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Diversity and Abundance of Oil-Degrading Bacteria and Alkane Hydroxylase (*alkB*) Genes in the Subtropical Seawater of Xiamen Island

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Abstract In this report, the diversity of oil-degrading bacteria and *alkB* gene was surveyed in the seawater around Xiamen Island. Forty-four isolates unique in 16S rRNA sequence were obtained after enrichment with crude oil. Most of the obtained isolates exhibited growth with diesel oil and crude oil. *alkB* genes were positively detected in 16 isolates by degenerate polymerase chain reaction (PCR). And for the first time, *alkB* genes were found in bacteria of *Gallaecimonas*, *Castellaniella*, *Paracoccus*, and *Leucobacter*. Additional 29 *alkB* sequences were retrieved from genomic DNA of the oil-degrading communities. Phylogenetic analysis showed that the obtained *alkB* genes formed five groups, most of which exhibited 60–80% similarity at the amino acid level with sequences retrieved from the GenBank database. Furthermore, the abundance of *alkB* genes in seawater was examined by real-time PCR. The results showed that *alkB* genes of each group in situ ranged from about 3×10^3 to 3×10^5 copies L^{-1}, with the homologs of *Alcanivorax* and *Pseudomonas* being the most predominant. Bacteria of *Alcanivorax*, *Acinetobacter*, and *Pseudomonas* are important oil degraders in this area; while those frequently reported in other area, like *Oleiphilus* spp., *Oleispira* spp., and *Thalassolituus* spp. were not found in our report. These results indicate that bacteria and genes involved in oil degradation are quite diverse, and may have restriction in geographic distribution in some species.

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Introduction

Alkane-degrading bacteria are ubiquitous in environments and play a crucial role in oil removal. In marine environments, they are frequently documented as, but not restricted to, *Alcanivorax* spp., *Oleiphilus* spp., *Oleispira* spp., and *Thalassolituus* spp. [3]. They can use alkanes, of various chain lengths, branched and/or straight, as sole carbon and energy sources [28, 29]. Recently, bacteria of *Brachybacterium, Idiomarina, Leifsonia, Martelella, Kordiimonas, Parvibaculum*, and *Tistrella* have been detected as new oil degraders from pelagic surface water of Atlantic Ocean [31].

While the mechanism of alkane mineralization has been not fully understood, it is known that alkane hydroxylase (AlkB) is one of the key enzymes in the process [15]. It has been detected in α-, β-, and γ-proteobacteria as well as the *Actinobacteria* [21, 27, 30]. Although several hydroxylases have been confirmed recently to be involved in alkane degradation, such as P450 [10], AlmA [23, 34], and Lad A [1], AlkB is most important and prevalent in aerobic oil-degrading bacteria. Actually, little is known about the diversity of *alkB* genes in marine environments. Recently, from the sediments of the Timor Sea, novel AlkB genes were retrieved, with identities averaged only 73% with those previously identified from marine α-proteobacteria [32]. Quite diverse *alkB* genes were also found in the bacteria isolated from oil-degrading communities across Atlantic Ocean; the *alkBs* from *Salinisphaera* isolates

formed a separate cluster and shared only 54–69% amino acid identity with that from *Nocardia farcinica* IFM10152 as a closest relative in GenBank [31].

AlkB gene can be used as molecular marker to detect the ecological role of oil-degrading bacteria in environments. All AlkB proteins are conserved in six hydrophobic stretches that are likely to span the cytoplasmic membrane [18], and eight to nine histidines that are essential for the alkane-hydroxylizing activity [26]. Based on these conserved moieties, degenerate primers for polymerase chain reaction (PCR) detection of *alkB* genes have been designed [21]. With these primers, the *alkB* gene has been detected in a variety of environments, including Mississippi shallow aquifers [20], California soil [19], Arctic and Antarctic soil [35], Brazilian soils [9], German barley fields and grassland soil [7], Mediterranean beaches [14], Alaskan and Antarctic marine sediments [6], and recently in the Timor Sea [32]. Moreover, the *alkB* gene can be used to predict the potential of bioattenuation of oil-polluted environments. A relationship between *alkB* gene abundance and *n*-alkane degradation has been confirmed [4, 17]. Quantification of *alkB* can be developed to monitor microbial community change during bioremediation of hydrocarbon-contaminated Antarctic soil [13]. The knowledge about the diversity of *alkB* gene as well as their hosting bacteria in certain area helps to evaluate the potential of this area to recover from an oil spill accident.

Oil spill occurs along the coastline here and there and unpredictably. Xiamen Island is a unique niche to examine marine oil-degrading bacterium, as it locates the subtropical area in the Taiwan Straits which links East China Sea and Southern China sea. Moreover, it is also a large international port, and the estuarine of Jiu-Long River. Pollutions of various sources are unavoidable and rouse public concerns [5]. The ranges for petroleum hydrocarbons in the sediments of Xiamen Harbour was 133–943 μg g^{-1} (dw) [12], and 10~14 μg L^{-1} in surface water (2005, unpublished). However, little is known about the oil-degrading bacteria in this area. In 2005, we collected the surface water around the island to examine the diversity of oil-degrading bacteria, as well as their abundance reflected by the copy numbers of *alkB* genes in situ.

Materials and Methods

Sampling

Surface seawater was collected with sterilized bottles in June 2005 around Xiamen Island, which is located at the mainland side of the Taiwan Straits (118.06–118.10°E, 24.40–24.50°N) (Fig. 1). Water samples were collected at 11 sites, one of which (arrowhead in Fig. 1) was used for quantification of the copy numbers of *alkB* genes. Other sites were used to

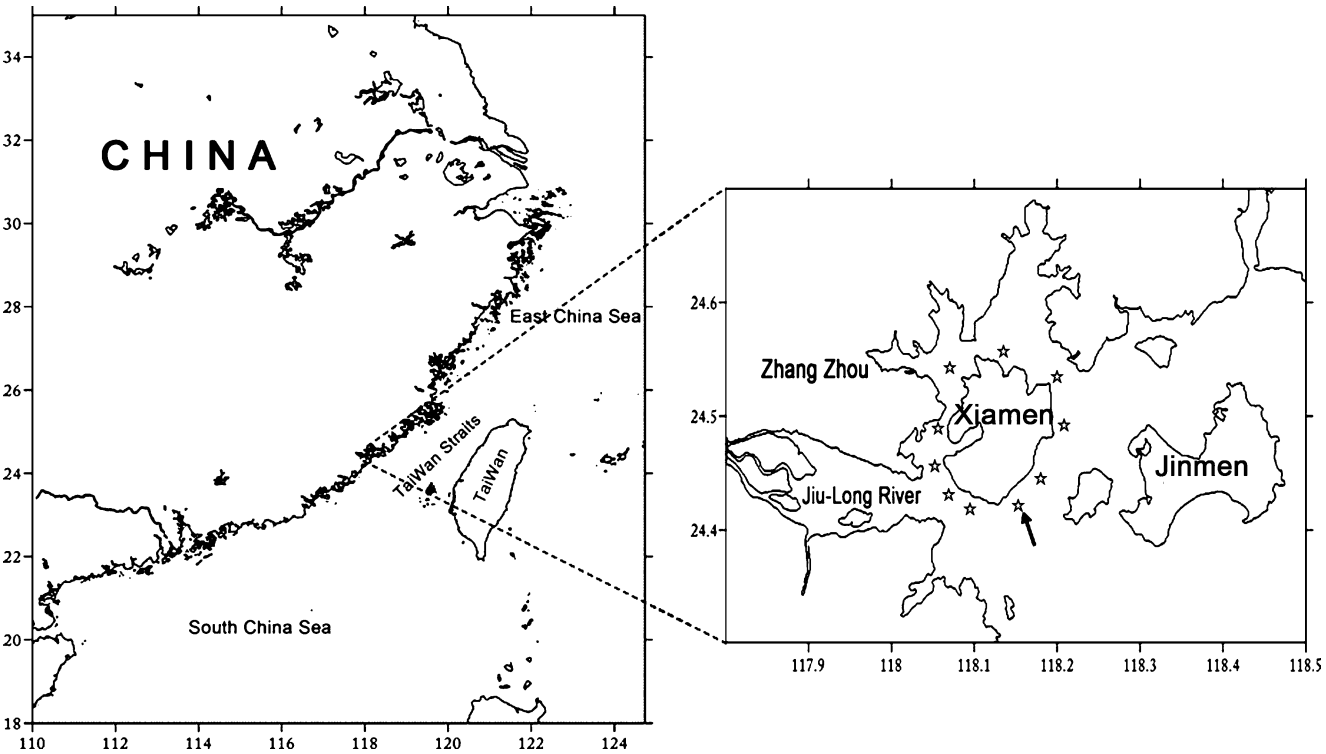

Figure 1 The location of sampling sites of Xiamen Island. *Left*, Taiwan Straits; *right*, Xiamen Island; pentacles indicate the sampling sites, the *arrowhead* points to the site sampling for *alkB* gene quantification

enrich oil-degrading bacteria. For quantification of *alkB* genes, three bottles of seawater samples were collected from the same site and treated separately throughout the experiments. The seawater quality parameters were measured according to universal methods [4, 33]: the chemical oxygen demand (COD) was 0.5~0.9 mg/L, the concentration of hydrocarbons was 10~14 μg/L, N in the form of nitrate was 2~4 mg/L, and chlorophyll a was 3~6 μg/L. Representatively, at the site labeled an arrow head: the pH was 7.8, the temperature was 27°C, the COD was 0.7 mg/L, the salinity was 2.9%, the concentration of hydrocarbons was 12 μg/L, N in the form of nitrate was 3 mg/L, and chlorophyll a was 4 μg/L (2005, unpublished).

Enrichment of Oil-Degrading Bacteria

About 1 ml of sterilized crude oil was added to a 100 ml sample in a 250-ml flask to start the enrichment of oil-degrading bacteria with a sterilized seawater treatment as a control. After 5 days, all samples exhibited oil degradation indicated by bacteria growth and oil emulsification, and 2% of the inoculum was transferred into 100 ml fresh artificial sea water medium (ASM) [8] with 1% crude oil as the carbon source and shaking (150 rpm) for 10 days at 28°C. Meanwhile, a control without inoculum was paralleled. Repeated transfers were done twice more. The bacteria were isolated on high-salt Luria–Bertani (HLB) agar plates [8] and characterized by the 16S rRNA gene.

Assays for Oil Biodegradation

Isolates were tested for their ability to utilize both 1% (*v*/*v*) sterilized crude and diesel oils in ASM, medium (no carbon sources) by measuring the cell density at 600 nm; growth was compared with a control containing no carbon source and a non-inoculated control. The isolates were first activated by growing in HLB liquid medium. Then, approximately 6×10^8 cells were collected, washed twice with sterilized ASM, and used to inoculate ASM medium. The assay for biodegradation was conducted in 100-ml Erlenmeyer flasks containing 30 ml sterile ASM medium, incubated at 25°C with shaking (150 rpm) for about a week.

Genomic DNA Extraction

Bacterial genomic DNA of isolates was extracted with the TIANamp Bacteria DNA kit (TIANGEN, China) according to the manufacturer's instruction. To extract the DNA from the enrichment communities, a 3-ml culture was centrifuged, and the cell pellet was resuspended in 120 μl 1× Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris, pH 7.5, 1 mM EDTA) before the addition of lysozyme at 37°C for 1 h. Then, 80 μl 10% sodium dodecyl sulfate was added, and the sample was incubated at 65°C for 1.5 h and then extracted with the same kit.

To quantify the copy number of the *alkB* gene in seawater, 3 l of surface seawater was collected and sieved through a phytoplankton net (10-μm mesh size, 23-cm mouth diameter, and 40-cm long) to remove large granules and through a nitrocellulose membrane (0.22 μm) to collect the remaining cells. The cell pellet was stored at −70°C and thawed, incubated with lysozyme at 37°C for 30 min. DNA was then extracted with TIANamp Bacteria DNA Kit according to the manufacturer's protocol (TianGen, China).

Bacterial 16S rRNA Gene Sequencing

The 16S rRNA gene was amplified by PCR using the universal primer set 16SF (positions 8~27 of the *Escherichia coli* numbering; 5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (positions 1,512~1,493; 5′-ACGGCTACCTTGTTACGACT-3′) [8]. Approximately 1.5-kb product was obtained, and sequenced by Invitrogen (Shanghai, China) using an ABI model 3730 DNA sequencer. The following thermal cycling parameters were used: a 5-min hot start at 95°C; followed by 32 cycles of denaturation for 1 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min; and a final extension of 20 min at 72°C.

Polymerase Chain Reaction Detection of *alkB* Genes

To survey the diversity of *alkB* genes, PCR was carried out with a pair of degenerate primers, *alkB*wf (5′-AAYAC NGCNCAYGARCTNGGVCAYAA-3′) and *alkB*wr(5′-GCR TGRTGRTCHGARTGNCGYTG-3′), which were designed based on well-conserved motifs of AlkB in the N-(NTXHELGHK) and C-terminal (LQRHSDHHA) domains [7]. In addition, another pair of degenerate primers, monf (5′-TCAAYACMGSNCAYGARCT-3′) and monr (5′-CCGTARTGYTCNAYRTARTT-3′), was also used, designed based on the same N-terminal motif (NTXHELGHK) as above and a different C-terminal motif (INYIEHYGLL) [8]. These primer pairs were expected to generate a PCR product of about 550 and 420 bp, respectively.

The PCR mix of 50 μl contained the following: 5 μl 10× buffer (provided with Taq polymerase), 1.5 μl $MgCl_2$ (50 mM), 4–5 μl dNTP mix (2.5 mM each), 5–6 μl of each primer (10 μM), 10–15 ng of purified DNA from cultured strains or 15–20 ng from total community DNA, and 2.5 U Taq DNA polymerase (Invitrogen, Karlsruhe, Germany). Cycling was performed with an initial denaturation for 5 min at 94°C followed by 32 cycles of 30 s at 94°C, 30 s at 50–55°C, and 45 s at 72°C and a final elongation step for 5 min at 72°C. PCR products were separated in a 1.0% agarose gel.

Polymerase Chain Reaction Product Cloning and Library Analysis

The PCR product of the *alkB* gene from each isolate was purified prior to cloning. The PCR product was run in an agarose gel, and the band of the expected size was cut out and extracted with the TIANGEN Mini Purification kit (TIANGEN, China). DNA was eluted in a final volume of 20 μl. The purified PCR products were cloned into a PMD-19 T vector (TaKaRa Bio, China) and transformed into *E. coli* DH5α cells. A fast screening of the transformants was performed by colony PCR [7]. Plasmid DNA was extracted with the Plasmid Mini kit (Qiagen, Hilden, Germany) and used for sequencing.

With the genomic DNA of each oil-enrichment community as template, *alkB* gene was PCR cloned to detect the more homologues other than those form isolates. The *alkB* PCR products from all the communites were blended, and cloned into a PMD-19 T vector and transformed into *E. coli* DH5α cells. The libraries were screened via restriction analysis by digestion with *Hae*III and *Msp*I (TaKaRa Bio, China), and clones with unique restriction patterns were sequenced. The sequencing processes were conducted with an ABI model 3730 DNA sequencer (Invitrogen, Shanghai, China).

Primer Design for PCR Quantification of *alkB* Genes

To quantify *alkB* in the surface seawater, five pairs of specific non-degenerate primers targeting different groups of *alkB* genes were designed for real-time PCR based on the results of the phylogenetic analyses in this report (Table 1). The specificity of these primers was examined by BLAST searches in public databases (NCBI) and further tested by standard real-time PCR using SYBR green with reference strains of known geno- or phenotype (Table 3). Additionally, the specificity of the above five pairs of primers for each *alkB* group was reconfirmed using the DNA extracted from surface seawater samples as a template by cloning, sequencing, and phylogenetic analysis of the PCR products. Briefly, purified PCR products (QIAquick PCR Purification Kit, Qiagen) were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen). A total of 100 clones were randomly chosen from the libraries of each Groups I, II, III, IV, and V for sequencing (MWG Biotech). In addition, melt curve analysis was used to check for the production of PCR products or secondary products, such as primer dimers, in these assays.

The amplification efficiency for the real-time PCR primer sets was estimated with a gradient of annealing temperatures (from 52 to 62°C) against DNA from reference strains of pure bacterial cultures (Table 4). Standards for quantative PCR (Q-PCR) calibration were constructed according to universal methods with reference to Powell and colleagues [13]. The standard curves for all assays were linear (r^2>0.95) over four orders of magnitude for the *alkB* assay. The r^2 values were consistently greater than 0.95 and usually greater than 0.99 for both assays. The primer concentration was optimized; the efficiency of the reactions was between 0.9 and 1.1 (or 90–110%). Runs that fell outside of these parameters (r^2<0.95; 0.9<efficiency<1.1) were repeated. There was no signal in the negative control (template absent) in the *alkB* assay.

Quantitative PCR

Real-time quantitative PCR was performed on an IQ™ 5 Multicolor Real-Time PCR Detection system (Bio-Rad,

Table 1 Characteristics of PCR primer sets used in this study

Primer	Target	Sequence 5′→3′	Size (bp)	Anneal. temp (°C)	Reference strains	Reference
*alkB*wf *alkB*wr	Degenerate	ATAAYACNGCNCAYGARCTNGGNCAYAA TGCRTGRTGRTCNGARTGNCGYTG	550	50	/	[7]
monf monr	Degenerate	TCAAYACMGSNCAYGARCT CCGTARTGYTCNAYRTARTT	420	52	/	[8]
*alkB*If *alkB*Ir	Group I-like[a]	GGA AAT TCG TAC TGC GGG AGA AGA AAG CGG TGC CGA GAA T	204	60	*Alcanivorax dieselolei* B-5	This study
*alkB*IIf *alkB*IIr	Group II-like[a]	ATGGAGCCTAGATAATGAAGT TAATTGGCACTGGTCAGC	147	56	*Alcanivorax borkumensis* SK2	This study
*alkB*IIIf *alkB*IIIr	Group III-like[a]	ACC GTG ATC GGC AGC CTT A CCA ACC ACA GCA TCA GGA CA	145	60	*A. dieselolei* B-5	This study
*alkB*IVf *alkB*IVr	Group IV-like[a]	GGGGCATAAGAAGGAAAGC GCACATGGTGGCCACGGTT	105	61	0	This study
*alkB*Vf *alkB*Vr	Group V-like[a]	TATAACCATTTCCGTGTAGA CCAACCTTGCAACAACTCATT	213	57	*Acinetobacter venetianus* M1	This study

[a] Used in quantitative RT-PCR of *alkB* gene copy numbers in situ.

California, USA) with the following program: 2 min at 50°C (uracil-N-glycosylase activation), 10 min at 95°C (activation of the Taq polymerase) and 45 cycles of denaturation (10 s, 95°C), annealing and elongation (30 s, 56–61°C). Fluorescence data were acquired at the end of the elongation step. The specificities of accumulated products were verified by melting curve analysis. The following reagents were added: 12.5 μl of 2× SYBR Green Supermix (Bio-Rad, California, USA), 1.0 μl of each forward and reverse primer (10 mM), 0.5 μl of template DNA, and PCR-grade water to a final volume of 25 μl. A negative treatment control (template absent) was included during each real-time PCR experiment to check the quality of the reagents used. For each gene, three biological replicates and three technical replicates were performed. Data shown represent the mean value from nine runs of real-time PCR and have a variation coefficient (CV = standard deviation value × 100/mean value) ranging from 6.2–24.5%, which corresponds to less than 0.2 Log errors for the quantification values. The statistical determination of significance (a=0.05) was determined with Microsoft Office Excel 2003 using a one sample Student's *t* test on the biological repeats of each experimental condition.

Sequencing, Phylogenetic and Diversity Analysis

Sequencing of the inserted fragments was carried out on a model 377 automated DNA sequencer using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The obtained sequences were aligned using DNAMAN (version 5.1, Lynnon Biosoft) with *alkB* sequences retrieved from GenBank. A phylogenetic tree of the derived protein sequences was constructed by the neighbor-joining method using the PHYLIP package [16] and DNAMAN programs. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data with 1,000 re-samplings. Jukes–Cantor evolutionary distances were calculated using DNADIST of the PHYLIP 3.68 package.

Sequences

Sequences of all 16S rRNA genes can be retrieved from the GenBank database under accession numbers GU593614 to GU593656. All *alkB* sequences were deposited in the GenBank database with accession numbers of GU593657 and from EU853379 to EU853422.

Results and Discussion

AlkB Genes from the Isolates and Enrichments

From the oil-enrichment communities, 44 strains were obtained that differed from each other in 16S rRNA gene sequence. Most of the obtained isolates exhibited oil degradation. They were affiliated to 26 genera, including *Alcanivorax*, *Acinetobacter, Pseudomonas, Flavobacterium, Corynebacterium, Gallaecimonas*, *Castellaniella*, *Paracoccus*, and *Leucobacter*, etc. Table 2 shows the isolates that were positively detected of *alkB* genes, including six isolates of *Alcanivorax*.Interestingly, *alkB* gene was detected for the first time according to our knowledge in the bacteria of following genera, *Gallaecimonas*, *Castellaniella*, *Paracoccus*, and *Leucobacter*; moreover, these isolates exhibited vigorous growth with crude oil (Table 2). However, other isolates which exhibited growth with crude oil were negatively detected of *alkB* genes, maybe harbored other alkane monooxygenases.

Additionally, from the *alkB* libraries derived from the community DNA, 45 different *alkB* sequences were obtained (Fig. 2), 16 of which were same to those obtained from the isolates (Table 2), others were most probably from bacteria in the community but not successfully isolated. All the sequences from both community and isolates contained eight to nine histidines that are essential for the alkane-hydroxylizing activity and well conserved. Phylogenetical analysis showed that they are clustered to *alkB* genes previously reported from *Pseudomonas putida*, *Pseudomonas fluorescens*, *Alcanivorax, Rhodococcus*, *Acinetobacter*, *Mycobacterium,* and *Nocardia.*

Most of the obtained *alkB* genes in this report exhibited 60–80% similarity at the amino acid level with sequences retrieved from the GenBank database. Compared to the surface water across the Atlantic Ocean [31], the *alkB* sequences in Xiamen coastal area were even more diverse (for more details, see "Phylogenetic Analysis" below). They are also different from those detected in soils; such as in land soil of Germany, *alkB* was detected mainly from *Rhodococcus, Acinetobacter*, *Mycobacterium*, and *Nocardia* [7]; while in Arctic and Antarctic soils, *alkB* sequences were closely related to those from *P. putida* GPo1, *Rhodococcus* sp. strain Q15, and *Acinetobacter* sp. strain ADP-1 [35].

Novel Oil-Degrading Bacteria in Xiamen Area

Several isolates as follows showed 16Sr RNA similarity below 98% with type strains. Isolate 1-C-1 showed only 94.5% similarity in 16S rRNA gene with *Flavobacterium indicum* GPTSA100-9^T. As a possible novel species of this genus, Isolate 1-C-1 exhibited vigorous growth with diesel oil and crude oil (Table 2); moreover, an *alkB* gene was detected, which is most closely related to that of *P. putida* strain GPo1 at the amino acid level, but only of 70% identity.

Isolate II-C-7 was also proved as oil degrader, and had 97% similarity in 16S rRNA gene with *Castellaniella ginsengisoli* DCY36^T. And for the first time of this genus, *alkB* gene was

Table 2 Growth characteristics of 44 bacterial isolates on oils and the occurence of *alkB*

Isolates	16SrDNA Acc. No.	Closest type strains	16S rDNA identity (%)	alkB genes Acc. No.	Diesel oil[a]	Crude oil[a]
1-C-1	GU593613	*Flavobacterium indicum* GPTSA100-9^T (AY904351)	1,319/1,396 (94.48%)	1-C-1*alkB*(EU853408)	++	++++
1-C-2	GU593614	*Paracoccus aestuarii* B7^T (EF660757)	1,276/1,299 (98.23%)	1-C-2*alkB*(EU853409)	+++	+++
1-C-4	GU593615	*Stappia indica* B106^T (EU726271)	1,448/1,448 (100%)	ND	+	++
1-D-2	GU593616	*Acinetobacter junii*LMG998^T (AM10704)	1,369/1,399 (97.85%)	1-D-2*alkB*(EU853410)	++++	++++
2-C-2	GU593617	*Oceanicola nanhaiensis* SS011B1-20^T (DQ414420)	1,424/1,424 (100%)	ND	+	++
2-C-3	GU593618	*Microbacterium oxydans* DSM 20578^T (Y17227)	1,385/1,387 (99.86%)	ND	++	+++
2-D-2	GU593619	*Pseudomonas pseudoalcaligenes* DSM 50188^T (Z76675)	1,424/1,462 (97.40%)	2-D-2*alkB*(EU853411)	++	++++
3-C-1	GU593620	*Gallaecimonas pentaromativorans* CEE_131^T (FM955223)	1,335/1,377 (96.95%)	ND	−	+
3-C-2	GU593621	*Gallaecimonas pentaromativorans* CEE_131^T (FM955223)	1,368/1,379 (99.20%)	ND	−	++
3-C-3	GU593622	*Alcanivorax dieselolei* B-5^T (AY683537)	1,389/1,497 (99.46%)	3-C-3*alkB* (EU853412)	++++	++++
3-C-4	GU593623	*Corynebacterium variabile* DSM 20132^T (AJ22815)	1,418/1,434 (98.88%)	3-C-4*alkB*(EU853413)	+	+++
3-D-1	GU593624	*Gallaecimonas pentaromativorans* CEE_131^T (FM955223)	1,345/1,377 (97.24%)	ND	−	−
4-C-1	GU593625	*Alcanivorax dieselolei* B-5^T (AY683537)	1,496/1,498 (99.86%)	NT	++++	++++
5-C-1	GU593626	*Leucobacter tardus* K 70/01^T (AM940158)	1,450/1,465 (98.97%)	5-C-1*alkB*(EU853414)	++	++++
5-C-2	GU593627	*Flavobacterium psychrolimnae* LMG 22018 ^{T} (AJ585428)	1,225/1,345 (91.37%)	ND	−	++
6-D-1	GU593628	*Alcanivorax dieselolei* B-5^T (AY683537)	1,458/1,464 (99.59%)	6-D-1*alkB* (EU853415)	++++	++++
6-D-2	GU593629	*Zunongwangia profunda* SM-A87^T (DQ855467)	1,472/1,482 (99.32%)	ND	−	++
6-D-3	GU593630	*Pseudomonas balearica* SP1402^T (U26418)	1,472/1,482 (99.64%)	ND	−	+
6-D-5	GU593631	*Ochrobactrum lupini* LUP21^T (AY45038)	1,360/1,360 (100.00%)	NT	NT	NT
6-D-6	GU593632	*Alcanivorax dieselolei* B-5^T (AY683537)	1,239/1,246 (99.44%)	6-D-6*alkB* (EU853416)	++++	++++
7-C-1	GU593633	*Alcanivorax venustensis* ISO4^T (AF328762)	1,467/1,497 (99.44%)	7-C-1*alkB* (EU853417)	+++	++
7-C-7	GU593634	*Alcanivorax venustensis* ISO4^T (AF328762)	1,411/1,486 (94.95%)	7-C-7*alkB* (GU593657)	++++	+++
7-D-1	GU593635	*Alcanivorax dieselolei* B-5^T (AY683537)	1,496/1,498 (99.86%)	NT	++++	++++
7-D-2	GU593636	*Ochrobactrum cytisi* ESC1^T (AY776289)	1,439/1,439 (100.00%)	ND	+	++
7-D-3	GU593637	*Tistrella mobilis* TISTR 1108^T (AB071665)	1,449/1,451 (99.86%)	ND	−	+
8-C-1	GU593638	*Bacillus stratosphericus* 41KF2a^T (AJ831841)	1,404/1,405 (99.93%)	ND	−	++
8-C-5	GU593639	*Phyllobacterium brassicacearum* STM 196^T (AY785319)	1,361/1,397 (97.42%)	ND	−	++
9-C-4	GU593640	*Gallaecimonas pentaromativorans* CEE_131^T (FM955223)	1,374/1,379 (99.62%)	9-C-4*alkB*(EU853418)	+	+++
9-C-5	GU593641	*Stappia indica* B106^T (EU72627)	1,448/1,448 (100.00%)	ND	−	++
10-C-2	GU593642	*Aerococcus viridans* ATCC 11563^T (M58797)	1,434/1,459 (98.29%)	ND	−	+++
10-C-6	GU593643	*Pusillimonas ginsengisoli* DCY25^T (EF672088)	1,416/1,454 (97.38%)	ND	NT	+++

Table 2 (continued)

Isolates	16SrDNA Acc. No.	Closest type strains	16S rDNA identity (%)	alkB genes Acc. No.	Diesel oil[a]	Crude oil[a]
10-D-2	GU593644	*Zunongwangia profunda* SM-A87^T (DQ855467)	1,388/1,459 (99.52%)	ND	+++	++
10-D-4	GU593645	*Idiomarina fontislapidosi* F23^T (AY526861)	1,400/1,419 (98.66%)	ND	+++	+++
10-D-5	GU593646	*Achromobacter xylosoxidans subsp. xylosoxidans* DSM 10346^T (Y14908)	1,448/1,451 (99.79%)	ND	+	++
I-C-2	GU593647	*Idiomarina loihiensis* L2TR^T (AE017340)	1,415/1,415 (100.00%)	ND	+	+++
I-C-3	GU593648	*Donghicola xiamenensis* Y-2 ^{T} (DQ120728)	1,419/1,419 (100.00%)	ND	−	+++
I-C-5	GU593649	*Joostella marina* En5^T (EF660761)	1,312/1,424 (92.14%)	ND	−	−
I-C-6	GU593650	*Microbacterium aoyamense* KV-492^T (AB234028)	1,430/1,443 (99.10%)	ND	+	++
I-D-3	GU593651	*Pseudomonas aeruginosa* LMG 1242^T (Z76651)	1,452/1,458 (99.79%)	I-D-3*alkB* (EU853419)	++	++
II-C-3	GU593652	*Nitratireductor aquibiodomus* NL21^T (AF534573)	1,307/1,321 (99.11%)	ND	+	−
II-C-7	GU593653	*Castellaniella ginsengisoli* DCY36^T (EU873313)	1,381/1,421 (97.25%)	II-C-7*alkB*1(EU853421) II-C-7*alkB*2(EU853420)	+++	++++
II-D-2	GU593654	*Castellaniella ginsengisoli* DCY36^T (EU873313)	1,441/1,441 (100.00%)	ND	+	++
II-D-3	GU593655	*Alcanivorax dieselolei* B-5^T (AY683537)	1,458/1,464 (99.50%)	II-D-3*alkB*(EU853422)	++++	++++
II-D-5	GU593656	*Pseudoalteromonas lipolytica* LMEB 39^T (FJ404721)	1,448/1,478 (97.97%)	ND	−	++

[a] Growth capability with diesel oil or crude oil in MM medium

ND not detected, *NT* not tested, (++++), (+++), (++), (+), and (+/−) indicating the growth capability from strong to weak with diesel oil or crude oil as sole carbon and energy source, measured by optical density at 600 nm, (++++) growth (OD_{600}>1) after a 4-day incubation at 25°C, (+++) growth (OD_{600}>0.6) after a 4-day incubation at 25°C, (++) growth (0.6>OD_{600}>0.2) after a 4-day incubation at 25°C, (+) growth (OD_{600}<0.2) after a 4-day incubation at 25°C, (−) no growth

detected; moreover, it harbored two *alkB* genes that had 97% and 68% similarities at the amino acid level with those of *Alcanivorax dieselolei* B-5^T and *P. putida* strain GPo1, respectively.

Isolate 7-C-7 is possibly a novel species of *Alcanivorax*. It is only of 94.9% similarity in 16S rRNA gene with *Alcanivorax venustensis* ISO4^T, while harbored an *alkB* homolog that had 80% similarity at the amino acid level with that of *P. fluorescens* CHA0.

Isolate 1-D-2 and 2-D-2 displayed 97.8% and 97.4% similarities in 16S rRNA gene with *Acinetobacter junii* LMG998^T and *Pseudomonas pseudoalcaligenes* subsp. *pseudoalcaligenes* DSM 50188^T. Both isolates exhibited vigorous growth with diesel oil and crude oil. Isolate 1-D-2 harbored an *alkB* gene of a high similarity with that of *Acinetobacter* sp. M-1. Isolate 2-D-2 harbored a quite novel *alkB* gene that had only 46% similarity at the amino acid level with that of *P. putida* (CAB54050) as the closest relative.

Phylogenetic Analysis

Our *alkB* homologs formed five major groups named groups I to V in the phylogenetic tree, base on their deduced amino acid sequences (Fig. 2). In addition, some sequences distantly related with the big clusters and formed several separate branches, named A to G in the tree. As mentioned above, these sequences are quite diverse and novel. Group I included 6 homologs derived from 6 isolates of *Alcanivorax*, *Castellaniella*, and *Flavobacterium*; they shared 73–75% similarity with the AlkB of *Marinobacter* sp. ELB17 (EAZ98470). Group II was composed of five AlkBs sharing 80–100% similarity with those of *P. putida* (CAB54050) and *Alcanivorax borkumensis* SK2 (BAC98365). For short, groups III, IV, and V were centered with previously reported AlkBs of *P. fluorescens* (CAB51045), *Rhodococcus* sp. Q15 (AF388182) and *Acinetobacter* spp., respectively (Fig. 2). The AlkB from *Acinetobacter* is also called AlkM, which has been proved

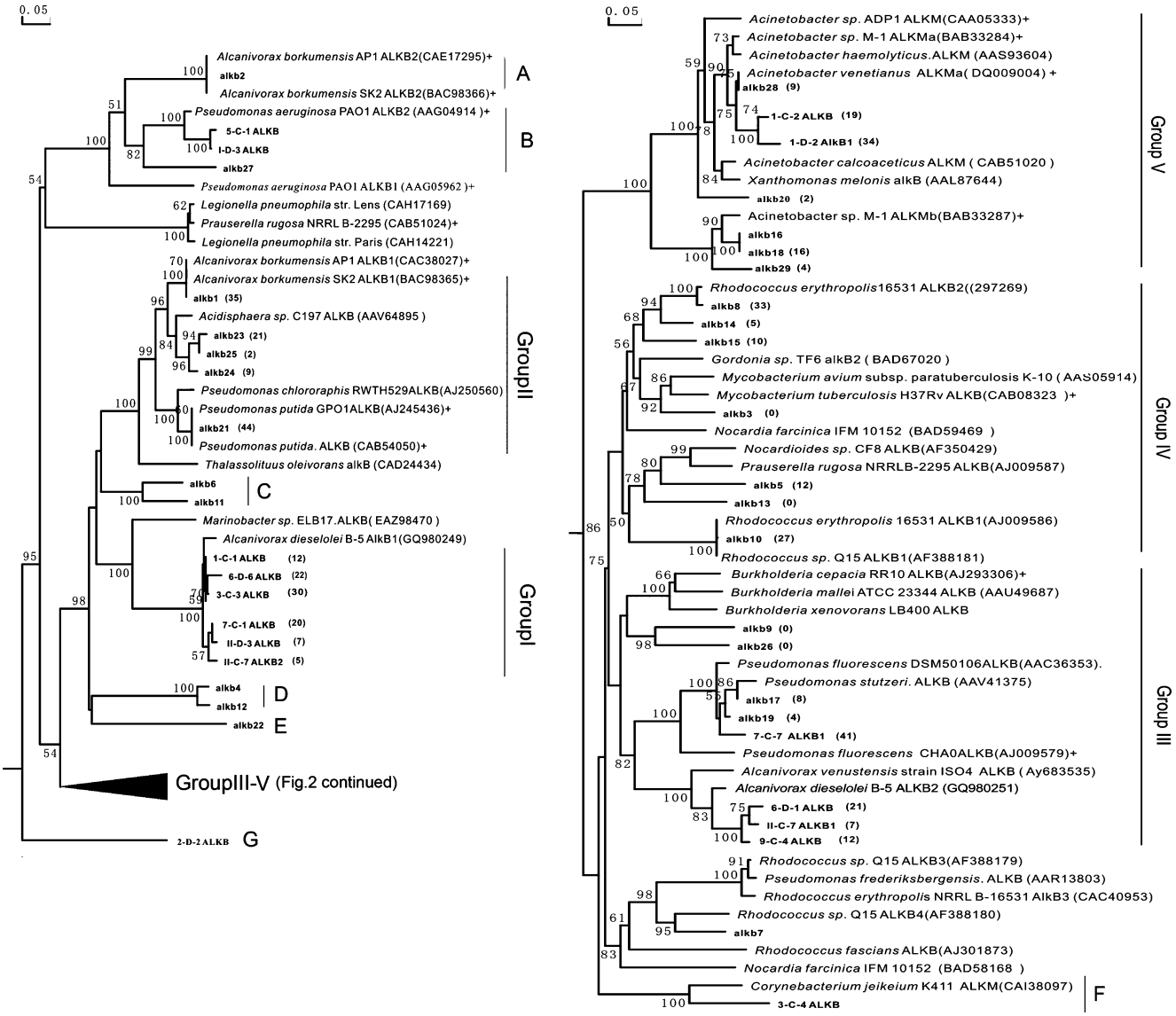

Figure 2 Phylogenetic tree based on amino acid sequences of AlkB homologs obtained from the Xiamen coastal area and references. AlkB reference sequences from the database are labeled with strain names and the GenBank accession numbers in parentheses (protein). AlkB proteins that have been confirmed for the function of alkane hydroxylase are indicated by (+). Sequences named *alkB*1 to 27 were derived from bacterial community DNA used in this report; others were from isolates and labeled with the isolate No. The numbers in parentheses are the clone number of each sequence in 100 clones derived with group-specific Q-PCR primers from surface seawater samples (see "Materials and Methods"). Scale bar, 0.05 substitutions per amino acid site

to act on long-chain alkanes, such as in encoded by *A. venetianus* strain M-1 and ADP1 [35].

Interestingly, distantly related bacteria can possess similar or even identical *alkB* genes, as observed in "group I", which contained nearly identical *alkB* genes. This is probably owing to horizontal gene transfer, as proposed previously [25].

Groups II and IV were mainly composed of those genes directly retrieved from the community libraries; other groups and branches also included some *alkB* genes directly retrieved from the community libraries. In other words, their host bacteria have not been isolated. Compared with *alkB* genes of isolates, those *alkB* genes were quite divergent. Moreover, they were divergent from the published sequences (Fig. 2).

Group Specificity of *alkB* Primers

The group specificity of *alkB* primers designed for quantification of *alkB* genes by Q-PCR in seawater was first examined with genomic DNA of both bacterial strains and seawater. Group-wise alignment of all *alkB* sequences obtained in this report allowed for the design of several specific primer pairs (Table 1; Electronic Supplementary

Material, Fig. S1). Then, each primer pair was tested on corresponding reference strains including our isolates (Table 3). All PCR products derived from alkane-degrading bacteria showed a single band of the expected size, while there was no amplification from non-target bacteria.

Furthermore, the group specificity was also tested on surface seawater samples. Cloning, sequencing and phylogenetic analysis of the PCR products were conducted for each group ("Materials and Methods"). In total, with Q-PCR, 96, 91, 93, 87, and 83 target *alkB* clones were obtained from the 5 libraries, respectively (Table 4). In general, the absolute majority of the sequences are identical to those detected by degenerate PCR described above in Fig. 2. For more details, with the *alkB*If/r primers, 30, 22, and 20 copies in Group I were identical or nearly identical to the *alkB*s of strain 3-C-3, 6-D-6 and 1-C-1, respectively. With *alkB*IIf/r primers of group II, *alkB* genes mainly composed of "alkb21" and "alkb1" with 44 and 35 copies, respectively, both were from uncultivated bacteria in Fig. 2. Similarly, in other three libraries, the group specificity was also confirmed. Overall, sequences close to *Pseudomonas, Alcanivorax, Rhodococcus*, and *Acinetobacter* occupied larger proportions in the five libraries, implies they are relatively abundant in the coastal area. However, "alkb3", "alkb9", "alkb13", and "alkb26" were not retrieved, indicating their low copy number in situ.

Worth to note, only two clones amplified with group I primers actually fell in group II; likely, in the other libraries, five to nine clones fell in non-targeted groups as well. However, only two, one, and two non-*alkB* sequences were found in groups I, IV, and V libraries, respectively (Table 4). In general, these results proved that the feasibility of these primer sets to quantify *alkB* genes with group specificity.

Valid Copy Number Range of *alkB* for Q-PCR

A preliminary Q-PCR experiment was conducted prior to in situ quantification. The results showed that for each group, *alkB* genes were amplified with the same efficiency (see "Materials and Methods"), and standard curves were linear for about six orders of magnitude (data not shown). This permits quantifying *alkB* in a range of four to six orders of magnitude. In details, the valid range for Group I, Group IV and Group V was 10^2 to 10^6 copies per tube (25 μl real-time PCR reagents), and 10^2 to 10^8 copies for groups II and III. These ranges were referred below in examination of *alkB* genes of in situ seawater.

Quantification of *alkB* Genes in the Coastal Surface Water

The validated Q-PCR assay was applied to quantification of *alkB* genes of seawater in situ. The real-time PCR quantification results are shown in Fig. 3. For each *alkB* group, the gene copy number (mean threshold values±S.D.) was determined ranging from about 3×10^3 to 3×10^5 L^{-1} in seawater. Specifically, *alkB* homologs in groups I to V were determined to be 4.19 (±0.26)×10^4, 3.27 (±0.57)×10^5, 4.70 (±0.54)×10^5, 4.75 (±0.93)×10^3, and 3.47 (±0.85)×10^3, respectively. Apparently, the copy numbers of groups II and III were significantly higher than the other groups.

Table 3 Bacterial strains used in validation of five pairs of RT-PCR primers

Strains	alkB If/r	alkB IIf/r	alkB IIIf/r	alkB IVf/r	alkB Vf/r	Reference
Alcanivorax dieselolei B-5	+	−	+	−	−	[8]
A. dieselolei 6-D-6	+	−	+	−	−	This study
Castellaniella sp. II-C-7	+	−	+	−	−	This study
A. dieselolei 3-C-3	+	−	−	−	−	This study
Alcanivorax borkumensis SK2	−	+	−	−	−	[2]
Psedomonas putida GPo1	−	+	−	−	−	[24]
Alcanivorax sp.7-C-7	−	−	+	−	−	This study
Alcanivorax dieselolei 6-D-1	+	−	+	−	−	This study
Gallaecimonas pentaromativorans 9-C-4	−	−	+	−	−	This study
Burkholdera cepacia RR10	−	−	−	+	−	[11]
Rhodococcus sp. strain Q15	−	−	−	+	−	[36]
Rhodococcus erythropolis 16531.	−	−	−	+	−	[36]
Acinetobacter sp.M-1	−	−	−	−	+	[22]
Acinetobacter sp.1-D-2	−	−	−	−	+	This study
Paracoccus sp.1-C-2	−	−	−	−	+	This study

(−) no amplification, (+) positive PCR amplification of expected product size

Table 4 Examination of group specificity of *alkB* primers for real-time PCR

alkB type	alkBIf/r	alkBIIf/r	alkBIIIf/r	alkBIVf/r	alkBVf/r
Group I	96	5	–	–	–
Group II	2	91	–	–	–
Group III	–	–	93	6	4
Group IV	–	–	2	87	5
Group V	–	–	3	2	83
Others[a]	–	4	2	4	6
None[b]	2	–	–	1	2

Five pairs of primers were tested on one site of in situ seawater in Xiamen area. In the table, the number represents the clones belong to different group

(–) not detected

[a] *alkB*s not in Group I to V

[b] Not *alkB* genes

As group I, II, and III contained *alkB* genes from *Alcanivorax* spp. and *Pseudomonas* spp., bacteria of two genera are deduced abundant correspondingly in seawater. Consistently, *A. dieselolei* was found to be one of the most predominant members in these oil-degrading communities (unpublished). In this report, four isolates of *A. dieselolei* of 16S rRNA gene similarity above 98% were obtained in the plate cultivation. These results indicate that *A. dieselolei* bacteria might play an important role in this area. However, *A. borkumensis,* which has been recognized to play an important role in oil removal from marine systems [2], was not isolated successfully. Interestingly, the clone numbers of Group I, III and V were significantly higher than the other groups in community libraries (data not shown). Group V contained *alkB* genes were obtained from *Acinetobacter* spp., which was also found to be one of the most predominant members in the oil-degrading communities (unpublished). Thus, *Acinetobacter* bacteria might play an important role in this area in case of oil spill occurrence.

Although the abundance of *alkB* genes partially reflected their role in alkane mineralization in situ, whether they actually function or dictate the synthesis of alkane monooxygenase requires further investigation, for example, by quantification of the mRNA or AlkB directly.

In addition to the pathway initiated by AlkB monooxygenase, other alkane monooxygenase systems should exist, such as cytochrome P450 [10], which also contribute to alkane degradation and oil removal in situ. In addition, AlmA has been confirmed as an alkane monooxygenase of the flavin-binding family and is involved in long-chain *n*-alkane metabolism [23, 34]. To gain an overview of the genes responsible for oil pollutant bioattenuation in the coastal area, P450 and *almA* genes, which have been confirmed as alkane monooxygenases, should also be taken into consideration.

In summary, the *alkB* genes were quite diverse in the coastal surface seawater around Xiamen Island. They exist in each milliliter of the surface water with about 1,000 copies, hosted in bacteria including *Alcanivorax*, *Acinetobacter, Pseudomonas*, *Gallaecimonas*, *Castellaniella*, *Paracoccus*, and *Leucobacter,* etc. These bacteria are thought active in removal of oil in situ, especially *Alcanivorax, Pseudomonas*, and *Acinetobacter* in this area. However, bacteria of *Oleiphilus* spp., *Oleispira* spp., and *Thalassolituus* spp., which frequently reported in other areas, were not found in our report. This indicates that oil-degrading bacteria may have restriction in geographic distribution in some species.

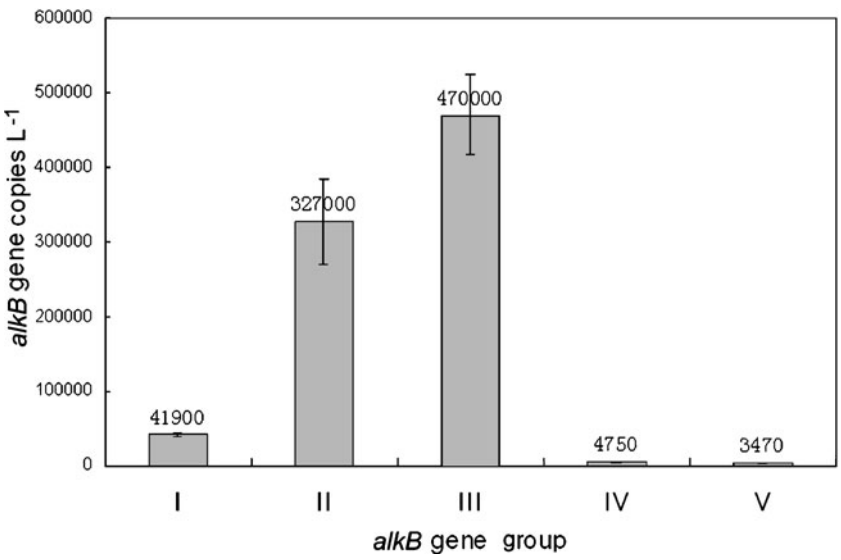

Figure 3 *alkB* gene copy numbers (mean threshold values±SD) in 1 l of surface seawater of the Xiamen coastal area quantified by real-time PCR. *I* Group I-like *alkB* gene, *II* group II-like *alkB* gene, *III* group III-like *alkB* gene, *IV* group IV-like *alkB* gene, and *V* group V-like *alkB* gene

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References

1. Feng L, Wang W, Cheng J, Ren Y, Zhao G, Gao C, Tang Y, Liu X, Han W, Peng X, Liu R, Wang L (2007) Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. Proc Natl Acad Sci USA 104:5602–5607
2. Golyshin PN, Martins Dos Santos VA, Kaiser O, Ferrer M, Sabirova YS, Lunsdorf H, Chernikova TN, Golyshina OV, Yakimov MM, Puhler A, Timmis KN (2003) Genome sequence completed of *Alcanivorax borkumensis,* a hydrocarbon-degrading bacterium that plays a global role in oil removal from marine systems. J Biotechnol 106:215–220
3. Head IM, Jones DM, Roling WF (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4:173–182
4. Heiss-Blanquet S, Benoit Y, Marechaux C, Monot F (2005) Assessing the role of alkane hydroxylase genotypes in environmental samples by competitive PCR. J Appl Microbiol 99:1392–1403
5. Islam KS, Rahman MM, Eda LEH (2009) Pollution status and sustainable management of Xiamen Bay in China: a brief review. Int J Ocean Syst Manage 1:155–168
6. Kuhn E, Bellicanta GS, Pellizari VH (2009) New alk genes detected in Antarctic marine sediments. Environ Microbiol 11:669–673
7. Kloos K, Munch JC, Schloter M (2006) A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. J Microbiol Methods 66:486–496
8. Liu C, Shao Z (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int J Syst Evol Microbiol 55:1181–1186
9. Luz AP, Pellizari VH, Whyte LG, Greer CW (2004) A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil. Can J Microbiol 50:323–333
10. Maier T, Forster HH, Asperger O, Hahn U (2001) Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. Biochem Biophys Res Commun 286:652–658
11. Marin MM, Smits TH, van Beilen JB, Rojo F (2001) The alkane hydroxylase gene of *Burkholderia cepacia* RR10 is under catabolite repression control. J Bacteriol 183:4202–4209
12. Ou S, Zheng J, Richardson BJ, Lam PK (2004) Petroleum hydrocarbons and polycyclic aromatic hydrocarbons in the surficial sediments of Xiamen Harbour and Yuan Dan Lake, China. Chemosphere 56:107–112
13. Powell SM, Ferguson SH, Bowman JP, Snape I (2006) Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. Microb Ecol 52:523–532
14. Quatrini P, Scaglione G, De Pasquale C, Riela S, Puglia AM (2008) Isolation of Gram-positive *n*-alkane degraders from a hydrocarbon-contaminated Mediterranean shoreline. J Appl Microbiol 104:251–259
15. Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 11:2477–2490
16. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
17. Sei K, Sugimoto Y, Mori K, Maki H, Kohno T (2003) Monitoring of alkane-degrading bacteria in a sea-water microcosm during crude oil degradation by polymerase chain reaction based on alkane-catabolic genes. Environ Microbiol 5:517–522
18. Shanklin J, Whittle E, Fox BG (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochem 33:12787–12794
19. Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K, Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Environ Microbiol 67:2469–2475
20. Stapleton RD, Ayler GS (1998) Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. Microbial Ecol: 36:49–361
21. Smits TH, Röthlisberger M, Witholt B, van Beilen JB (1999) Molecular screening for alkane hydroxylase genes in gram-negative and gram-positive strains. Environ Microbiol 1:307–317
22. Tani A, Ishige T, Sakai Y, Kato N (2001) Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. J Bacteriol 183:1819–1823
23. Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB (2007) Identification of novel genes involved in long-chain *n*-alkane degradation by *Acinetobacter* sp. strain DSM 17874. Appl Environ Microbiol 73:3327–3332
24. van Beilen JB, Wubbolts MG, Witholt B (1994) Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 5:161–174
25. van Beilen JB, Smits THM, Whyte LG, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser KH, Witholt B (2002) Alkane hydroxylases in gram-positive strains. Environ Microbiol 4:676–682
26. van Beilen JB, Duetz WA, Schmid A, Witholt B (2003) Practical issues in the application of oxygenases. Trends Biotechnol 21:170–177
27. van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci Technol 58:427–440
28. van Beilen JB, Funhoff EG (2005) Expanding the alkane oxygenase toolbox: new enzymes and applications. Curr Opin Biotechnol 16:308–314
29. van Beilen JB, Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21
30. Vomberg A, Klinner U (2000) Distribution of alkB genes within *n* alkane-degrading bacteria. J Appl Microbiol 89:339–348
31. Wang L, Wang W, Lai Q, Shao Z (2010) Gene diversity of CYP153A and AlkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean. Environ Microbiol 12 (5):1230–1242
32. Wasmund K, Burns KA, Kurtboke DI, Bourne DG (2009) Novel alkane hydroxylase gene (alkB) diversity in sediments associated with hydrocarbon seeps in the Timor Sea, Australia. Appl Environ Microbiol 75:7391–7398
33. WEF AA (1998) Standard Methods for the Examination of Water and Wastewater (20th ed.), APHA-AWWA-WEF, Washington, DC
34. Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB, Throne-Holst M (2007) Bacterial metabolism of long-chain *n*-alkanes. Appl Microbiol Biotechnol 76:1209–1221
35. Whyte LG, Schultz A, Van Beilen JB, Luz AP, Pellizari D, Labbé D, Greer CW (2002) Prevalence of alkane monooxygenase genes in arctic and antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol Ecol 41:141–150
36. Whyte LG, Smits TH, Labbe D, Witholt B, Greer CW, van Beilen JB (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. Appl Environ Microbiol 68:5933–5942