Extremophiles (2008) 12:255–262
DOI 10.1007/s00792-007-0122-x

ORIGINAL PAPER

Isolation and characterization of alkane hydroxylases from a metagenomic library of Pacific deep-sea sediment

Meixiang Xu · Xiang Xiao · Fengping Wang

Received: 18 September 2007 / Accepted: 29 October 2007 / Published online: 18 December 2007

Abstract Two clones 9E7 and 21G8 in a metagenomic library of the east Pacific deep-sea sediment were found to contain alkane hydroxylase genes (*alkB*). The whole insert sequences of the two cosmid clones were determined. The insert sequences of 9E7 and 21G8 are 40 and 35 kb, respectively. Besides *alkB*, several alcohol/aldehyde dehydrogenase genes were also determined. A homolog of rubredoxin 2 of *Pseudomonas putida* was identified on 9E7 immediately downstream the *alkB* gene, but was lacking on 21G8. Unlike previous reports, the *alkB* genes on 9E7 and 21G8 have opposite transcription directions to those of linked alcohol/aldehyde dehydrogenase genes. Phylogenetic analysis put these two deep-sea AlkBs into a unique branch of integral membrane hydroxylases. The two *alkB* genes (9E7–*alk*B and 21G8-*alk*B) were cloned into pCom8 and introduced into two *alk*B expression host systems *P. fluorescens* KOB2Δ1 and *P. putida* GPo12 (pGEc47ΔB). The transformed strains can grow on the *n*-alkanes from C5 to C16, indicating that both 9E7-AlkB and 21G8-AlkB have a wide substrate range. The data further indicate that the deep sea would be a rich resource for exploring novel alkane-degrading strains and genes.

Communicated by K. Horikoshi.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-007-0122-x) contains supplementary material, which is available to authorized users.

M. Xu · X. Xiao
Key Laboratory of Marine Biogenetic Resources,
Third Institute of Oceanography, State Oceanic Administration,
Xiamen 361005, People's Republic of China

F. Wang (✉)
Daxue Road 178#, Xiamen 361005, People's Republic of China
e-mail: fengpingw@yahoo.com

Keywords Alkane hydroxylases · Deep sea · Metagenomic library

Introduction

Bacterial oxidation of *n*-alkanes is a very common phenomenon in nature (Bühler and Schindler 1984). It is estimated to amount to several million tons per year from natural oil seepage and oil spills alone (Rosenberg and Ron 1996). The biodegradability of alkanes by microorganisms is well established. Alkanes are chemically quite inert and have to be activated to allow further metabolic steps to take place. The first step in the bacterial aerobic degradation of alkanes is that it is catalyzed by oxygenases (Labinger and Bercaw 2002). Alkane hydroxylase genes have received increasing attention as markers to predict the potential of different environments for oil degradation (van Beilen and Funhoff 2005). In addition, alkane hydroxylases are versatile biocatalysts, which carry out a wide range of useful oxidation reactions (Li et al. 2001; Witholt et al. 1990). Therefore, considerable interest is being devoted to using bacterial alkane oxidation systems as biocatalysts for the production of fine chemicals and pharmaceuticals (Whyte et al. 2002b). *Pseudomonas putida* GPo1 alkane hydroxylase system is the best known of the systems, which consists of an integral membrane hydroxylase (AlkB, a monooxygenase) and two soluble proteins rubredoxin (AlkG) and rubredoxin reductase (AlkT). Biochemical and genetic studies on *Pseudomonas putida* GPo1 alkane hydroxylase propose that this enzyme is the prototype of a very diverse collection of related non-heme iron integral membrane oxygenases (Smits et al. 2002). Genes that are closely related to the alkane hydroxylase gene (*alkB*) of GPo1 have been detected in a large fraction of the microbial population (van Beilen et al. 2003, 2005).

To date, the AlkBs were found in various environments including Alaskan sediments (Sotsky et al. 1994), contaminated soil (Knaebel and Crawford 1995), cold ecosystems (Whyte et al. 1996), fuel oil-contaminated sites (Guo et al. 1997), shallow aquifers (Stapleton and Ayler 1998), bulk soil (Siciliano et al. 2001), land treatment units (van Beilen et al. 2003) and Arctic and Antarctic soil (Whyte et al. 2002a). It would be especially useful to explore AlkBs from some extreme environments, as the AlkBs in these environments may possess uncommon characteristics. The deep sea represents the largest and most unexplored ecosystem on earth, most of which is cold and under high pressure. It may harbor rich bio-resources both for scientific research and industrial utilization. Alkane-degrading bacteria have been isolated and frequently detected in the deep-sea sediments (Liu and Shao 2005; ZZ Shao, personal communication). In our project to explore deep-sea gene resources, we constructed a cosmid library from a Pacific deep-sea sediment ES0303 site (Xu et al. 2007). In this study, we report our work on isolation and characterization of *alkB*s found in the deep-sea cosmid library.

Materials and methods

Collection of deep-sea sediment

The deep-sea sediment was collected by multi-core sampler on September 2003, at the ES0303 station of east Pacific nodule province (8°21′11″N, 145°24′09″W), during the DY105-12/14 cruise of R/V DaYang Yihao. The depth of this site is 5,274 m, temperature 1.5°C and the salinity 35‰. The sediment core was kept at −20°C for shipping and storing in the lab until usage.

Strains, plasmids and media

The bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Pseudomonas putida* GPo12 (pGEc47ΔB), *P. putida* GPo1 (OCT) and *P. fluorescens* KOB2Δ1 were grown on Luria-Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 28°C and maintained at 4°C. *Escherichia coli* series were grown on LB medium at 37°C and maintained at 4°C. *E. coli* strains with plasmids were supplemented with appropriate antibiotics (tetracycline, 12.5 μg/mL; ampicillin, 100 μg/mL; gentamicin, 10 μg/mL). *P. putida* GPo12 (pGEc47ΔB) is an *alkB* deletion derivative and cannot grow on *n*-octane unless an equivalent *alkB* gene is supplied. *P. fluorescens* KOB2Δ1 is an *alkB1* deletion derivative of *P. fluorescens* CHA0, which no longer grows on C_{12} to C_{16} *n*-alkanes but still can grow on $C_{18–32}$. *P. fluorescens* KOB2Δ1 can be complemented for growth on these alkanes by pCom8 derivatives containing *alkB* genes from other bacteria (Smits et al. 2002). Plasmid pCom8 is a broad host-range expression vector for *alkB* genes based on pUCP25 and the *P. putida* GPo1 *alkBp* promoter (Smits et al. 2001). Modified M9 salts minimal medium (12.8 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.0152 g/L $CaCl_2 \cdot 2H_2O$), which were added with different *n*-alkanes as the sole carbon sources and supplemented with $FeSO_4$ to a final concentration of 10 μM (*E. coli*, host strain) or 30 μM (*Pseudomonas* sp., host strain) and 0·001% thiamine were used throughout in Erlenmeyer flasks at 30°C (*Pseudomonas* sp.) or 37°C (*E. coli*) in a gyratory shaker set at 200 rpm (Smits et al. 2002).

Screening of alkane oxygenase genes

Degenerate primer set MonF (TCAAYACMGSNCAYG ARCT) and MonR (CCGTARTGYTCNAYRTARTT; Liu and Shao 2005), which was designed based on the conserved regions between histidine boxes of alkane hydroxylase gene sequences, were used to screen the positive *alk*B gene containing cosmid clones. For an initial screening, 12 clones in one line of the 96-well plate were mixed and DNA was isolated for PCR screening. Then the single clones from the mixed clone samples, which had positive PCR amplification band were screened again to determine the positive single clone. The following PCR program was used: 3 min at 95°C and 35 cycles of 30 s at 95°C, 1 min at 58°C and 1 min at 72°C, 10 min at 72°C and then stopped at 4°C. The PCR product from the putative *alkB* containing cosmid clones was cloned into the pMD18-T vector (Takara Bio, China) and sequenced. The obtained sequence was analyzed and compared with the *alkB* sequences in database with blast program. Then, the confirmed *alkB* gene fragment was labeled as DNA probe to hybridize with the selected cosmid clones to further verify the putative positive clones. DNA probe preparation and dot blot/Southern blot hybridization were performed according to the manufacturer's instructions of the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland).The positive colonies, which were proved to contain *alkB* genes were selected for next experiment.

Analysis of cosmid clone sequences

Shotgun sequencing method was used to determine the whole insert sequences of the selected cosmid clones. One pUC18 library of random 2 kb fragments obtained by sonication was constructed and clones were sequenced

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli EPI100	Host cloning strain	EPICENTRE, Madison, Wisconsin
PWEB::TNC^{TM}	Cosmid vector for metagenomic library construction	EPICENTRE, Madison, Wisconsin
9E7	Deep-sea microbial genome cosmid clone	This study
21G8	Deep-sea microbial genome cosmid clone	This study
E. coli DH10B	Cloning strain	Our lab
E. coli CC118 (RK600)	Helper strain for triparental matings	Ditta et al. 1980
P. putida GPo1	C_5 to C_{12} *n*-alkane-degrading strain	Schwartz and McCoy 1973
P. putida GPo12(pGEc47ΔB)	Alk$^-$ (*alkB Bam*HI deletion); Tet^r	Smits et al. 2002
P. fluorescens KOB2Δ1	*alkB*1 deletion: C_{12}–C_{16} Alk$^-$; C_{18}–C_{28} Alk$^+$	Smits et al. 2002
pCom8	*alkB* genes expression vector, PalkB; Gm^r (gentamicin); oriT; alkS; broad host range	Smits et al. 2001
pCom8-*alkB*-9E7	pCom8 with *alkB* from cosmid clone 9E7	This study
pCom8-*alkB*-21G8	pCom8 with *alkB* from cosmid clone 21G8	This study
P. fluorescens KOB2Δ1 (*alkB*-9E7)	*P. fluorescens* KOB2Δ1 supplemented with pCom8-*alkB*-9E7 plasmid	This study
P. fluorescens KOB2Δ1 (*alkB*-21G8)	*P. fluorescens* KOB2Δ1 supplemented with pCom8-*alkB*-21G8 plasmid	This study
P. putida GPo12 (pGEc47ΔB; *alkB*-9E7)	*P. putida* GPo12 (pGEc47ΔB) supplemented with pCom8-*alkB*-9E7 plasmid	This study
P. putida GPo12 (pGEc47ΔB; *alkB*-21G8)	*P. putida* GPo12 (pGEc47ΔB) supplemented with pCom8-*alkB*-21G8 plasmid	This study

from both ends using ABI3700 sequencer (Applied Biosystem Inc., USA). The sequence was assembled using the program sequencer. Open reading frame (ORF) analysis was performed using the GeneMark program (http://opal.biology.gatech.edu/GeneMark/). The putative ORF and relative sequences were blasted on NCBI, SwissProt, and EMBL database 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/). The analysis results were summarized and the genomic map of each clone was predicted. The relevant genes involved in the alkane hydroxylase system of the cosmid clones were arranged, compared and analyzed.

Construction and functional expression of *P. fluorescens* KOB2Δ1 (9E7*alkB*) and (21G8*alkB*) and *P. putida* GPo12 (pGEc47ΔB; 9E7*alkB*; and 21G8*alkB*) recombinants

The *alkB* genes on clone 9E7 and 21G8, which were screened out from the cosmid library were amplified using primers 9E7alkBF (5′-ACGCGAGAATTCGATATGGG-3′), 9E7alkBR (5′-AACGAAGCTTCATTCAGGGC-3′), 21G8alkBF (5′-TTGAATTCCCAGTAGCCG-3′) and 21G8alkBR (5′-ACGTAAGCTT ATTCCGGACA-3′), into which the restriction enzyme sites of *Eco*RI or *Hin*dIII were integrated, respectively. PCRs were carried out using pfu DNA polymerase (Fermentas, Vilnius, Lithuania). The following PCR program was used: 3 min at 95°C and 25 cycles of 30 s at 95°C, 1 min at annealing temperature 58°C, and 2 min at 70°C; 5 min at 72°C and then stopped at 4°C. PCR products were digested with the respective enzymes *Eco*RI and *Hin*dIII and purified over a 1% agarose gel. The digested PCR products were cloned into pCom8 vector and transformed into *E. coli* DH10B. *E. coli* strains harboring plasmids were grown with appropriate antibiotics (tetracycline, 12.5 μg/mL; ampicillin, 100 μg/mL; gentamicin, 10 μg/mL). The transformants containing the desired genes were identified by PCR and verified by direct sequencing using ABI3700 sequencer (Applied Biosystem Inc., USA). The sequencing primer set of pCom8 vector is bp3702 (5′-ACACTACCATCGGCGCTACG) and bp4033C (5′-CAAGCGTCCGATTAGCTCAG). Nucleotide and amino acid sequences were analyzed and compared using DNAMAN (version 5.1, Lynnon Corporation, Canada). The pCom8 derivatives pCom8-9E7-alkB and pCom8-21G8-alkB were isolated and then transformed into *P. fluorescens* KOB2Δ1 according to the method described before (HΘjberg et al. 1999). The plasmids were introduced into another host strain *P. putida* GPo12 (pGEc47ΔB) by tri-parental mating with *E. coli* DH10B harboring plasmids as the donor, and *E. coli* CC118 (RK600) as the helper strain (Ditta et al. 1980).

Transconjugants were selected on LB medium containing the appropriate antibiotics. For *P. fluorescens* KOB2Δ1 recombinants, gentamicin was used at 100 μg/mL. For *P. putida* GPo12 recombinants, tetracycline (12.5 μg/mL) and gentamicin (100 μg/mL) were used. The final selected positive recombinants were designated as *P. fluorescens* KOB2Δ1 (9E7-alkB), *P. fluorescens* KOB2Δ1 (21G8-alkB), *P. putida* GPo12 (pGEc47ΔB; 9E7-alkB) and *P. putida* GPo12 (pGEc47ΔB; 21G8-alkB).

The recombinants were monitored for growth on various alkanes as the sole carbon and energy source. The *n*-alkanes of $C_{5–11}$ were provided as vapor in a closed system and the *n*-alkanes of $C_{14,16,18}$ were provided at a concentration of 1% (vol/vol) into the M9 medium. Alkanes that are solid at 30°C ($C_{22,24,26,28,32,36}$) were dissolved to 10% (vol/vol) in dioctylphthalate and then added into the M9 medium as a second phase at 2% (vol/vol) (Smits et al. 2002). All cultures were grown aerobically at 30°C (*Pseudomonas* sp.) or 37°C (*E. coli*). The tested strains were first cultured in the LB liquid medium and then concentrated separately. Before adding into the alkane-degrading detection M9 medium, the concentrated strain pellets were washed thrice with the M9 medium without alkanes to remove the possibly remaining carbon sources from LB. All cultivations were performed at least in triplicate at the same conditions.

Results

Screening of *alkB* genes from the deep sea cosmid library

The average size of the inserts of clones in the deep-sea sediment cosmid library was estimated to be about 35 kb. The library contained at least 122 Mbp genomic DNA of the deep-sea organisms (Xu et al. 2007). PCR screening with degenerate primer set MonF and MonR and further southern hybridization verification were used successfully to obtain two cosmid clones 9E7 and 21G8, which contain putative *alkB* genes. The PCR products were cloned and sequenced as described in "Materials and methods". Sequences analysis showed that the PCR fragments of these two clones both contained part of the histidine motifs (Hist-1, Hist-2) and part of the HYG-motif conserved in *alkB* genes (data not shown).

Genomic information on clones 9E7 and 21G8

The whole genomic sequences of both cosmid clones were determined by shotgun sequencing method. The sequences generated had around sevenfold coverage of the inserted genomic DNA. Open reading frames (ORFs) were predicted with the GeneMark Program. The putative ORFs and their nucleotide sequences were blasted and analyzed as described in "Materials and methods". The detail analysis results of ORFs with their closest neighbors in the database are summarized in supplementary Table S1 and S2. The gene organization and genes possibly involved in the alkane hydroxylase system are illustrated in Fig. 1 a, b. It is clearly shown in Fig. 1 that *alkB* genes have the opposite transcription direction to alcohol/aldehyde dehydrogenase genes on both genomes. A gene encoding a 54 amino acid (aa) protein, which is homologous to rubredoxin 2 of *P. putida* was identified immediately downstream the *alkB* gene on the 9E7 genome. On the 21G8 genome, only one *alkB* gene and several alcohol and aldehyde dehydrogenase genes involved in alkane hydroxylase system were found. No rubredoxin reductase genes were found in both 9E7 and 21G8 genomes, which is consistent with previous reports (van Beilen et al. 2003).

The deep-sea *alkB* genes found in the ES0303 cosmid library were named 9E7-*alkB* and 21G8-*alkB*. 9E7-*alkB* is composed of 424 aa and 21G8-*alkB* is composed of 460 aa. The nucleotide sequence identity between 9E7-*alkB* and 21G8-*alkB* is 63.57% and amino acid identity is 66.67%. The most homologous AlkB to 9E7-AlkB in the data bank is Alkane 1-monooxygenase of *Alcanivorax borkumensis* with 55% identical match and 73% positive match (see Table S1). To 21G8-AlkB, the best-matched homologous AlkB is Alkane-1 monooxygenase from *Pseudomonas putida* with 56% identical match and 72% positive match (see Table S2). More detailed data are listed in Tables S1 and S2. It is well known that four histidine-containing motifs (Hist1, HE[L/M]XHK; Hist2, EHXXGHH; Hist3, LQRH[S/A]DHHA; and HYG motif, NYXEHYG[L/M]) were well conserved in bacterial alkane hydroxylases (Whyte et al. 2002b). We located all the four conserved histidine motifs on both the 9E7-AlkB and 21G8-AlkB (Fig. 2). The most significant variety found in 9E7-AlkB and 21G8-AlkB compared with other AlkBs existed in Hist-3 box (LQRH[S/A]DHHA), which is the longest and almost perfectly conserved stretch in all alkane hydroxylases. In 9E7-AlkB and 21G8-AlkB, the Hist-3 boxes were composed of L**E**RH[S/A]DHHA, while in other AlkBs were composed of L**Q**RH[S/A]DHHA (the difference is indicated by bold character).

A phylogenetic tree was constructed based on the alignment of partial amino acid sequences of membrane-bound alkane hydroxylases including short-chain-length alkane degradation AlkBs and long-chain-length alkane degradation AlkBs and the best-matched homologous AlkBs of 9E7-AlkB and 21G8-AlkB (Fig. 3). Only the segment corresponding to the fragments covered from histidine box 1 to histidine box 4 were used for the alignment. Previous

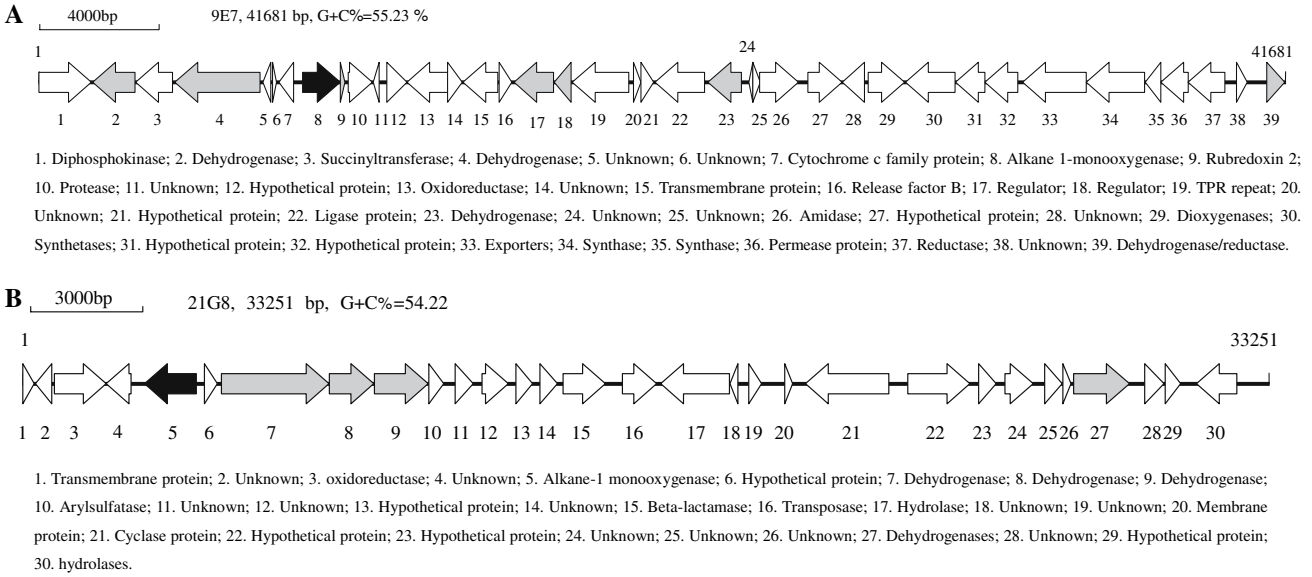

Fig. 1 Genomic map showing the gene organization of cosmid clone 9E7 (**a**) and 21G8 (**b**). The complete genomic sequences of cosmid clones 9E7 and 21G8 were determined by short-gun sequencing as described in the "Materials and methods". GeneMark Program was used to perform open reading frame (ORF) analysis. Clone 9E7 (**a**) is 41681bp with 39 predicted ORFs, which were represented by 1–39, while clone 21G8 (**b**) is 33251bp with 30 predicted ORFs and represented by 1–30. The detailed information of the ORFs of clones 9E7 and 21G8 is presented in the supplementary Table S1 and S2, respectively. The transcription direction of the ORFs is represented by the direction of the *arrow*. The *alkB* gene is marked in *black*, other genes possibly involved in the alkane hydroxylase system are marked in *gray* and the other genes in *white*

analysis indicated that there was no clear linkage between the diversity of the *alk*B genes and bacterial phylogenetic lines (van Beilen et al. 2003). The phylogenetic tree we built also proved the view. The phylogenetic tree (Fig. 3) showed that 9E7-AlkB and 21G8-AlkB were assembled together into a unique branch. They seem to be the hybrids of the two major groups of membrane-bound alkane hydroxylases, which suggest that these two AlkBs may have a wide *n*-alkane substrate degrading range.

Function verification of 9E7-alkB and 21G8-alkB

Two primer sets designed based on the obtained sequences were used to PCR amplify the *alkB* genes from the cosmid clones 9E7 and 21G8. After beimg digested with two introduced enzymes *Eco*RI and *Hin*dIII, the entire *alkB* genes were cloned into the expression vector pCom8. The positive clones harboring *alkB* genes pCom8-9E7-alkB and pCom8-21G8-alkB were selected by PCR amplification and confirmed by sequencing. By tri-parental mating or electroporation ("Materials and methods"), plasmids pCom8-9E7-*alkB* and pCom8-21G8-*alkB* were introduced into the host strain *P. putida* GPo12 (pGEc47ΔB) and *P. fluorescens* KOB2Δ1. The final selected positive recombinants *P. fluorescens* KOB2Δ1 (9E7-*alkB*) and *P. fluorescens* KOB2Δ1 (21G8-*alkB*) were cultured on the LB plates with 100 μg/mL gentamicin. *P. putida* GPo12 (pGEc47ΔB; 9E7-*alkB*) and *P. putida* GPo12 (pGEc47ΔB; 21G8-*alkB*) were cultured on the LB plates with 12.5 μg/mL tetracycline and 100 μg/mL gentamicin. To detect the alkane-degrading activities of both AlkBs, C_5–C_{11}, $C_{14,16,18,20,22,24,28,32,36}$ alkanes were used as the sole carbon sources in the M9 medium. The strains *P. putida* GPo1, *P. putida* GPo12 (pGEc47ΔB), *P. fluorescens* KOB2Δ1, 9E7, 21G8 and cosmid clone host *E. coli* EPI100 were all tested at the same time as the control strains (see "Materials and methods"). The functional expression results of alkane-degrading activities of these four recombinants and the control strains are listed in Table 2 except of strains 9E7, 21G8 and *E.coli* EPI100, which had no growth in all tested media. *P. putida* GPo12 (pGEc47ΔB; 9E7-*alkB*) could have light growth in *n*-alkanes C_5–C_8, $C_{11,}$ C_{14} and good growth in *n*-alkanes C_9. *P. fluorescens* KOB2Δ1 (9E7-alkB) could grow in most *n*-alkanes tested in our study except C_5 and C_6. *P. putida* GPo12 (pGEc47ΔB; 21G8-alkB) had light growth in *n*-alkanes C_5–C_8, C_{11} and C_{14}, and good growth in *n*-alkanes $C_{9.}$ *P. fluorescens* KOB2Δ1 (21G8-alkB) had good growth in all *n*-alkanes. Because hydroxylase system of *P. fluorescens* KOB2Δ1 can grow on *n*-alkanes from C_{18} to C_{32} and $C_{36,}$ and *P. putida* GPo12 (pGEc47ΔB) could not grow on *n*-alkanes, it is obvious that these two novel AlkBs really have a wide range of *n*-alkanes utilization range. Based on the functional expression results, the 9E7-AlkB and 21G8-AlkB can grow at least in

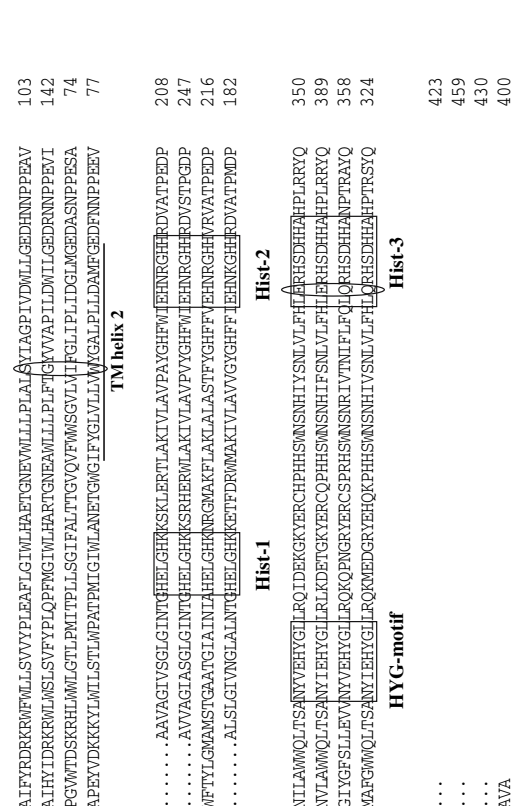

Fig. 2 Manual alignment of predicted amino acid sequences of 9E7-AlkB and 21G8-AlkB together with referenced sequences of AlkBs from other strains. The conserved histidine motifs (Hist-1, Hist-2, Hist-3 and HYG motif) are indicated with *box*. The difference of Hist-3 box among the AlkBs sequences is indicated with *ellipse*. The putative transmembrane helix2 based on *P. putida* GPo1 AlkB sequence is *underlined* and the position of residue W55 of *P. putida* GPo1 AlkB is also indicated with *ellipse*. The accession number of *P. putida* GPo1 AlkB is CAB54050 and *P. fluorescens* CHA0 AlkB is CAB51045. The alignment result was created by the alignment program of DNAMAN 5.1

n-alkanes C_5 to C_{16} and possibly may grow on even longer-chain-length *n*-alkanes.

Discussion

Though numerous alkane hydroxylase systems have been found in various environments such as soils and aquifers (van Beilen et al. 2003), this is the first report of a detailed genetic characterization of alkane hydroxylase systems in a deep-sea environment. As only less than 1% of the microorganisms can readily be cultivated in the natural environment, cultivation-independent molecular technology has largely expanded our knowledge of the microbial community in nature. Increasing genetic information is becoming available for the still uncultivated microorganisms, and novel genes for bio-utilization have been cloned through a metagenomic approach (Henne et al. 2000; Knietsch et al. 2003; Rondon et al 2003). Studies on alkane hydroxylase gene diversity and relative substrate range should help to optimize the biodegradative activity of *n*-alkanes-degrading strains and benefit alkane hydraoxylase biocatalytic applications (Ditta et al. 1980; Fernando 2005; Smits et al. 2002; van Beilen et al. 2003, 2005; van Beilen and Funhoff 2005). In this study, two novel *alkB* genes, together with several novel alcohol/aldehyde dehydrogenase genes, were identified on the DNA fragments of the two cosmid clones 9E7 and 21G8. Many alkane-degrading strains possess homologues of the *P. putida* GPo1 alkane hydroxylase. The novel AlkBs found in this study are unexceptional the homologues of *P. putida* GPo1 alkane hydroxylase. The deep-sea 9E7-AlkB (424 aa) and 21G8-AlkB (460 aa) have 64–67% identical matches between each other and 55–56% identical matches to homologues in the data bank. Phylogenetic analysis further indicated that these two AlkBs belong to a unique branch of integral membrane hydroxylases.

Functional expression showed that the isolated deep-sea AlkBs could degrade medium-chain-length and long-chain-length (C_{5-16}) *n*-alkanes. van Beilen et al. (2005) have proposed a specific residue theory that an amino acid position of AlkBs determines whether long-chain-length alkanes can be hydroxylated. They found that when longer alkanes could be degraded, W55 of *P. putida* AlkB or W58 of *A.borkumensis* AlkB1 had changed to a much less bulky amino acid, usually S (serine) or C (cysteine). They also found that the corresponding position in the alkane hydroxylases from other bacteria that oxidize alkanes longer than C_{13} is always occupied by a less bulky hydrophobic residue such as A, V, L or I (van Beilen et al. 2005). Sequences analysis results show that the corresponding residues on the TM helix 2 of 9E7-AlkB and 21G8-AlkB are S and G (Fig. 2), which are less bulky residues and partly compatible with the theory.

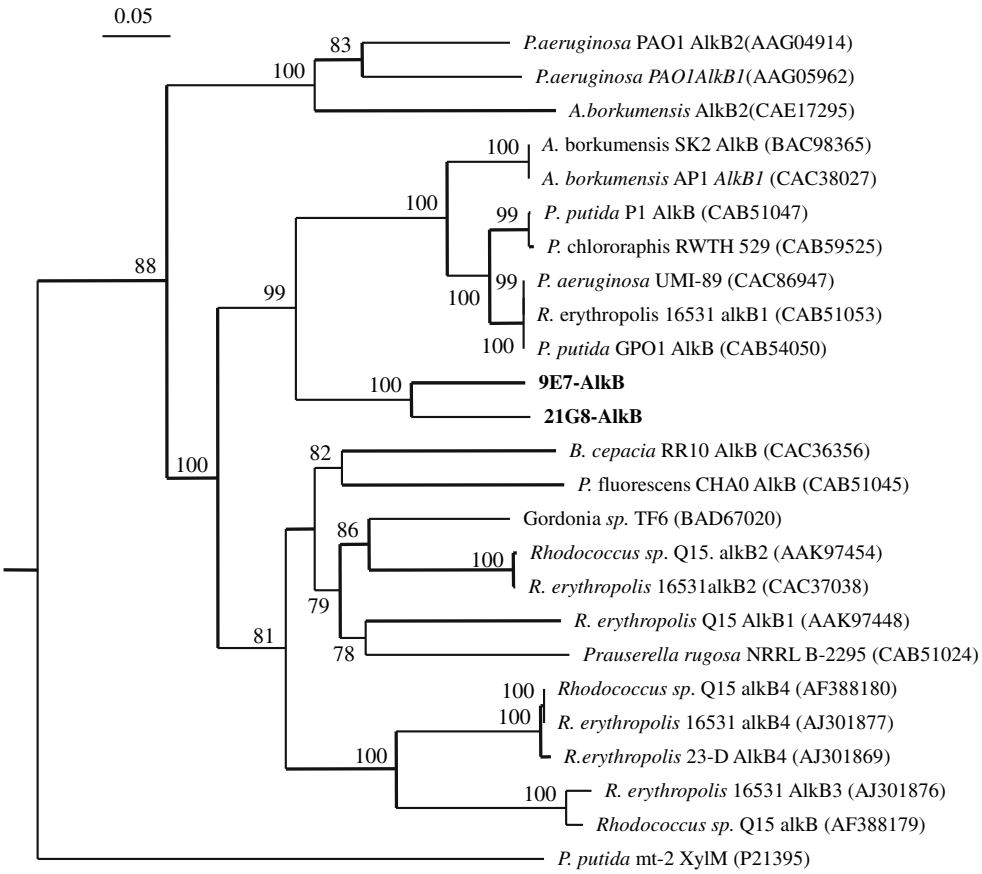

Fig. 3 Phylogentic tree based on partial amino acid sequences of membrane-bound alkane hydroxylases. Only the segment corresponding to the 550 base pair fragments between histidine box 1 and histidine box 4 was used for the alignment. The dendrogram was constructed by the neighbor-joining method using DNAMAN 5.1 program. Only bootstrap values above 50 from 1,000 replicates are shown. The scale bar represents 0.05 substitution per amino acid site

Table 2 Heterologous expression of the *alk*B genes in host system of *P. putida* GPo12 (pGEc47ΔB) and *P. fluorescens* KOB2Δ1 as measured by growth on *n*-alkanes

Strains	Growth detection on alkane															
	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{28}	C_{32}	C_{36}
P. putida GPo12 (pGEc47ΔB)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. putida Gpo1	++	++	++	++	++	+	+	–	–	–	–	–	–	–	–	–
P. fluorescens KOB2Δ1	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	+
P. putida GPo12 (pGEc47ΔB; 21G8-alkB)	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–
P. putida GPo12 (pGEc47ΔB; 9E7-alkB)	+	++	+	+	++	+	+	–	–	–	–	–	–	–	–	–
P. fluorescens KOB2Δ1 (21G8-alkB)	+	+	+	++	++	++	++	++	++	++	++	++	++	++	+	+
P. fluorescens KOB2Δ1 (9E7-alkB)	+	+	+	+	+	++	+	+	+	++	++	++	++	++	+	+

–, No growth; +, light growth; ++, good growth

Experiments such as site-directed mutagenesis of this position combined with the substrate range growth selection might further validate the theory.

The organization of genes involved in alkane oxidation varies strongly among the different alkane-degrading bacteria (van Beilen et al. 2001, 2003). Some locate in the chromosome DNA, while others locate in mega plasmids. In most strains, genes involved in alkane degradation seem to be distributed over the genome. Most rubredoxin genes are located immediately downstream of the alkane hydroxylase genes. A small 54 aa homolog of rubredoxi was also determined on 9E7, while absent on 21G8. There seems no fixed pattern of the *alk* genes arrangement. But all the previous *alk*s arrangements have an unambiguous phenomenon that *alkB* gene always have, the same transcription direction with alcohol or aldehyde dehydrogenase genes. Though the *alks* are also distributed randomly on the genomes of 9E7 and 21G8, the *alkB* genes found in the two cosmid clones have an opposite transcription direction to those alcohol/aldehyde dehydrogenase genes. This is the

first report that the *alkB* transcribed oppositely to the alcohol or aldehyde dehydrogenase genes. It is regarded that diversity of alkane hydroxylase is due to horizontal gene transfer (van Beilen et al. 2003). However, the evolution of diverse families of alkane hydroxylases with essentially the same function is not clear yet, it remains to be one of the major challenges in future research.

Acknowledgments We would like to thank Dr. Jan B van Beilen for generously providing us the *alk*B gene expressed system including strains and vector of *P. putida* GPo1, *P. putida* GPo12 (pGEc47ΔB), *P. fluorescens* KOB2Δ1, *E. coli* RK600 and pCom8. We are indebted to Dr. Zongze Shao for his kind recommendations and helpful discussions. Our thanks are due to the crew on the R/V Dayang Yihao for help in collecting the samples. This work was supported by the NSF China Award (40476054), COMRA fund (DY7000M-02).

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