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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 (C_5–C_{36}) 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 C_8 to C_{36}. Notably, the expression of the *p450* and *almA* genes was only upregulated in the presence of medium-chain (C_8–C_{16}) or long-chain (C_{22}–C_{36}) *n*-alkanes, respectively; while *alkB1* and *alkB2* responded to both medium- and long-chain *n*-alkanes (C_{12}–C_{26}). 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 C_5–C_{36}) (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 (C_1–C_4), while membrane-bound non-haem iron (AlkB) (van Beilen *et al.*, 1994) or cytochrome P450 monooxygenases oxidize medium-chain alkanes (C_5–C_{16}). 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* PAO1 (*alkB1 and alkB2*), *Rhodococcus erythropolis* Q15 (*alkB1–4*) and *Acinetobacter* sp. M-1 (*alkMa* and *alkMb*). The soluble class

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II-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 enzyme systems oxidize medium-chain alkanes; particularly *alkB1*, which acts on C_5–C_{12}; *alkB2*, which acts on C_5–C_{16} (van Beilen *et al*., 2004); and *P450*s, which oxidizes C_6–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_{20} 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 ($> C_{16}$) 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 (LadA) 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 *Sau*3AI-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).

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′–3′)
alkBf	AAY ACW GSN CAY GAR MTN GGN CAY AA
alkBr	TGR TGR TCB GAR TGN CGY TG
almAwf	GGNGGNACNTGGGAYCTNTT
almAwr	ATRTCNGCYTTNAGNGTCC
alkB1f	ACG AAT ACA TAT GAA GAC CGT AAC C
alkB1r	TTA GAA GCT TGA GGT GGT CAA TT
alkB2f	AGC ATA TGA GCA AGG CAG GTG AGT TCA
alkB2r	TAA AGC TTA GTG TGC TGG GCG CCA CCG C
p450f	ACT TAA TTA ATG CAC TTA CAG ATC AAG
p450r	GCC AAT TGA GTT AAA AAA CGC CAG TTG
almApF	TACATATGCCAACTTTGCACGCCTGC
almApR	ATAAGCTTGAGCCATGCGCGC
2inf	TTC CAG TTG ACT TCC GCC A
2inr	GAA TTT CCA GAT GCT TTC GC
RT-gyrF	GTG AAG GTG CCT GAT CCC AAG
RT-gyrR	GGG CTG CAT CGA TAA TCT TCT G
RT-p450F	GGA GTT GCG GGT AGT GTC ATT
RT-p450R	ACA AGG AGG CAC GAC GCA A
RT-alk1F	ACC GTG ATC GGC AGC CTT A
RT-alk1R	CCA ACC ACA GCA TCA GGA CA
RT-alk2F	GGA AAT TCG TAC TGC GGG AGA
RT-alk2R	AGA AAG CGG TGC CGA GAA T
RT-almAF	ACA GTG GCA AAC GGG TGG
RT-almAR	CGG TGG CTA CAT AGG TGG G

In the GenBank, the deduced full-length AlkB1 sequence (394 aa) shared the highest levels of identity with the β-proteobacteria AlkBs (for example 63% with *Ralstonia pickttii* 12J and 61% with *Burkholderia cenocepacia* J2315) and significantly lower levels of homology with all the other AlkBs (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 in the β-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.

Sequence analysis showed that the gene organization of *A. dieselolei* B-5 *alkB1* region is different from those of other well-known *alkB* operons. Notably, a putative alcohol dehydrogenase gene (73% identity to *Marinobacter algicola* DG893 zinc-containing alcohol dehydrogenase) is positioned immediately upstream of *alkB1* and is orientated in the opposite direction (indicated as *orf4*; Fig. 2A). Downstream of *orf4* and oriented in the opposite direction, an ORF (*orf3*) was present that encodes a protein of 65% identity to *A. borkumensis* SK2 hypothetical protein ABO_2698. Upstream of *orf3* and in the opposite orientation, there is an ORF (*orf2*) that encodes a

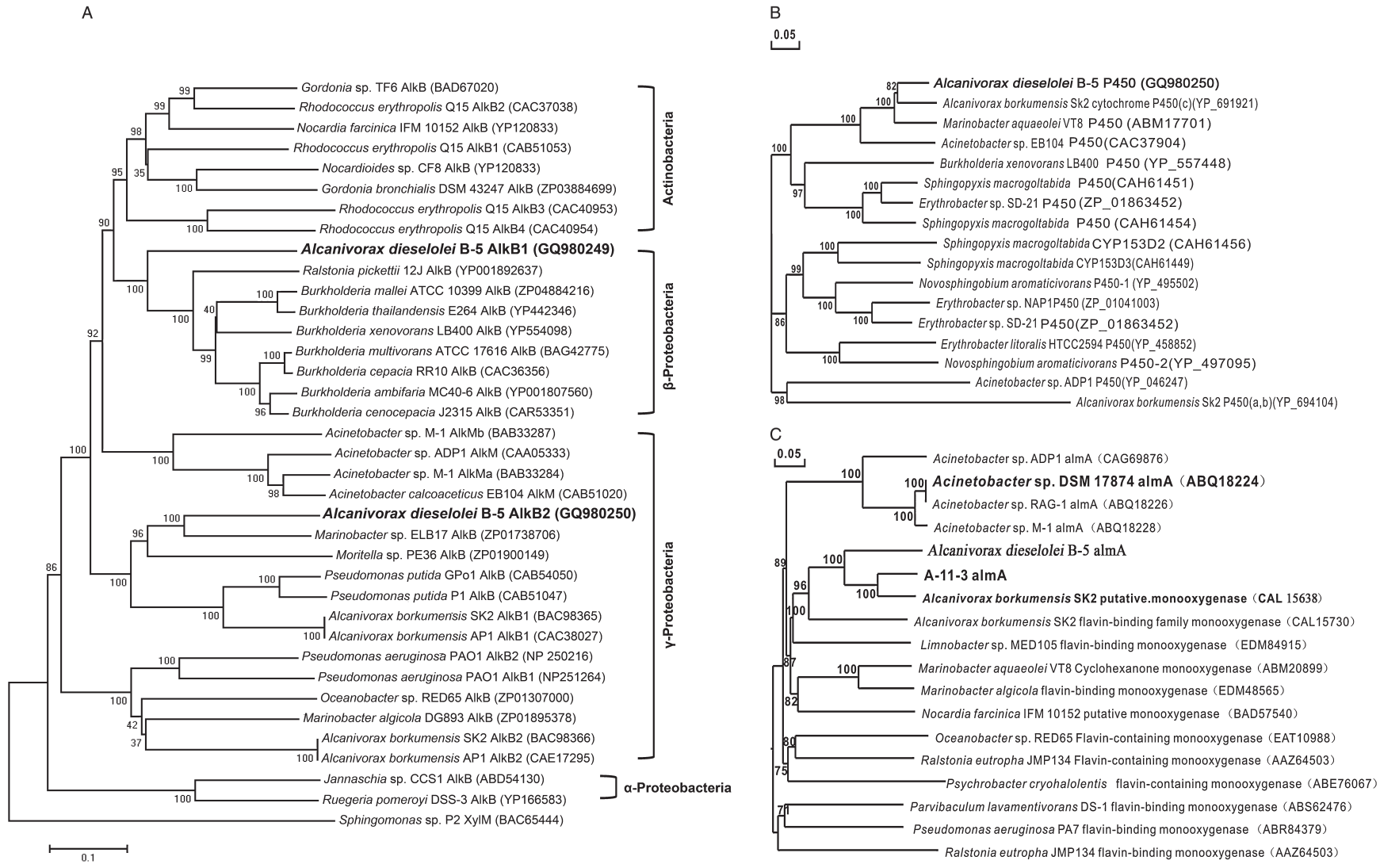

Fig. 1. Neighbour-joining phylogenetic trees based on the alignment of amino acid sequences of alkane monooxygenases from *A. dieselolei* B-5 and reference strains.
A. Neighbour-joining phylogenetic tree based on the alignment of amino acid sequences of *alkB*-like alkane monooxygenases. The tree was based on almost full-length AlkBs comprising 378–483 amino acids. The XylM of *Sphingomonas* sp. P2 was used as an outgroup. AlkBs from *A. dieselolei* B-5 are 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 reference flavin-binding monooxygenase sequences were retrieved form NCBI database. The scale bar denotes divergence percentage between sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

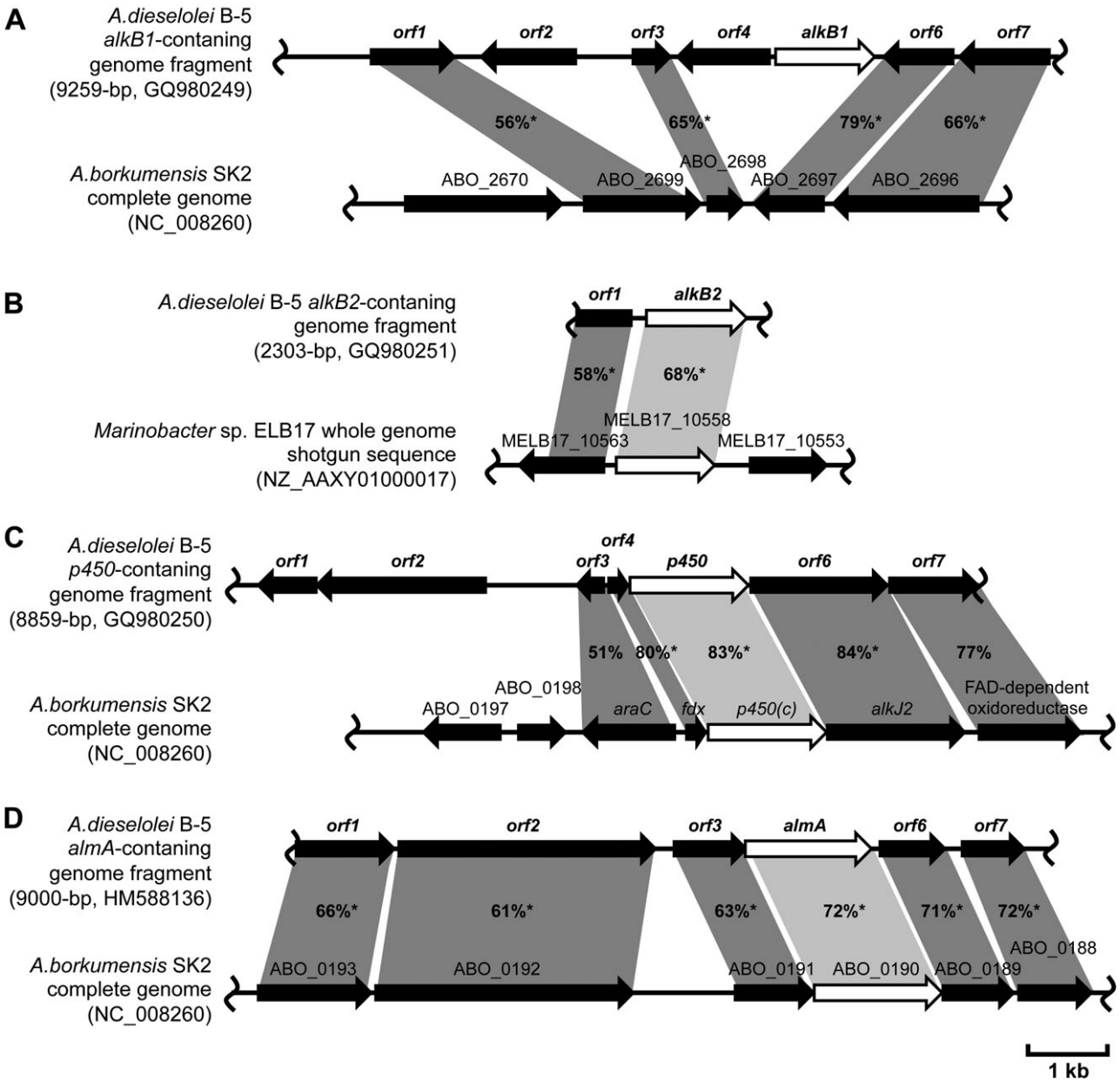

Fig. 2. Organization of alkane hydroxylase gene segments from *A. dieselolei* B-5 aligned to homologous genes from *A. borkumensis* SK2 or *Marinobacter* sp. ELB17.
A. *alkB1* gene cluster, gene designations: *alkB1*, alkane hydroxygenase; ABO_2696, short-chain dehydrogenase; ABO_2697, hypothetical protein; ABO_2698, hypothetical protein; ABO_2699, phospholipase D; ABO_2670, hypothetical protein.
B. *alkB2* gene cluster, MELB17-10563, AraC family transcriptional regulator; MELB17-10558, alkane-1-monooxygenase; MELB17-10553, putative glycine betaine-binding ABC transporter.
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_0198, TetR family transcriptional regulator.
D. *almA* gene cluster, *almA*, flavin-binding monooxygenase; 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 monooxygenase; 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 (GTCACTT) 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 *orf3*, *orf4* and *p450*. 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 *Sau*3AI-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 monooxygenase 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 monooxygenase 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

Table 2. Growth rates (h^{-1}) of *Pseudomonas* recombinants containing the *A. dieselolei* B-5 alkane hydroxylase genes.

Pseudomonas recombinants	C_6	C_8	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{16}
P. putida GPo12 (pGEc47ΔB)								
pCom8B5-*alkB1*	0.08	0.13	0.19	0.31	0.36	0	0	0
pCom8B5-*alkB2*	0.11	0.24	0.39	0.49	0.57	0.10	0	0
pCom12B5-*p450*	0.37	0.48	0.42	0.36	0.35	0.09	0	0
P. fluorescens KOB2Δ1								
pCom8B5-*alkB1*	0	0	0.020	0.029	0.032	0.030	0.277	0.294
pCom8B5-*alkB2*	0	0	0.034	0.034	0.098	0.123	0.198	0.241
pCom8B5-*almA*	0	0	0.004	0.019	0.047	0.058	0.089	0.129
pCom12B5-*p450*	0	0	0.093	0.085	0.137	0.129	0.235	0.206

almA and in the same orientation, there is an ORF (*orf3*) that encodes a protein of 63% identity to *A. borkumensis* SK2 alpha/beta fold family hydrolase (ABO_0191). Two polypeptides showing 66% (*orf1*) and 61% (*orf2*) amino acid identities to *A. borkumensis* SK2 proteins were found upstream of *orf3* (Fig. 2D).

Heterologous expression of alkB1*,* alkB2*,* p450 *and* almA

To determine whether the *alkB1* and *alkB2* genes encoded functional alkane hydroxylases, we cloned the B-5 *alkB1* and *alkB2* into a broad-host-range expression vector pCom8, respectively, placing it under the control of the *P. putida* GPo1 *alkB*-promoter. The resulting plasmid, pCom8B5-*alkB1* and pCom8B5-*alkB2* were introduced into *P. putida* GPo12 (pGEc47ΔB), a strain that lacks part of *alkB* gene, but contains all other genes necessary for growth on *n*-alkanes (Smits *et al*., 2002). The recombinant harbouring *alkB1* was able to grow on C_6–C_{12} *n*-alkane vapour, and had a substantial growth rate on C_8–C_{12} (Table 2). The shortest doubling time of 2.8 h was observed with C_{12}. Like many other alkane hydroxylases tested (Smits *et al*., 2002; van Beilen *et al*., 2004), the introduction of B-5 *alkB1* did not allow *P. putida* GPo12 cells grow on *n*-alkanes longer than C_{12}. In Table 2, *P. putida* recombinants harbouring *alkB2* showed an increasing growth rate (up to 0.57 h^{-1}) from C_6 to C_{12}. Unlike *alkB1*, the introduction of *alkB2* allowed *P. putida* recombinants grow on C_{13}. Subsequently, pCom8B5-*alkB1* and pCom8B5-*alkB2* were introduced into *P. fluorescen*s KOB2Δ1 (Smits *et al*., 2002) to test its catalytic ability towards alkanes shorter than C_{18} respectively. This recombinant strain harbouring *alkB1* grew slowly on C_{10}–C_{13} with doubling time of up to 31 h, while thrived on C_{14}–C_{16} *n*-alkanes (Table 2). The *P. fluorescens* KOB2D1 strain harbouring *alkB2* grew on C_{10}–C_{16} alkanes, with the fastest growth rate on C_{16}. Taken together, these results indicated that B-5 *alkB1* and *alkB2* encode functional alkane hydroxylases, which catalyses the hydroxylation of *n*-alkanes ranging from C_6 to C_{16} at the very minimum.

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 C_6–C_{12} *n*-alkanes. Although both *P. putida* recombinants grew on C_5, 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 C_{13}. On the other hand, in *P. fluorescens* KOB2Δ1, *p450* allowed a substantial growth of the host on C_{10}–C_{16}, with the fastest growth rate on C_{14}.

To determine whether the *almA* 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 C_{18} was tested. The results showed that the recombinant strain had a low but substantive growth rate on C_{12}–C_{16} (Table 2). The shortest doubling time of 5.37 h was observed on C_{16}.

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 ($C_{19}H_{40}$) and phytane ($C_{20}H_{42}$), 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, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and C_{26} significantly induced the expression of *alkB1* compared with controls. The largest

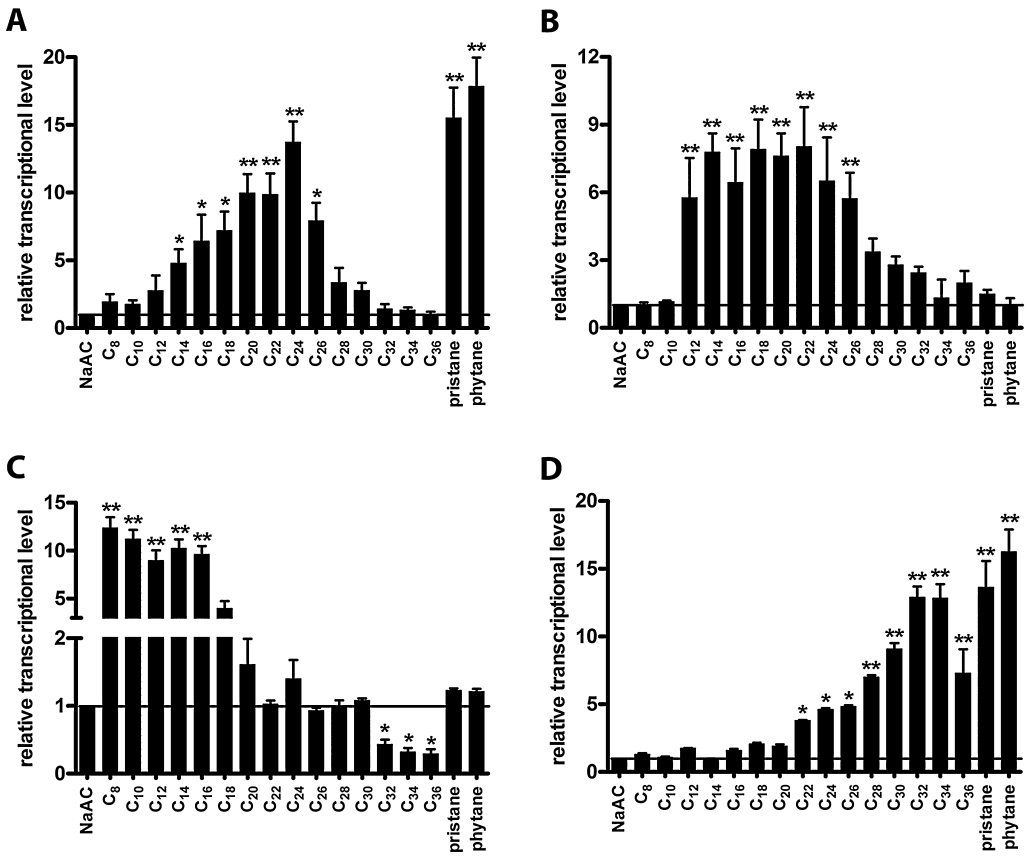

Fig. 3. Expression levels of *alkB1* (A), *alkB2* (B), *p450* (C) and *almA* (D) in *A. dieselolei* B-5 cells grown in different substrate as sole carbon and energy source. *gyrB* was used as the reference gene, and cells grown with sodium acetate were used as the control state. Substrates used are indicated. Relative expression levels were determined with real-time RT-PCR. $*P < 0.05$; $**P < 0.01$ versus control. Data are representative of three independent experiments.

increase (13.7-fold) was observed with C_{24}. Interestingly, *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 *alkB2* was induced by C_{12}–C_{26} *n*-alkanes to similarly high levels (5.7- to 8.0-fold increases). The inclusion of branched alkanes, or *n*-alkanes shorter than C_{12} or longer than C_{26} did not produce significant responses in *alkB2* transcription (Fig. 3B). Only *n*-alkanes shorter than C_{16} significantly increased *p450* mRNA level. The highest increase (12.4-fold) was observed for C_{12}. On the contrary, when grown on longer-chain alkanes (C_{32}, C_{34} and C_{36}), the expression of *p450* was remarkably depressed, compared with basal expression levels within control cultures (Fig. 3C).

Unlike the results obtained for the *alkB1, alkB2* and *p450* genes, *almA* mRNA levels responded to long-chain *n*-alkanes (C_{22}–C_{36}). Notably, longer-chain *n*-alkanes (C_{28}–C_{36}) produced high increases (7.0- to 12.9-fold). Branched alkanes (pristane and phytane) also elicited strong transcriptional increase of *almA* (Fig. 3D).

Discussion

Being a cosmopolitan marine bacterium, *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, *A. dieselolei* B-5 possesses at least four functional alkane hydroxylase genes: *alkB1*, *alkB2*, *p450* and *almA* that jointly provide this bacterium with the ability to utilize a wide substrate spectrum of alkanes.

Neighbour-joining phylogenetic analyses of *alkB*-type alkane hydroxylases showed four distinct clusters that separated well from each other (Actinobacteria, β-, γ- and α-proteobacteria). In case of *A. dieselolei* B-5, AlkB2 branched with other γ-proteobacteria AlkBs, while AlkB1 is located in the β-proteobacteria cluster and is closely related to *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* (zp01738706). 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 (Sabirova *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 C_5–C_{12} and C_5–C_{16} alkanes, respectively; P450s oxidized C_6–C_{10} substrates (van Beilen *et al*., 2004; 2006; Schneiker *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 (C_{12}–C_{26}). The substrate spectrum of *A. dieselolei* B-5 *almA* (C_{22}–C_{36}) 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 C_{12}–C_{20} alkanes in the transcriptional analysis (Fig. 3D), the *Pseudomonas* recombinant harbouring *almA* showed a substantive growth on C_{12}–C_{16} 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, C_{28}–C_{36} (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 C_{22}–C_{36}.

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 (Schneiker *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

Table 3. Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
Alcanivorax dieselolei B-5	Type strain, grows on C_5–C_{36} alkanes	Liu and Shao (2005)
Pseudomonas fluorescens KOB2Δ1	*alkB* knockout strain of *P. fluorescens* CHA0	Smits *et al.* (2002)
Pseudomonas putida Gpo12	*P. putida* Gpo1 cured of the OCT plasmid	Smits *et al.* (2002)
E. coli CC118(RK600)	Helper strain for triparental mating	Ditta *et al.* (1980)
E. coli DH10B	Donor strain for triparental mating	Invitrogen
E. coli DH5α	Cloning strain	Lab stock
Plasmid		
pGEc47ΔB	*alkFGHJKL, alkST*(Gpo1) in pLAFR1	van Beilen *et al.* (1992)
pCom8	Broad-host range expression vector, Gm^r Pro_{alk} *oriT alkS*	Smits *et al.* (2001)
pCom12-PxF200R1500	P450 expression vector, pCom8 containing the *Mycobacterium* sp. HXN200 ferredoxin and ferredoxin reductase.	van Beilen *et al.* (2006)
pCom8B5-*alkB1*	*A. dieselolei* B-5 *alkB1* gene in pCom8	This study
pCom8B5-*alkB2*	*A. dieselolei* B-5 *alkB2* gene in pCom8	This study
pCom8B5-*almA*	*A. dieselolei* B-5 *almA* gene in pCom8	This study
pCom12B5-*p450*	*A. dieselolei* B-5 *p450* gene in pCom12-PxF200R1500	This study

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 (van Beilen *et al.*, 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 method (Saitou and Nei, 1987), maximum-likelihood (Felsenstein and Churchill, 1996) 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 data by performing 1000 resamplings.

Heterologous expression of alkane hydroxylase genes

According to the method described by van Beilen and colleagues (van Beilen *et al.*, 2004), 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* GPo12 (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. Approximate 1×10^8 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 $P < 0.05$.

Sequences

The sequences of the four *A. dieselolei* B-5 alkane hydroxylase genes and flanking DNA have been deposited in the NCBI database with the following Accession Numbers: *alkB1*, GQ980249; *alkB2*, GQ980251; *p450*, GQ980250; and *almA*, HM588136.

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