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Genes involved in alkane degradation in the *Alcanivorax hongdengensis* strain A-11-3

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Abstract *Alcanivorax hongdengensis* A-11-3 is a newly identified type strain isolated from the surface water of the Malacca and Singapore Straits that can degrade a wide range of alkanes. To understand the degradation mechanism of this strain, the genes encoding alkane hydroxylases were obtained by PCR screening and shotgun sequencing of a genomic fosmid library. Six genes involved in alkane degradation were found, including *alkB1*, *alkB2*, *p450-1*, *p450-2*, *p450-3* and *almA*. Heterogeneous expression analysis confirmed their functions as alkane oxidases in *Pseudomonas putida* GPo12 (pGEc47ΔB) or *Pseudomonas fluorescens* KOB2Δ1. Q-PCR revealed that the transcription of *alkB1* and *alkB2* was enhanced in the presence of *n*-alkanes C_{12} to C_{24}; three *p450* genes were up-regulated by C_8–C_{16} *n*-alkanes at different levels, whereas enhanced expression of *almA* was observed when strain A-11-3 grew with long-chain alkanes (C_{24} to C_{36}). In the case of branched alkanes, pristane significantly enhanced the expression of *alkB1*, *p450*-3 and *almA*. The six genes enable strain A-11-3 to degrade short (C_8) to long (C_{36}) alkanes that are straight or branched. The ability of *A. hongdengensis* A-11-3 to thrive in oil-polluted marine environments may be due to this strain's multiple systems for alkane degradation and its range of substrates.

Keywords Marine oil pollution · Biodegradation · Alkane hydroxylase · Gene cluster · *Alcanivorax hongdengensis*

Introduction

Alkanes are highly reduced compounds of straight, branched or cyclic chains of various lengths. In nature, bacterial oxidation of *n*-alkanes has been identified worldwide (Head and Jones 2006; Smits et al. 2002; van Beilen et al. 2004, 2006; Wang et al. 2010; Whyte and Smits 2002). Members of the genus *Alcanivorax* seem to play a particularly pivotal role in bioremediation of oil spills (Liu and Shao 2005; Schneiker et al. 2006; Wu et al. 2009). *Alcanivorax* spp. are found not only in oil-impacted environments (Head and Jones 2006) but also in the open sea (Wang et al. 2010). The genus *Alcanivorax* currently includes six species including *A. borkumensis*, *A. jadensis*, *A. venustensis*, *A. dieselolei*, *A. hongdengensis* and *A. balearicus*; all of these species were isolated from marine environments except *A. balearicus*, which was isolated from a subterranean saline lake (Head and Jones 2006; Liu and Shao 2005; Schneiker et al. 2006; Wu et al. 2009). *A. borkumensis* metabolizes a wide range of alkanes, including linear alkanes (C_{10}–C_{32}), cyclo-alkanes and isoprenoids (Hara et al. 2003, 2004). *A. dieselolei* is a promising candidate for oil pollution mitigation due to its wide substrate range and hydrocarbon-degrading abilities (Liu and Shao 2005; Liu et al. 2011). The success of the genus *Alcanivorax* may be due to the ability of the members of this genus to use branched-

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chain alkanes more effectively than other hydrocarbon-degrading bacteria, giving these species a selective advantage (Hara et al. 2003; Liu et al. 2011).

In prokaryotes, several enzyme systems are involved in alkane degradation. Integral membrane di-iron alkane hydroxylase (AlkB) was the first enzyme discovered to have a role in alkane oxidation (van Beilen et al. 1994). Subsequently, cytochrome P450 of the CYP153 family was found to be involved in the oxidation of C_5 to C_{16} *n*-alkanes in *Acinetobacter* (Maier and Forster 2001; van Beilen et al. 2006). For long-chain alkanes, two oxidases have been identified: the soluble flavoprotein alkane monooxygenase LadA in *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007) and the flavin-binding monooxygenase AlmA in *Acinetobacter* sp. DSM 1784 (Throne-Holst et al. 2007).

The *A. hongdengensis* strain A-11-3 was isolated from the surface water of the Malacca and Singapore Straits (Wu et al. 2009). Like other hydrocarbonoclastic bacteria, *A. hongdengensis* is present at low or undetectable levels in unpolluted environments but blooms dramatically after an oil spill, becoming the predominant microbe in polluted marine waters (unpublished). Members of this species have been frequently detected in surface water, deep water and sediments of the Pacific Ocean, Atlantic Ocean, Indian Ocean, South China Sea, Sea of Japan and the Straits of Taiwan by our group and others (unpublished). The wide substrate range and broad geographic distribution of this species indicate its important role in marine oil degradation. This report aimed to identify the key enzymes of *A. hongdengensis* that are responsible for alkane degradation.

Materials and methods

Bacterial strains and growth conditions

A. hongdengensis A-11-3 ^{T} (LMG 24624) was originally isolated from the surface water of the Malacca and Singapore Straits (Wu et al. 2009). Table 1 lists other bacterial strains that were used as recipient strains and for gene cloning, as well as plasmids used in this study.

Strain A-11-3 was incubated at 28 °C with shaking at 200 rpm unless noted otherwise. *Escherichia coli* and *Pseudomonas* strains were grown at 30 °C in Luria–Bertani broth medium (Luria et al. 1960) and M9 salt medium (Sambrook et al. 1989), respectively. The recombinants of *Pseudomonas putida* and *Pseudomonas fluorescens* were grown under conditions previously described by van Beilen et al. (2004).

Degradation experiments

To test its oil-degrading ability, strain A-11-3 was cultivated first in M2 medium as previously described (Liu and Shao 2005); cells were then collected from a 10-ml culture and inoculated in 100 ml no carbon source medium ASM (Liu and Shao 2005) in 300-ml Erlenmeyer flasks with 2% (w/v) crude oil or liquid paraffin (mainly composed of C_{12}–C_{18} straight alkanes) as the sole carbon sources and then incubated at 28 °C on a rotary shaker (180 rpm for 20 days). Individual flasks were selected at intervals (gently vortex for 5 min to mix), and decimal dilutions of the cultures were made and plated on M2 agar plates for determination of the colony-forming unit (CFU) number.

To test the range of *n*-alkane substrates, ASM medium was supplemented with 1% (w/v) *n*-alkanes from C_8 to C_{36} or pristane as the sole carbon source, respectively. For *n*-alkanes ranging from C_{20} to C_{36}, 0.001% (w/v) of plysurf A-210 G was added into the medium as a dispersant. A set of uninoculated bottles was treated in parallel and served as the control. The bottles were incubated at 28 °C on a rotary shaker (180 rpm) for 7 days. In each bottle, residual *n*-alkanes were analyzed by gas chromatogram (GC). All experiments were performed in duplicate.

Gas chromatogram (GC) analysis

Residual *n*-alkanes were extracted using a continuous liquid–liquid extraction technique. The cultures were transferred to the liquid–liquid extractor. The interior of the flask was rinsed with hexane, and the washings were poured into the central well of the extractor, which allowed the solvent to rise slowly up through the sample and overflow into the round bottom reflux flask. The sample was extracted for 24 h, and then, the extract was adjusted to the same volume as that of the original culture, supplemented with 0.005% (w/v) of squalane as an internal standard. Finally, the sample was analyzed by GC for *n*-alkanes less than C_{36}. The extraction recovery rate was calculated by comparing the concentration of a specific alkane before and after extraction and was 93.2±3.72% for C_8, 94.7±3.32% for C_{12}, 95.7±2.27% for C_{16}, 94.7±3.63% for C_{24}, 92.7±4.27% for C_{32}, 93.5±4.21% for C_{36} and 92.6±3.07% for pristane.

GC analysis was performed using an Agilent Technologies 6820 N gas chromatograph equipped with an on-column injection, FID detector and SPBTM-5 capillary column (30 m× 0.53 mm i.d., 1.5 lm thickness). Nitrogen was used as a carrier and was set at a constant flow rate of 35 ml/min. The oven temperature was set at 150 °C for 5 min and then increased to 280 °C at a rate of 15 °C/min. The injector and detector temperatures were 280 °C and 350 °C, respectively.

Genomic fosmid library construction and analysis

A genomic library was constructed from strain A-11-3 DNA using the CopyControl^{TM} HTP fosmid library production kit with the pCC2FOS vector and the phage T1^{R}-resistant

EPI300 *E. coli* strain (Epicentre, Madison, WI). Briefly, high-molecular weight DNA was isolated from cells grown in M2 using a Wizard genomic DNA purification kit (Promega, Madison, WI) and sheared to ~40 kb by pipetting. The fragments were end repaired, ligated into the fosmid vector and packaged. The host cells were grown overnight according to the kit's instructions. Colonies were selected and transferred to LB plates containing chloramphenicol.

The degenerate primer set alkBwf (AAYACNGCNCAY GARCTNGGNCAYAA) and alkBwr (GCRTGRTGR TCNGARTGNCGYTG) (Kloos et al. 2006), which was designed based on the conserved regions between histidine boxes of alkane hydroxylase gene sequences, was used to screen the fosmid clones containing the *alkB* gene. The degenerate primer set CF (ATGTTYATHGCNATGGAYCCNC) and CR (NARNCKRTTNCCCATRCANCKRTG) (Kubota and Nodate 2005), which was designed based on the well-conserved N-terminal (MFIAMDPP) and C-terminal (HTCMGNRL) domains of CYP153 subfamily genes, was used to screen fosmid clones for the *P450* gene. For the initial screening, ten clones in one line of the 96-well plate were mixed, and DNA was isolated for PCR screening. From these mixed clone samples, the single clones that yielded positive PCR amplification bands were screened again for confirmation. The following PCR program was used: 3 min at 95 °C and 35 cycles of 30 s at 95 °C, 1 min at 52 °C to 55 °C and 1 min at 72 °C, 10 min at 72 °C and then stopped at 4 °C. The PCR products from the putative *alkB*- or *P450*-containing fosmid clones were cloned into the pMD18-T vector (TaKaRa Bio, China) and sequenced. The sequences were analyzed and compared with the *alkB* or *P450* sequences in the database using the blast program (http://www.ncbi.nlm.nih.gov/BLAST/). Then, the confirmed *alkB* or *P450* gene fragment was labeled and used as a DNA probe to hybridize with the selected gene. Fosmid clones were used to further screen positive clones containing different fragment inserts. DNA probe preparation and dot blot/Southern blot hybridization were performed using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer's instructions. The positive colonies containing the *alkB* or *P450* genes were selected for the next experiment.

Shotgun sequencing was used to determine the whole insert sequences of the selected fosmid clones. A pUC18 library of random 2-kb fragments obtained by sonication was constructed, and clones were sequenced from both ends using an ABI3700 sequencer (Applied Biosystem Inc., USA). The sequence was assembled using the program sequencer (Bioeditor 1.6.1). The open reading frame (ORF) analysis was performed using the GeneMark program (http://opal.biology.gatech.edu/GeneMark/). The putative ORF and relative sequences were compared to sequences in the NCBI, SwissProt and EMBL databases by blastN (http://www.ncbi.nlm.nih.gov/BLAST/), blastX (http://www.ncbi.nlm.nih.gov/BLAST/) and Wu-blast (http://www.ebi.ac.uk/blast2/), respectively. The results were summarized, and the genomic map of each clone was predicted. The relevant genes involved in the alkane hydroxylase system of the fosmid clones were arranged, compared and analyzed.

Heterologous expression of alkane hydroxylase genes

According to the method described by van Beilen et al. (2004), the *A. hongdengensis* A-11-3 *alkB1*, *alkB2* and *almA* genes were amplified using the appropriate primer pairs. Each amplification product was cloned into pCom8 (Smits et al. 2001) between the *NdeI* or *BamHI* and *HindIII* sites, respectively. The *P450* genes were amplified using the p4501, p4502 and p4503 forward and reverse primer pairs and cloned into pCom12-PxF200R1500 (van Beilen et al. 2006) between the *MfeI*, *EcoRI* and *PacI* sites, respectively. The PCR primers used in this study are listed in Table 2. The resulting plasmids pCom8-*alkB1*, pCom8-*alkB2*, pCom12-*P450-1*, pCom12-*P450-2* and pCom12-*P450-3* were transferred to *P. putida* GPo12 (pGEc47DB) by triparental mating as described previously (Hara et al. 2004) using *E. coli* DH10B as the donor and *E. coli* CC118 (RK600) as the helper strain (Ditta et al. 1980). The plasmids were transferred to *P. fluorescens* KOB2Δ1 by electroporation (Dower et al. 1988). In addition, pCom8-*almA* was transferred to *P. fluorescens* KOB2Δ1 by electroporation. The features of the plasmids used in this study are described in Table 1. The growth of the transformants on *n*-alkanes was tested as previously described by Smits et al. (2002).

Real-time PCR

A. hongdengensis A-11-3 was cultivated in ASM medium supplemented with sodium acetate or various alkanes as the sole carbon source (from C_8 to C_{36}, pristane) until the mid-exponential phase. Approximately 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). Total RNA yield was estimated using a Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE, USA). Approximately 4-μg RNA was reverse transcribed using 20-ng random primer (Invitrogen, Carlsbad, CA, USA) and the PrimeScript™ Reverse Transcriptase enzyme (TaKaRa, Dalian). 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

Table 1 Cloning strains, host strains and plasmids used or constructed in this work

Strain or plasmid	Relevant characteristics (genotype)	Reference or source
P. putida GPo12 (pGEc47ΔB)	alk$^-$ (alkB BamHI deletion); Tet^r	(Smits et al. 2002)
P. fluorescens KOB2Δ1	alkB1 deletion; C12–C16 Alk$^-$; C18–C28 Alk$^-$	(Smits et al. 2002)
E. coli DH5a	Cloning strain	Invitrogen
E. coli CC118 (RK600)	Helper strain for triparental mating	(Ditta et al. 1980)
pCom8	Broad-host-range expression vector with P*alkB*; Gm^r *oriT alkS*	(Smits et al. 2001)
pGEc47ΔB	pGEc47, deletion in *alkB*	(van Beilen et al. 1992)
pCom12-Px$F_{200}R_{1500}$	As pCom8-PA7F200R1500, with the CYP153 gene cloned between the *EcoRI* and *PacI* sites	(van Beilen et al. 2006)
pCom8-*alkB1*	1,380-bp *NdeI-hidIII* fragment from pTQ15alkB1 cloned in pCom8/*NdeI-HidIII*	This study
pCom8-*alkB2*	1,149-bp *NdeI-hidIII* fragment from pTQ15alkB2 cloned in pCom8/*NdeI-HidIII*	This study
pCom8-*almA*	1,527-bp *BamHI-hidIII* fragment from pTQ15alkB2 cloned in pCom8/*BamHI -HidIII*	This study
pCom12-$P_{450\text{-}1}F_{200}R_{1500}$	1,341-bp *MfeI-PacI* fragment from pTQ15P450-1 cloned in pCom12/*MfeI-PacI*	This study
pCom12-$P_{450\text{-}2}F_{200}R_{1500}$	1,383-bp *EcoRI-PacI* fragment from pTQ15P450-2 cloned in pCom12/*EcoRI -PacI*	This study
pCom12-$P_{450\text{-}3}F_{200}R_{1500}$	1,356-bp *MfeI-PacI* fragment from pTQ15P450-3 cloned in pCom12/*MfeI-PacI*	This study

were synthesized by Invitrogen (Carlsbad, CA, USA). Quantitative real-time PCR was performed using IQ^{TM}SYBR Green supermix and an IQ^{TM} 5 Multicolor Real-time PCR Detection System (both from Bio-Rad, CA, USA). The 16S rRNA housekeeping gene was used as a reference gene to normalize gene expression in strain A-11-3. The relative fold change in mRNA quantity was calculated for the gene of interest in each sample using the ΔΔCt method. For each

Table 2 Oligonucleotides used in this study

Oligonucleotides	Sequence (5′–3′)
alkB1f	AGCATATGGCTACATTAGAACAAGGC
alkB1r	TAAAGCTTCCGCAGTCGGGGCA
alkB2f	TAAAGCTTACGCGATATTGTTGACT
alkB2r	ATCATATGTTCGAGAATATGAATGCC
p4501f	GCTTAATTAATTTTTTGCCGTCAGTTTG
p4501r	GACAATTGATGCATTTTCAGATTAAAACGA
p4502f	GCGAATTCATGTCACTGAAATCCACCGCGA
p4502r	CTTAATTAATTATTTTTTCGCTGTCAGCTGCACC
p4503f	ATCAATTGATGGAAGTCGCCCAGCAAGAGG
p4503r	CGTTAATTAATTATCGCTTCTCCAGCACCACC
almAf	ATGGATCCATTACGCCAA GTTTGCACGC
almAr	ATAAGCTTGAATTAGAACTCGGTACGCG
16SRTf	GCACAAGCGGTGGAGCAT
16SRTr	AACCCAACATCTCACGACACGA
alkB1RTf	TTCCTGCCGCGCACCATGAT
alkB1RTr	GCAGGCCGTAGTGCTCCATGTAGTT
alkB2RTf	GGCTCTGGTTGTAACGGCTTGA
alkB2RTr	TGGATGGGCGGTTTGCTG
P4501RTf	GAGAATTTACCGACGAAGATG
P4501RTr	CCAACGATAAGCAGAGCC
P4502RTf	TACGAACTGCGGCACAAG
P4502RTr	CATCAGGCTAATCAAATCAAAC
P4503RTf	TACCGACGGGCTAATGCT
P4503RTr	CTGCCGATGTGGCTGTTC
almARTf	ATAGGTTAAATACGGTTCTCTGCAG
almARTr	CAGCACTGGCCAGATAACTACG

The underlined entries are restriction enzyme cleavage sites sequence

RNA preparation, at least three independent real-time PCR experiments were conducted. The sequences of gene-specific primers are listed in Table 2.

Statistical analysis

Data were analyzed by 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 $P<0.05$.

Sequence accession numbers

The sequences of the five *A. hongdengensis* A-11-3 alkane hydroxylase genes and flanking DNA have been deposited in the NCBI database with the following accession numbers: JF747236 through FJ747240.

Results

Utilization of oil and alkanes by the A-11-3 strain

The A-11-3 strain was inoculated into ASM medium supplemented with crude oil or liquid paraffin as the sole carbon source. Growth on liquid paraffin started on day 1 (2%), and growth on crude oil started on day 2 (Fig. 1). During the lag stage, crude oil-containing medium was emulsified, indicating the production of an emulsifying agent (data not shown). In fact, the strain produced lipopeptides as its surface-active compounds (Wu et al. 2009). When tested with C_6 to C_{40} and pristane, degradation occurred from C_8 to C_{36} and pristane (Fig. 2). Vigorous degradation occurred from C_8 to C_{28} and pristane. The most degradation occurred in C_{16}, which was degraded by approximately 90% in 7 days.

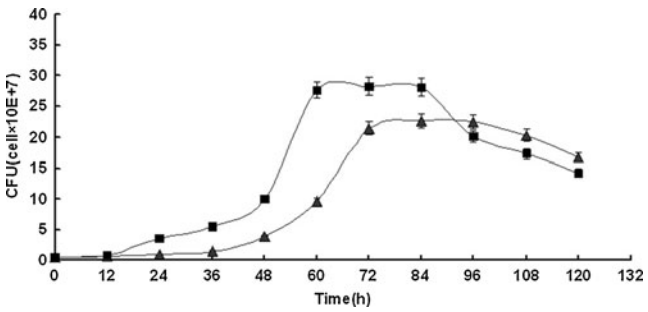

Fig. 1 Growth of A-11-3 in mineral medium ASM supplemented with 2% (w/v) crude oil (*filled triangles*) and liquid paraffin (*filled squares*) as the sole carbon source. Cells were grown at 28 °C on a rotary shaker (180 rpm). Data are the means of duplicate flasks

Sequence analysis of fosmid clones containing *AlkB* and *P450* sequences

In the A-11-3 strain genomic fosmid library, after PCR screening with the degenerate primer set alkBwf and alkBwr and further verification by Southern hybridization, two fosmid clones that contained putative *alkB* genes, named *alkB1* and *alkB2,* were successfully obtained. Similarly, using degenerate primers CF and CR and further Southern hybridization, three clones containing putative *p450* genes, named *p450-1, p450-2* and *p450-3,* were obtained. Using the appropriate probes, Southern blotting analysis revealed that only one copy each of these *alkB* and *p450* genes was carried on the A-11-3 chromosome (data not shown).

These fosmid clones contained DNA inserts of approximately 40 kb in size. Flanking regions of approximately 19- and 25-kb DNA around *alkB1* and *alkB2,* respectively, and of approximately 21- to 36-kb DNA around three *P450* genes were sequenced. The putative ORFs and their nucleotide sequences were blasted and analyzed. The detailed annotations are summarized in supplementary Tables S1 to S5. The gene clusters putatively involved in alkane degradation are illustrated in Figs. 4 and 6 and described in detail in the following discussion.

Sequence analysis of *alkB* gene clusters

Four conserved histidine-containing motifs, including Hist1 (HE[L/M]XHK), Hist2 (EHXXGHH), Hist3 (LQRH[S/A] DHHA) and the HYG motif (NYXEHYG[L/M]), were found in both AlkB1 and AlkB2, which are well conserved in the bacterial AlkB family (Whyte and Smits 2002). A phylogenetic tree was constructed based on the alignment of amino acid sequences of membrane-bound alkane hydroxylases (Fig. 3). The amino acid sequence identity between AlkB1 and AlkB2 was very low (38.6%). AlkB2 is closely related to AlkB2 of *A. borkumensis* SK2. However, AlkB1 shares little sequence identity with all reported AlkB, 49.6% sequence identity with AlkM of *Acinetobacter* sp. ADP1, 47.5% with AlkMa of *Acinetobacter* sp.M-1 and less than 45% with all other alkane hydroxylases. The detailed ORF annotations are provided in Tables S1 and S2.

In the *alkB1* gene cluster (Fig. 4a), five *orf*s were probably involved in alkane degradation including dehydrogenase (*orf6*), rubredoxin protein (*orf8*), rubredoxin reductase (*orf9*), *orf7* and *ofr10*. *Orf7* encodes an alkane hydroxylase-rubredoxin fusion protein of *alkB1* and *rubA1* that is joined by a 3′-end-5′-end ATGA sequence overlap. *Orf6* encodes an alcohol dehydrogenase (ADH), which is involved in the second step of alkane oxidation by converting alcohol into aldehyde. *Orf8* encodes a rubredoxin protein, which shuttles

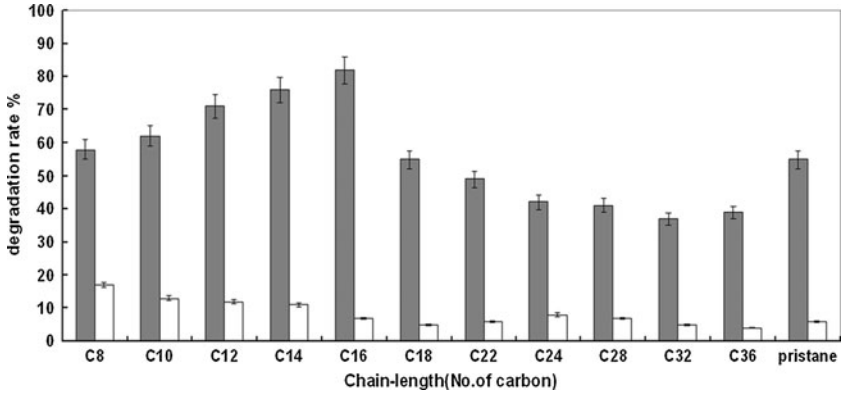

Fig. 2 Degradation of individual *n*-alkanes by A-11-3. The degradation rates of individual *n*-alkanes after addition of A-11-3 (*hatched bars*) and without addition of any bacteria (*open bars*) are shown. As previously described, the cells were grown in 100 ml of ASM medium supplemented with 0.01% yeast extract and 1% (w/v) individual *n*-alkanes in 300-ml culture bottles at 28 °C on a rotary shaker (180 rpm) for 7 days. Individual bottles were selected, and the residual *n*-alkanes were extracted and analyzed by GC. Data are the means of duplicate culture bottles

electrons from rubredoxin reductase. *Orf9* encodes a rubredoxin reductase, which is an essential component of the alkane hydroxylase system. *Orf10* encodes a polypeptide similar to a TetR family transcriptional regulator.

The *alkB2* region in strain A-11-3 is most similar to the *alkB2* operon in *A. borkumensis* SK2 (Fig. 4b, Table S2). Upstream of *alkB2, Orf20* encodes GntR, a bacterial regulatory protein with 87% sequence identity with its ortholog in *A. borkumensis* SK2.

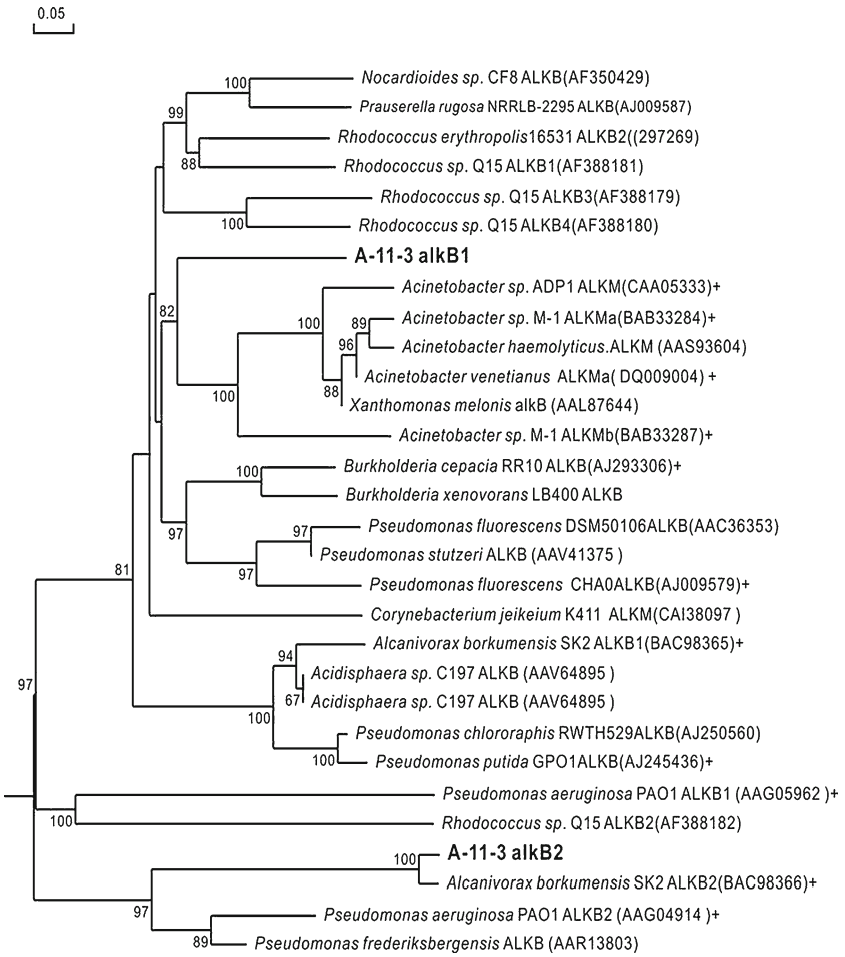

Fig. 3 Rooted phylogenetic tree based on an alignment of full-length amino acid sequences of AlkB obtained from A-11-3 and references. Alkane hydroxylases that were shown to be functional by heterologous expression or gene knockouts are indicated by (+). *Scale bar*, 0.05 substitutions per amino acid site

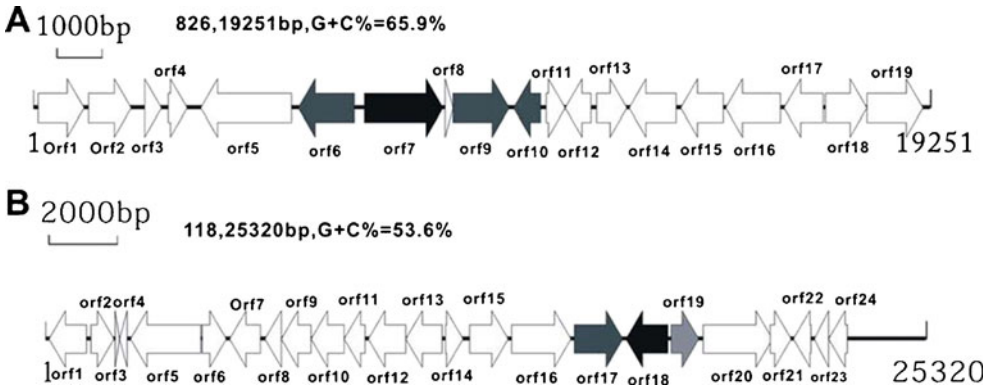

Fig. 4 Genomic map showing the gene organization of fosmid clone 826 and 118. **a** Fosmid clones 826 and **b** 118 containing *alkB1* and *alkB2,* respectively. The complete genomic sequences of fosmid clones 826 and 118 were determined by short-gun sequencing as described in the "Materials and methods" section. The GeneMark program was used to perform open reading frame (ORF) analysis. Clone 826 is 19,251 bp with 19 predicted ORFs, which were represented by orf 1–19. Clone 118 is 25,320 bp with 24 predicted ORFs, which were represented by orf 1–24. The detailed information for the ORFs of clones 826 and 118 is presented in Tables S1 and S2, respectively. The transcriptional direction of the ORFs is represented by the direction of the *arrow*. The *alkB* gene is marked in *black*, genes possibly involved in the alkane hydroxylase system are marked in *gray*, and other genes are in *white*

Sequence analysis of *P450* gene clusters

Strain A-11-3 harbors three *P450* genes of full-length fragments from 1,342 to 1,383 bp, encoding peptides with >75% identities to previously described P450 proteins. P450-1 and P450-3 are closest to P450 (b) and P450 (a) of *A. borkumensis* SK2, respectively. The *P450-1* gene displays 84% identity to P450 (b) of strain SK2. P450-3 shares 92% identity with P450 (a) of SK2. P450-2 was branched with *Marinobacter* sp. EB104 P450, and these genes shared 75% identity (Fig. 5).

Analysis of genes upstream and downstream of *P450-1* and *P450-3* revealed that the two gene clusters share the highest identity with strain SK2 protein and formed a very similar gene cluster to that of SK2 *p450(c)* or *(a)* (Fig. 6). Notably, the downstream gene of *P450-1* shares the highest identity (85%) with a putative flavin-containing monooxygenase gene of *A. borkumensis* and is least related to other *almA* genes. For example, it shares only 53.6% with the *almA* of *Acinetobacter* sp. DSM 17874. A putative alcohol dehydrogenase gene (84% identity to *A. borkumensis* SK2 dehydrogenase) is positioned immediately downstream of *almA* and is orientated in the same direction (indicated as *orf22*; Fig. 6a). Downstream of *orf22* and oriented in the same direction, an ORF (*orf23*) is present, which encodes a protein of 86% identity to the *A. borkumensis* SK2 putative metal hydrolase (YP_691908). Upstream of *almA* and in the same orientation, there is an ORF (*orf20*) that encodes a protein with 79% identity to the *A. borkumensis* SK2 alpha/beta fold family of hydrolases (YP_691911). However, no *orf*-encoding enzyme involved in the conversion of alkane to fatty acid was found in the flanking regions of *P450-3.* In addition, the gene organization of the *P450-2* region is different from that of *P450-1* and *P450-3* (Fig. 6b). The detailed ORF annotations are provided in Tables S3, S4 and S5.

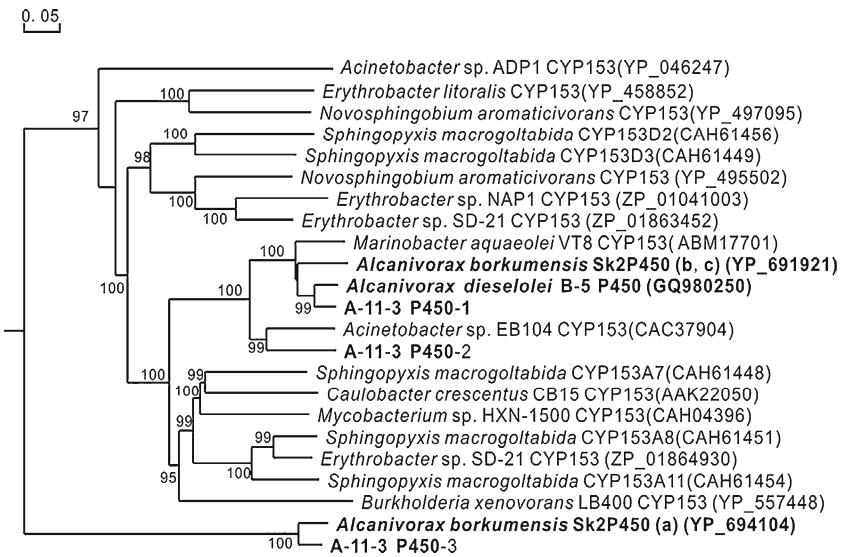

Fig. 5 Rooted phylogenetic tree based on an alignment of full-long amino acid sequences of P450 obtained from A-11-3 and references. *Scale bar*, 0.05 substitutions per amino acid site

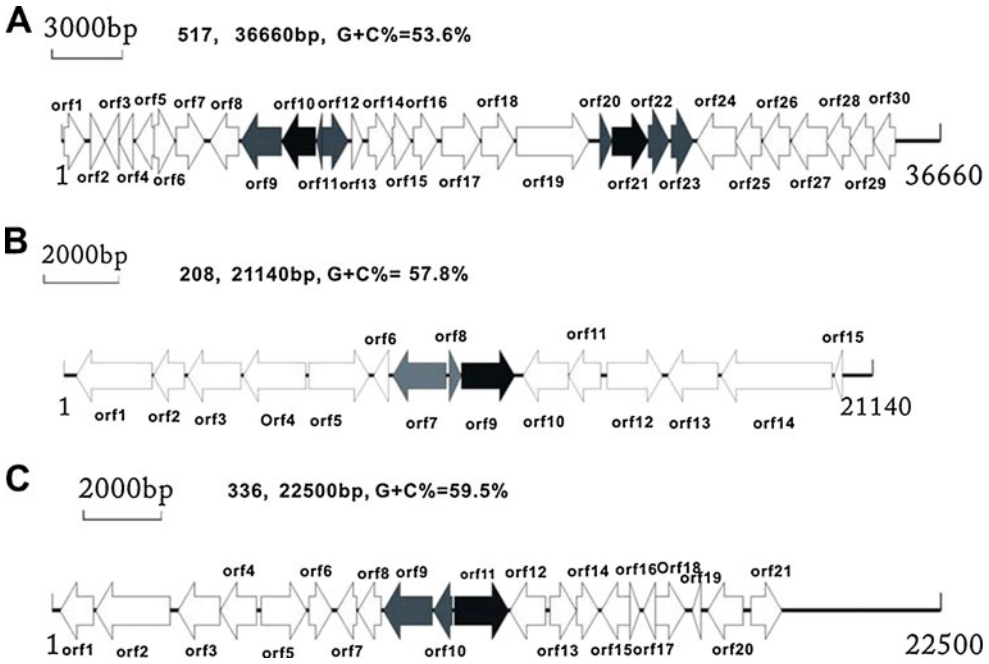

Fig. 6 Genomic map showing the gene organization of fosmid clones 517, 208 and 336**. a** Fosmid clone 517 containing *P450-1* (orf10) and *almA* (orf21); **b** clone 208 containing *P450-2* (orf 9); **c** clone 336 containing *P450-3* (orf11). The complete genomic sequences of fosmid clones 517, 208 and 336 were determined by short-gun sequencing as described in the "Materials and methods" section. The GeneMark program was used to perform open reading frame (ORF) analysis. Clone 517 (**a**) is 36,660 bp with 30 predicted ORFs, which were represented by orfs 1–30. Clone 208 (**b**) is 21,140 bp with 15 predicted ORFs and represented by orfs 1–15. Clone 336 (**c**) is 22,500 bp with 21 predicted ORFs and represented by orfs 1–21. The detailed information of the ORFs of clones 517, 208 and 336 is presented in supplementary Tables S3, S4 and S5, respectively. The transcription direction of the ORFs is represented by the direction of the *arrow*. The *P450* gene is marked in *black*, other genes possibly involved in the alkane hydroxylase system are marked in *gray*, and the other genes are marked in *white*

Heterologous expression of *alkB*, *P450* and *almA*

For functional confirmation, the putative *alkB* genes were inserted into the broad-host-range expression vector pCom8, placing it under control of the *P. putida* GPo1 *alkB* promoter. The resulting recombinant plasmids, pCom8-*alkB1* and pCom8-*alkB2,* were introduced into *P. putida GPo12* (pGEc47ΔB), a strain that lacks part of the *alkB* gene but contains all other genes necessary for growth on *n*-alkanes (Smits et al. 2002). Subsequently, pCom8-*alkB1* and pCom8-*alkB2* were introduced into *P. fluorescens* KOB2Δ1 (Smits et al. 2002) to test their catalytic abilities on alkanes shorter than C_{18}. As a result, the plasmid pCom8-*alkB1* provided the *alkB*-deficient mutant *P. putida* GPo12 (pGEc47ΔB) with the capability to grow on C_6 to C_{12} *n*-alkanes (Table 3); the pCom8-*alkB1* enabled the recombinant *P. fluorescens* KOB2Δ1 to grow vigorously on C_{13} to C_{16} *n*-alkanes (Table 3). These results indicate that *alkB1* catalyzes the hydroxylation of *n*-alkanes ranging from C_6 to C_{16} at the very minimum. Similarly, the *alkB2* recombinant strains of the *P. putida* GPo1 and *P. fluorescens* can grow on C_6 to C_{16} *n*-alkanes. The shortest doubling time was 1.5 h with C_{12}. However, the *P. putida* GPo1 and *P. fluorescens* cannot degrade branched alkanes (data not shown).

Similarly, the *p450* genes were inserted into pCom12-PxF200R1500, which encodes ferredoxin and ferredoxin reductase (van Beilen et al. 2006), and transformed into corresponding recipient strains. The plasmids pCom12-PxF200R1500 containing *p450-1* and *p450-2* genes enabled the strains of *P. putida* GPo12 and *P. fluorescens* KOB2Δ1 to grow on C_6 to C_{16} *n*-alkanes, respectively (Table 3). In the case of *P450-1,* octane (C_8) was the best substrate for growth of the recombinant strain *Pseudomonas* with the shortest doubling time of 1.47 h among C_6–C_{16}. In contrast, the recombinant containing *p450-2* exhibited comparably fast growth with C_{10} to C_{12} *n*-alkanes. However, the *P450-3* recombinant strain was not active in alkane degradation and showed only slow growth on C_6 to C_{11} *n*-alkanes. Thus, they showed different selections on alkanes from C_6 to C_{16}.

The gene *almA* was inserted in vector pCom8, and the resulting recombinant plasmid pCom8A-*almA* was transformed into *P. fluorescens* KOB2Δ1. The catalytic ability was tested with alkanes shorter than C_{18}. As a result, the recombinant strain had a low growth rate on C_{12} to C_{16} (Table 3). The shortest doubling time, 6.58 h, occurred with C_{16}.

Gene expression induced by different alkanes

Quantitative real-time PCR analysis showed that *alkB1* expression was strongly induced by C_{12}–C_{24} and pristane. C_{16}

Table 3 Growth rates of *P. fluorescens* KOB2Δ1, *P. putida* GPo12 (pGEc47ΔB) and derived recombinants on *n*-alkanes ranging from C_6 (hexane) to C_{16} (hexadecane)

Strain or plasmid	Growth rate (h^{-1}) on							
	C_6	C_8	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{16}
P. putida GPo12 (pGEc47ΔB)								
pCom8-*alkB*1	0.13	0.21	0.39	0.41	0.52	0.08	0	0
pCom8-*alkB*2	0.16	0.33	0.27	0.33	0.46	0.10	0	0
pCom12-*P450*-1	0.43	0.47	0.44	0.37	0.39	0.09	0	0
pCom12-*P450*-2	0.28	0.30	0.41	0.43	0.48	0	0	0
pCom12-*P450*-3	0.11	0.16	0.21	0.25	0.39	0	0	0
pCom8	0	0	0	0	0	0	0	0
pCom12	0	0	0	0	0	0	0	0
P. fluorescens KOB2Δ1								
pCom8-*alkB*1	0	0	0.054	0.053	0.096	0.103	0.178	0.227
pCom8-*alkB*2	0	0	0.032	0.030	0.105	0.112	0.191	0.241
pCom8-*almA*	0	0	0	0.025	0.039	0.082	0.107	0.158
pCom12-*P450*-1	0	0	0.027	0.031	0.129	0.134	0.274	0.103
pCom12-*P450*-2	0	0	0.024	0.019	0.102	0.114	0.297	0.209
pCom12-*P450*-3	0	0	0	0	0.107	0.135	0.210	0.227
pCom8	0	0	0	0	0	0	0	0
pCom12	0	0	0	0	0	0	0	0

and pristane increased *alkB1* expression by 8.7-fold and 5.8-fold, respectively. However, *alkB1* expression was only weakly induced by the short-chain alkane (C_8), and there was no obvious response to C_{28}, C_{32} or C_{36} (Fig. 7a). The *alkB2* gene was induced significantly by C_{12} to C_{18} (4.4- and 9.4-fold, respectively) and moderately by C_8 and C_{24} to C_{36} (approximately 1.4-fold). In contrast to *alkB*1, *alkB*2 was not obviously induced by pristane (Fig. 7a).

The expression profiles of three *P450* genes were somewhat different (Fig. 7b). None was sensitive to *n*-alkanes longer than C_{18}, and all were induced significantly by C_{12} to C_{16}. *P450*-1 was induced most significantly by C_{12} (9.8-fold increase) and was not induced by pristane. The *P450-2* gene was significantly induced by C_8 to C_{16}, and C_{14} induced the highest increase (9.6-fold). The *P450-2* gene was not sensitive to pristane. Surprisingly, the *P450-3* gene, which was most significantly induced by C_{16}, was also significantly induced by pristane (6.7-fold increase).

Unlike the results obtained for the *alkB1*, *alkB2* and three *p450* genes, *almA* mRNA levels responded to long-chain *n*-alkanes (C_{18}–C_{36}). Notably, longer-chain *n*-alkanes (C_{24}–C_{36}) produced high increases (8.8- to 24.5-fold). However, the induction expression was not observed in the case of C_8–C_{16} alkanes. Branched alkanes (pristane) also elicited strong transcriptional increase of almA (Fig. 7c).

Discussion

Bacteria of the genus *Alcanivorax* play a key role in bioremediation of oil spills (Schneiker et al. 2006). *A. hongdengensis* A-11-3 utilizes alkanes ranging from C_8 to C_{36} but not those less than C_8, indicating that strain A-11-3 has evolved a mechanism for degradation of medium- or long-chain *n*-alkanes but not short chains ($<C_6$). In this report, the characterization of six alkane hydroxylases participating in the catabolism of saturated hydrocarbons in strain A-11-3 was unambiguously identified, including two homologs of AlkB (AlkB1 and AlkB2), three P450 homologs (P4501–3) and an AlmA-like (AlmA) alkane hydroxylase. Heterologous expression and Q-PCR analysis showed that the three P450 were involved in the oxidation of medium-chain (C_8–C_{16}) substrates. The AlmA involved in the oxidation of long-chain (C_{18}-C_{36}) *n*-alkanes, and AlkB1 and AlkB2 oxidize both medium- and long-chain *n*-alkanes (C_{12}–C_{24}). These multiple alkane hydroxylase systems ensure that strain A-11-3 can utilize alkane substrates of a broad chain length range.

The presence of multiple systems for alkane degradation may be a common feature of *Alcanivorax* spp. *A. borkumensis* SK2 is the first and most extensively studied member of this genus and harbors two *alkB* genes and three *P450* genes (Schneiker et al. 2006). Recently, in the type strain *A. dieselolei* B-5, four genes, including two *alkBs*, one *P450* and one *almA*, have been found to be involved in alkane assimilation (Liu et al. 2011). However, variations exist among these homologs in different strains not only in the number of homologs but also in their sequence and gene organization, indicating the diversity that has evolved in genes responsible for alkane assimilation.

In the strain *A. borkumensis* SK2, both *alkB*1 and *alkB*2 can be induced by *n*-alkanes of short to medium chains (C_5–C_{16}) (van Beilen et al. 2004, 2006). In the case of *A.*

dieselolei B-5, the *alkB1* and *alkB2* genes were induced by C_{14}–C_{26} and C_{12}–C_{26} alkanes, respectively (Liu et al. 2011). In this report, the *alkB*1 and *alkB*2 genes of strain A-11-3 can be induced by alkanes of medium- to long-chain *n*-alkanes (C_{12}–C_{24}). Interestingly, *alkB*1 of strain A-11-3 catalyzes the oxidation of branched alkanes, which is a similar function to that of *alkB*2 of strain SK2 and *alkB*1 of strain B-5 (Liu et al. 2011; Schneiker et al. 2006; van Beilen et al. 2004). However, *alkB*2 of strain A-11-3 has no response to branched alkanes despite its grouping in the same phylogenetic branch with *alkB*2 of strain SK2.

P450 systems seem to be essential in the assimilation of short- to medium-length alkanes. In strain SK2, the two genes P450 (b) and P450 (c) can oxidize C_6–C_{14} alkanes (Schneiker et al. 2006; van Beilen et al. 2004, 2006). In strain B-5, the *P450* genes can oxidize C_8–C_{16} substrates (Liu et al. 2011). In strain A-11-3, the three *P450* genes can oxidize C_8–C_{16} substrates. Phylogenetically, the two *P450* genes of strain SK2, the two *P450* genes of strain A-11-3 and the *P450* genes of strain B-5 are grouped in the same cluster with those of *Acinetobacter* sp. EB104 (Fig. 5). These proteins catalyze the oxidation of short- to medium-chain *n*-alkanes (C_8–C_{16}). The *P450-3* gene of strain A-11-3 was grouped in another

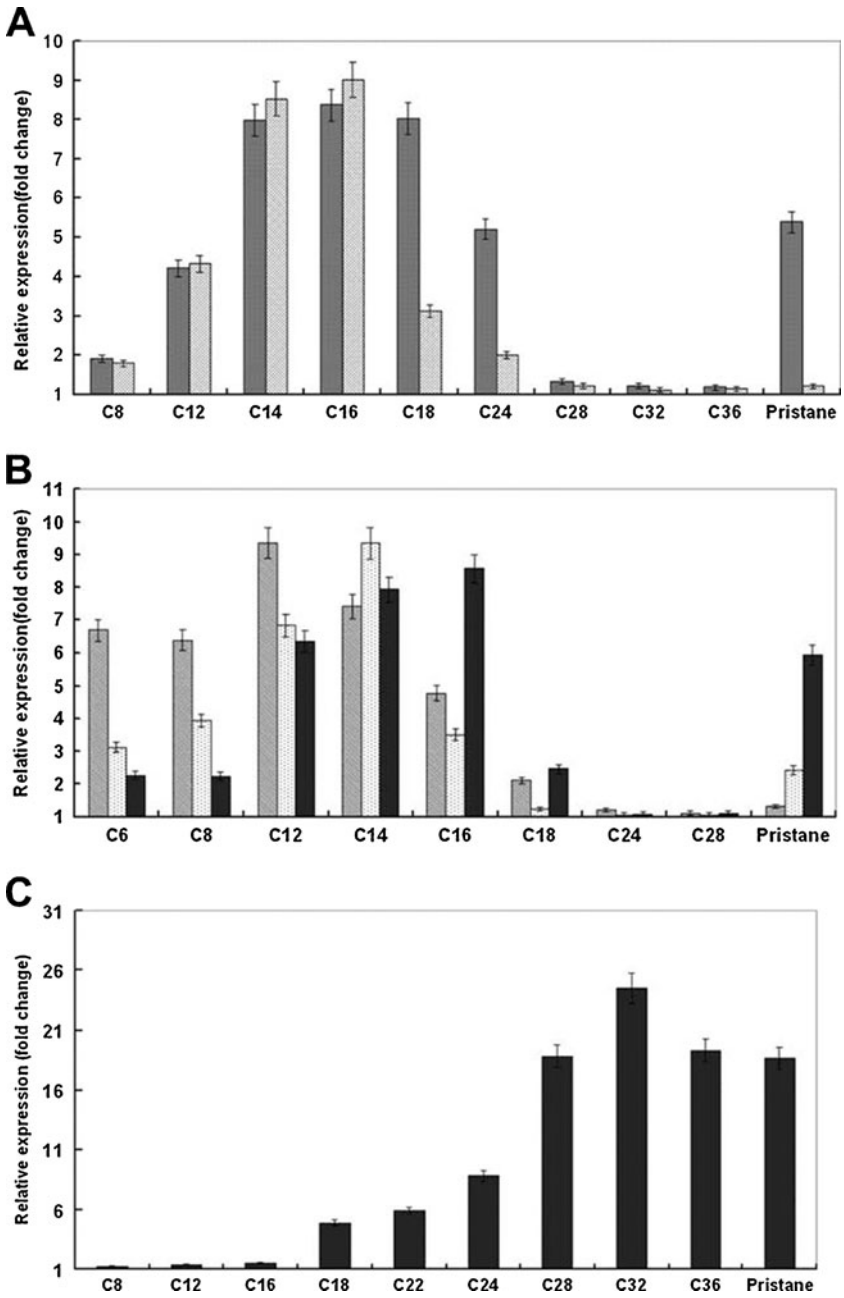

Fig. 7 Transcript levels of *alkB, P450* and *almA* genes in A-11-3 during incubation with 0.5% (v/v) individual *n*-alkanes. **a** Transcript levels of *alkB1* (*black columns*) and *alkB2* (*striped columns*) genes; **b** Transcript levels of *P450-1* (*striped columns*), *P450-2* (*punctate columns*) and *P450-3* (*black columns*) genes; **c** Transcript levels of *almA* genes

branch with P450 (a) of strain SK2, both of which can catalyze the oxidation of medium-chain *n*-alkanes (C_{12}–C_{16}) and branched alkanes (pristane or phytane).

The *almA* gene was found downstream of *P450-1* in strain A-11-3. The expression of *almA* is enhanced by the presence of long-chain *n*-alkanes (C_{18} to C_{36}) and branched alkanes (pristane) in strain A-11-3, and the *Pseudomonas* recombinant harboring *almA* showed substantial growth on C_{12}–C_{16} alkanes (Table 3), suggesting that A-11-3 *almA* encodes alkane hydroxylase. This evidence complements the substrate ranges of *alkB* and *p450* genes, and these results clearly indicated that the ability of strain A-11-3 to degrade long-chain *n*-alkanes is dependent on the expression of *almA*.

Branched-chain alkanes are thought to be more difficult to degrade than linear alkanes (Pirnik et al. 1974). However, increasing numbers of bacteria have been found to degrade branched-chain alkanes such as isooctane (Solano-Serena et al. 2004) and pristane (Koopmans et al. 1999). In *A. borkumensis* SK2, isoprenoid hydrocarbon (phytane) acts as a strong inducer of *P450* (a) and *alkB*2 (Liu et al. 2011; Schneiker et al. 2006; van Beilen et al. 2004). Recently, we found that both pristane and phytane activate the expression of *alkB*1 and *almA* in *A. dieselolei* B-5 (Liu et al. 2011). In this report, we found that pristane selectively activates the expression of *alkB1, P450-3* and *almA* in strain A-11-3. The superior ability of *Alcanivorax* bacteria to degrade branched alkanes would increase their usefulness in oil-containing seawater, as proposed previously (Hara et al. 2003).

The *alkB1* cluster in strain A-11-3 is special among *Alcanivorax* bacteria due to its gene organization. However, it is very similar to the *alkB1* cluster in *Rhodococcus* sp. Q15 (Whyte and Smits 2002) and the *alkB*6 cluster in *Geobacillus* sp. strain MH-1 (Liu et al. 2009). Despite the obvious difference in gene organization compared to other *Alcanivorax* strains, the ORFs in the *alkB1* cluster shared high sequence homologies with their homologs in strain SK2.

In summary, the results of this report confirmed that *Alcanivorax* bacteria thrive in oil-polluted marine environments by employing different kinds of alkane monooxygenases, including AlkB, P450 and AlmA, to assimilate a variety of alkanes varying from short to long and from straight to branched chains. However, their homologs varied in both sequence similarity and gene organization.

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