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PAH Degradating Bacterial Communities in Coal Tar Contaminated Soil

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Coal tar is one of the organic pollutants that are most hazardous to human health and are generally difficult to treat. Indigenous microorganisms that are capable of degrading contaminants may play an important role in the removal of pollutants and environmental remediation. Samples were collected from Beijing Coking and Chemistry and investigated for polycyclic aromatic hydrocarbon (PAH) content and microbial community structures at different depth of the drilling sites using 16S rRNA amplification and polymerase chain reaction denaturing gradient gel electrophoresis. The correlation between the distribution of contaminant components and the number of microbes in terms of the depth of the drilling sites were studied. The main contaminants in the site were naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene. The direct count of microbes from 68 samples showed that the microbial distribution varied within each borehole and increased with the PAH contaminant concentration. Microbial concentration ranged between 10^6 and 10^8. Based on the sequencing and comparison of the dominant and special DGGE bands, 14 indigenous prokaryotic microbes in coal tar contaminated sites were identified, and were phylogenetically classified into four classes, Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Actinobacteria. The microbes play an important role in the biodegradation process, and are valuable references for the remediation of contaminated soil.

Key words: coal tar, PAHs, microbial community, PCR-DGGE.

Coal tar is an organic pollutant that is difficult to treat. Various toxic substances are present in coal tar, which includes aromatic hydrocarbons that can quickly enter the sedimentary environment and stay for a long time because of their hydrophobicity and low water solubility. Therefore, aromatic hydrocarbons have strong biological toxicity, especially PAHs (Krauss *et al.*, 2000; Menzie *et al.*, 1992).

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Considering its contaminants are difficult to remove, its special physicochemical properties, and its long residence time, coal tar is harmful to the ecology, crops, and human health in contaminated areas. Thus, removing polycyclic aromatic hydrocarbon (PAH) compounds from the environment quickly and effectively is essential to minimize their adverse effects. Currently, the principal processes for the successful removal are microbial transformation and degradation (Gibson *et al.*, 1975; Knapp *et al.*, 2005; Nicole *et al.*, 2006). Microorganisms in the soil environment are considered indicators of soil pollution. Microorganisms are generally very sensitive and respond rapidly to low pollutant concentrations. Their activity and diversity reflect the quality of the soil. Coal tar in the soil can change the soil

microbial amount, microbial activity, community structure, population components, and microbial diversity. Other indicators can also be considered indicators of environmental quality assessment in contaminated sites. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) reveals the microbial species, numbers, population size, and other information to resolve the community structure, function and dynamics of the samples (Smalla *et al.*, 2007; Christine *et al.*, 2010). Using DGGE profiles based on the 16S rRNA genes, the bacterial communities in soil contaminated with mostly 2-, 3- and 4-ring PAHs produced from coal tar distillation were assessed, and the persistence of a bacterial consortium was determined, as represented by Gram-negative bacterial strains that belong mainly to Gammaproteobacteria, particularly *Pseudomonas* and *Enterobacte*r. These strains have strong PAH-degrading capacities that persist throughout the biotreatment. Thus, the presence of *Pseudomonas* and *Enterobacter* strains in this type of PAH-contaminated soil are good bioindicators for the potential biodegradation of 2-, 3- and 4-ring PAHs. Increasing attention is currently focused the correlation between degrading functions and indigenous bacteria colony effect. Verrhiest *et al*.(Verrhiest *et al*., 2002) show that PAH induces perturbations in the density and the metabolism of microbial communities. Indigenous bacteria may also be used in toxicity assessment.

The present study was conducted in Beijing at a site that had been contaminated with coal tar pollution for a long time. The current research aims to analyze the pollution of the contaminated soil samples at different depths and various locations and to determine the biomass within the 40 m × 25 m area. A PCR-DGGE analysis targeting 16S rRNA gene was used to resolve the space succession process of bacterial communities in the polluted soils. The DGGE bands with the representative DNA were cleaved and sequenced to identify the dominant bacteria. The characterization of the microbial community and the dominant bacterial populations in the polluted soil would provide a basis for improving the biological evaluation and bioremediation of contaminated sites.

MATERIALS AND METHODS

Soil sampling

A 40 m × 25 m area of the site was selected to determine the contamination, pollutant type, and extent of pollution. Six holes with an average depth of 12.74 m were drilled. Samples of 68 soils were collected at different vertical depths on the subsurface at the frequency of one sample per 1 m.

Analysis of PAHs

The freeze-dried soil samples were extracted and cleaned followed Method 3541 (automated Soxhlet extraction), Method 3630C (silica gel cleanup) recommended by the US EPA (1996).

The PAH composition was analyzed using gas chromatography-mass spectrometry (Therm Finnigan, USA) equipped with a DB-5 chromatographic column (0.25 mm × 30 m × 0.25 μm).

While PAH concentrations were analyzed using high-performance liquid chromatography (HPLC) (DIONEX, USA) with UV detection at 254 nm.

Counting of microorganisms

In the present study, dichloro-triazinylamino fluorescein (DTAF) fluorescence microscopy was used to count directly the number of microorganisms in the 68 soil samples (Bloem *et al*., 2004).

Total DNA extraction

The total community DNA was extracted from 68 soil samples using an Omega Soil DNA Kit (Omega Bio-tek, USA). DNA was extracted from 500 mg of soil according to the manufacturer's instructions. The DNA concentrations in the extracts were measured spectroscopically and were adjusted for PCR amplification.

Nested PCR amplification of 16S rRNA gene

The first PCR amplification: The bacterial 16S rRNA was amplified using primers 8f and 1492r. Initial denaturation was performed at 94 °C for 10 min. Amplification was carried out using 25 cycles including denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and DNA extension at 72 °C for 90 s, with final extension at 4 °C for 10 min. Then, 5 μL of the PCR product was electrophoresed in 1.5% agarose gel at 120 V for 40 min. The gels were then stained with 0.5 mg/L ethidium bromide solution for 20 min and destained in 1× Tris–acetate–EDTA

(TAE) buffer for 15 min. After these procedures, the samples were photographed.

The second PCR amplification: The PCR products of the first PCR amplification were amplified using primers GC_341f and 907r. The second PCR amplification used a touchdown PCR program (Muyzer *et al.*, 2004). Initial denaturation was performed at 94 °C for 5 min. Amplification was carried out using 10 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 60 s and DNA extension at 72 °C for 90 s. The annealing temperature of the reaction was decreased by 1 °C every cycle from 65 °C to a 'touchdown' at 55 °C. This was followed by 15 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 60 s, and DNA extension at 72 °C for 90 s. Further DNA extension was conducted at 72 °C for 90 s, with a final extension at 4 °C for 10 min. Then, 5 µL of the PCR product was run in 1.5% agarose gel at 120 V for 40 min. The gels were then stained with 0.5 mg L^{-1} ethidium bromide solution for 20 min, and destained in 1× TAE buffer for 15 min. The gel was then photographed.

Denaturing Gradient Gel Electrophoresis

DGGE was performed using the Dcode™ Universal Mutation Detection System (BioRad, USA). The PCR products were electrophoresed directly on 6% polyacrylamide gel in 1× TAE buffer containing a linear gradient ranging from 30% to 60% denaturant (Hendrickx *et al.*, 2006; Pickup *et al.*, 2001). Electrophoresis was run at 60 °C with a constant voltage of 80 V. After 16 h of electrophoresis, the gel was stained with $AgNO_3$ (Cairns *et al*., 1994), and then was visualized using a scanner (VILBER INFINITY3000, France).

DGGE finger-print, sequencing and phylogenetic analysis

Prominent and representative DGGE bands were excised from DGGE polyacrylamide gel for 16S rRNA fragment sequencing. DNA sequences were compared to GenBank for a similarity analysis using the Advanced Blast Search program (GenBank, NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1990). The distributions of prokaryotic microbial populations at different vertical depths in the underground environment were analyzed. A distance-based evolutionary tree was constructed using Jukes and Cantor's corrected similarity values with the neighbor-joining algorithm of Saitou and Nei (Saitou *et al*., 1987) to study the classified and the evolutionary relationships of major prokaryotic microbial populations that existed in the coal tar contamination of the underground environment.

RESULTS AND DISCUSSION

PAHs composition and concentration

Monitoring of PAHs composition

Qualitative analysis of coal tar in the soil samples was performed by GC-MS. Therefore, the target pollutants in the study of coal tar contaminated sites were naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene of the PAHs.

Monitoring of PAHs concentration

The naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene in soil samples were quantitatively analyzed using HPLC. The concentrations of the six PAHs at each sampling point (ZK1–ZK6) were summed up and the results are shown in Figures 1a-1f.

The concentrations of the six PAH pollutants in the six drill samples indicated that the ZK3 sample was the least contaminated whereas the ZK5 sample was the most contaminated. Of the 68 sampling points, the minimum total concentration of the six PAH pollutants was 0.09 mg/kg, which was observed in the 4.1 m deep ZK1 sample. The maximum total PAH concentration was 923.90 mg/kg, which was observed in the 2.3 m deep ZK5 sample. In summary, the vertical distribution of the total PAH concentration among the six drillings did not exhibit any pattern. However, the vertical distribution of the total PAH concentration within each drilling exhibited its own pattern.

Analysis of environmental microbial quantities

Figure 1a to 1f show the direct count of microbes from the 68 samples from ZK1 to ZK6. The results show that the microbial distribution varied in each borehole and increased with the contaminant concentration. Microbial concentration ranged between 10^6 and 10^8. The minimum concentration was 8.90×10^6 CFU/g, which was observed at a depth of 4.10 m in ZK1, whereas the maximum concentration, 1.422×10^8 CFU/g, was observed at a depth of 6.80 m in ZK2. This distribution may have resulted from the

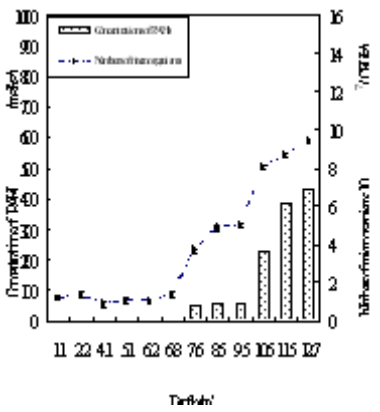

Fig. 2(a). ZK1

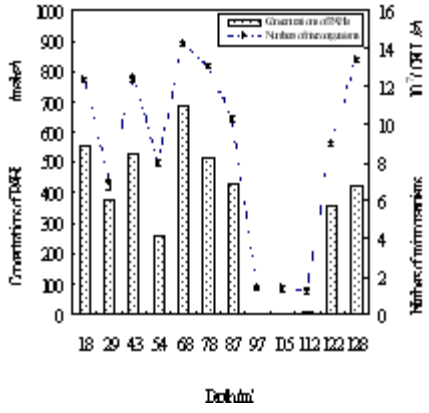

Fig. 2(b). ZK2

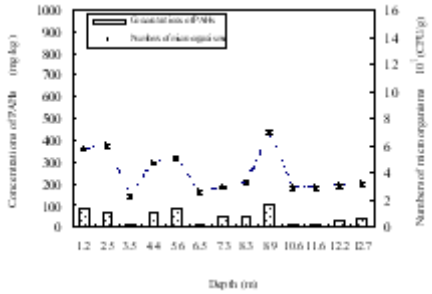

Fig. 2(d). ZK4

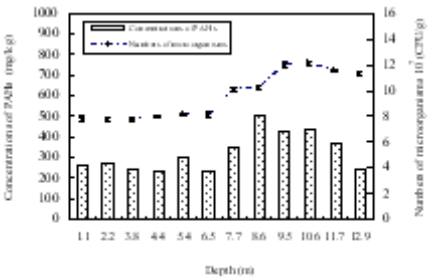

Fig. 2(c). ZK3

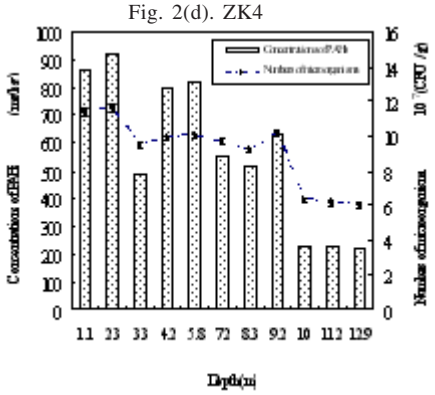

Fig. 2(e). ZK5

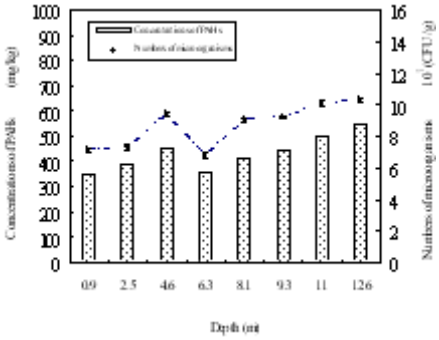

Fig. 2(f). ZK6

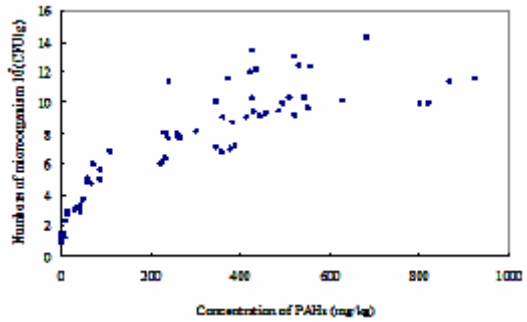

Fig. 2(g). ZK7

Fig. 2. Relationship between the quantities of microorganisms and the pollutant concentrations at different sampling points

prolonged exposure of the sites to coal tar pollution, and the indigenous microorganisms are able to survive after natural selection under the selective pressure of the coal tar-contaminated environment. The microorganisms in this site survived well and were well adapted to the coal tar, which suggests that the number of indigenous microorganisms in the local underground environment may be related to the coal tar pollutants.

To determine whether the distribution of pollutant concentrations is correlated with the

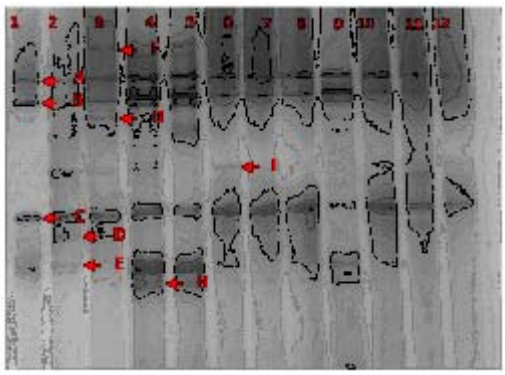

Fig. 3(a). DGGE fingerprint of prokaryotic microorganisms in ZK1

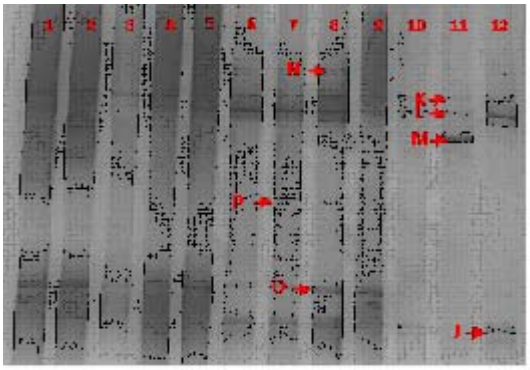

Fig. 3(b). DGGE fingerprint of prokaryotic microorganisms in ZK2

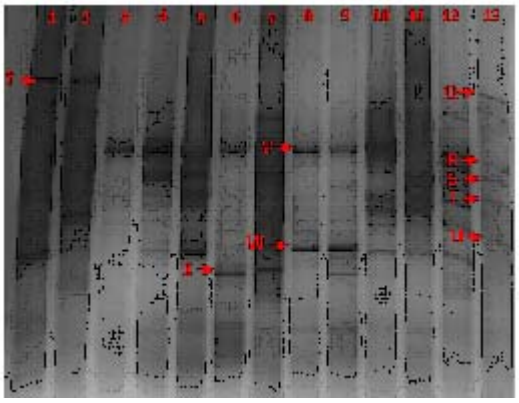

Fig. 3(c). DGGE fingerprint of prokaryotic microorganisms in ZK3

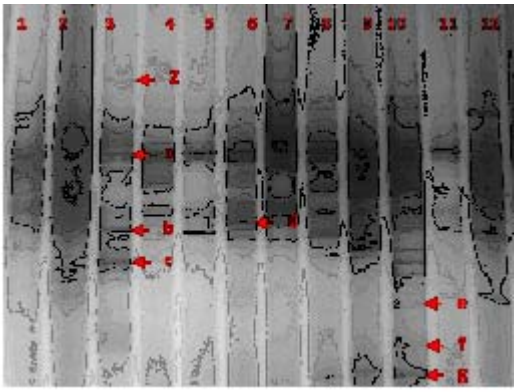

Fig. 3(d). DGGE fingerprint of prokaryotic microorganisms in ZK4

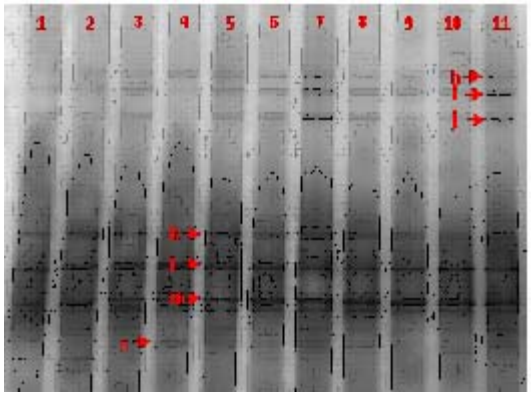

Fig. 3(e). DGGE fingerprint of prokaryotic microorganisms in ZK5

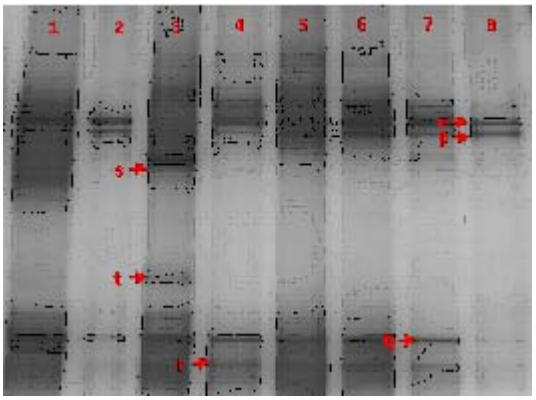

Fig. 3(f). DGGE fingerprint of prokaryotic microorganisms in ZK6

number of microorganisms in the underground environment, the number of microorganisms in each drilling and the distribution of the total PAH concentration in terms of vertical depth were compared (Figures 1a–1f).

The distribution of microorganisms at different depths within the same drilling was clearly correlated with the pollutant concentration. The numbers of microorganisms increased with increasing pollutant concentrations. The maintenance of adequate microbial biomass in the soil, with a high microbial activity, could have increased soil resistance to degradation factors and are thus of paramount importance to ecosystem sustainability. The growth trend of the microorganisms increased as the PAH concentration increased (Fig.2), which is consistent with the findings by Mlynarz and Ward (Mlynarz *et al*.,1995) and Davis *et al.* (Davis *et al*.,1998). The results of the present study show that the characteristics of the distribution of microorganisms in contaminated soil are important biological factors that affect the natural attenuation and biological degradation of organic coal tar pollutants. The selection of coal tar contamination on soil microorganisms enhances the adaptability of microorganisms and their capacity for degrading PAHs, as well as increasing the number of indigenous PAH-degrading bacteria.

DGGE fingerprint analysis of prokaryotic microorganisms

The DGGE fingerprints of prokaryotic microorganisms in ZK1 to ZK6 are shown in Figures 3a-3f.

The structure of the prokaryotic microbial community changed with the depth at every point in ZK1 and ZK5, but the differences were not significant. Prokaryotic microbial community structure changed significantly with the depth at every point in ZK2, ZK3, ZK4, and ZK6. Bands A, B, and C were commonly owned by 12 lanes in ZK1. Band M was unique in lane 11 (12.20 m) and band P occurred in lanes 7 and 8 (8.70 m to 9.70 m) in ZK2.

In ZK1 to ZK6, the structure of the prokaryotic microbial community changed slightly with the depth of the site. The results show that the species composition of the prokaryotic microbial community changed which are related with the changes of chemical compounds in different depth of the site.

Prokaryotic microorganisms

The monitoring and the analysis of the sequence advantages and the specific bands in the DGGE fingerprints show that 14 indigenous prokaryotic microorganisms were found in the underground environment of pollution site, namely *Bacillus* sp. EPI-R1 (*Bacillus* genus EPI-R1 strains), *Bacillus* cereus 03BB102, *Bacillus thuringiensis (Bacillus thuringiensis*), *Bacillus* sp. SA, Ant14 *(Bacillus* SA Ant14 strains), *Bacillus weihenstephanensis* KBAB4 (*Wechsler Bacillus* KBAB4), Uncultured *Bacillus* sp. (uncultured *Bacillus* certain bacteria), and *Ralstonia pickettii* (*Petri Ralston* bacteria), *Nocardioides* sp. (*Nocardia* certain bacteria), *Azoarcus* sp. BH72 (nitrogen-fixing *Vibrio* genus

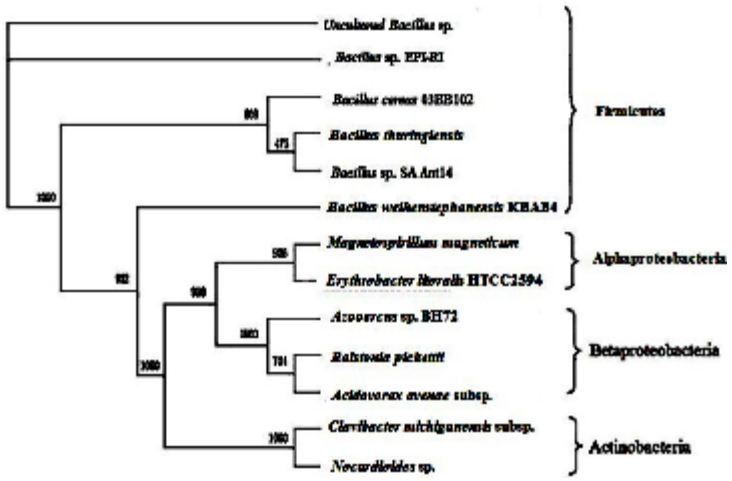

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences of the prokaryotic microorganisms. The tree was tested for branching order confidence by maximum-parsimony analysis and a round of 100 bootstraps

BH72 strain), *Erythrobacter litoralis* HTCC2594 (*Erythrobacter* genus HTCC2594 strain), *Magnetospirillum magneticum* (*Acidovorax Willems* genus magnetotactic spiral strains), *Acidovorax avenae* subsp. (food acid subspecies of the genus avenae strain), *Clavibacter michiganensis* subsp. (subspecies of *Michigan* stick rod-shaped bacteria) and an unknown new species had not been reported in GenBank.

In the current research, the existing bacteria in the underground environment of contaminated sites can be classified into four groups[1]. *Bacillus cereus* had an absolute advantage and was found at different depth of the drilling. *Bacillus* sp. EPI-R1; *Bacillus cereus* 03BB102; *Bacillus thuringiensis*; *Bacillus* sp. SA, Ant14; and *Bacillus weihenstephanensis* KBAB4 were also present at various depths in all locations. Thus, these five *Bacillus* strains share a common living space. However, one *Bacillus* strain, designated as uncultured *Bacillus* sp., was different from the other strains. These bacteria survived at depths ranging from 6.30 m to 12.90 m. The uncultured *Bacillus* sp. was not grown in the laboratory; thus, it is anaerobic. (2) *Actinobacteria*, *Nocardioides*, and *Clavibacter* were the dominant Actinobacteria in the contaminated environment. *Nocardioides* sp. was widely distributed at different depths in ZK1, ZK3, and ZK5. *Clavibacter michiganensis* subsp. was widely distributed at different depths in ZK4 and ZK6. *Nocardioides* sp. and *Clavibacter michiganensis* subsp. were widely distributed at the vertical depth from the surface to the deepest. These results indicate that these two actinomycetes have highly viability and they could live in both aerobic and anaerobic environments. (3) Alphaproteobacteria, *Magnetospirillum*, and Alphaproteobacteria, *Erythrobacter* were the dominant bacteria at depths ranging from 12.20 m to 12.70 m in ZK3. *Erythrobacter litoralis* HTCC2594 and *Magnetospirillum magneticum* were present in the aqueous medium near the groundwater level, which indicates that these two Alphaproteobacteria are anaerobic. (4) *Azoarcus* sp. BH72 was widely distributed at different depths in ZK1, ZK2, ZK4, and ZK6. *Ralstonia pickettii* was widely distributed at different depths in ZK1, ZK2, and ZK6. *Acidovorax avenae* subsp. was widely distributed at different depth in ZK3 and ZK4. Overall, the three bacteria were widely present within the entire depth distribution of the contaminated sites, which indicates that these three Betaproteobacteria could survive under both aerobic and anaerobic conditions.

Phylogenetic tree based on 16S rDNA gene sequences of the prokaryotic microorganisms is shown in Fig. 4. The phylogenetic analysis indicates that the dominant prokaryotic microbes could be classified into 4 classes: Firmicutes, Alphaproteobacteria, Betaproteobacteria, and Actinobacteria. The genetic relationship was associated with the phylogenetic distance. The 14 bacteria were the dominant groups monitored from the long-term PAH pollution. The physiologic functions of these bacteria were related with PAH degradation. Degradation fragments of PAHs always appeared in the degradative plasmids, which could be transposed between the dominant flora. These groups played a specific role in the symbiotic system of degradation of PAHs. The characteristics of the degradation of PAHs of the strains had not been analyzed in depth because of the difficulty in obtaining pure cultured strains. However, the information of the community structure obtained in the current experiment provided valuable basis for enhanced bioremediation of the contaminated sites.

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