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Diversity and abundance of *n*-alkane degrading bacteria in the near surface soils of a Chinese onshore oil and gas field

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Abstract

Alkane degrading bacteria have long been used as an important biological indicator for oil and gas prospecting, but their ecological characteristics in hydrocarbon microseep habitats are still poorly understood. In this study, the diversity and abundance of *n*-alkane degrading bacterial community in the near surface soils of a Chinese onshore oil and gas field were investigated using molecular techniques. Terminal restriction fragment length polymorphism (T-RFLP) analyses in combination with cloning and sequencing of *alkB* genes revealed that trace amount of volatile hydrocarbons migrated from oil and gas reservoirs caused a shift of the *n*-alkane degrading bacterial community from Gram-positive bacteria (*Mycobacterium* and *Rhodococcus*) to Gram-negative genotypes (*Alcanivorax* and *Acinetobacter*). Real-time PCR results furthermore showed that the abundance of *alkB* genes increased substantially in the surface soils underlying oil and gas reservoirs even though only low or undetectable concentrations of hydrocarbons were measured in these soils due to efficient microbial degradation. Our findings broadened the knowledge on the ecological characteristics of alkane degrading community in hydrocarbon microseeps and may provide a new approach for microbial prospecting for oil and gas (MPOG).

1 Introduction

Hydrocarbon microseepage is a widely distributed natural phenomenon in the geochemical carbon cycle (Etiope and Ciccioli, 2009). Driven by reservoir pressure, some volatile components from oil and gas reservoirs can vertically penetrate the cover above and rise to the surface of the earth. These gaseous and volatile hydrocarbons will affect the distribution and growth of the microbial community in the near surface soil (Klusman and Saeed, 1996). The technology of microbial prospecting for oil and gas (MPOG) is based on this theory to forecast the existence of oil and gas deposits. In recent years, the microbial prospecting method can be integrated with geological and

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geophysical methods to evaluate the hydrocarbon prospect of an area and to prioritize the drilling locations, thereby reducing drilling risks and achieving higher success in petroleum exploration (Rasheed et al., 2012; Wagner et al., 2002).

In general, the indicator bacteria for MPOG can be classified into two major groups: methane oxidizing bacteria (methanotrophs) and C_{2+} alkane degrading bacteria In the last few years, the abundance, distribution and community composition of methane oxidizing bacteria have been relatively well studied in various methane seeps (Deutzmann et al., 2011; Håvelsrud et al., 2011; Kadnikov et al., 2012). Despite methane is by far the most abundant hydrocarbon gas associated with petroleum in the reservoir, bacterial oxidation of C_{2+} alkanes is of infinitely more value in petroleum prospecting than methane oxidation (Muyzer and van der Kraan, 2008). Since methane is also a common product of the anaerobic digestion of organic matter, it is obvious that the presence of methanotrophs in a soil sample is less likely to be indicative of leakage from a subsurface reservoir than the presence of the more specific C_{2+} alkane-degrading bacteria (Shennan, 2006).

Therefore, recently, the ecological characteristics of alkane degrading bacteria at various hydrocarbon macroseeps (active seeps with large concentrations of migrated hydrocarbons) have been surveyed, such as marine hydrocarbon seeps (Redmond et al., 2010; Wasmund et al., 2009) and oil spill zones (Valentine et al., 2010; Wang et al., 2011). These studies revealed that the presence of high concentrations of hydrocarbons significantly affect the biogeographical distribution and phylogenetic diversity of alkane degrading bacteria in the near surface waters and sediments. Nevertheless, the knowledge on the ecological characteristics of alkane degrading bacteria at hydrocarbon microseeps (passive seeps with low concentrations of migrated hydrocarbons) is still lacking to date. After long-term acclimation to trace and continuous hydrocarbons supply, are there any differences in alkane degrading community between the oil and gas field and the surrounding soils? It is a critical issue for the success of MPOG.

Recent advances in microbial molecular biology have significantly improved our knowledge of the genes and enzymes associated with alkane metabolism as well as

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the microbiology of C_{2+} alkane degraders. Alkane hydroxylase (*alkB*) is one of the key enzymes of the degradation of aliphatic alkanes under aerobic conditions (Beilen and Funhoff, 2007). This enzyme is most important and prevalent in aerobic oil degrading bacteria (Rojo, 2009; Smits et al., 1999) In this study, by using *alkB* gene as the *n*-alkane degrading biomarker, we chose a Chinese onshore oil and gas field as a hydrocarbon microseep model system in order to: (1) investigate the *n*-alkane degrading community composition in oil and gas field soils and compare it to the compositions of the communities in the surrounding soils by a combination of clone library and terminal restriction fragment length polymorphism (T-RFLP) analysis; (2) evaluate the effect of trace amount of hydrocarbons migrated from oil and gas reservoirs on the abundance of *n*-alkane degraders using quantitative real time PCR.

2 Materials and methods

2.1 Study site and sampling

Soil samples were collected from the Shaozhuang Oil and Gas Field (37° 7′ N, 118° 14′ E) within the Shengli Area, Shandong, China, in June 2011. The soil is classified based on the FAO (Food and Agriculture Organization of the United Nations) system as a stagno-fiuvic gleysol on loamy-sandy sediments over gley. The pH of surface soil (0–200 cm) in water (1 : 2) is 7.6 ~ 9.0. The underground gas and oil reservoir, covering 3.7 km^2, is 1100 ~ 1270 m beneath the earth's surface. The oil and gas bearing bed is approximately 15 m thick. Soils were collected aseptically in pre-sterilized whirl pack bags under aseptic conditions from a depth of 60 cm. The detailed sample collection scheme is shown on the geological map (Fig. 1). Soil pH, water content, electrical conductivity, total headspace n-alkanes (C_{2+}) and total adsorbed n-alkanes (C_{2+}) are shown in Table 1. All of these chemical properties were determined as previously described (Schumacher, 1996; Xu et al., 2010).

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2.2 DNA extraction

DNAs were extracted in duplicate from the soil samples using a modified procedure (Xu et al., 2010) and FastDNA Spin kit for soil (MP Biomedicals) according to the manufacturer's instructions. The products of duplicate extractions were pooled and used for further PCR amplification.

2.3 Cloning, sequencing and phylogenetic analysis

Three clone libraries of partial *alkB* genes were constructed to provide a better resolution for differentiating the individual T-RFs as phylogenetic lineages. Partial *alkB* genes were amplified using the forward primer alkBf (5′-AAYACNGCNCAYGARCTNGGNCAYAA-3′) and the reverse primer alkBr (5′-GCRTGRTGRTCNGARTGNCGYTG-3′) (Kloos et al., 2006). This primer set yields a PCR product of approximately 550 bp. A Mastercycler nexus PCR cycler (Eppendorf) with a TaKaRa PCR Kit was used for the DNA amplification. The reaction mixture contained, in a total volume of 50 µl, 5 × PCR buffers, 4.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM each primer, and 2 U of Ex-Taq DNA polymerase, and 1 µl (10–20 ng) of the genomic DNA was added as the template. The PCR program consists of an initial 5 min denaturation step at 95 °C, 30 cycles of repeated denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s, and extension at 72 °C for 1 min, followed by final extension step of 10 min at 72 °C.

Amplicons were gel-purified using Gel Extraction Kit (TaKaRa) according to the manufacturer's instructions. Ligation into pGEM-T Easy vector (Promega) and transformation into *Escherichia coli* JM109 were performed according to the manufacturer's instructions. Clones were selected randomly and sequenced by ABI 377 DNA sequencer (Applied Biosystems). The RDP chimera check program was used to screen for potential chimeras. Sequences are available under the GenBank accession numbers: JX276475-JX276506. Nucleotides sequences from PCR products and reference strains were analyzed using the MEGA software (Tamura et al., 2011). The

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neighbor-joining method, with complete deletion of gaps and missing data and poisson correction for multiple substitutions, was used to calculate the distances and to construct phylogenic trees.

2.4 Terminal restriction fragment length polymorphism analysis

For terminal restriction fragment (T-RF) profiles, partial *alkB* genes were amplified with 5′ fluorescently labeled forward primer (alkBf-labeled with 6-carboxyfluorescein) and a reverse primer (alkBr). Two independent 25 µl PCRs were performed for each sample and the products were combined and purified with Gel Extraction Kit (TaKaRa). Aliquots of the purified amplicons were then digested with 10 U of *Msp* I (TaKaRa) for 3 h at 37 °C. Each 200 µl tube contained 16 µl of amplicons, 2 µl of the incubation buffer, and 2 µl of restriction enzyme made up to total volume of 20 µl. The digested amplicons were mixed with GeneScan 500 ROX size standards (Applied Biosystems) and analyzed by capillary electrophoresis with GeneScan software (Applied Biosystems). Signals with a peak area that was less than 1000 relative fluorescence units were regarded as background noise and excluded from the analysis. The relative abundance of a detected terminal restriction fragment (T-RF) within a given T-RFLP pattern was calculated as the respective signal height of the peak divided by the peak height of all peaks of the T-RFLP pattern. The size of each *n*-alkane degrading species T-RF peak corresponded to the value for that species determined by in silico analysis of clone library with DNASTAR software. Both the presence/absence and relative abundance of T-RFs were considered in data analysis.

2.5 Ordination analyses of T-RFLP fingerprints

The ordination analyses of T-RFLP profiles were performed using CANOCO 4.5 software (Microcomputer Power) as previously described (Rui et al., 2009). The detrended correspondence analysis (DCA) was firstly run to estimate the gradient length of variables. It was found that the longest gradient was shorter than 3.0. Thus, the principal

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component analysis (PCA) was chosen for analysis, because it performed better than the unimodal approaches under such conditions according to the CANOCO manual (Ter Braak and Smilauer, 1998). The settings of CANOCO 4.5 are as follows: inter-sample distance scaling, no post-transformation of scores, log data transformation (no offset), and center by species.

2.6 Quantitative real-time PCR

Copy numbers of partial *alkB* genes of *n*-alkane degrading bacteria were quantified with the real-time PCR analysis (primer set alkBf-alkBr). The real-time PCR assay was carried out in the CFX96 Real-Time PCR System (BioRad) with SYBR *Premix Ex Taq* Perfect Real Time (TaKaRa) according to the manufacturer's instructions. A three-step PCR protocol was used, with an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and 5 s at 80 °C for denature, annealing, elongation, and data acquisition, respectively. Melting curves were obtained at 60 to 96 °C at a 0.5 % heating rate. The Genbank accession number of qPCR DNA standard was JN106044. To evaluate the abundance of *n*-alkane degrading bacteria relative to total bacteria, the percentages of *alkB* genes in proportion to 16S rRNA were also calculated as previously described (Xu et al., 2009).

2.7 Data analysis

Statistical analysis of data was performed by ANOVA, with differences determined by the method of least significant differences at the 5 % level ($P < 0.05$). All statistical analyses were run with STATISTICA 6.0 (StatSoft).

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3 Results and discussion

3.1 Phylogenetic diversity of *n*-alkane degrading bacteria

In order to assess the diversity of *alkB* at the phylogenetic level, three clone libraries representative of samples from oilfield, gasfield and the reference site were constructed (sampling sites see Fig. 1). Approximately 83–95 % of the more abundant *n*-alkane degrading phylotypes in the soil libraries were identified from S_{Chao1} and S_{ACE} richness estimators (Fig. S1) Phylogenetic analyses of 136 clones revealed that the obtained *alkB* genes in this study exhibited 69–99 % similarity at the amino acid level with sequences retrieved from the GenBank database (Table S1). More than half of clones showed highest affinity to either *Acinetobacter* strains or the Gamma-proteobacterial marine hydrocarbon-degrading bacterium, such as *Alcanivorax dieselolei* and *Marinobacter aquaeolei* VT8. Interestingly, only a small part of clones were grouped in a large cluster that were most closely related to Gram-positive actinobacterial *Rhodococcus*, *Mycobacterium*, and *Nocardia*, which are well-known for their ability to degrade short-chain and gaseous alkanes (Pérez-de-Mora et al., 2011; Shennan, 2006). A group of three OTUs from the study zone formed a separate cluster (cluster A; Fig. 2) with very little sequence identity to *alkB* sequences from known organisms (only 71 % with *Burkholderia cepacia*) and other unknown uncultured bacteria (79 % identity with uncultured bacterium DQ288068).

3.2 *n*-alkane degrading community structure above oil and gas reservoirs

In this study, we used T-RFLP fingerprinting based on *alkB* genes to characterize the *n*-alkane degrading community structure above oil and gas reservoirs. Only those with an Ap (percentage abundance) higher than 1 % in at least one profile were selected as the signature T-RFs for further analyses. This cutoff was adopted from a previous study in order to minimize the amount of data noise (Noll et al., 2005) Seven fragments (33 bp, 70 bp, 74 bp, 120 bp, 133 bp, 142 bp and 340 bp) were detected as

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major peaks in the T-RFLP profiles (Fig. 2). A combination of in silico sequence analyses and T-RFLP fingerprinting of the representative clones showed that five terminal restriction fragments (T-RFs) could be assigned to a single lineage, i.e. 33 bp to *Salinisphaera*, 70 bp to *Alcanivorax*, 120 bp to uncultured *Actinobacteria*, 142 and 340 bp to unclassified *n*-alkane degrading bacteria, whereas two T-RFs (74 bp and 133 bp) were associated with more than one lineage. The 74-bp T-RF represented mostly Gamma-proteobacterial *Acinetobacter* and *Marinobacter* but occasionally also uncultured bacteria in hydrocarbon seep (OTU-17). The 133-bp T-RF was characteristic of actinobacterial *Mycobacterium* and *Rhodococcus* and a unclassified *n*-alkane degrading bacterium (OTU-2)

As shown in Fig. 3, T-RFLP profiles revealed that long-term and continuous hydrocarbons supply considerably influenced the structure of *n*-alkane degrading community. The most notable differences were the relative abundances rather than the fragments lengths. In the background soil, the 133 bp T-RF showed a high abundance (up to $32 \sim 45$ %), indicating that the actinobacterial *Mycobacterium* and *Rhodococcus* was a predominant group on *alkB* gene level. By contrast, in the oil and gas field soils, the *Actinobacteria* became less frequent, and the niche vacated by this dominant group was gradually filled with proteobacterial *Alcanivorax* and *Acinetobacter*. The replacement of *Proteobacteria* was more pronounced in oilfield soil as compared with gasfield soil (Fig. 3).

Principal component analyses (PCA) furthermore showed that the samples from oilfield and gasfield were clearly separated from those from reference site, although the difference between oilfield and gasfield was indiscernible (Fig. 4). Peaks 133 (putative actinobacterial *Mycobacterium* and *Rhodococcus*) had large loadings in the direction in which non-oil and gas reference sample cluster was localized. On the contrary, in oil and gas cluster, peaks 70 (putative *Alcanivorax*), 74 (putative γ-proteobacterial *Acinetobacter* and *Marinobacter*) seemed to be positively correlated with the trace and continuous hydrocarbons.

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How can the trace amount of hydrocarbons migrated from oil and gas reservoirs cause a shift of the *n*-alkane degrading bacterial community from Gram-positive bacteria to Gram-negative genotypes? As mentioned above, Gram-positive bacteria possess the ability to utilize short-chain and gaseous alkanes (C_1–C_5) in contrast to other hydrocarbon-degrading bacteria (Pérez-de-Mora et al., 2011; Shennan, 2006). However, dominance of *Actinobacteria* has also been previously reported in non-hydrocarbon-affected soils (Luz et al., 2004; Margesin et al., 2003), possibly owing to their metabolic versatility which includes n-alkanes and a wide range of organic compounds (Rojo, 2009). That's why Gram-positive *Actinobacteria* can be detected in both oil and gas field and background zones in the present study (Fig. 3). On the contrary, medium- and long-chain hydrocarbons (C_{13+}) have long been considered to be difficult to penetrate the caprock of oil deposits and rise to the surface of the earth, thereby being ignoring in most surface geochemical surveys. Recently, however, Gore corporation has successfully detected thermogenic hydrocarbon compounds from C_2 to C_{20} in the near surface soils of oil and gas reservoirs with a highly sensitive passive diffusion module (Anderson, 2006), indicating that volatile heavy hydrocarbons are able to migrate to the surface in a geologic time scale. Interestingly, Gram-negative *Acinetobacter* and *Marinobacter* are well-known for their ability to degrade medium- and long-chain n-alkanes ranging from C_{13} to C_{30} (Doumenq et al., 2001; Sakai et al., 1994). The predominance of Gram-negative bacteria over Gram-positive bacteria was also observed in other alkane-rich habitats, such as marine hydrocarbon macroseeps (Wasmund et al., 2009) and heavy hydrocarbon contaminated soils (Kaplan and Kitts, 2004). Therefore, Gram-negative *n*-alkane degraders seemed to be a good indicator population for MPOG. Next step, mRNA-based pyrosequencing experiments will be carried out to verify this conclusion.

3.3 Quantitative analysis of *n*-alkane degrading bacteria

Quantitative real-time PCR was used to estimate the abundance of *n*-alkane degrading bacteria at the different locations. In order to minimize environmental interference,

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such as noise caused by variations in soil type, we normalizes the abundances of *alkB* genes against the total abundance of 16S rRNA genes, sample-by-sample. The biogeographical distribution of the relative abundances of *n*-alkane degrading bacteria in soil samples collected from east-west direction survey line was shown in Fig. 5. In the oil and gas field soil samples, the relative abundances of *alkB* genes were significantly higher than in all other soil samples (4.7 % ± 0.3 % and 7.4 % ± 1.1 %, respectively). In the reference samples, the ratios were 2.2 to 3.4-fold lower, in the range from 0.7 % to 4.6 %. The high value area was basically consistent with the areas of the oil and gas accumulation (Fig. 5).

However, to our surprise, hydrocarbon concentrations in soils above oil and gas fields were relatively low or even undetectable (Table 1). We speculate that efficient biodegradation of hydrocarbons was occurring and that this degradation removed detectable quantities of hydrocarbons before they can be measured by geochemical analyses. Similar effect has been previously reported in the study of marine hydrocarbon macroseeps (visible seeps) (Wasmund et al., 2009). Therefore, the quantification of *alkB* gene copy numbers in soils provides an insight into the microbial response to the microseepage of hydrocarbons and acts as a useful complementary tool for understanding this habitats response to trace hydrocarbons in addition to geochemical measurements.

In the preliminary study, we tried to isolate and enumerate *n*-alkane degrading bacteria using standard plate count method (data not shown). However, quantitative distribution of culturable species was confused and cannot be used to forecast oil and gas reservoirs. This phenomenon might be caused by: (i) the existence of a large quantity of uncultured alkane degraders in soils (Kloos et al., 2006) such as the 142 and 340 bp T-RFs in our case (Fig. 2) and (ii) the contradiction between short-term culture (days) in laboratory under high-concentration alkanes and long-term acclimation (years) to trace alkanes in natural environments. Therefore, culture-independent approaches using *alkB* gene to detect the presence and abundance of *n*-alkane degrading bacteria

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taken directly from soil samples without cultivation can significantly improve the accuracy rate of MPOG.

4 Conclusions

In this study, the diversity and abundance of *n*-alkane degrading bacterial community in the near surface soils of a onshore oil and gas field were investigated using molecular techniques. The determination of *alkB* gene-based T-RFLP profiles and subsequent affiliation to clone sequences and PCA ordination showed that trace amount of hydrocarbons migrated from oil and gas fields influenced not only the quantity but also the structure of *n*-alkane degrading bacterial community. The predominance of Gram-negative *Proteobacteria* (*Alcanivorax* and *Acinetobacter*) over Gram-positive *Actinobacteria* (*Mycobacterium* and *Rhodococcus*) was observed in oil and gas field soils. Real-time PCR results furthermore showed that the abundance of *alkB* genes increased substantially in the surface soils underlying oil and gas reservoirs even though only low concentrations of hydrocarbons were measured in these soils due to efficient biodegradation. Our findings broadened the knowledge about the ecological characteristics of alkane degrading community in hydrocarbon microseeps and may provide a new approach for MPOG. Next step, RNA-based stable isotope probing experiments will be carried out to link microbial community *n*-alkane metabolism with the phylogenetic identities of key organisms.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/9/14867/2012/bgd-9-14867-2012-supplement.pdf.

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Table 1. General soil properties, headspace and adsorbed n-alkanes content of research area.

Sample code	Geographical description	Water content (%)	pH value	Electrical conductivity ($\mu S\,cm^{-1}$)	Total headspace n-alkanes (C_{2+}) ($\mu l\,l^{-1}$)	Total adsorbed n-alkanes (C_{2+}) ($\mu l\,l^{-1}$)
B1	Background	52	8.83	909	0.74	2.48
B3	Background	59	8.99	1139	0.74	3.79
B5	Background	57	9.00	672	0.79	2.35
B7	Background	54	9.07	539	0.73	0.75
B10	Background	45	8.83	892	0.20	1.40
B14	Oil field zone	60	8.09	644	0.21	1.08
B16	Oil field zone	28	8.69	355	0.22	1.35
B17	Oil field zone	43	8.93	629	0.47	1.09
B18	Oil field zone	56	9.36	554	0.00	1.22
B20	Oil field zone	56	8.58	238	0.00	1.74
B21	Oil field zone	57	8.58	580	0.73	0.89
B30	Gas field zone	57	8.50	530	0.64	1.44
B31	Gas field zone	54	9.09	380	0.38	1.08
B32	Gas field zone	53	8.55	644	0.20	0.85
B34	Gas field zone	53	8.61	923	0.35	1.14
B35	Gas field zone	56	8.38	1128	0.36	0.83
B37	Background	56	8.19	839	0.77	1.75
B39	Background	53	8.11	763	0.77	2.40
B41	Background	52	8.67	861	0.70	1.41

BGD
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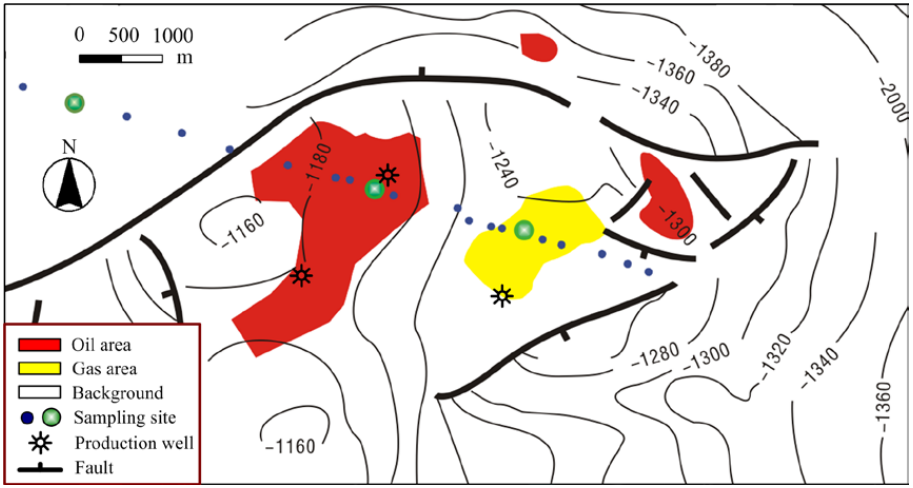

Fig. 1. Geological base of Shaozhuang oil and gas field and sampling scheme for spatial analysis of *n*-alkane degrading bacterial community. Sampling positions for clone library analyses of alkane hydroxylase (*alkB*) gene diversity at three different sites are marked with green circles. Small blue circles indicate the soil samples for T-RFLP and qPCR analysis.

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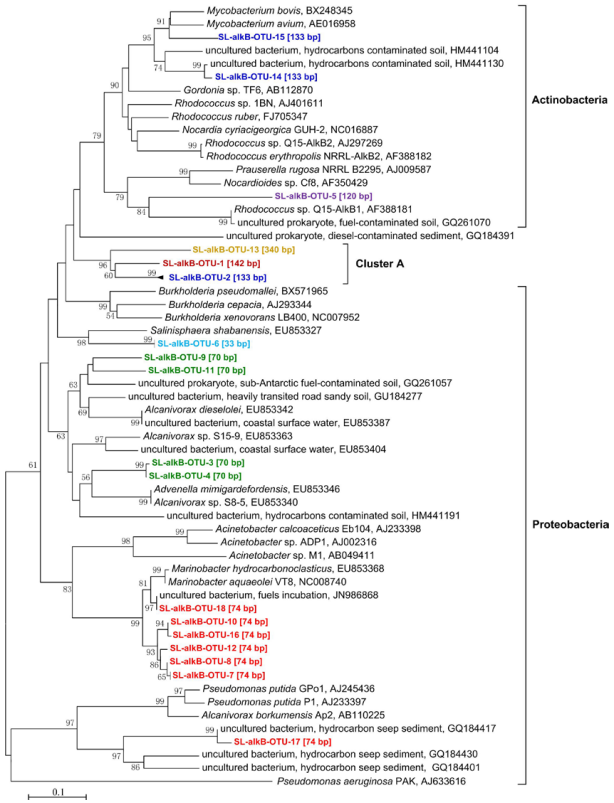

Fig. 2. Phylogenetic relationship of deduced *alkB* sequences (182 amino acids) generated from different soil samples. The tree was constructed using the neighbor-joining method in MEGA software. The scale bar represents 10 % sequence divergence; values at the nodes are the percentages of 1000 bootstrap replicates supporting the branching order; bootstrap values below 50 % are not shown. Representative clones obtained in this study are shown in different colors which represent different *n*-alkane degrading groups, and the in silico T-RF size is given in square brackets. Reference sequences are shown in black.

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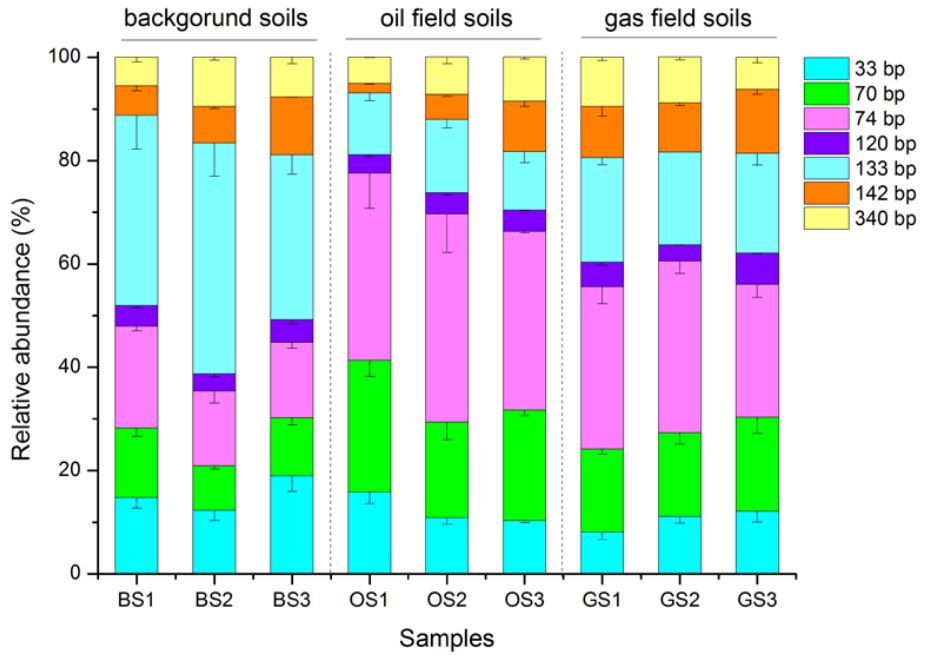

Fig. 3. Comparison of *alkB* gene based T-RFLP profiles obtained from different soil samples. Values are mean ±S.D. (n = 3). *Msp* I was used as restriction enzyme. OS = oilfield surface soil; GS = gasfield surface soil, BS = background soil (numbers 1–3 indicate field replicates from the corresponding sites).

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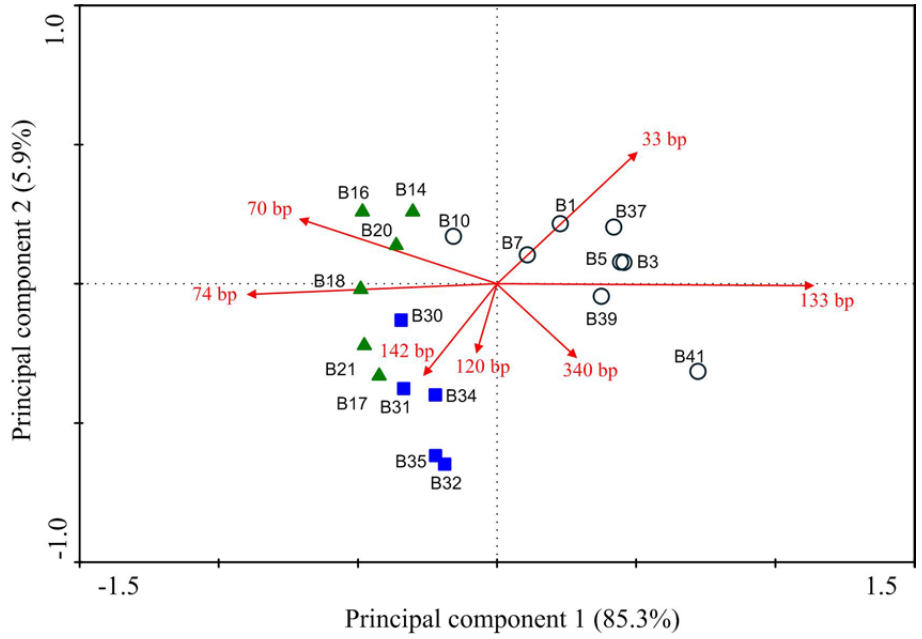

Fig. 4. PCA ordination plot for the samples and T-RFs based on *alkB* T-RFLP data from different soil samples. Symbols: red arrows = T-RFs (the size in base pairs of forward T-RFs by *Msp* I); green triangles, blue squares, black circles and associated numbers indicate oilfield surface soils, gasfield surface soils, background soils and sample serial numbers, respectively.

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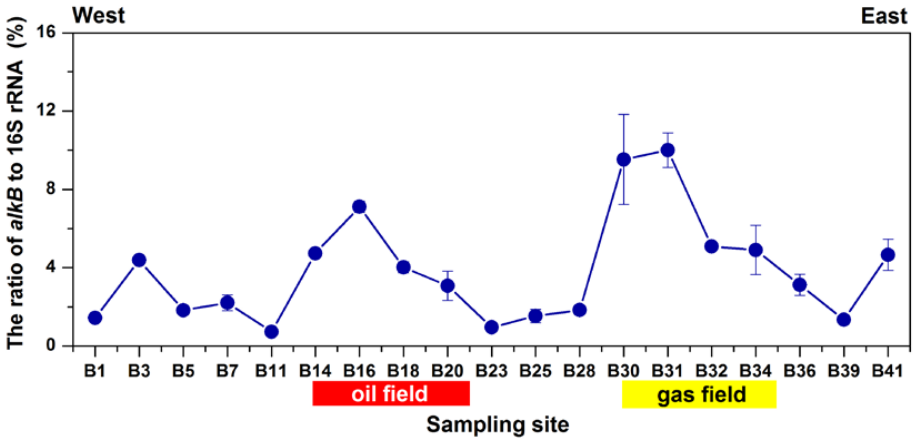

Fig. 5. Biogeographical distribution of the percentages of *alkB* gene copy numbers (representing the number of *n*-alkane degrading bacteria) in proportion to 16S rRNA gene copy numbers (representing the number of total *Eubacteria*) of soil samples collected from east-west direction survey line of Shaozhuang oil and gas field (n = 3).

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