



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

K. Xu^{1,2}, Y. Tang², C. Ren², K. Zhao², and Y. Sun¹

¹Department of Earth Science, Zhejiang University, Hangzhou 310027, China

²Wuxi Research Institute of Petroleum Geology, Research Institute of Petroleum Exploration & Production, SINOPEC, Wuxi, Jiangsu 214151, China

Correspondence to: K. Xu (xukewei8201@gmail.com) and Y. Sun (ygsun@zju.edu.cn)

Received: 9 September 2012 – Published in Biogeosciences Discuss.: 29 October 2012

Revised: 5 February 2013 – Accepted: 11 March 2013 – Published: 25 March 2013

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 Gram-negative genotypes (*Alcanivorax* and *Acinetobacter*) dominated *n*-alkane-degrading bacterial communities in the near-surface soils of oil and gas reservoirs, while the dominant microbial communities were Gram-positive bacteria (*Mycobacterium* and *Rhodococcus*) in background soil. Real-time quantitative polymerase chain reaction (PCR) results furthermore showed that the abundance of *alkB* genes increased substantially in the surface soils above oil and gas reservoirs even though only low or undetectable concentrations of hydrocarbons were measured in these soils. The results of this study implicate that trace amounts of volatile hydrocarbons migrate from oil and gas reservoirs, and likely result in the changes of microbial communities in the near-surface soil.

etrate 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 was integrated with geological and 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 C₂₊ 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 that methane is by far the most abundant hydrocarbon gas associated with petroleum in the reservoir, bacterial oxidation of C₂₊ 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 C₂₊ alkane-degrading bacteria (Shennan, 2006).

Therefore, recently, the ecological characteristics of alkane-degrading bacteria at various hydrocarbon

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 pen-

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 affects 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. It remains to be shown whether there is any difference in alkane-degrading community between the oil and gas field and the background soils, which 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 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 (van Beilen and Funhoff, 2007). This enzyme is highly relevant and representative 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 investigate the *n*-alkane-degrading community composition in oil and gas field soils and compare it to the compositions of the communities in the background soils by a combination of clone library and terminal restriction fragment length polymorphism (T-RFLP) analysis. The abundance of *n*-alkane degraders was determined by using quantitative real-time polymerase chain reaction (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-fluvic Gleysol on loamy-sandy sediments over gley. The pH of surface soil (0–200 cm) in water (1 : 2) is in the range of ~8.09–9.36. The underground gas and oil reservoir, covering 3.7 km², is ~1100–1270 m beneath the earth's surface. The oil- and gas-bearing bed is approximately 15 m thick. Soils were collected 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

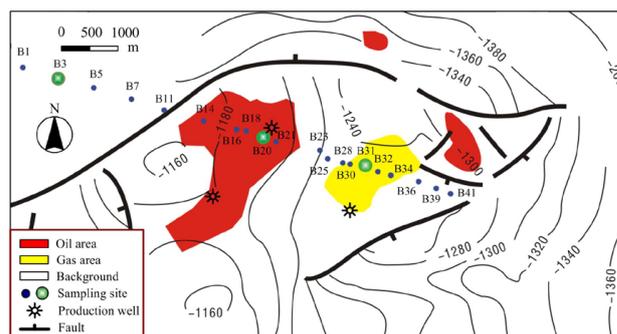


Fig. 1. Geological base of Shaozhuang oil and gas field and sampling scheme for spatial analysis of *n*-alkane-degrading bacterial community. Sampling positions for clone library analyses of alkane hydroxylase (*alkB*) gene diversity at three different sites are marked with green circles. Small blue circles indicate the soil samples for T-RFLP and qPCR analysis. The thinner black lines and related negative 4-digit numbers are contour lines. Soils were collected at a depth of 60 cm.

properties were determined as previously described (Schumacher, 1996; Xu et al., 2010).

2.2 DNA extraction

DNA was 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 cyclor (Eppendorf) with a TaKaRa PCR kit was used for the DNA amplification. The reaction mixture contained, in a total volume of 50 μ L, 5 \times PCR buffers, 4.0 mM MgCl₂, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.5 mM each primer, and 2 U of *Ex-Taq* DNA polymerase, and 1 μ L (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 $^{\circ}$ C, 30 cycles of repeated denaturation at 94 $^{\circ}$ C for 45 s, annealing at 53 $^{\circ}$ C for 45 s, and extension at 72 $^{\circ}$ C for 1 min, followed by final extension step of 10 min at 72 $^{\circ}$ C.

Amplicons were gel-purified using a 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). *AlkB* sequences were aligned manually with *alkB* sequences obtained from the GenBank database and checked for chimeras by bisecting and drawing two sub-phylogenetic trees from the bisects of each sequence. The sequences that showed different topologies among the two sub-trees were regarded as chimeric and removed from the libraries. Sequences are available under the GenBank accession numbers JX276475–JX276506. Nucleotide sequences from PCR products and reference strains were analyzed using the MEGA software (Tamura et al., 2011). The 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 phylogenetic 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 μ L 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 in triplicate with 10 U of *Msp* I (TaKaRa) for 3 h at 37 °C. Each 200 μ L tube contained 16 μ L of amplicons, 2 μ L of the incubation buffer, and 2 μ L of restriction enzyme made up to a total volume of 20 μ L. The digested amplicons were mixed with GeneScan 500 ROX size standards (Applied Biosystems) and analyzed by capillary electrophoresis. After electrophoresis, the sizes of the 5'-terminal restriction fragments (T-RF) and the intensities of their fluorescence emission signals were automatically calculated by the GeneScan analysis 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 EditSeq software (by searching for the first restriction enzyme site "C/CGG" of *Msp* I) of the DNASTAR package. All predicted and observed T-RF matches were within 2 bp from each other. 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 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 Real-time quantitative PCR

Copy numbers of partial *alkB* genes of *n*-alkane-degrading bacteria were quantified with the real-time quantitative PCR analysis (primer set *alkBf*–*alkBr*). Real-time PCR of tenfold dilutions of extracted DNA was carried out in the CFX96 real-time PCR system (Bio-Rad) with SYBR Premix *Ex Taq* Perfect Real Time Kit (TaKaRa) in triplicate 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 real-time PCR standard was generated using plasmid DNA from one representative clone containing *alkB* gene (GenBank accession number: JN106044), and a dilution series of standard template over seven orders of magnitude was used to optimize the real-time PCR conditions.

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. Bacterial 16S rRNA genes were quantified as previously described (Xu et al., 2009) with slight modification by using primer set 515f (5'-GTGCCAGCMGCCGCGG-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3'). The standard curves of 16S rRNA genes were made in a similar manner to *alkB* genes as described above. Real-time quantitative PCR was performed in triplicate with 25 μ L reaction mixture containing 12.5 μ L SYBR Premix *Ex Taq*, 0.5 μ M of each primer, 11 μ L of H₂O and 1.0 μ L of DNA template.

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).

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 oil field, gas field and the reference site were constructed (for 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. More than half of clones showed highest affinity to either *Acinetobacter* strains or γ -proteobacterial marine hydrocarbon-degrading bacteria, 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 (operational taxonomic units) 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 were detected as 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*, 12 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 γ -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 hydrocarbon supply considerably influenced the structure of *n*-alkane-degrading community. The most notable differences were the relative abundances rather than the fragment lengths. In the background soil, the 133 bp T-RF showed a high abundance (up to ~32–45 %), indicating that the actinobacterial *Mycobacterium* and *Rhodococcus* were 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 oil field soil as compared with gas field soil (Fig. 3). Comparing the T-RFLP results to *alkB* gene copy numbers determined by qPCR (Fig. S2), it also indicated that *Proteobacteria* are becoming more frequent in the oil and gas field (Table S1).

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

We speculate that the trace amounts of hydrocarbon 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 hydrocarbon-unaffecting 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 is 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 cap rock of oil deposits and rise to the surface of the earth, thereby being ignored in most surface geochemical surveys. Recently, however, W. L. Gore & Associates, Inc. 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 on 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}

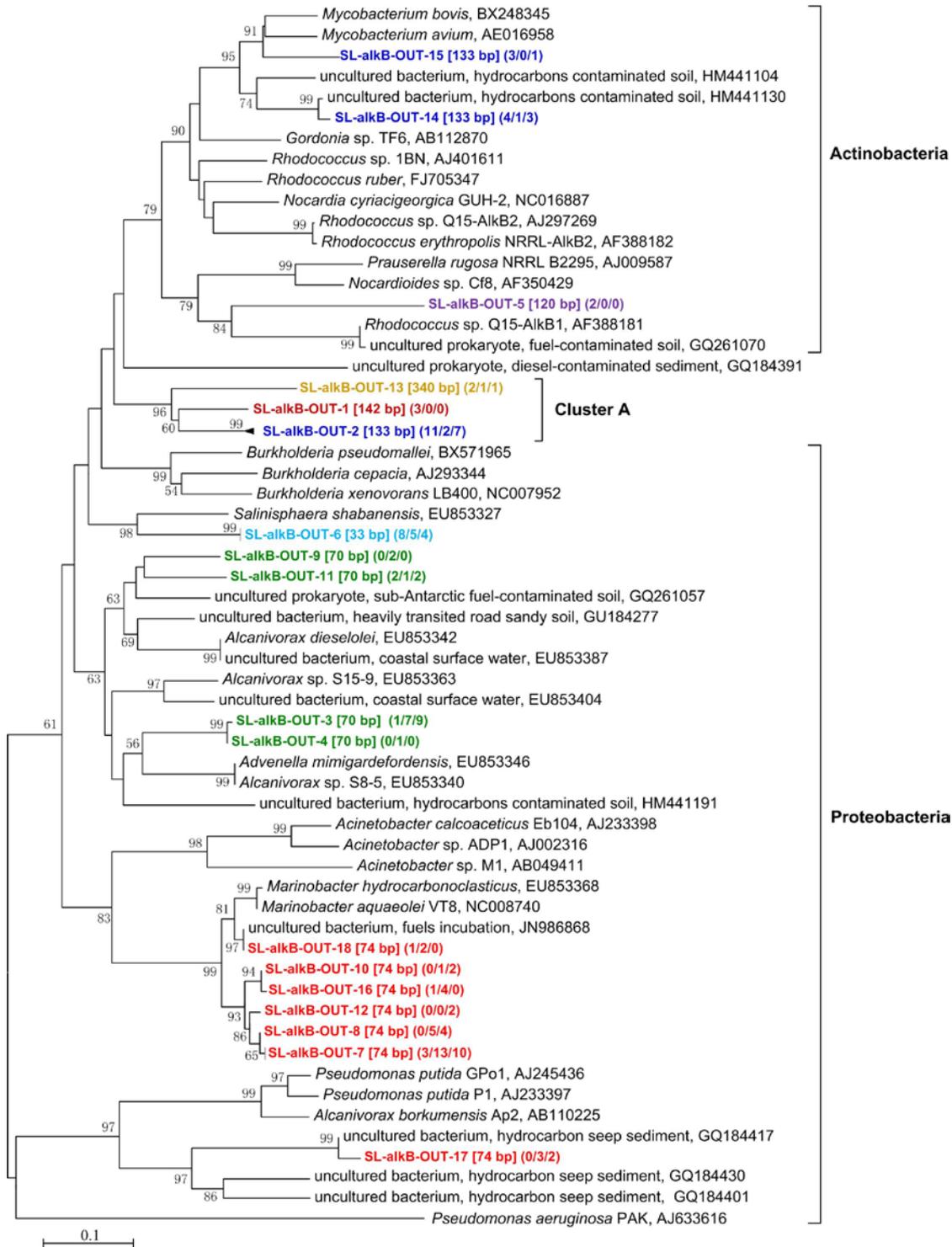


Fig. 2. Phylogenetic relationship of deduced *alkB* sequences (182 amino acids) generated from different soil samples. The scale bar represents 10 % sequence divergence; values at the nodes are the percentages of 1000 bootstrap replicates supporting the branching order; bootstrap values below 50 % are 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. A similarity cutoff of 97 % is used to define an OTU (operational taxonomic unit). The number of clones for each representative OTU is given in parentheses (reference soil/oil field surface soil/gas field surface soil). Reference sequences are shown in black.

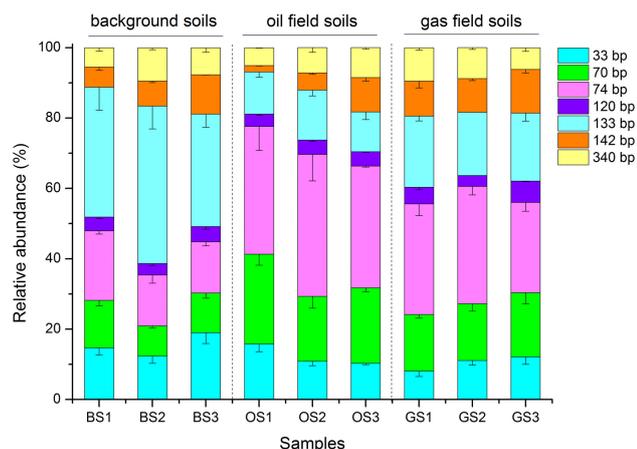


Fig. 3. Comparison of *alkB*-based T-RFLP profiles obtained from different soil samples. Values are mean \pm S.D. ($n = 3$). *Msp* I was used as restriction enzyme. OS = oil field surface soil; GS = gas field surface soil; BS = background soil (B3, B14 and B34). Numbers 1–3 indicate field replicates from the corresponding sites.

(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.

3.3 Quantitative analysis of *n*-alkane-degrading bacteria

Real-time quantitative PCR was used to estimate the abundance of *n*-alkane-degrading bacteria at the different locations. In order to minimize environmental interference, such as noise caused by variations in soil type, we normalized 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 is shown in Fig. 5 (the absolute numbers of *alkB* gene were shown in Fig. S2). In the oil and gas field soil samples, the relative abundances of *alkB* genes were significantly higher than in all other soil samples ($4.7\% \pm 0.3\%$ and $7.4\% \pm 1.1\%$, 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 could be measured by geochemical analyses. A similar effect has been previously reported in a study of marine hydro-

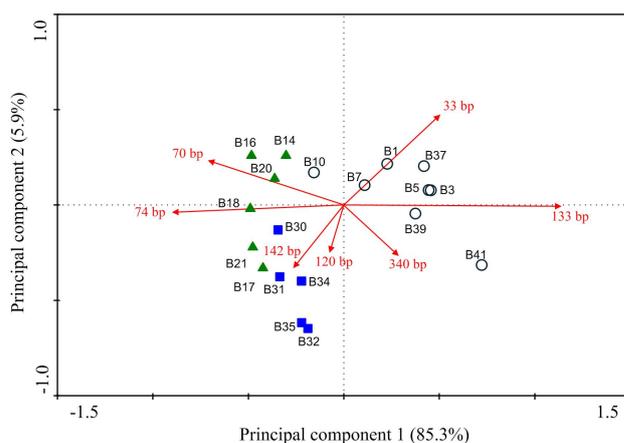


Fig. 4. PCA ordination plot for the samples and T-RFs based on *alkB* T-RFLP data from different soil samples. Symbols: red arrows = T-RFs (the size in base pairs of forward T-RFs by *Msp* I); green triangles, blue squares, black circles and associated numbers indicate oil field surface soils, gas field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

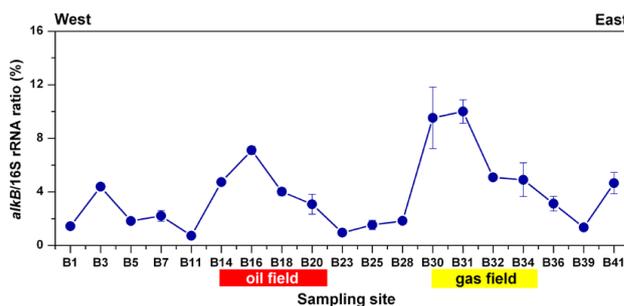


Fig. 5. Biogeographical distribution of the percentages of *alkB* gene copy numbers (representing the number of *n*-alkane-degrading bacteria) in proportion to 16S rRNA gene copy numbers (representing the number of total *Eubacteria*) of soil samples collected from east–west direction survey line of Shaozhuang oil and gas field ($n = 3$). Soils were collected at a depth of 60 cm.

carbon 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 habitat response to trace hydrocarbons in addition to geochemical measurements (Wasmund et al., 2009).

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/or (ii) the

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

Sample code	Geographical description	Water content (%)	pH value	Electrical conductivity ($\mu\text{s cm}^{-1}$)	Total headspace <i>n</i> -alkanes (C_{2+}) ($\mu\text{L L}^{-1}$)	Total adsorbed <i>n</i> -alkanes (C_{2+}) ($\mu\text{L L}^{-1}$)
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

* Soils were collected at a depth of 60 cm.

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 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 amounts 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 above oil and gas reservoirs even though only low concentrations of hydrocarbons were measured in these soils.

Supplementary material related to this article is available online at: <http://www.biogeosciences.net/10/2041/2013/bg-10-2041-2013-supplement.pdf>.

Acknowledgements. We thank Xu Yang for help with terminal restriction fragment length polymorphism (T-RFLP) analysis and Wanmeng Wang for support with real-time quantitative PCR analysis. This work was financially supported by the National Science Foundation of China (41202241 and 41072099), State key laboratory of petroleum resources and prospecting (prp/OPEN-1208) and the research project of SINOPEC (P11058).

Edited by: Z. Jia

References

- Anderson, H. S.: Amplified geochemical imaging: an enhanced view to optimize outcomes, *First Break*, 24, 77–81, 2006.
- Deutzmann, J. S., Wörner, S., and Schink, B.: Activity and diversity of methanotrophic bacteria at methane seeps in eastern Lake Constance sediments, *Appl. Environ. Microb.*, 77, 2573–2581, 2011.
- Doumenq, P., Aries, E., Asia, L., Acquaviva, M., Artaud, J., Gilewicz, M., Mille, G., and Bertrand, J. C.: Influence of *n*-alkanes and petroleum on fatty acid composition of a hydrocarbonoclastic bacterium: *Marinobacter hydrocarbonoclasticus* strain 617, *Chemosphere*, 44, 519–528, 2001.

- Etiopio, G. and Ciccioli, P.: Earth's degassing: a missing ethane and propane source, *Science*, 323, 478–478, 2009.
- Håvelsrud, O. E., Haverkamp, T. H., Kristensen, T., Jakobsen, K. S., and Rike, A. G.: A metagenomic study of methanotrophic microorganisms in Coal Oil Point seep sediments, *BMC Microbiol.*, 11, 221, doi:10.1186/1471-2180-11-221, 2011.
- Kadnikov, V. V., Mardanov, A. V., Beletsky, A. V., Shubenkova, O. V., Pogodaeva, T. V., Zemskaya, T. I., Ravin, N. V., and Skryabin, K. G.: Microbial community structure in methane hydrate-bearing sediments of freshwater Lake Baikal, *FEMS Microbiol. Ecol.*, 79, 348–358, 2012.
- Kaplan, C. W. and Kitts, C. L.: Bacterial succession in a petroleum land treatment unit, *Appl. Environ. Microb.*, 70, 1777–1786, 2004.
- Kloos, K., Munch, J. C., and Schloter, M.: A new method for the detection of alkane monooxygenase homologous genes (*alkB*) in soils based on PCR-hybridization, *J. Microbiol. Methods*, 66, 486–496, 2006.
- Klusman, R. and Saeed, M.: Comparison of light hydrocarbon microseepage mechanisms, in: *Hydrocarbon migration and its near surface expression*, edited by: Schumacher, D. and Abrams, M., AAPG Memoir, 157–168, 1996.
- Luz, A. P., Pellizari, V. H., Whyte, L. G., and Greer, C. W.: A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil, *Can. J. Microbiol.*, 50, 323–333, 2004.
- Margesin, R., Labbe, D., Schinner, F., Greer, C. W., and Whyte, L. G.: Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils, *Appl. Environ. Microb.*, 69, 3085–3092, 2003.
- Muyzer, G. and van der Kraan, G. M.: Bacteria from hydrocarbon seep areas growing on short-chain alkanes, *Trends Microbiol.*, 16, 138–141, 2008.
- Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W.: Succession of bacterial community structure and diversity in a paddy soil oxygen gradient, *Environ. Microbiol.*, 7, 382–395, 2005.
- Pérez-de-Mora, A., Engel, M., and Schloter, M.: Abundance and diversity of *n*-alkane-degrading bacteria in a forest soil co-contaminated with hydrocarbons and metals: a molecular study on *alkB* homologous genes, *Microbiol. Ecol.*, 62, 959–972, 2011.
- Rasheed, M. A., Kalpana, M. S., Prasanna, M. V., Lakshmi, M., T. Madhavi, Mani, D., Patil, D. J., Dayal, A. M., and Raju, S. V.: Geo-microbial and light gaseous hydrocarbon anomalies in the nearsurface soils of Deccan Syncline Basin, India: Implications to hydrocarbon resource potential, *J. Petrol. Sci. Eng.*, 84–85, 33–41, 2012.
- Redmond, M. C., Valentine, D. L., and Sessions, A. L.: Identification of novel methane-, ethane-, and propane-oxidizing bacteria at marine hydrocarbon seeps by stable isotope probing, *Appl. Environ. Microbiol.*, 76, 6412–6422, 2010.
- Rojas, F.: Degradation of alkanes by bacteria, *Environ. Microbiol.*, 11, 2477–2490, 2009.
- Rui, J., Peng, J., and Lu, Y.: Succession of bacterial populations during plant residue decomposition in rice field soil, *Appl. Environ. Microb.*, 75, 4879–4886, 2009.
- Sakai, Y., Maeng, J. H., Tani, Y., and Kato, N.: Use of long-chain *n*-alkanes (C₁₃-C₄₄) by an isolate *Acinetobacter* sp M-1, *Biosci. Biotechnol. Biochem.*, 58, 2128–2130, 1994.
- Schumacher, D.: Hydrocarbon-induced alteration of soils and sediments, in: *Hydrocarbon migration and its near-surface expression*, edited by: Schumacher, D. and Abrams, M. A., 66, AAPG Memoir, Tulsa, 71–89, 1996.
- Shennan, J. L.: Utilisation of C₂-C₄ gaseous hydrocarbons and isoprene by microorganisms, *J. Chem. Technol. Biotechnol.*, 81, 237–256, 2006.
- Smits, T. H., Röthlisberger, M., Witholt, B., and van Beilen, J. B.: Molecular screening for alkane hydroxylase genes in gram-negative and gram-positive strains, *Environ. Microbiol.*, 1, 307–317, 1999.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S.: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 28, 2731–2739, 2011.
- Ter Braak, C. J. F. and Smilauer, P.: *Canoco reference manual and user's guide to Canoco for Windows: software for canonical community ordination (version 4)*, Microcomputer Power, Ithaca, NY, 1998.
- van Beilen, J. B. and Funhoff, E. G.: Alkane hydroxylases involved in microbial alkane degradation, *Appl. Microbiol. Biotechnol.*, 74, 13–21, 2007.
- Valentine, D. L., Kessler, J. D., Redmond, M. C., Mendes, S. D., Heintz, M. B., Farwell, C., Hu, L., Kinnaman, F. S., Yvon-Lewis, S., Du, M., Chan, E. W., Tigreros, F. G., and Villanueva, C. J.: Propane respiration jump-starts microbial response to a deep oil spill, *Science*, 330, 208–211, 2010.
- Wagner, M., Wagner, M., Piske, J., and Smit, R.: Case Histories of microbial prospecting for oil and gas, AAPG studies in Geology 48 and SEG Geophysical References Series, 11, 453–479, 2002.
- Wang, W., Wang, L., and Shao, Z.: Diversity and abundance of oil-degrading bacteria and alkane hydroxylase (*alkB*) genes in the subtropical seawater of Xiamen Island, *Microbiol. Ecol.*, 62, 959–972, 2011.
- Wasmund, K., Burns, K. A., Kurtböke, D. I., and Bourne, D. G.: Novel Alkane Hydroxylase Gene (*alkB*) Diversity in Sediments Associated with Hydrocarbon Seeps in the Timor Sea, Australia, *Appl. Environ. Microb.*, 75, 7391–7398, 2009.
- Xu, K., Liu, H., Du, G., and Chen, J.: Real-time PCR assays targeting formyltetrahydrofolate synthetase gene to enumerate acetogens in natural and engineered environments, *Anaerobe*, 15, 204–213, 2009.
- Xu, K., Liu, H., and Chen, J.: Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion, *Bioresour. Technol.*, 101, 2600–2607, 2010.