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Keywords: Anthracene; Phenanthrene; Fluoranthene; DNA Stable-Isotope Probing; Biodegradation

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Abstract: Information on microorganisms possessing the ability to metabolize different polycyclic aromatic hydrocarbons (PAHs) in complex environments helps in understanding PAHs behaviour environment and developing bioremediation strategies. In the present study, stable-isotope probing (SIP) was applied to investigate degraders of PAHs in a forest soil with the addition of individually 13C-labeled phenanthrene, anthracene, and fluoranthene. Three distinct phenotypes were identified as the active phenanthrene-, anthracene- and fluoranthene-degrading bacteria. The putative phenanthrene degraders were classified as belonging to the genus Sphingomona, previously identified in the degradation of anthracene/fluoranthene by DNA-SIP and phenanthrene by PLFA-SIP. For anthracene, bacteria of the genus Rhodanobacter were the putative degraders, and in the microcosm amended with fluoranthene, the putative degraders were identified as belonging to the phylum Acidobacteria. Our results from DNA-SIP are the first to directly link Rhodanobacter- and Acidobacteria-related bacteria with anthracene and fluoranthene degradation, respectively. The results also illustrate the specificity and diversity of three- and four-ring PAHs degraders in forest soil, contributes to our understanding of natural PAHs biodegradation processes, and also proves the feasibility and practicality of DNA-based SIP for linking functions with identity especially uncultured microorganisms in complex microbial biota.

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Dear Editor,

We would like to submit our manuscript "Bacteria **capable of degrading anthracene, phenanthrene, and fluoranthene as revealed by DNA based stable-isotope probing in a forest soil**" for possible publication in *Journal of Hazardous Materials*. The total word count of the manuscript is 4537.The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:
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The present study investigated microorganisms responsible for degradation of anthracene, phenanthrene, and fluoranthene in forest carbon-rich soils sample by DNA-based stable isotope probing. 16S rRNA sequencing of the ^{13}C-enriched fraction suggests bacteria in genus *Rhodanobacter*, genus *Sphingomona*, and phylum *Acidobacteria* were responsible for the degradation of anthracene, phenanthrene, and fluoranthene respectively, which is also firstly reported by DNA-based stable isotope probing. The finding illustrates the specificity of three- and four-ring PAHs degraders in forest soil, contributes to our understanding of PAHs behaviour environment and biodegradation processes. I believe the results presented in this manuscript will be of interest for a wide range of researchers of environmental microbiology ecology and bioremediation.

I would be very grateful if you could let me know the results of the review process in the near future. Thank you very much for your help. I am looking forward to hearing from you soon.

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Sincerely yours,

Chunling

Highlights

- ✧ Investigate PAHs degraders in forest carbon-rich soils via DNA-SIP
- ✧ *Rhodanobacter* is identified to metabolite anthracene for the first time
- ✧ The first fluoranthene degrader belongs to *Acidobacteria*
- ✧ Different functions of PAHs degraders in forest soils from contaminated soils

Novelty statement

Information about the microbes in forest carbon-rich soil with the ability of degrading POPs is crucial for POPs fate in soil reservoir. The present study applied DNA-SIP to investigate three- and four-ring PAHs degraders in a forest soil for the first time. Our results are the first to directly link *Rhodanobacter*- and *Acidobacteria*-related bacteria with anthracene and fluoranthene degradation, and the finding further proved that the PAHs degrading microorganisms in forest carbon-rich soils are similar to those of heavily contaminated soils, but their functions vary a lot. The result contributes to deeper understanding on PAHs natural attenuation processes and bioremediation.

Bacteria capable of degrading anthracene, phenanthrene, and fluoranthene as revealed by DNA based stable-isotope probing in a forest soil

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ABSTRACT

Information on microorganisms possessing the ability to metabolize different polycyclic aromatic hydrocarbons (PAHs) in complex environments helps in understanding PAHs behaviour environment and developing bioremediation strategies. In the present study, stable-isotope probing (SIP) was applied to investigate degraders of PAHs in a forest soil with the addition of individually ^{13}C-labeled phenanthrene, anthracene, and fluoranthene. Three distinct phenotypes were identified as the active phenanthrene-, anthracene- and fluoranthene-degrading bacteria. The putative phenanthrene degraders were classified as belonging to the genus *Sphingomona*, previously identified in the degradation of anthracene/fluoranthene by DNA-SIP and phenanthrene by PLFA-SIP. For anthracene, bacteria of the genus *Rhodanobacter* were the putative degraders, and in the microcosm amended with fluoranthene, the putative degraders were identified as belonging to the phylum *Acidobacteria*. Our results from DNA-SIP are the first to directly link *Rhodanobacter*- and *Acidobacteria*-related bacteria with anthracene and fluoranthene degradation, respectively. The results also illustrate the specificity and diversity of three- and four-ring PAHs degraders in forest soil, contributes to our understanding on natural PAHs biodegradation processes, and also proves the feasibility and practicality of DNA-based SIP for linking functions with identity especially uncultured microorganisms in complex microbial biota.

Keywords: Anthracene; Phenanthrene; Fluoranthene; DNA Stable-Isotope Probing; Biodegradation; Forest soil

1. Introduction

Soils contaminated by polycyclic aromatic hydrocarbons (PAHs) have attracted great concerns for long because of their genotoxic/carcinogenic potential and ubiquitous presence in the environment [1]. PAHs contamination results primarily from the incomplete combustion of organic matters at high temperature during human activities. Besides, PAHs can also enter environmental systems by means of natural processes, such as forest fires and direct biosynthesis. Widespread released into the environment, PAHs have been detected in various media, especially the soil as the major repository for these compounds [2]. The existence of PAHs in the tissue of vegetables and rice near PAHs contaminated soil increases human exposure chances and health risks [1]. Hence, the study of efficient techniques to remove PAHs from soils has become a major research topic among environmental scientists.

The primary process of PAHs attenuation in soil is microbial degradation, and bioremediation provides an environmentally friendly and cost-effective option for removing these compounds from the soil environment [3]. To date, both culture dependent and independent techniques have been used to study the organisms responsible for PAHs degradation [4-7]. Bacterial species possessing the ability to metabolize PAHs are readily isolated from contaminated soils or sediments. These studies have enhanced our knowledge of PAHs metabolic pathways and mechanisms under laboratory conditions [8-12]. However, PAHs are normally present as complex mixtures in soils and sediments, and the traditional methods of isolating and cultivating targeted bacteria in the laboratory cannot reproduce the actual condition to unravel the complex interactions within microbial communities and represent the correlations with the specific environment. Furthermore, it is difficult to isolate a large range of organisms from their native environment, and direct-cultivation [13] underestimates the microbial diversity of functional microorganisms [14] and leads to a distorted picture of the microbial communities. In contrast, culture-independent techniques circumvent the issues associated with cultivation and thus overcome the drawbacks of traditional methods.

Stable-isotope probing (SIP) is one such culture-independent technique, able to link identities with functions in complex systems [15]. SIP is suitable for identifying microbial populations within complex communities that are responsible for the degradation of targeted contaminants [6, 7, 15, 16]. To date, SIP has been successfully applied to identify soil bacteria capable of metabolizing BTEX toluene [7], biphenyl [17, 18], benzoate [17], and PAHs (naphthalene [4, 6], anthracene [19, 20], phenanthrene [4], pyrene [5, 9], fluoranthene [21], and benz[a]anthracene [21]). Particularly for PAHs which are always found as complex mixtures in soils, some identified PAHs degraders can utilize a wide range of PAHs as sole carbon sources [22], or in some cases are only capable of degrading specific PAHs molecules. For example, Jones et al. investigated some PAHs degraders in a contaminated soil via SIP, including naphthalene, phenanthrene, pyrene, fluoranthene and benz[a]anthracene, and found that the bacterial guilds possessing the ability to metabolize individual compounds were different but shared several members [21].

However, most SIP studies address PAHs degraders in heavily contaminated soils [4, 16, 23] and little attention is paid on the PAHs metabolism in forest soils. From global distillation theory [24], persistent organic pollutants (POPs) undergo long-range transport at global scale and eventually accumulated in medias with organic carbon-rich reservoirs. Forest soils with rich carbon and abundant precipitation are reported to attenuate the grasshopper effect of POPs and stabilize them in undisturbed media [24, 25]. Thus, mountain-forest organic-rich soil is one of the main reservoirs of POPs, including PAHs, where the microbial response to and degradation of PAHs is a critical factor affecting the global fate of PAHs. Besides, microbial diversities in forest soils are more dynamic and sensitive than those of contaminated soils due to their low historical exposure and less tolerance to toxic PAHs pollutants [26-28]. Forest soils are therefore hypothesized with unique PAHs degraders compared to those in contaminated soils and have the necessities to be investigated for yet-unknown PAHs metabolic genes and pathways. Till now, only limited efforts have been attempted to uncover the microorganisms possessing the ability to degrade a wide variety of PAHs in forest soils by SIP.

In the present study, ^{13}C-DNA targeted SIP was applied to identify the bacteria with the ability of degrading anthracene (ANT), phenanthrene (PHE), and fluoranthene (FLT) individually in mountain forest soil, and to determine the relationships among the degraders, with the aim of providing deeper understanding on the fate of PAHs in organic-rich sink and useful information for enhancing the practicability of bioremediation in complex community.

2. Materials and methods

2.1. Soil samples

The soil in this study was collected from the forest of Mt. Dabie (31°5'24"N, 115°46'12"E) in Hubei Province, China. The pH of the soil was determined as previously described [29]. Total organic carbon (TOC) in the soil was analyzed with an elemental analyzer (CHNS Vario Ei III, Elementar) after removal of carbonates with HCl as described previously [30]. The pH value and TOC of the studying soil were 5.3 and 13.5%, respectively. After transfer to the laboratory, the soil samples were briefly air dried, followed by removing large objects and crushing chunks manually. The samples were subsequently blended and sieved through a 2-mm pore-size screen to exclude stones and other debris for further analysis.

2.2. PAHs degradation

Microcosms were set up individually for the three PAHs (ANT, PHE and FLA), as follows. Three grams of soil (dry weight) was placed in a 150-mL serum bottle containing 10-mL phosphate-buffered mineral medium [31]. After sealing with rubber stoppers and aluminum seals, unlabeled PAHs (99%, Cambridge Isotope Laboratories, Inc.) or ^{13}C-labeled PAHs (ring-$^{13}C_6$, 99%, Cambridge Isotope Laboratories, Inc.) were added to the bottles via gas-tight syringe to obtain a final concentration of 10 mg kg^{-1} for each PAHs. The cover of the bottles was opened each day for 1 hour to keep oxygen in the media close to natural environments. The four treatments included: sterile controls (nonbioactive, with PAHs additive), negative controls without PAHs

addition, unlabeled PAHs amended treatments (^{12}C-ANT, ^{12}C-PHE and ^{12}C-FLT), and ^{13}C labeled PAHs amended treatments (^{13}C-ANT, ^{13}C-PHE and ^{13}C-FLT). Soils were γ-irradiated (50 kGy) for 2 h to prepare the sterile control. SIP microcosms were harvested at 3, 9 and 14 days and the whole microcosms were sacrificed for PAHs analysis and DNA extraction. All the treatments were prepared in triplicate and incubated at room temperature (~20°C) with shaking at a speed of 120 rpm min^{-1}.

2.3.PAHs analysis

There was no significant difference in PAHs removal between samples amended with ^{13}C-labeled and unlabeled PAHs [7, 21, 32-34], and all the PAHs analysis in this study was then carried out for soil samples amended with unlabeled PAHs. More precisely, the soil samples (sterilize control and unlabeled PAHs amended treatment) were sacrificed on Day 3, 9 and 14 after the initiation of cultivation, according to previous instruction [32, 33]. Briefly, soil samples were freeze-dried, homogenized, pulverized, spiked with 1,000 ng of deuterated PAHs as surrogate standards, and extracted into dichloromethane (DCM) in a Soxhlet apparatus for 48 h, with the addition of activated copper to remove sulfur. The extract was concentrated to ~0.5 mL after solvent exchange to hexane. The soil extracts were purified on a multilayer silica-gel/alumina column filled with anhydrous Na_2SO_4, neutral silica gel (3% w/w; deactivated), and neutral alumina (3% w/w; deactivated; from top to bottom) via elution with 15-mL hexane/DCM (1:1, v/v). After concentrated to ~50 μL under a gentle stream of N_2, 1,000 ng of hexamethylbenzene was added as an internal standard prior to analysis.

PAHs were detected using an Agilent 7890 gas chromatograph equipped with a capillary column (DB-5MS, 30 m, 0.25 mm, 0.25μm) and a mass spectrometric detector (MSD, Agilent 5975). One microliter of sample was injected in splitless mode with a 10-min solvent delay time. High purity helium was used as the carrier gas with a flow rate of 1.83 mL min^{-1}. The temperature of the injector and transfer lines was 290°C and 300°C, respectively. The initial oven temperature was set at 60°C for 1 min, raised to 290°C at a rate of 3°C min^{-1}, and then held for 20 min.

PAHs standards were used to quantify PAHs in soil samples. Instrumental performance was subjected to quality control calibration, using the standards after each set of eight samples had been analyzed. Six PAHs standard concentrations were used to derive calibration curves. The concentrations in the samples were corrected by referring to surrogate recovery levels.

2.4. DNA extraction, isopycnic ultracentrifugation and gradient fractionation

On Day 3, 9 and 14, samples amend with ^{13}C labeled and unlabeled PAHs (ANT, PHE and FLA) were sacrificed for DNA extraction. Total genomic DNA was extracted using a MoBio power soil DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA) according to the manufacturer's instruction. DNA concentrations were determined using an ND-2000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then DNA was prepared as previously for isopycnic ultracentrifugation and gradient fractionation [32, 33]. Briefly, approximately 10,000-ng DNA was added to Quick-Seal polyallomer tubes (13×51 mm, 5.1 ml, Beckman Coulter), along with a Tris-EDTA (TE, pH 8.0)/CsCl solution with a final buoyant density of ~1.77 g mL^{-1}. After sealed with a cordless quick-seal tube topper, the tubes were transferred to a Beckman ultracentrifuge and centrifuged at 178,000 × g (20°C) for 48 h. Following centrifugation, 150-μL fractions were collected from each tube using a fraction recovery system (Beckman). The BD value of each fraction was then measured. CsCl was removed by glycogen-assisted ethanol precipitation [35].

2.5. PCR and TRFLP analysis

The ultracentrifugation fractions were subjected to terminal restriction fragment length polymorphism (TRFLP) analysis using standard procedures [7]. Briefly, DNA extracts were amplified with 27F-FAM (5′-AGAGTTTGATCMTGGCTCAG; 5′ end-labeled with carboxyfluorescein) and 1492R (5′-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) using the following polymerase chain reaction (PCR) program: initial melting at 94°C for 5 min; 30 amplification cycles of 94°C for 30 s,

55°C for 30 s, and 72° for 1.5 min; final extension at 72°C for 10 min. After amplification, the presence of PCR products was confirmed by 1% agarose gel electrophoresis and the subsequent staining of the gel with ethidium bromide. PCR products were purified using an Omega ENZA Cycle-Pure kit (Omega Bio-Tek, Inc.) following the manufacturer's instructions, and then digested with HaeIII (New England Biolabs) for 4-5 h at 37°C. One nanogram of each labeled PCR product was analyzed on an ABI 3730 Genetic Analyzer running the Peak Scanner software version 1.0. A GeneScan™ ROX 500™ set of internal standards (Applied Biosystems) was used. The percentage abundance of each fragment was determined as described previously [35]. The microbes responsible for ^{13}C assimilation were identified by comparing the relative abundances of specific terminal restriction fragments (TRFs) from the unlabeled samples (amended with ^{12}C-unlabeled PAHs) with those from the labeled samples (amended with ^{13}C-labeled PAHs) on Day 3, 9 and 14 for each ultracentrifugation fraction.

2.6. Sequencing of 16S rRNA genes

16S rRNA genes from the heavy fraction of the ^{13}C-labeled DNA genes were cloned and sequenced. Briefly, 16S rRNA amplification followed similar process described above, with the exception that the 27F-FAM primer was replaced by the 27F primer. The purified PCR products were cloned into a pGEM-T Easy Vector and transformed into *Escherichia coli* JM 109 competent cells. The plasmids of positive clones were extracted using an EZNA plasmid mini-kit (Omega Bio-Tek, Inc.) and the 16S rRNA inserts were sequenced with an ABI 3730 genetic analyzer. Sequence similarity searches and alignments were performed with the aid of the Basic Local Alignment Search Tool (BLAST) algorithm (National Center for Biotechnology Information, Bethesda, Md.) and Molecular Evolutionary Genetics Analysis (MEGA 5.1). Phylogenic trees for the 16S rRNA gene sequences along with the closest matches in GenBank were obtained with the neighbor-joining method using MEGA 5.1.

2.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in this study are available in GenBank under the respective accession numbers for 204-bp TRF (KM267483), 240-bp TRF (KM267484) and 291-bp TRF (KM267485).

3. Results

3.1. PAHs biodegradation in soils

The residual percentages of ANT, PHE and FLT in forest soil from Mt. Dabie on 3, 9 and 14 d are listed in Table 1. Each PAHs compound showed a slight decrease with time in sterile controls, whereas in non-sterile samples, the residual percentage of each PAHs also showed a well-defined decrease but significantly lower than that in the sterile controls. The PAHs removal in the non-sterile samples resulted mostly from the activities of microorganisms in the soil. The degradation removal efficiency at 14 days followed the order: PHE (49.7%) > ANT (43.4%) > FLT (18.9%). The majority of FLT degradation occurred over the period from 9 to 14 d, with a corresponding degradation efficiency of 5.3% (3 d), 7.4% (9 d) and 18.9% (14 d), respectively.

3.2. Microbial structure analysis via SIP and TRFLP

In the PHE-amended microcosms, a 291-bp *Hae*III TRF was involved in the PHE degradation at 3 d, 9 d and 14 d, as revealed by the TRFLP results (Fig. 1A). The 291-bp TRF was present and enriched in both the heavy fractions from the ^{13}C-PHE and the light fractions from the ^{12}C-PHE treatments. Furthermore, the degree of enrichment increased from 3 d to 14 d, and its buoyant density (BD) in the ^{13}C-PHE treatment increased over time. The BD values of the relative abundance peaks of 291-bp TRF were 1.7280 g ml^{-1}, 1.7306 g ml^{-1}, and 1.7365 g ml^{-1} at 3 d, 9 d, and 14 d, whereas such increase was not observed in the ^{12}C-PHE treatment. Although several other TRFs (70-bp and 269-bp) were also enriched in the heavy fractions from the ^{13}C-PHE samples, the same enrichment was observed in ^{12}C-PHE treatments. Furthermore, the relative abundance peak of the TRFs remained constant, regardless of the time (data not shown). These results indicate that microorganisms represented by 291-bp TRF assimilated the ^{13}C-labled PHE and were responsible for PHE degradation in the forest soil.

To identify the PHE-degraders based on the TRFLP results and obtain taxonomic information on the corresponding bacteria, a 16S rRNA clone library was constructed from the heavy fractions from the ^{13}C-PHE treatment. Clones with the predicted cut sites matching TRFLP cut sites of the corresponding TRFs were selected for phylogenetic analysis. Previous studies have shown that small differences (2–3 bases) often occur between the measured fragment lengths and those predicted using sequence data [36-38]. Clones with predicted cut sites matching 291-bp TRF (based on the analysis of the clone sequences, each with a predicted *Hae*III cut site of 294 bp) accounted for the majority of microbes in the clone library. Clones with predicted cut sites of 269-bp and 70-bp TRF were also numerous, but less dominant than those of 291-bp TRF (Table 2). Bacteria represented by 291-bp TRF were classified as genus *Sphingomona* (>98% sequence identity), and those represented by 269-bp and 70-bp TRF fell into the genus *Aquabacterium* order *Burkholderiales*, as identified by the RDP analysis tool "classifier".

In the microcosms amended with ANT, a 204-bp TRF was involved in the degradation process. At 9 d with ~22 % ANT degradation, a significant increase in the BD value was noted in the 204-bp TRF abundance peak in the ^{13}C-ANT microcosms compared with the ^{12}C-ANT one, which indicated their roles in ^{13}C-ANT assimilation (see Fig. 2A). After 14 d remediation with an ANT degradation of ~43%, the increase in the BD value associated with the 204-bp TRF abundance peak and its enrichment of the relative abundance of 204-bp TRF in the heavy fractions were more pronounced in samples from the ^{13}C-ANT microcosms, whereas in ^{12}C-ANT treatment, the enrichment of the 204-bp TRF relative abundance occurred in the light fractions. This scenario indicated that microorganisms represented by 204-bp TRF were responsible for the ANT degradation. According to the 16S rRNA clone library data, clones with the predicted cut site matching 204-bp TRF fragments were affiliated with the genus *Rhodanobacter* within the family *Xanthomonadaceae* (>98% sequence identity). Furthermore, the presence of clones with predicted cut sites of 69-, 219-, and 76-bp were also observed (Table 2). They were classified in the family *Xanthomonadaceae*, genus *Burkholderia* and *Gp1*, and class *Acidobacteria_Gp1*, but

their relative abundance was consistently high in the heavy fractions. Thus, in the present study, only bacteria represented by the 204-bp TRF fragment were correlated with the ANT degradation.

In microcosms inoculated with FLT, a 240-bp TRF was enriched in the heavy fractions derived from the ^{13}C-FLT treatment, but not in the heavy fractions from ^{12}C-FLT microcosms at 3, 9 and 14 d (Fig. 3A). Furthermore, the relative abundances of the TRF in the heavy fractions from the ^{13}C-FLT samples increased with time, and the BD values of the relative abundance peaks were 1.7153 g ml^{-1}, 1.7184 g ml^{-1}, and 1.7198 g ml^{-1}, with corresponding relative abundances of 5.3%, 7.8% and 11.3% at 3 d, 9 d and 14 d, respectively, indicating the increasing ^{13}C-labeled carbon atom uptake with time. In contrast, in the ^{12}C-FLT microcosms, the enrichment of 240-bp TRF occurred in the light fractions, and the BD value of its maximum relative abundance remained almost constant (Fig. 3A). Compared to the treatments amended with PHE, the smaller increment in BD value and relative abundance of the peaks in the heavy fractions from ^{13}C-FLT treatment may be attributed to lower FLT removal at the sampling time (~5.3%, ~7.4%, and ~18.9% FLT depletion at 3 d, 9 d, and 14 d, respectively), and the less ^{13}C carbon labeled ration in FLT compound (6 labeled carbon atoms). The 16S rRNA clone library data revealed that the bacteria represented by 240-bp TRF were correlated with organisms in the phylum *Acidobacteria*, sharing 92% and 91% sequence similarity with *Candidatus Solibacter usitatus strain Ellin6076* and *Bryobacter aggregatus strain MPL3*, respectively (Fig. 3B). Additionally, organisms affiliated to the family *Holophagaceae* (203-bp TRF), *Acidobacteria_Gp2* (77-bp TRF) in phylum *Acidobacteria*, and family *Xanthomonadaceae* (69-bp TRF) in phylum *Gammaproteobacteria* were also observed in the 16S rRNA clone library. The phylum *Acidobacteria* has been linked to the degradation of PAHs [39] and polychlorinated biphenyls (PCBs) [40] from contaminated soil and sediment. Microorganisms in the family *Xanthomonadaceae* have also been linked to the degradation of PAHs in soils using SIP and other methods [21, 41].

4. Discussion

Forest and other carbon-rich soils retard the long-range transport of POPs (including PAHs), and became important reservoirs of these compounds emitted in the past decades [24]. Information about the microbes in forest soil with the ability of degrading POPs is crucial for the POPs fate in soil reservoirs, even on a global scale. Currently, most of the works on PAHs biodegradation and the responsible degrading microorganisms were limited to the contaminated soils. Given the final destination of PAHs in the carbon-rich forest soils, new exploration in the microbial response to low level PAHs exposure and their capabilities of PAHs degradation contributes to deeper understanding of their natural attenuation processes. Furthermore, the microbial diversity in forest soils, far away from the human activities and disturbance, is expected to be higher than the contaminated soils which were usually suppressed by the existence of toxic organic pollutants [26, 28]. In the present study, DNA-SIP was applied to a China forest soil to study the PHE, ANT and FLT degradation and the organisms responsible for the degradation in this complex microbial biota.

The removal efficiency of PHE, ANT and FLT after 14 days degradation differed in the following order: PHE> ANT> FLT, consistent with the increment in BD values and the relative abundance of the peaks of 291-, 204- and 240-bp TRFs in the heavy fractions derived from ^{13}C-labeled treatments amended with PHE, ANT, and FLT individually. The biodegradation of PAHs in soil is often limited by the low bioavailability and follows an inverse relationship with increasing number of aromatic rings and molecular weights [42]. The bioavailability of PAHs in soil is determined by their Log *Kow* (octanol–water partition coefficient). Although both PHE and ANT have three rings, ANT has higher Log *Kow* (4.68) in the cultivation medium than PHE (4.50), which resulted in the lower degradation efficiency; while the lowest degradation efficiency of FLT among the three PAHs is attributed to its four aromatic rings and highest Log *Kow* (5.16). Similar results were observed in other studies [43-47].

The degradation rates of PAHs obtained in the present study were comparable with previous reports[45], but considerably higher than those reported by Zeng et al.

(2010), and lower than those in the systems supplemented with pure cultivated bacteria and isolated microbial consortia [44, 46]. The soil environment is complex and the difference in degradation rates may be attributed to differences in the soil properties, experimental conditions, and the concentration and composition of contaminants in the tested system.

SIP coupled with TRFLP revealed that different bacteria were involved in the PHE, ANT and FLT degradation in the same forest soil. Bacteria represented by 291-bp TRF were members of the genus *Sphingomonads*, and responsible for PHE degradation. *Sphingomonad*s are known to utilize broad range of carbon substrates including both substituted and unsubstituted mono- and poly-aromatic hydrocarbons up to five rings [21]. Furthermore, the capability of *Sphingomonad*s to degrade ANT and FLT has been demonstrated using DNA-SIP in previous studies [21]. Johnsen et al. showed that *Sphingomonads* were related to PHE degradation using PLFA-SIP [48]. The present study is the first to link PHE degradation with *Sphingomonad*-related microorganisms using DNA-SIP. *Burkholderia*-related bacteria are well known for their ability to degrade PAHs and biphenyls in soils, and their important roles in the bioremediation of petroleum hydrocarbon contaminated soil have been documented in several studies [49-51]. In the present study, *Burkholderia*-related bacteria were also observed in the SIP analyses in samples amended with PHE, but the TRFLP results showed that these bacteria were not directly involved in PHE degradation.

Bacteria represented by 204-bp TRF were correlated with ANT degradation and assigned to the genus *Rhodanobacter* within the family *Xanthomonadaceae*. Additionally, other sequences related to members of the family *Xanthomonadaceae* and genus *Burkholderia* also appeared in the clone library, which indicated that these bacteria may be associated with the metabolism of ANT. *Xanthomonadaceae*- and *Burkholderiaceae*-related bacteria have been observed frequently in PAHs-contaminated soils [41, 52]. Jones et al. reported that *Pseudoxanthomonas* were related to naphthalene and anthracene degradation using SIP [21]. As a member of *Xanthomonadaceae*, *Rhodanobacter* was first reported as a lindane-degrading bacterium in 1999 by Nalin et al. [53]. Subsequently, a bacterium sharing high

similarity with *Rhodanobacter* was isolated from the slurry, exhibiting a powerful ability to degrade high-molecular-weight PAHs [54]. A new member of the genus *Rhodanobacter* named BPC1 was also observed as a major degrader in microbial consortia from soil possessing the ability to efficiently metabolize benzo[a]pyrene (BaP) [55]. To our knowledge, no SIP studies associated with ANT degradation by *Rhodanobacter* have been reported. Our results, demonstrated by direct observation of the ANT degradation ability of *Rhodanobacter* in forest soil, confirmed the contribution of strains in this genus to ANT degradation.

The bacteria responsible for FLT degradation were affiliated most closely with the phylum *Acidobacteria*. Bacteria with various genotype and metabolic pathways in this phylum have been found in a number of environmental samples, such as soil, sediment, activated sludge, freshwater, and seawater [56, 57], since the phylum was first identified in 1997 by Ludwig et al. [58] and accounts for ~20% of the soil bacterial community. *Acidobacteria*-related bacteria have been proved in the degradation of petroleum [59], PAHs [41, 59], and PCBs [40, 59] in various habitats. To date, SIP has been successfully applied to investigate the ability of *Acidobacterium* to metabolize propionate [60], biocide [61], herbicide [62], and benzene [36]. Our recent SIP research also demonstrated that *Acidobacteria*-related bacteria were related to the degradation of PHE in forest soil [32]. However, no previous SIP studies have linked *Acidobacteria*-related bacteria with FLT metabolism; our results are the first to show that bacteria in this phylum are directly involved in the degradation of FLT in the complex microbial community of forest soils.

It is interesting that the microorganisms capable of metabolizing PHE, ANT and FLT in this study were different from previous investigations. The genus *Sphingomonads* is PHE degrader in this study but linked to ANT and FLT metabolisms in other studies [21]. Our ANT degrader (genus *Rhodanobacter*) was reported with the ability of degrading high-molecular-weight PAHs [54], and the FLT degrader (*Acidobacteria*-related bacteria) was linked with PHE degradation in other soils [32]. The results hinted that the PAHs degraders in forest carbon-rich soils are unique compared to those in PAHs heavily contaminated soils, possibly attributing to

low PAHs exposure history and higher microbial diversity. Previous research has shown several environmental factors affecting the growth and functions of various microbes in soils, such soil type, nutrients content, incubation conditions, and PAHs exposure history [63-65], resulting in the preferential enrichment of PAHs degrading microorganisms. For example, the amount of phenanthrene in soils influenced the diversity of *Sphingomonas* which is commonly existed in PAHs-contaminated environments [4]. Besides, the microbial growth and metabolism might be also affected by the profile and bioavailability of the targeted PAHs compounds. The PAHs degraders in PAHs-contaminated soils have the selectivity among PAHs substrates, and they have simultaneous or sequential utilization of various PAHs compounds under different conditions [34]. Our findings further proved that the PAHs degrading microorganisms in forest carbon-rich soils are similar to those of heavily contaminated soils, but their functions vary to a great extent.

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Legends of tables and figures

Tables

Table 1 Percentage of PAHs residual in soil over cultivation time

Table 2. Summary of heavy-fraction 16S rRNA gene clone library in microcosms constructed with PHE, ANT and FLT.

Figures

Fig. 1. A: Percent relative abundance of TRF 291-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with phenanthrene (PHE) after 3, 9, 14 days.

B: Phylogenetic tree of the bacteria corresponding 291-bp TRF from microcosms amended with phenanthrene (PHE) along with the closest matches in GenBank.

Fig. 2. A: Percent relative abundance of TRF 204-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with anthrancene (ANT) after 3, 9, 14 days.

B: Phylogenetic tree of the bacteria corresponding 204-bp TRF from microcosms amended with anthracene (ANT) along with the closest matches in GenBank.

Fig. 3. A: Percent relative abundance of TRF 240-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with fluoranthene (FLT) after 3, 9, 14 days.

B: Phylogenetic tree of the bacteria corresponding 240-bp TRF from microcosms amended with fluoranthene (FLT) along with the closest matches in GenBank.

Table

Table 1 Percentage of PAHs residual in soil over cultivation time

Time (days)	PHE[1](%)		ANT[2](%)		FLT[3](%)	
	Sterile control	Sample	Sterile control	Sample	Sterile control	Sample
3	92.3±1.8	70.7±0.7	80.6±2.4	64.9±1.9	95.6±1.4	90.3±1.7
9	89.4±2.1	59.9±3.2	83.1±3.7	61.4±0.8	90.6±1.6	83.2±0.9
14	85.1±1.2	35.3±2.8	79.0±1.1	35.6±3.7	85.3±2.5	66.4±1.4

[1]PHE represents phenanthrene; [2]ANT represents anthracene; [3]FLT represents fluoranthene.

Table 2 Summary of heavy-fraction 16S rRNA gene clone library in microcosms constructed with PHE, ANT and FLT.

Compound/T-RFs (bp)	Enriched in ^{13}C heavy fractions	Clone frequency	taxa
PHE[1]			
291 (KM267485)	Yes	35	*Sphingomonads*
269 (KT697632)	No	5	*Aquabacterium*
70 (KT697633)	No	5	*Aquabacterium*
ANT[2]			
204 (KM267483)	Yes	36	*Rhodanobacter*
219 (KT697634)	No	4	*Burkholderia*
69 (KT697635)	No	6	*Xanthomonadaceae*
76 (KT697636)	No	4	*Gp1*
FLT[3]			
240 (KM267484)	Yes	31	*Acidobacteria*
77 (KT697637)	No	7	*Acidobacteria_Gp2*
69 (KT697638)	No	6	*Xanthomonadaceae*
203 (KT697639)	No	6	*Holophagaceae*

[1]PHE represents phenanthrene; [2]ANT represents anthracene; [3]FLT represents fluoranthene.

Figure

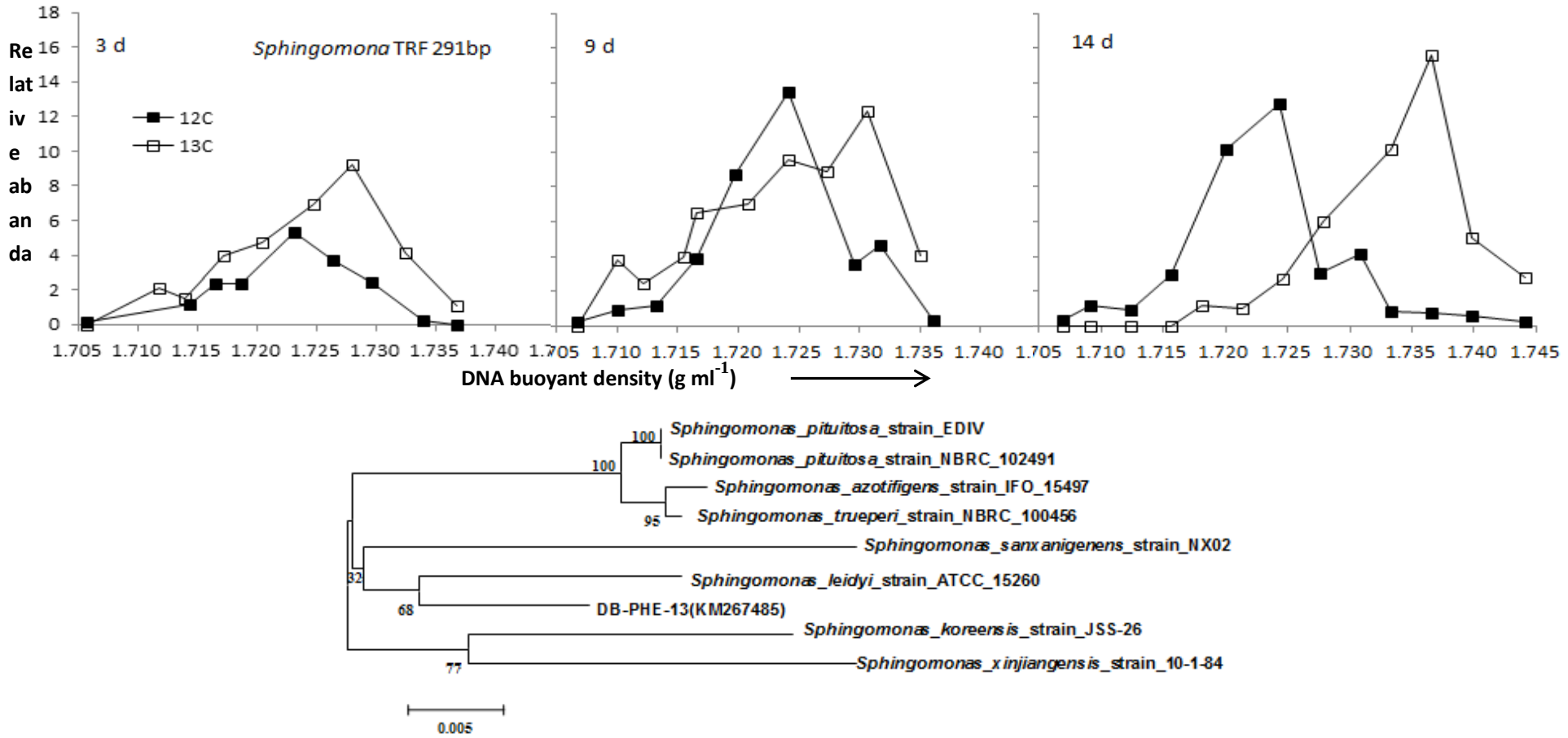


Fig. 1. A: Percent relative abundance of TRF 291-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with phenanthrene (PHE) after 3, 9, 14 days. Figure symbols: □ ^{13}C-PHE; ■ ^{12}C-PHE.B: Phylogenetic tree of the bacteria corresponding 291-bp TRF from microcosms amended with phenanthrene (PHE) along with the closest matches in GenBank, constructed with MEGA 5.0 software using the neighbor-joining method. The tree is based on 16S rRNA sequence and produced by MEGA 5.0.

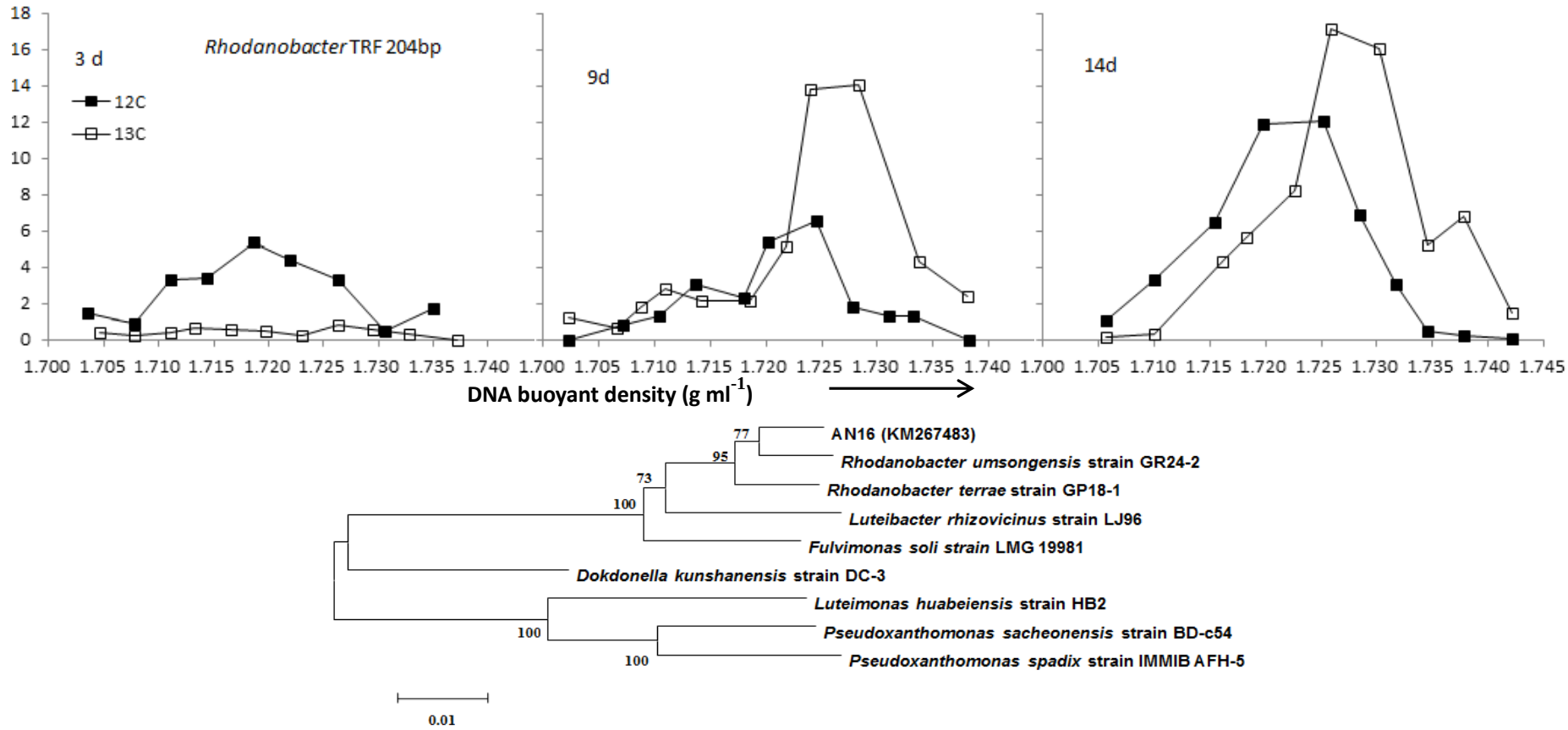
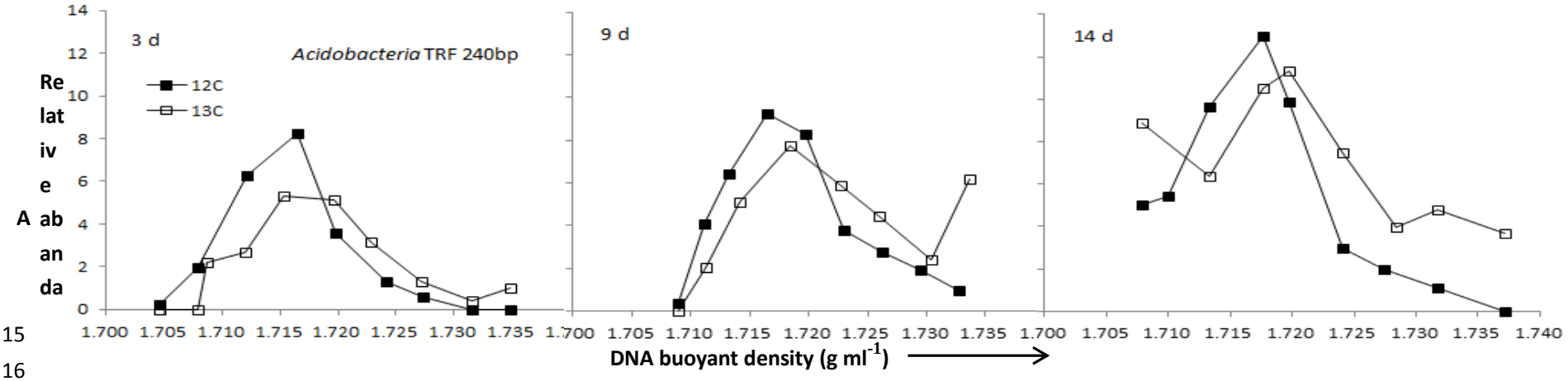


Fig. 2. A: Percent relative abundance of TRF 204-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with anthrancene (ANT) after 3, 9, 14 days. Figure symbols: □ ^{13}C-ANT; ■ ^{12}C-ANT.B: Phylogenetic tree of the bacteria corresponding 204-bp TRF from microcosms amended with anthracene (ANT) along with the closest matches in GenBank, constructed with MEGA 5.0 software using the neighbor-joining method. The tree is based on 16S rRNA sequence and produced by MEGA 5.0.



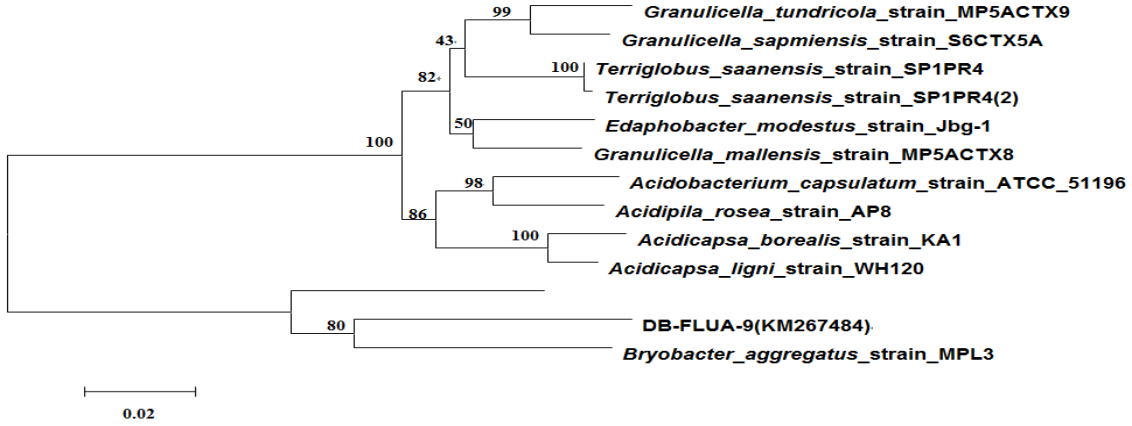


Fig. 3. A: Percent relative abundance of TRF 240-bp fragments (digested by HaeIII) within buoyant density gradients of bacterial DNA extracted from microcosms amended with fluoranthene (FLT) after 3, 9, 14 days. Figure symbols: □ ^{13}C-FLT; ■ ^{12}C-FLT. B: Phylogenetic tree of the bacteria corresponding 240-bp TRF from microcosms amended with fluoranthene (FLT) along with the closest matches in GenBank, constructed with MEGA 5.0 software using the neighbor-joining method. The tree is based on 16S rRNA sequence and produced by MEGA 5.0.