we found that branched alkanes, pristane and phytane, were capable of activating alkB1 and almA (Fig. 3). The significant increase in the transcripts of these two genes indicates that A. dieselolei B-5 has an ability to degrade branched alkanes. Taken together with its ability to degrade a wide spectrum of n-alkanes, our results suggest that this strain has the potential to predominate in an oil-polluted marine environment.

**Experimental procedures**

**Bacterial strains and growth conditions**

Alcanivorax dieselolei B-5 (DSM 16502) was grown in HLB (Liu and Shao, 2005) or in SM1 medium (Yakimov et al., 1998) supplemented with the required substrates. Cultures
were incubated at 28°C and shaken at 200 r.p.m. unless noted otherwise. Other bacterial strains used in this study are listed in Table 3. *Escherichia coli* and *Pseudomonas* strains were grown at 30°C in LB broth medium (Luria et al., 1960) or M9 salts medium (Sambrook et al., 1989). The growth conditions of *P. putida* and *P. fluorescens* recombinants containing *B*-5 alkane hydroxylase genes are as described by van Beilen and colleagues (2004).

**Colony hybridization**

Colony hybridizations were performed as described (Grunstein and Hogness, 1975), using the same hybridization conditions for Southern blot. For Southern blotting analysis, total DNA from *A. dieselolei* B-5 was isolated according to the method of Ausubel (Ausubel, 1995), followed by treatment with restriction endonucleases and separation by agarose gel electrophoresis. Resolved DNA fragments were blotted onto nylon membranes, probed by digoxigenin (DIG)-labelled fragments, and then detected by using anti-DIG antibody and CSPD (Roche Diagnostics, Mannheim, Germany).

**Sequence analysis**

Sequence analysis was performed using the visualization and annotation tool Artemis (Rutherford et al., 2000) to identify coding sequences (CDSs) in the DNA. BLAST (Altschul et al., 1990) and conserved domain searches were performed in attempts to assign putative functions to the CDSs. Sequences were aligned using MUSCLE version 3.7 (Edgar, 2004) with nucleotide sequences obtained from the NCBI GenBank. Phylogenetic dendrograms of genes were constructed by three different algorithms: neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1978) and maximum-parsimony (Fitch, 1971) methods using the MEGA4 program (Tamura et al., 2007) with the p-distance model and pairwise deletion of gaps/missing data, substitution model PAM Matrix (Dayhoff). Bootstrapping analysis was used to evaluate the tree topology of the three algorithms by performing 1000 resamplings.

**Heterologous expression of alkane hydroxylase genes**

According to the method described by van Beilen and colleagues (van Beilen et al., 1994), the *A. dieselolei* B-5 *alkB1*, *alkB2* and *almA* genes were amplified using the *alkB1f* and *alkB1r*, the *alkB2f* and *alkB2r* and the *almApF* and *almApR* primer pairs respectively. They each were cloned into pCom8 (Smits et al., 2001) between the *NdeI* and HindIII sites. The PCR primers used in this study are listed in Table 1. The p450 gene was amplified using the p450f and p450r primers, and cloned into pCom12-PxF200R1500 (van Beilen et al., 2006) between the EcoRI and PacI sites. The resulting plasmids, pCom8B5-*alkB1*, pCom8B5-*alkB2*, pCom8B5-*almA* and pCom12B5-*p450*, were transferred to *P. putida* Gp01 (pGEc47DB) by triparental mating as described previously (Hara et al., 2004), with *E. coli* DH10B as the donor, and *E. coli* CC118 (RK600) as the helper strain (Ditta et al., 1980). Moreover, pCom8B5-*alkB1*, pCom8B5-*alkB2* and pCom12B5-*p450* were transferred to *P. fluorescens* KOB2D1 by electroporation (Dower et al., 1988). The features of the plasmids used in this study are described in Table 3. The growth on *n*-alkanes of the transformants was tested as described by Smits and colleagues (Smits et al., 2002).

**Real-time PCR**

*Alcanivorax dieselolei* B-5 was cultivated in SM1 medium supplemented with sodium acetate or various alkanes as the sole carbon source until the mid-exponential phase. Approximately 1 × 10⁶ cells were mixed with RNA Bacteria Protect Reagent (Qiagen, Valencia, CA, USA). Total RNA was then extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol, followed by treatment with DNase I (Invitrogen, Carlsbad, CA, USA). RNA yield was estimated using a Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE, USA). About 4 μg of RNA was reversely transcribed by using 20 ng of random primer (Invitrogen, Carlsbad, CA, USA) and the PrimeScript Reverse Transcriptase enzyme (TaKaRa, Dalian, China). Control reactions without reverse transcriptase were conducted to verify the absence of genomic DNA. The primers for real-time PCR were designed with the Primer premier 5.0.
software package (http://www.premierbiosoft.com/). Gene-specific primers were synthesized by Invitrogen (Carlsbad, CA, USA). Quantitative real-time PCR was performed using IQSYBR Green supermix and an IQTM 5 Multicolor Real-time PCR Detection System (both from Bio-Rad, CA, USA). Expression of the housekeeping gene, gyrB, was used as reference gene to normalize tested genes in A. dieselolei B-5. The relative fold change in mRNA quantity was calculated for the gene of interest in each sample using the ΔΔCt method. For each RNA preparation, at least three independent real-time PCR experiments were conducted. The sequences of gene-specific primers are listed in Table 1.

Statistical analysis

Data was analysed with unpaired two-tailed Student's t-tests or one-way ANOVA followed by Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA, USA). Data were expressed as mean ± SD derived from at least three independent experiments. Differences were considered significant at P < 0.05.

References


