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ORIGINAL PAPER

Microbial functional genes reveal selection of microbial community by PAHs in polluted soils

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Abstract This report shows an increase of PAH-related microbial functional genes with PAH concentration in soils. Adaptation of microbial communities to organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) is a crucial issue. However, there is little knowledge on mechanisms ruling microbial community selection. Here, we studied microbial functional genes in soils contaminated by PAHs. We used GeoChip, an advanced functional gene array for gene analysis. Soil PAH concentrations were measured and microbial functional genes were categorized. PAH-related microbial functional genes, *bph, nah, nidA, phd, dfb*, and *qor*, were quantitatively expressed. Total microbial functional genes and PAH-related microbial functional genes were compared with PAH concentration by cluster analysis and curve-fitting analysis. We found that the average abundance of PAH-related microbial functional genes increased from 0.13 to 0.33, whereas that of total microbial functional genes decreased from 0.22 to 0.10 when PAHs concentration increased from 1.01 to 164.28 mg kg^{-1}. It was also found that the classification of microbial community structure characteristics based on PAH-related microbial functional genes was closely similar to the classification based on PAHs concentration. Findings reveal that PAH stress promotes the dominance of PAH-related microbial communities.

Keywords Polycyclic aromatic hydrocarbons · Microbial functional genes · Environmental stress · Microarray · GeoChip · Microbial community selection

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of persistent organic pollutants that have been extensively found in petrochemical-related sites in China (Mai et al. 2001; Guo et al. 2007; Liu et al. 2010a, b). Due to their carcinogenic properties, PAHs are of great concern. During the past few years, the attention of their environmental behavior has been positively received. The natural attenuation of PAHs is critically dependent on indigenous microbial communities. Therefore, the microbial response is regarded as a considerable factor for site assessment and prediction.

Due to the rapid development of molecular technologies, the knowledge of microbial communities under stressful condition has been well advanced. Microbial functional genes are prevailing alternatives to elaborate the link between phylogenetic diversity and physiological functions of microbial communities. Functional gene array is a helpful tool using specific probes targeting sequences unique to genes within families of interest, which provides a novel way to reveal the characteristics of contaminated

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site. GeoChip, a robust functional gene array, was developed to pursue a high-throughput and powerful genomic technology for investigating biogeochemical, ecological, and environmental processes (He et al. 2007; Zhou et al. 2008; He et al. 2010). In principle, functional gene array contains probes for corresponding gene encoding enzymes of specific function, which would allow the simultaneous determination of numerous genes at one time. Functional gene array provides information regarding the potential functional capabilities of microbial communities and higher taxonomic understanding at the species level.

Past studies have applied GeoChip in several areas, such as oxidation and biodegradation of crude oil (Liang et al. 2010a, b) uranium bioreduced region (Van Nostrand et al. 2009; Wu et al. 2010; Xu et al. 2010), heavy metal-contaminated groundwater (Hemme et al. 2010), hydrogen-producing bioreactor (Huang et al. 2010), microbial electrolysis (Liu et al. 2010a, b), etc. However, comprehensive information regarding indigenous microbial functional diversity on PAHs-contaminated site is rarely reported.

The objective of this study is to explore the relationship between microbial functional genes and PAHs in a typical contaminated site. Based on collected information, the mechanism is due to be revealed involving the response of microorganisms to contamination.

Experimental

Soil samples analysis

Nine soil samples, labeled as DH01, DH02, DH03, DH04, DH05, DH06, DH07, DH08, and DH09, were obtained from 3 different locations (spot 1: N38°57′20″, E121°36′08″; spot 2:N38°57′43″, E121°37′03″; and spot 3: N38°57′13″, E121°36′00″) in the depth range of 0–1, 1–2, 2–3m, respectively, at a typical PAHs-contaminated site in Dalian, Liaoning Province, China, where a group of petrochemical plants was located previously. The soil in this place was of clay loam texture and with moisture content between 6 and 10 % (w/w). All the samples were sealed and stored in case filled with dry ice during delivery to the laboratory. The soil samples were divided into two parallel parts for the analysis of total PAHs concentration and microbial functional genes, respectively.

The total PAHs concentration was calculated as the sum of 16 PAHs including naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]flouranthene, benzo[a]pyrene, dibenz[ah]anthracene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene.

The soil samples were pretreated in 200 mL sample vials by mixing with carbon disulfide according to the ratio of 1:1 (w/w). All the sample vials were sealed and placed in a bioshaker at a speed of 100 rpm overnight. Then, the mixtures were let stand, and the supernatant was syringed for quantitative analysis by gas chromatograph with flame ionization detector (GC–FID) device with detective limit of 0.01 mg kg^{-1}. The standard curve was calibrated using a working standard mixture of 16 PAHs (Sigma Aldrich kit) at a series of diluted concentration gradient. This method was applied based on the comparison with common method in the previous study (Zhang et al. 2010).

GeoChip analysis

The microbial community genomic DNA was extracted from soil samples according to the method described elsewhere (Hurt et al. 2001). Briefly, the soil samples were well mixed by combining freeze grinding and sodium dodecyl sulfate for cell lysis. The DNA was purified by agarose gel electrophoresis, followed by phenol−chloroform−butanol extraction. The purified DNA was quantified by an ND-1000 spectrophotometer (Nanodrop Inc., USA). One hundred ng of purified DNA was amplified in triplicate using the TempliPhi kit (Amersham Biosciences, USA) in a modified buffer containing single-strand-binding protein at concentration level of 200 ng μL^{-1} and spermidine at 0.04 mM to increase the sensitivity of amplification and was incubated at 30 °C for 3 h (Liang et al. 2010a, b). The amplified DNA was denatured and fluorescently labeled and purified (Wu et al. 2008). The labeled products were dried and resuspended in 130 μL hybridization solution containing 50 % formamide, 3× saline-sodium citrate, 0.3 % sodium dodecyl sulfate, 0.7 μg μL^{-1} herring sperm DNA, 0.02 mM dithiothreitol, and water. Hybridization to GeoChip3.0 was performed on an HS4800 Hybridization Station (Tecan US, USA) in triplicate at 42 °C for 10 h (He et al. 2010). Microarray analysis was performed on ScanArray 5000 (PerkinElmer, USA) at 95 % laser power and 68 % photomultiplier tube gain.

Data process

The scanned image was processed using ImaGene (BioDiscovery, USA), and raw data were obtained and upload to the data analysis pipeline in the microarray data manager (link: http://ieg.ou.edu/microarray/) for normalization and analysis according to the following steps. Spots flagged as poor quality and with a signal-to-noise ratio of <2.0 were removed. The signal-to-noise ratio was expressed as signal intensity minus background and then divided by standard deviation of background. The normalized intensity of each spot was calculated by dividing the signal intensity of each spot by the mean intensity of the effective spots of the

array. Any replicate with its signal intensity minus mean signal intensity of more than two times the standard deviation was removed as an outlier. Meanwhile, at least two spots out of three replicates were required for the positive identification of corresponding gene.

The average abundance was calculated using the average value of effective signal data set with normalized signal intensity of corresponding microbial functional genes (He et al. 2010). The PAHs-related microbial functional genes were chosen for cluster analysis and statistical seriation on a free software named PermutMatrix (Caraux and Pinloche 2005). The cluster of PAHs was executed by comparing the similarity of component formation as well as their concentrations. The dissimilarity of Euclidean distance was calculated according to the linkage rule of McQuitty's method and seriation rule of multiple-fragment heuristic method.

Results and discussion

The goal of this study was to reveal the relationship between microbial functional genes and PAHs concentration and explain the mechanism involving in microbial community selection by PAHs in contaminated soils. PAHs concentration of each soil sample was analyzed and the microbial functional genes were detected and categorized. Total microbial functional genes and PAHs-related microbial functional genes were compared with PAHs concentration by cluster analysis and curve-fitting analysis.

PAHs concentration

The PAHs concentration of each soil sample was analyzed. The result of PAHs concentration of each soil sample is shown in Table 1. The components of 16 PAHs are categorized into 2,3,4,5,6-ring, respectively.

Microbial functional genes

The detected microbial functional genes were divided into the following categories: carbon cycle, nitrogen cycle, phosphorus cycle, metal reduction, organic degradation, energy transfer, and others (He et al. 2007). In the category of organic degradation, there were types of aromatic, chlorinated, hydrocarbons, pesticides, herbicides, and others, while aromatic carboxylic acid, 'benzene, toluene, ethylbenzene, and xylenes' (BTEX), PAHs, heterocyclic aromatic hydrocarbons, chlorinated aromatic hydrocarbons, and others were child categories of aromatic type. Among all the soil samples, the detected microbial functional genes were shown in Table 2.

The results showed that 7,714 individual microbial functional genes were discovered with average abundance of 0.19. Although PAHs-related microbial functional genes only possessed 1.36 % of total microbial functional genes, the average abundance of PAHs-related microbial functional genes was relatively high, which was 0.23. This implied that the environmental stress of PAHs on microorganisms created superior circumstance for the growth of PAHs-related microorganisms.

The PAHs-related microbial functional genes included 6 kinds: *bph, nah, nidA, phd, dfb*, and *qor*. These typical microbial functional genes have been discovered to play important roles in the metabolism pathway of PAHs, whose details could be found in KEGG database (http://www.genome.jp/kegg/).

Table 2 also showed the information of their proportion to total PAHs-related microbial functional genes and their average abundance. The results showed that *bph* was of the greatest proportion (58.09 %), while *nidA* was of the highest average abundance (0.34) among all the genes. The

Table 1 PAHs concentration detected in soil samples

Location	DH01 Spot 1	DH02 Spot 1	DH03 Spot 1	DH04 Spot 2	DH05 Spot 2	DH06 Spot 2	DH07 Spot 3	DH08 Spot 3	DH09 Spot 3
Depth (m)	0–1	1–2	2–3	0–1	1–2	2–3	0–1	1–2	2–3
2-ring (%)	7.73	4.80	4.04	5.67	9.40	3.78	13.86	8.88	23.40
3-ring (%)	27.64	33.47	39.90	31.29	34.29	41.50	80.20	30.84	21.00
4-ring (%)	43.33	38.41	38.83	40.94	34.24	37.85	2.97	36.45	33.21
5-ring (%)	16.19	17.77	13.53	17.73	19.30	12.70	1.98	18.69	13.59
6-ring (%)	5.11	5.55	3.70	4.37	2.77	4.17	0.99	5.14	8.80
Total PAHs (mg kg^{-1})	13.71	21.27	14.86	164.82	19.48	15.35	1.01	2.14	12.95

Note that DH04 was attributed to high contamination (164.82 mg kg^{-1}); DH07 and DH08 were attributed to low contamination (1.01 and 2.14 mg kg^{-1}, respectively); meanwhile, DH01, DH02, DH03, DH05, DH06, and DH09 were attributed to moderate contamination (13.71, 21.27, 14.86, 19.48, 15.35, and 12.95 mg kg^{-1}, respectively). Three- and four-ring PAHs were the main components. This showed the spatial difference between the soil samples. DH was the code for the soil sample identifier

PAHs polycyclic aromatic hydrocarbons

Table 2 Microbial functional genes of soil samples

	Total number	Percentage of total genes	Percentage of PAHs-related genes	Average abundance
Total microbial functional genes	7,714	100.00	–	0.19
Subcategory: organic degradation	3,231	41.88	–	0.19
Sub-subcategory: aromatic	2,425	31.43	–	0.19
Sub-subcategory: PAHs	105	1.36	100.00	0.23
bph	61	0.79	58.09	0.22
nah	13	0.17	12.38	0.24
nidA	3	0.04	2.86	0.34
phd	25	0.32	23.81	0.24
dfb	2	0.03	1.90	0.26
qor	1	0.01	0.95	0.15

Note that the PAHs-related microbial functional genes occupied 1.36 % of total genes but with relatively high average abundance of 0.23. The trend indicated the superiority of PAHs-related microbial functional genes. Six typical PAHs-related functional genes, *bph, nah, nidA, phd, dfb,* and *qor*, were compared. Note that *nidA* was of the highest average abundance (0.34) among all the genes. The result revealed the dominant existence of microorganisms with *nidA* responsible for the degradation of PAHs

PAHs polycyclic aromatic hydrocarbons

nidA gene is responsible for the synthesis of large subunit of PAH dioxygenase during degradation of PAHs such as phenanthrene, pyrene, benzo[a]pyrene, etc. (Pagnout et al. 2007). The dioxygenase produced by *nidA* is regarded to be crucial in the degradation pathway with the role of promoter in initializing the metabolism of PAHs. The other genes (*bph, nah, phd, dfb*, and *qor*) are mainly responsible for 2,3-dihydroxybiphenyl 1,2-dioxygenase (Taguchi et al. 2004), naphthalene 1,2-dioxygenase (McLeod et al. 2006), hydratase–aldolase (Saito et al. 2000), angular dioxygenase (Schuler et al. 2008),and putative quinoline 2-oxidoreductase (Pohlmann et al. 2006), respectively. These enzymes were considered as significant for certain steps of metabolism of PAHs. The discovery of these genes also implied the potential existence of typical PAHs-degrading bacteria. The result of average abundance indicated that *nidA* was the most abundant PAHs-related microbial functional genes. This could be ascribed to dominant existence of microorganisms with *nidA* responsible for the degradation of PAHs.

Comparison of microbial functional genes and PAHs concentration

The cluster analysis and curve-fitting analysis were applied for the comparison between total microbial functional genes/PAHs-related microbial functional genes and PAHs concentration. Figure 1 showed the cluster results of PAHs-related microbial functional genes and the concentration of PAHs between different soil samples. The similarity of the structure of microbial functional genes was displayed highly consistence along with that of the concentration of PAHs. Among all the soil samples, DH04 was attributed to high contamination; DH07 and DH08 were attributed to low contamination; meanwhile, DH01, DH02, DH03, DH05, DH06 and DH09 were attributed to moderate contamination. The microbial community responsible for the degradation of PAHs was likely to be consisted of *Rodococcus* sp., *Mycobacterium* sp., *Burkholderia* sp., *Pseudomonas* sp., *Nocardioides* sp., etc., as identified by GeoChip (He et al. 2010).

The total microbial functional genes and PAHs-related microbial functional genes were compared with the concentration of PAHs by curve-fitting method. The results (Fig. 2) showed that the average abundance of PAHs-related microbial functional genes increased from 0.13 to 0.33; meanwhile, the average abundance of total microbial functional genes decreased from 0.22 to 0.10 when PAHs concentration increased from 1.01 to 164.28 mg kg^{-1}.

This indicated that environmental stress created by contamination would make negative impact on the microbial communities, or the higher the contamination was, the less the microbial diversity was represented. However, the response of PAHs-related microorganisms was positive with the corresponding contamination, or the higher the contamination was, the more actively the specific microorganisms act.

This provided evidence that specific microorganisms would survive and grow well under corresponding environmental stress. As little information of microbial functional genes in PAHs-contaminated site has been reported, the results could contribute to the knowledge of microbial process in PAHs-contaminated site. Besides, this is an important application of GeoChip and would extend the understanding of mechanism involving in the natural attenuation, self-cleaning, and emergency response of

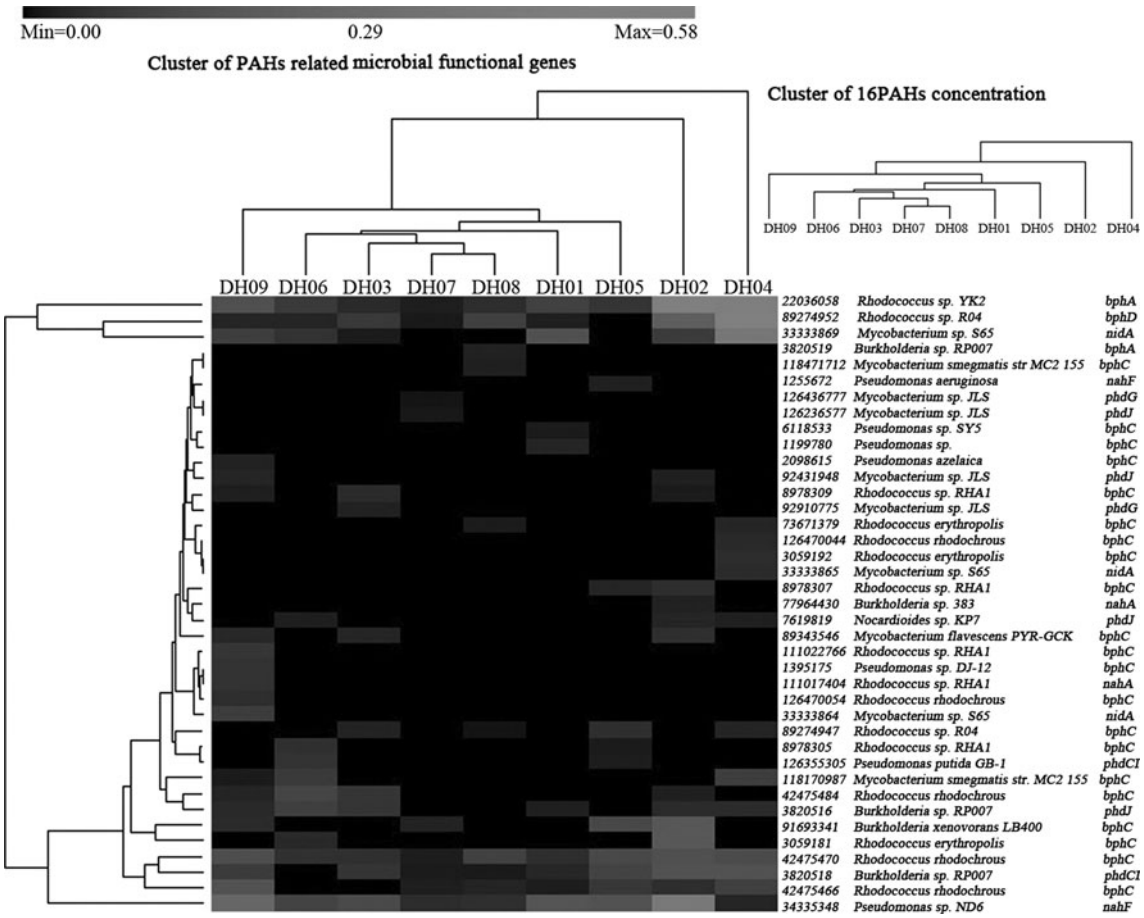

Fig. 1 Cluster result of PAHs-related microbial functional genes and 16 PAHs concentration. Euclidean distance calculation with the linkage rule of McQuitty's method and seriation rule of multiple-fragment heuristic method was applied. Note the similarity between PAHs-related microbial functional genes and 16 PAHs concentration, which indicated that PAHs played an important role in microbial community selection. *PAHs* polycyclic aromatic hydrocarbons

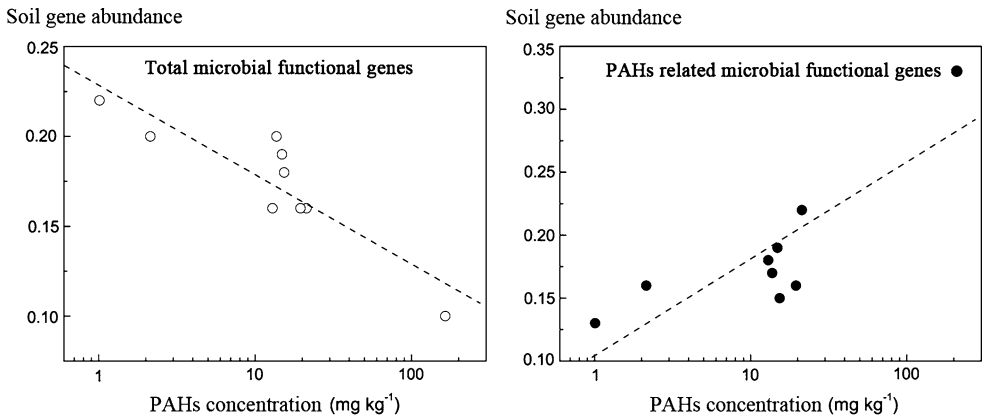

Fig. 2 Total microbial functional genes (*left*) and PAHs-related microbial functional genes (*right*) versus PAHs concentration. Note that the average abundance of PAHs-related microbial functional genes increased; meanwhile, the average abundance of total microbial functional genes decreased when PAHs concentration increased. The equations of curve-fitting result were $y = -0.05 \log x + 0.23$ with $r^2 = 0.7929$, and $y = 0.08 \log x + 0.10$ with $r^2 = 0.6658$, respectively. This trend showed a selection of microbial functional genes by PAHs concentration. *PAHs* polycyclic aromatic hydrocarbons

microecology, etc., which could help improve the site remediation strategy.

Conclusion

Little knowledge has been reported in the past few years about the impact of PAHs in contaminated soils on microbial community selection with detailed microbial information. Here, we demonstrated the intensive relationship between microbial functional genes and PAHs concentration with detailed information of GeoChip results. In summary, the environmental stress effect of PAHs selectively promoted the dominance of PAHs-related microbial communities. The results could refresh the current knowledge of microbial mechanism involving in PAHs-contaminated site.

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