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SHORT COMMUNICATION

# Diverse bacteria isolated from microtherm oil-production water

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**Abstract** In total, 435 pure bacterial strains were isolated from microtherm oil-production water from the Karamay Oilfield, Xinjiang, China, by using four media: oil-production water medium (Cai medium), oil-production water supplemented with mineral salt medium (CW medium), oil-production water supplemented with yeast extract medium (CY medium), and blood agar medium (X medium). The bacterial isolates were affiliated with 61 phylogenetic groups that belong to 32 genera in the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Except for the *Rhizobium*, *Dietzia,* and *Pseudomonas* strains that were isolated using all the four media, using different media led to the isolation of bacteria with different functions. Similarly, nonheme diiron alkane monooxygenase genes (*alkB*/*alkM*) also clustered according to the isolation medium. Among the bacterial strains, more than 24 % of the isolates could use *n*-hexadecane as the sole carbon source for growth. For the first time, the alkane-degrading ability and *alkB*/*alkM* were detected in *Rhizobium*, *Rhodobacter*, *Trichococcus*, *Micrococcus*, *Enterococcus*, and *Bavariicoccus* strains, and the *alkM* gene was detected in *Firmicutes* strains.

**Keywords** Oil-production water · Alkane monooxygenase · Microtherm · Culturability

## Introduction

Along with the depletion of easily recoverable crude oil deposits, microbial enhanced oil recovery (MEOR) has been gaining increasing interest because it is environmentally friendly and cost-efficient (Lazar et al. 2007). An oil reservoir is a very special environment containing high pressure and few nutrients, which accommodates specific microorganisms. The low-temperature strata are often found in shallow oil reservoirs with depths ranging from 200 to 2,000 m. At relatively low temperatures, crude oil in the low-temperature strata is often poor in fluidity, which hampers the oil recovery. Therefore, the microbial degradation of petroleum hydrocarbons becomes more important for efficient oil recovery in microtherm oilfields.

Recently, many studies have investigated the microbial community in oil-production water from oil

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reservoirs (Dahle et al. 2008; Kaster et al. 2009; Li et al. 2006; Tang et al. 2012; Zhao et al. 2012). Simultaneously, many attempts have been made to isolate microbial strains from oil reservoirs as well as oil-production water (Kaster et al. 2009; Miroshnichenko et al. 2001). However, majority of the investigations and isolations were conducted on the mesotherm high-temperature oil reservoirs and oil-production water (Kaster et al. 2009; Wang et al. 2011), and only a few studies have addressed microtherm oil-production water.

Therefore, we investigated the isolation of bacterial strains from low-temperature oil-production water, which were obtained from the low-temperature oil stratum, by using four types of media at 25 °C. We also screened the strains that were able to degrade oil components and detected the phenol hydroxylase and alkane monooxygenase genes.

## Materials and methods

### Strain isolation and identification

The oil-production water was collected from the low-temperature oil stratum in Liuzhong Block, Karamay Oilfield, Xinjiang Uygur Autonomous Region, China, and transported to the laboratory at 4 °C. The temperature of the block strata and the production water was 20.6 °C. Other characteristics of this block have been previously described (Zhao et al. 2012).

First, cells in the production water were collected with a filter (0.22 μm pore size) under aseptic conditions. Then, the cells were resuspended with a sterile saline solution and diluted to $10^{-3}$ to make an inoculating suspension. Four types of media were used: (i) Cai medium (1,000 mL production water, 20 g agar, sterilized); (ii) CW medium (1,000 mL production water, 1.0 g $NH_4HO_3$, 1.0 g NaCl, 1.5 g $K_2HPO_4$, 0.5 g $KH_2PO_4$, 20 g agar, sterilized); (iii) CY medium (1,000 mL production water, 5.0 g yeast extract, 20 g agar, sterilized); and (iv) X medium (blood agar, Beijing Sybrisk Science & Technology Co., Ltd). The inoculating suspensions (100 μL) were plated on the four media and statically incubated at 25 °C in the dark for 9 days. All colonies that grew on the plates were picked, purified, and stored for further investigation. The isolates were named by the medium name and the colony series numbers. For example, the 1st and 10th strains isolated from Cai medium were named as Cai-1 and Cai-10, respectively.

DNA extraction, amplification, and analysis of the 16S rRNA gene from the purified isolates were conducted using previously described protocols (Wang et al. 2007). Before sequencing, the isolates were affiliated to different groups according to the colony phenotype and restriction fragment length polymorphism according to a previously described protocol (Yu et al. 2011).

### Degradation of petroleum components by bacterial strains

After cells of different strains were grown in liquid LB medium (5.0 g $L^{-1}$ yeast extract, 10.0 g $L^{-1}$ tryptone, 10 g $L^{-1}$ NaCl; pH 7.0) at 25 °C for 2 days, they were harvested as pellets after centrifugation (2,000×*g* at 4 °C for 10 min). Then, the pellets were washed twice and resuspended in an aseptic saline solution to prepare the inoculating suspension for further physiological experiments.

To investigate the petroleum degrading ability of different strains, the inoculum suspensions were added to 30 mL of mineral salt medium (MSM: 1.0 g $L^{-1}$ $NH_4NO_3$, 1.0 g $L^{-1}$ NaCl, 1.5 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ $KH_2PO_4$, 0.2 g $L^{-1}$ $MgSO_4{\cdot}7H_2O$) supplemented with phenol (100 mg $L^{-1}$, final concentration), *n*-hexadecane (100 mg $L^{-1}$, final concentration), or phenanthrene (100 mg $L^{-1}$, final concentration) as the sole carbon and energy source. Then, the cultures were incubated at 25 °C and shaken at 150 rpm in the dark. On the 5th day, the cultures were sampled for the detection of residual organic compounds as well as cell growth.

The concentrations of phenol and *n*-hexadecane were detected by using the HPLC–UV and GC-FID with the previously described methods and protocols (Sun et al. 2011; 2012). The residual phenanthrene in the culture was extracted with dichloromethane. Briefly, the samples were thoroughly extracted with an equal volume of dichloromethane. From the dichloromethane layer, 2 mL of the mixture was collected and dried by anhydrous $Na_2SO_4$. Then, 1 mL of the dried mixture was transferred into a new tube for volatilization. The dichloromethane residue was dissolved in 0.5 mL of methanol. Then, methanol was used to determine the concentration of phenanthrene following the same protocol as that used for

**Table 1** Primer sets used in the present study

| Genes | Primer | Primer sequence |
|---|---|---|
| Multiple-components phenol hydroxylase genes (*pheN*) (Xu et al. 2001) | Phe1 | 5′-AGGCATCAAGATCACCGACTG-3′ |
| | Phe2 | 5′-CGCCAGAACCATTTATCGATC-3′ |
| Nonheme diiron alkane monooxygenase gene (*alkB/alkM*) (Kloos et al. 2006) | AlkB-1f | 5′-AAYACNGCNCAYGARCTNGGNCAYAA-3′ |
| | AlkB-1r | 5′-GCRTGRTGRTCNGARTGNCGYTG-3′ |
| Cytochrome P450 enzymes gene (CYP153A) (Wang et al. 2010) | P450FS | 5′-TGTCGGTTGAAATGTTCAT-3′ |
| | P450RS | 5′-TGCAGTTCGGCAAGGCGGTT-3′ |
| Flavin-binding monooxygenase gene (*almA*) (Wang and Shao 2011) | AlmAdf | 5′-GGNGGNACNTGGGAYCTNTT-3′ |
| | AlmAdr | 5′-ATRTCNGCYTTNAGNGTCC-3′ |

determining the phenol concentration except that the results were recorded at a wavelength of 250 nm.

### Analysis of phenol hydroxylase and alkane monooxygenase genes

The previous described primers used to amplify phenol hydroxylase genes (*pheN*) as well as alkane monooxygenase genes, including nonheme diiron alkane monooxygenase gene (*alkM*/*alkB*), Cytochrome P450 enzymes gene (*CYP153A*), and Flavin-binding monooxygenase gene (*almA*) (Kloos et al. 2006; Wang et al. 2010; Wang and Shao 2011; Xu et al. 2001) and are listed in Table 1. DNA fragments corresponding with the correct target size for each gene were cloned into the pMD19-T vector (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China) and sequenced.

The obtained DNA sequences were aligned in GenBank by using the BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequences of alkane monooxygenase and phenol hydroxylase were translated using the MEGA software package version 5.0 according to the universal codon (Tamura et al. 2011). The reference sequences were retrieved from GenBank. After multiple sequence alignment of the sequences by CLUSTAL X and manually correction, the phylogenetic tree based on the phenol hydroxylase or alkane monooxygenase gene sequences were constructed using neighbour-joining method (Saitou and Nei 1987) in the MEGA software package version 5.0 (Tamura et al. 2011). The stability of tree topology was evaluated with maximum-likelihood and maximum parsimony algorithms.

## Results

### Phylogenetic distribution of the isolates from different media

After cultivation on four different kinds of media at 25 °C in the dark, X and Cai media led to the fastest and slowest growth, respectively, whereas CY and CW media showed the highest number of colonies (Fig. S1). At the end of the cultivation period (9 days), 435 colonies were isolated and purified from CY (134 isolates), CW (140 isolates), X (119 isolates), and Cai (42 isolates) media. Screened according to colony topologies, these 435 isolates were classified into 246 representative strains. Then, 246 representative strains were selected for further analyses including 16S rRNA gene sequencing. These 246 strains were classified into 61 groups according to 16S rRNA gene sequences and 16S rRNA gene fragment restriction digestion patterns, which could be further assigned to 32 genera belonging to the *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria* (Fig. 1).

Among the 61 groups of bacterial strains, 13, 15, 27, and 25 groups were isolated from Cai, CW, CY, and X media, respectively (Table 2; Figs. S2–S6). Furthermore, 1 (3 isolates), 4 (6 isolates), 3 (6 isolates), and 2 (2 isolates) groups isolated from Cai, CW, CY, and X media, respectively, which accounts for 7.7, 26.7, 11.1, and 7.7 % of the total groups in the corresponding media, potentially represented novel bacterial taxonomical groups because they shared <98 % 16S rRNA gene sequence identity with validly published bacterial species (Supplementary Table S2). Considerably more

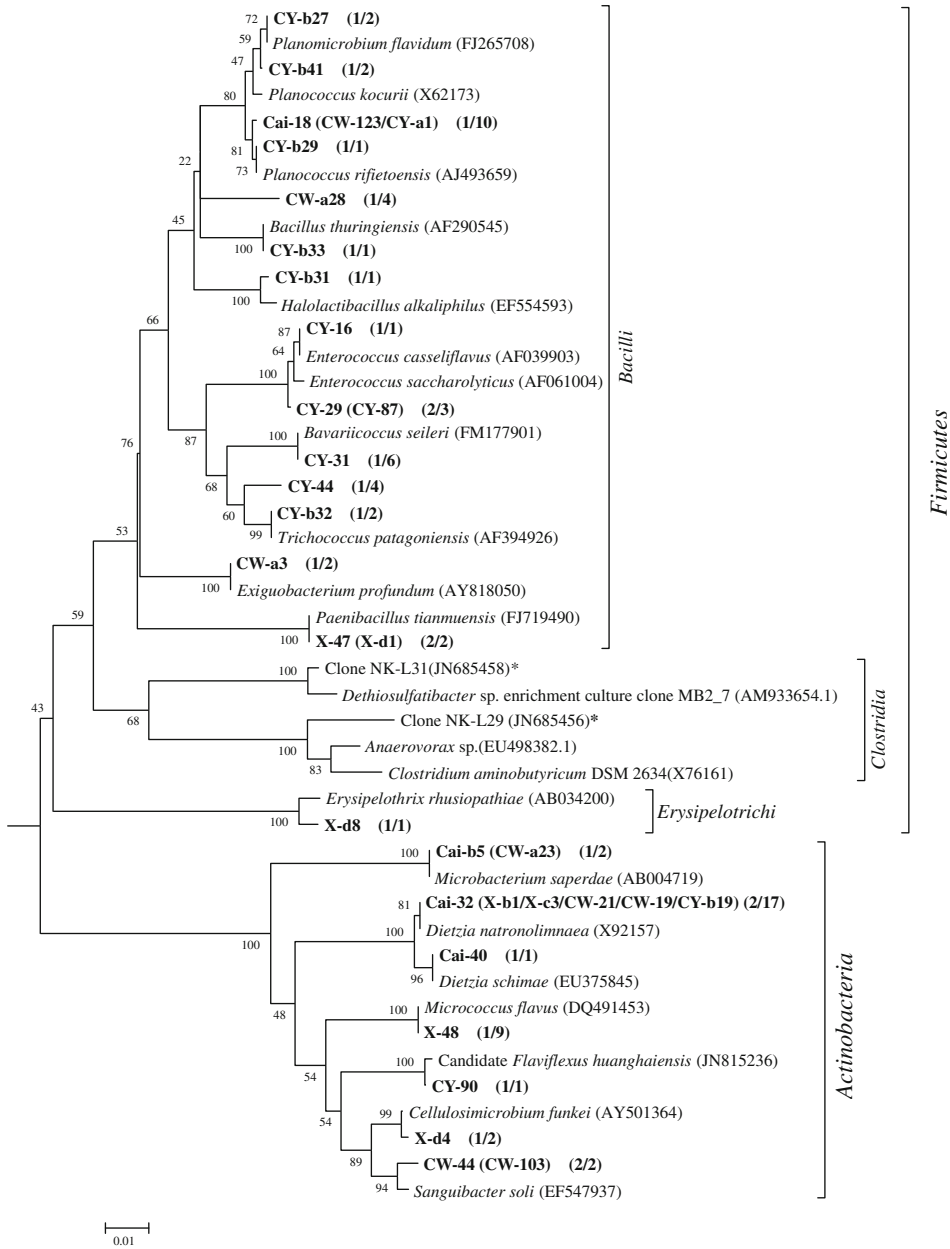
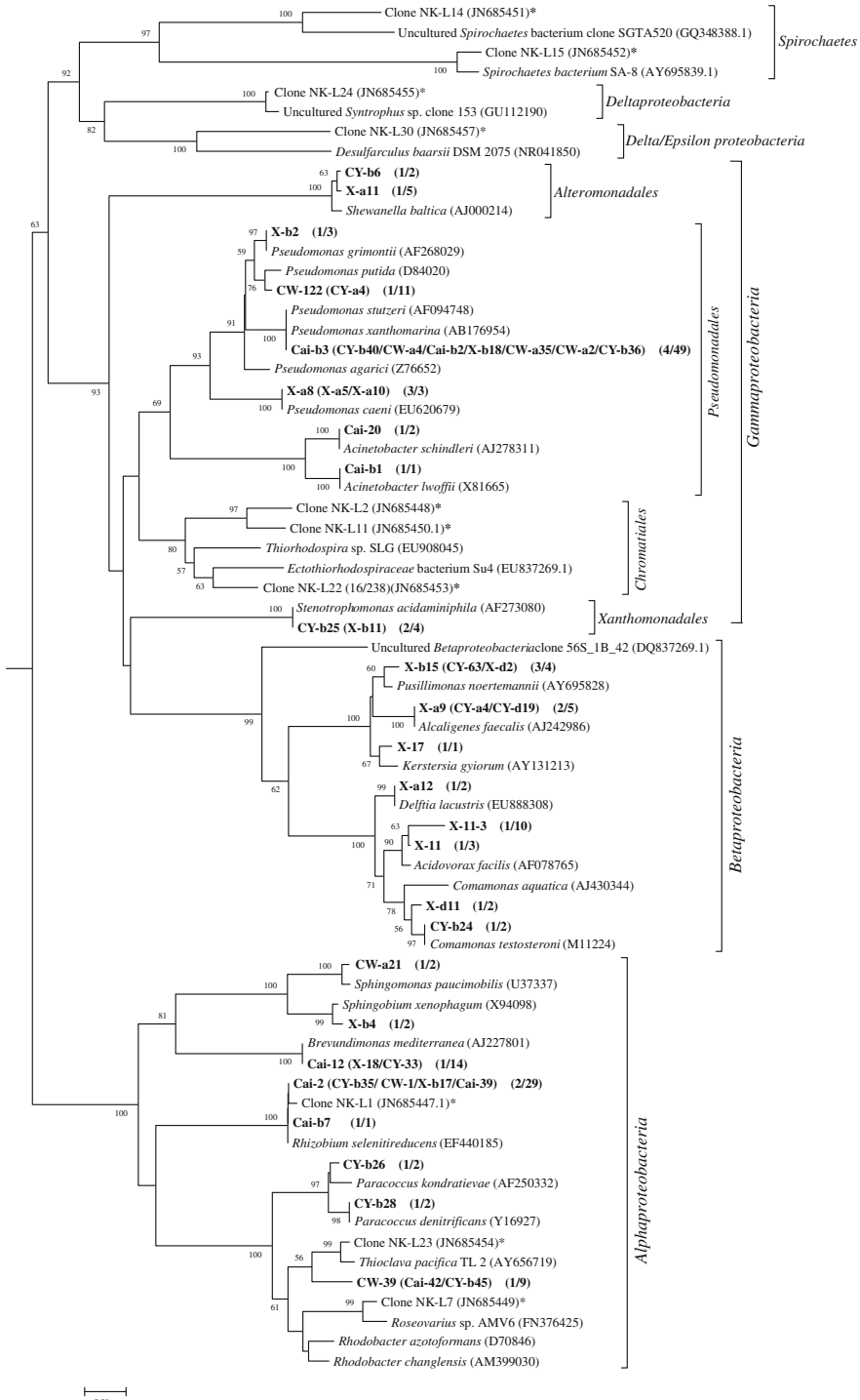



**Fig. 1** Phylogenetic tree based on the 16S rRNA gene of the representative strains of the 61 groups was constructed by using neighbor-joining method, with the tree topology evaluated by bootstrap analysis based on 1,000 resampling replicates with MEGA software package version 5.0. The bootstrap *values* (%) are indicated at the nodes. The *scale bars* represent 0.01 substitutions per site. The strains with overstriking names were isolated in the present study, while the sequences with *asterisks* are the sequences from a clone library analysis of the same stratum production water (Zhao et al. 2012). The *numbers* in brackets after the strains are the numbers of phylogenetic group (prior) (The *1* means all the stains in this pattern sharing the same 16S rRNA gene, and similar hydrocarbon-degrading genes, clone topology, and hydrocarbon-degrading characteristics; *2*, *3*, and *4* mean the strains in this pattern forming 2, 3 and 4 groups respectively, all sharing the same 16S rRNA gene but with different functional characteristics) and numbers of the isolates within this pattern (later) obtained in the present study

**Fig. 1** continued




bacteria belonging to *Proteobacteria* were isolated from the high-nutrient (CY and X) media than from the relatively low-nutrient (Cai and CW) media. *Acinetobacter* strains were isolated only from Cai medium. *Sanguibacter*, *Exiguobacterium*, and *Sphingobium* strains were isolated only from CW agar.

**Table 2** Distribution of bacterial strains from different medium on genus and phylum levels

| Taxonomy | Media | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cai | | | | CW | | | | CY | | | | X | | | |
| | Total | Degrading strains | | | Total | Degrading strains | | | Total | Degrading strains | | | Total | Degrading strains | | |
| | | Hex | Phe | Phen | | *n*-Hex | Phe | Phen | | *n*-Hex | Phe | Phen | | *n*-Hex | Phe | Phen |
| *Actinobacteria* | | | | | | | | | | | | | | | | |
| *Actinomycetaceae*[a] | – | – | – | – | – | – | – | – | 1 (1) | 0 | 0 | 0 | – | – | – | – |
| *Cellulosimicrobium* | – | – | – | – | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 |
| *Dietzia* | 2 (4) | 2 | 0 | 0 | 2 (6) | 2 | 0 | 0 | 1 (5) | 1 | 0 | 0 | 2 (3) | 2 | 0 | 0 |
| *Microbacterium* | 1 (1) | 0 | 0 | 0 | 1 (1) | 0 | 0 | 0 | – | – | – | – | – | – | – | – |
| *Micrococcus* | – | – | – | – | – | – | – | – | – | – | – | – | 1 (9) | 1 | 0 | 0 |
| *Sanguibacter* | – | – | – | – | 2 (2) | 0 | 0 | 0 | – | – | – | – | – | – | – | – |
| *Firmicutes* | | | | | | | | | | | | | | | | |
| *Bacillus* | – | – | – | – | 1 (4) | 0 | 0 | 0 | 1 (2) | 0 | 0 | 0 | – | – | – | – |
| *Bavariicoccus* | – | – | – | – | – | – | – | – | 1 (5) | 1 | 0 | 0 | – | – | – | – |
| *Enterococcus* | – | – | – | – | – | – | – | – | 3 (4)[b] | 1 | 1 | 0 | – | – | – | – |
| *Erysipelotrichaceae*[a] | – | – | – | – | – | – | – | – | – | – | – | – | 1 (1) | 0 | 0 | 0 |
| *Exiguobacterium* | – | – | – | – | 1 (2) | 0 | 0 | 1 | – | – | – | – | – | – | – | – |
| *Halolactibacillus* | – | – | – | – | – | – | – | – | 1 (1) | 0 | 0 | 1 | – | – | – | – |
| *Paenibacillus* | – | – | – | – | – | – | – | – | – | – | – | – | 2 (2) | 0 | 0 | 1 |
| *Planococcus* | 1 (2) | 0 | 0 | 0 | 1 (6) | 0 | 0 | 0 | 3 (5) | 2 | 0 | 0 | – | – | – | – |
| *Planomicrobium* | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 | – | – | – | – |
| *Trichococcus* | – | – | – | – | – | – | – | – | 2 (6) | 1 | 0 | 0 | – | – | – | – |
| *Alphaproteobacteria* | | | | | | | | | | | | | | | | |
| *Brevundimonas* | 1 (6) | 0 | 0 | 0 | – | – | – | – | 1 (5) | 0 | 0 | 0 | 1 (3) | 0 | 0 | 0 |
| *Paracoccus* | – | – | – | – | – | – | – | – | 2 (4) | 1 | 0 | 0 | – | – | – | – |
| *Rhizobium* | 3 (16) | 2 | 0 | 0 | 1 (7) | 1 | 0 | 0 | 1 (6) | 1 | 0 | 0 | 1 (1) | 1 | 0 | 0 |
| *Rhodobacter* | 1 (7) | 1 | 0 | 0 | 1 (1) | 1 | 0 | 0 | 1 (1) | 1 | 0 | 0 | – | – | – | – |
| *Sphingomonas* | – | – | – | – | 1 (2) | 0 | 0 | 0 | – | – | – | – | – | – | – | – |
| *Sphingobium* | – | – | – | – | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 |
| *Betaproteobacteria* | | | | | | | | | | | | | | | | |
| *Acidovorax* | – | – | – | – | – | – | – | – | – | – | – | – | 2 (13) | 1 | 0 | 0 |
| *Alcaligenes* | – | – | – | – | – | – | – | – | 2 (2) | 0 | 2 | 0 | 1 (3) | 0 | 1 | 0 |
| *Alcaligenaceae*[a] | – | – | – | – | – | – | – | – | – | – | – | – | 1 (1) | 0 | 0 | 1 |

**Table 2** continued

| Taxonomy | Media | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cai | | | | CW | | | | CY | | | | X | | | |
| | Total | Degrading strains | | | Total | Degrading strains | | | Total | Degrading strains | | | Total | Degrading strains | | |
| | | Hex | Phe | Phen | | *n*-Hex | Phe | Phen | | *n*-Hex | Phe | Phen | | *n*-Hex | Phe | Phen |
| *Comamonas* | – | – | – | – | – | – | – | – | 1 (2) | 0 | 1 | 0 | 1 (2) | 0 | 0 | 0 |
| *Delftia* | – | – | – | – | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 |
| *Pusillimonas* | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 | 2 (2) | 0 | 0 | 0 |
| *Gammaproteobacteria* | | | | | | | | | | | | | | | | |
| *Acinetobacter* | 2 (3) | 1 | 0 | 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| *Pseudomonas* | 2 (2)[c] | 1 | 0 | 1 | 4 (45)[c] | 3 | 0 | 1 | 3 (12) | 1 | 0 | 0 | 5 (7) | 1 | 0 | 0 |
| *Shewanella* | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 | 1 (5) | 0 | 0 | 0 |
| *Stenotrophomonas* | – | – | – | – | – | – | – | – | 1 (2) | 0 | 0 | 0 | 1 (2) | 0 | 0 | 0 |
| Sum | 13 (41) | 7 | 0 | 1 | 15 (76) | 6 | 0 | 2 | 27 (69) | 10 | 5 | 1 | 25 (60) | 6 | 1 | 2 |
| Genera | 8 | – | – | – | 10 | – | – | – | 19 | – | – | – | 17 | – | – | – |
| Unique species | 1 | – | – | – | 3 | – | – | – | 6 | – | – | – | 7 | – | – | – |

Taxa name marked with [a] is family name; the number in table are the number of strains, in bracket are the total isolates, –represents no strain

[b] *Enterococcus* sp. strain CY-29 could simultaneously degrade phenol and *n*-hexadecane

[c] *Pseudomonas* sp. strain Cai-b3 (=CW-a35) could simultaneously degrade *n*-hexadecane and phenanthrene

*Microbacterium* strains were only isolated from Cai and CW media, whereas no *Betaproteobacteria* strains were obtained from these 2 media. *Halolactibacillus*, *Enterococcus*, *Planomicrobium*, *Paracoccus*, and one *Actinomycetaceae* strains (with the 16S rRNA gene sequence identities <94 % with all of the known species) were unique species isolated from CY agar. *Cellulosimicrobium*, *Micrococcus*, *Paenibacillus*, *Sphingobium*, *Delftia* strains, in addition to 2 strains belonging to the genera *Erysipelotrichaceae* and *Alcaligenaceae* (with identical 16S rRNA gene sequence identities with 2 known genera of *Alcaligenaceae*: *Kerstersia* and *Bordetella*) were unique species isolated from X agar.

Three genera, *Dietzia* (18 isolates), *Rhizobium* (30 isolates), and *Pseudomonas* (66 isolates), could be isolated from all four media. In all, 4, 6, 5, and 3 *Dietzia* strains; 16, 7, 6, and 1 *Rhizobium* strains; and 2, 45, 12, and 7 *Pseudomonas* strains isolated from Cai, CW, CY, and X agars, respectively (Table S2).

### Distributions of isolates with different hydrocarbon-degrading abilities as well as alkane monooxygenase and phenol hydroxylase genes

Among the 61 representative strains, four strains from CY and X media belonging to *Alcaligenes*, *Comamonas*, and *Enterococcus* genera could use phenol as the sole carbon source for growth (Table S1). In contrast, no strains from Cai and CW agars showed phenol-degrading ability. Five strains belonging to *Halolactibacillus*, *Exiguobacterium*, *Paenibacillus*, *Pseudomonas,* and *Alcaligenaceae* (a potential novel genus) and isolated from the four media could use phenanthrene as the sole carbon and energy source for growth (Tables 2, S1). It is notable that no study has described *Enterococcus* spp. using phenol and *Exiguobacterium* and *Halolactibacillus* spp. using phenanthrene as the sole carbon and energy sources for growth. Four phenol hydroxylase genes (*pheN*) responsible for the hydroxylation of phenolic compounds were obtained from four strains isolated from CY and X media that belonged to *Alcaligenes*, *Sphingobium*, and *Comamonas*. The four *pheN* were grouped into different clusters in the phylogenetic tree (Fig. S7). Although the *pheN* gene was PCR amplified from strain *Sphingobium* sp. X-b4, no phenol-degrading ability was detected. In contrast, no *phe* gene was detected in the phenol-degrading *Enterococcus* sp. CY-29 (Table S1).

Compared with the small amount of strains able to degrade phenol and phenanthrene, 46.2, 40.0, 33.3, and 24.0 % of the total strains from Cai, CW, CY, and X media were able to degrade *n*-hexadecane, respectively (Table S2). These included *Planococcus*, *Rhizobium*, *Acinetobacter*, *Pseudomonas*, *Rhodobacter*, and *Dietzia* strains from Cai medium; *Pseudomonas*, *Rhodobacter*, *Planococcus*, and *Dietzia* strains from CW medium; *Pseudomonas*, *Paracoccus*, *Planococcus*, *Trichococcus*, *Enterococcus*, *Bavariicoccus*, and *Dietzia* strains from CY medium; and *Pseudomonas*, *Acidovorax*, *Micrococcus*, *Dietzia* strains from X medium. The genes encoding alkane monooxygenase, which is responsible for alkane hydroxylation, were also detected using PCR amplification from the isolates. The *alkB*/*alkM* could be clustered into three groups (Fig. 2). Cluster I represents the *alkB* genes from all of the *Dietzia* strains. Cluster II represents the *alkM* genes from *Trichococcus*, *Micrococcus*, *Enterococcus*, *Paracoccus*, *Pseudomonas,* and *Bavariicoccus*, which is also clustered with *alkM* from *Acinetobacter* strains (Ratajczak et al. 1998; Sun et al. 2012). Cluster III represents the *alkB* genes from *Rhizobium*, *Rhodobacter*, and *Acinetobacter*, which is also clustered with the *alkB* gene from *Pseudomonas putida* Gpo1 (van Beilen et al. 2001). *CYP153A*, another medium-chain alkane hydroxylation gene (Nie et al. 2013b), and *almA*, a long-chain alkane hydroxylation genes, were detected only in *Dietzia* strains (data not shown).

## Discussions

It is believed that environmental conditions drive microbial community to evolve relevant functions or to select special microorganisms to adapt the environments. In a microtherm oil reservoir, such as the Liuzhong Block of the Karamay Oilfield, microorganisms should be able to use the crude oil components for growth and adapt to temperatures as low as 22 °C. Therefore, it is reasonable that more than 24 % of the bacteria isolated from this oil-rich environment have the ability to degrade petroleum hydrocarbons, including *n*-hexadecane and phenanthrene. Among them, strains belonging to *Rhizobium*, *Rhodobacter*, *Trichococcus*, *Micrococcus*, *Enterococcus*, and *Bavariicoccus* genera were detected, for the first

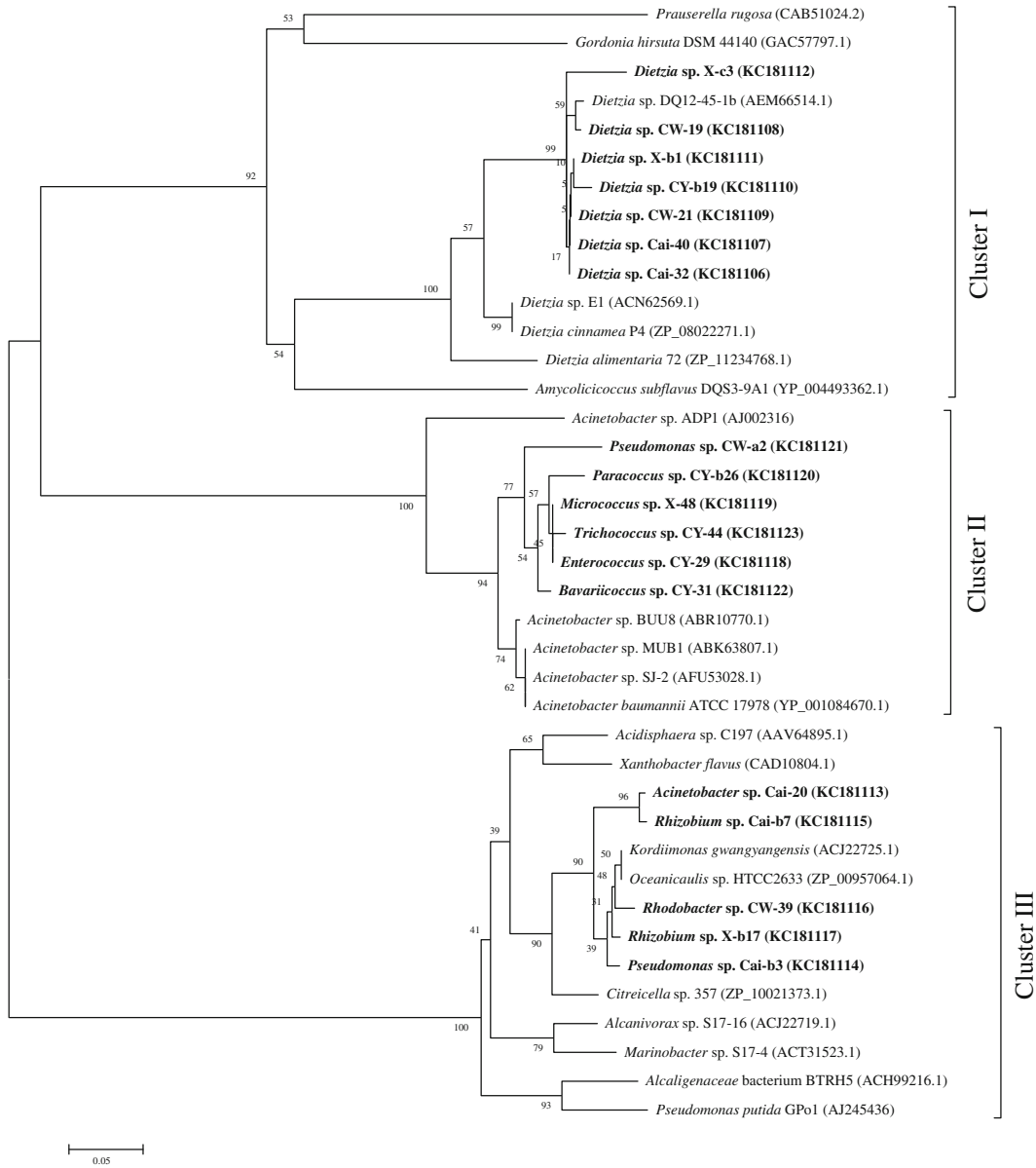



**Fig. 2** Phylogenetic tree based on the partial amino acid sequences of the alkane monooxygenase was constructed by using the neighbor-joining method. The tree topology was evaluated by bootstrap analysis based on 1,000 resampling replicates with the MEGA software package version 5.0. The bootstrap *values* (%) are indicated at the nodes. The *scale bars* represent 0.05 substitutions per site

time, with the ability to degrade *n*-hexadecane, as well as contain the *alkB/alkM* genes. Phylogenetically distant but related bacteria, including *Micrococcus*, *Trichococcus* and *Enterococcus*, *Paracoccus* and *Bavariicoccus*, and *Pseudomonas*, had closely related *alkB/alkM* genes (Fig. 2). In addition, *alkM* genes, usually detected in *Acinetobacter* (Ratajczak et al. 1998; Sun et al. 2012), *Actinobacteria* (Alonso-Gutiérrez et al. 2011; Shen et al. 2010), and *Proteobacteria* (Tesar et al. 2002; Wang et al. 2010), were first detected in *Firmicutes* strains. It is notable that although *Planococcus* spp. CW-123 and CY-b41 as well as *Pseudomonas* spp. CW-122 and X-b2 could use *n*-hexadecane as the sole carbon and energy source for vigorous growth, none of the *alkB*/*alkM*, *CYP153A* and *almA* genes could be detected by using PCR method.

It is interesting that so many *Rhizobium* strains were isolated from the microtherm oil-rich environment, which is in consistent with the results analyzed by clone library analysis of the 16S rRNA genes (Zhao et al. 2012). *Rhizobium* strains are usually isolated from soil or aquatic environments, especially in the plant rhizosphere (Yoon et al. 2010), although a few *Rhizobium* strains were recently obtained from a bioreactor (Hunter et al. 2007; Quan et al. 2005; Wen et al. 2011). Recently, some *Rhizobium* strains were reported to be capable of utilizing aromatic compounds such as phenanthrene (Wen et al. 2011; Zhang et al. 2012). In the present study, most of the *Rhizobium* strains isolated from Cai medium could efficiently degrade *n*-hexadecane and harbored a special alkane monooxygenase gene.

These phenomena may be ascribed to the horizontal transfer of the alkane monooxygenase genes between different bacteria, as argued by Nie et al. (2013a). However, further investigation is needed to explain the phenomena as well as to understand the behaviors of bacterial strains under mesophilic conditions.

As artificial environmental selecting pressure, each medium led to the isolation of different and unique bacterial strains. For example, seven groups of bacteria were isolated only from X medium, 6 groups from CY medium, three groups from CW medium, and one group from Cai medium (Table 2). In addition, crude oil constituents from oil production could enhance the ability of Cai, CW, and CY media to grow more bacterial strains that are able to degrade petroleum hydrocarbons such as *n*-hexadecane and phenanthrene. In contrast, the yeast extract in CY and X media resulted in the isolation of phenol-degrading strains. A similar pattern was also found in the distribution of the *alkB/alkM* genes. Except for those from the *Dietzia* and *Rhizobium* strains (isolated from four media simultaneously), *alkB* genes clustered with the media: Cai-strains harbored Cluster III (*alkB*) nonheme diiron alkane monooxygenase and CY-strains harbored Cluster II (*alkM*) nonheme diiron alkane monooxygenase, regardless of the phylogenetic differences among the host bacterial strains. Further investigation is needed to address whether the medium selection of the isolates with specific functions is a universal phenomenon.

Although four kinds of media were used, they were obviously not enough to isolate all the bacteria in the production water. As revealed by the clone library analyses, some bacterial lineages could not be isolated by the four media, including bacteria belonging to *Deltaproteobacteria* and *Spirochaetes* (Zhao et al. 2012). In contrast, *Betaproteobacteria* and *Actinobacteria* which were isolated by cultivation were not detected by the clone library analyses. In addition, the *Clostridia* relatives were only detected by the culture-independent method, while *Bacilli* and *Erysipelotrichi* strains were isolated (Fig. 1). The common predominant bacteria detected with both methods were *Pseudomonas*.

In summary, 435 pure bacterial strains were isolated from microtherm oil-production water by using four different media, which were affiliated with 61 phylogenetic groups belonging to phyla of *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Only *Rhizobium*, *Dietzia,* and *Pseudomonas* strains were commonly isolated from the 4 media. Different medium selected bacterial strains with different *n*-hexadecane degradation abilities with *alkB*/*alkM* genes being clustered according to the media. In addition, the alkane-degrading abilities and *alkB*/*alkM* genes were detected in *Rhizobium*, *Rhodobacter*, *Trichococcus*, *Micrococcus*, *Enterococcus*, and *Bavariicoccus* strains.

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