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Reconstructed metagenomes reveal changes of microbial functional profiling during PAHs degradation along a rice (*Oryza sativa*) rhizosphere gradient

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Abstract

Aims: The response of microbial metagenome to polycyclic aromatic hydrocarbons (PAHs) degradation in the rice rhizosphere remains poorly understood. We investigated the spatial and temporal variations of microbial communities and reconstructed metagenomes along the rice rhizosphere gradient during PAHs degradation.

Methods and Results: The experiment was performed in rhizoboxes, in which the rhizosphere region was divided into five 1-mm thick layers. Based on denaturant gradient gel electrophoresis profiling and sequencing of bacterial and archaeal 16S rRNA genes, predicted metagenomes were reconstructed. The microbial communities in the rice rhizosphere were influenced by the PAHs concentration and distance from the root surface during PAHs degradation. Correlation network analysis showed that archaea played an important role in PAHs degradation. Predicted metagenomes can be clustered into two groups with high and low PAHs degrading potential, respectively. The relative abundance of genes for defense mechanisms, replication, recombination and reparation was significantly higher in samples with high PAHs degrading potentials. The relative abundance of the dioxygenase gene was greater near the root surface of the rice. However, the abundance of aldolase and dehydrogenase was constant in rhizosphere soils at different distances from the root surface.

Conclusions: Distance from root surface and PAH concentrations affected the microbial communities and metagenomes in rice rhizosphere. The abundance of dioxygenase genes relating to PAH degradation in metagenomes mirrored the PAH degradation potential in rice rhizosphere.

Significance and Impact of the Study: Our findings suggested that the predicted metagenomes reconstructed from 16S rRNA marker gene sequences provide further insights into the spatial variation and dynamics of microbial functioning that occur during bioremediation.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) enter the environment through anthropogenic activities, e.g. as industrial byproducts and incomplete combustion (Zhang and Tao 2009). Most PAHs compounds have strong mutagenic and carcinogenic properties (Nisbet and LaGoy 1992). Because of the high partition coefficients of PAHs between soil and the atmosphere, a high percentage of PAHs in the environment tends to be adsorbed by soils (Johnson *et al.* 2005). Anthropogenic PAHs in contaminated soils pose a significant risk to the environment and human health in terrestrial ecosystems (Nadal *et al.* 2004), and PAH-polluted-soils require effective

remediation. Bioremediation, which has potential advantages over physical or chemical remediation approaches, has the ability for the complete degradation of the pollutants, with low cost, low risk and little soil disturbance (Mackova *et al.* 2006). The rice ecosystem is one of the most widely distributed agronomic ecosystems in the world, occupying about 150 million ha worldwide, equivalent to about 20 percent of agricultural land (Lobell and Field 2007). The phytoremediation potential of the rice ecosystem for organic pollutants has received close attention recently (Su and Zhu 2008).

Plants have been widely reported to enhance the degradation of PAHs in the rhizosphere (Ma *et al.* 2010). The stimulatory effects of plants on PAHs degradation depend upon the interactions between plant root activities and microbial metabolism in the rhizosphere (Mackova *et al.* 2006). The main reason for rhizosphere formation is the environmental gradient formed due to the release of organic acids in the form of root exudates from roots and the absorption of mineral nutrients (Joner *et al.* 2002). The root exudates have the potential to enhance the degradation of PAHs by promoting the growth of soil micro-organisms (Yoshitomi and Shann 2001). Root exudates can also increase the solubility and bioavailability of PAHs in soil (Gao *et al.* 2010), and enhance the PAH biodegradation rate with micro-organisms. As the components of root exudates can vary between plant species or genotypes, the microbial communities in the rhizosphere of different plant species vary (Jones and Darrah 1994).

There is a redox gradient in the rhizosphere caused by radial oxygen loss from the root surface (Wu *et al.* 2012). The most important factors that influence PAHs degradation, microbial communities and electron acceptors, will shift spatially along the redox gradient (Aaen *et al.* 2011). However, most studies have compared the communities in the rhizosphere with those in the bulk soil, but the heterogeneity in the rhizosphere was ignored (Sipila *et al.* 2008). He *et al.* (2005) constructed a rhizobox system to divide the rhizosphere with mesh into one mm layers to investigate the spatial changes of pentachlorophenol degradation along the rhizosphere gradient. The degradation rates of pentachlorophenol in the ryegrass rhizosphere did not decrease linearly with distance from the root surface, and the highest degradation rate was detected in the rhizosphere soil at three mm from the root surface (He *et al.* 2009).

Given that little direct degradation of PAHs by plants, the stimulatory effect on PAHs degradation in the rhizosphere is mainly due to enhanced microbial degradation (Su and Zhu 2008). Recent advances in molecular microbial ecology have permitted the resolution of the diversity of microbial communities in the environment during PAHs degradation. Profiling phylogenetic marker genes, such as the 16S ribosomal RNA gene, is a key tool for representing microbial communities, however, it does not provide direct evidence of a community function for PAH-degrading bacteria communities. A Metagenome analysis provides insight into the physiological potential of the microbial community and enables the identification of the different metabolic pathways in an ecosystem under different environmental conditions (Guazzaroni *et al.* 2013; van de Vossenberg *et al.* 2013). The first metagenome study of the rhizosphere of rice plants was concerned with bacterial and archaeal communities in the rhizosphere and phyllosphere of rice, and revealed a remarkable consistency with respect to the dominant functional profiling (Knief *et al.* 2012). However, the metagenome related to PAHs degradation in the rice rhizosphere remains poorly understood. Metagenomic analysis requires large-scale sequencing data, which is still costly at present (Davenport and Tummler 2013). Okuda *et al.* (2012) and Langille *et al.* (2013) proposed a novel method for rapidly and efficiently predicting the functional composition of a metagenome without using large scale genomic sequencing. This approach maps 16S rRNA gene sequences to fully sequenced genomes to predict metagenome of bacteria and archaea communities. The predicted metagenome links phylogeny and function profiling, and provides useful insights into uncultivated microbial communities for which only marker gene surveys are currently available. Comparing with a published metagenome, Okuda *et al.* (2012) validated a predicted metagenome reconstructed with 16S rRNA gene sequences obtained from denaturing gradient gel electrophoresis analysis, and showed that the predicted metagenome successfully evaluated genetic dynamics in actual environmental samples.

In this study, bacteria and archaea 16S rRNA genes in the rice rhizosphere soils spiked with different PAH concentrations were amplified and separated by denaturing gradient gel electrophoresis. The predominant bands in the gel were excised and sequenced to identify major microbial species in the microbial community. These 16S rRNA gene sequences were mapped to fully sequenced genomes to reconstruct a metagenome, which can be used for predicting the functional profiling changes in rice rhizosphere soils during PAHs degradation.

Materials and methods

Soil sampling and DNA extraction

Sampling of rhizosphere soil was performed as described in our previously paper (Okuda *et al.* 2012). In brief, paddy soils were sampled then spiked with PAHs at either high or low rate, respectively. The spiked PAHs

were a mixture of PAHs dissolved in acetone to give high and low phenanthrene, pyrene and benzo[a]pyrene concentrations of 200, 100 and 20 mg kg^{-1} (H-PAH), and 20, 10 and 2 mg kg^{-1} (L-PAH), respectively. The dissolved solution were comprehensively mixed with 20 g soils firstly, and further mixed with left soils after the acetone volatilized. The rhizosphere soils were divided into five 1-mm thick layers by use of a nylon mesh with 40 μm pore sizes. Rice (*Oryza sativa* L.) seedlings were transplanted into three replicated rhizoboxes 1 week after germination. Rhizosphere soil samples were taken from three replicated rhizoboxes at 15, 30 and 45 days after transplanting, respectively. Total DNA for each soil sample was isolated from 0·5 g soils using the FastDNA Kit (MP Biomdecals, Inc., Santa Ana, CA) and FastPrep Instrument (MP Biomdecals, Inc., Santa Ana, CA), for 40 s at a speed setting of 6·0. Extracted DNA for three replicated soil samples were pooled and stored at −20°C until use.

Bacterial and archaeal community profiling and sequencing

The microbial communities in the rice rhizosphere were profiled using PCR-DGGE. The forward primer F338 and reverse primer R518 were used for amplifying a 180-bp fragment of the V3 region of the 16S rRNA gene of Bacteria (Muyzer and Smalla 1998). The 188-bp-long 16S rRNA genes of Archaea were amplified with the forward primer Arch334F and reverse primer Univ522R (O'Connell *et al.* 2003). The forward primers were synthesized with a GC-clamp at the 5′ end. The PCR procedure was as follows: an initial cycle of 5 min at 95°C, followed by 20 cycles of 45 s at 94°C, 1 min at 63·5, 61°C with a touchdown of 0·5°C per cycle for bacteria and archaea, and 45 s at 72°C, then followed by 10 cycles of 45 s at 94°C, 45 s at 53·5 and 51°C for bacteria and archaea, respectively, and 45 s at 72°C, with a final extension of 10 min at 72°C. Triplicates were conducted to minimize the bias in amplification.

The PCR products were pooled and were performed electrophoresis in a 8% (w/v) polyacrylamide gel with a denaturant gradient ranging from 20 to 60% at 60°C for 15 min at 30 V, then 280 min 165V in1× Tris-acetate-EDTA buffer with a Dcode Universal Mutation Detection system (Bio-Rad, Hercules, CA). The gels were stained by SYBR Green I and analysed with Image J 1.42q (http://rsb.info.nih.gov/ij).

Twenty-seven bacterial bands and eighteen archaeal bands in the DGGE gel were excised for analysis, transferred to a 1·5-ml Eppendorf tube and dispersed in 20 ml sterile deionized water to release the DNA. The recovered DNA was then amplified and the PCR productions were checked on DGGE gels under the same conditions. With 2 μl of the eluted DNA as the template and primes without a GC clamp, PCR was performed to generate more target DNAs for cloning. The PCR products were cloned into the pEASY-T1 simple clone vector (TransGen Biotech, Beijing, China) and sequenced using the M13 primers by Shanghai Invitrogen. The sequenced bacterial and archaeal clones from the DGGE profiles are listed in Table S1 and S2. The sequences longer than 150 bp have been deposited in the Genbank with the accession numbers from JQ943926 to JQ943951.

Determination of 16S rRNA gene copy numbers with real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed to quantify the bacterial and archaeal 16S rRNA genes, respectively, in the soil samples. The non-GC-clamped versions of the primers used in DGGE analysis were used for qPCR. The reactions with TransStartTM Green qPCR SuperMix (TransGen Biotech, Beijing, China) were performed on an Roche LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) using the following protocol: 50°C for 2 min, 95°C for 15 min, then 40 cycles of denaturing at 94°C for 15 s and annealing at 56°C for 30 s for the bacterial and archaeal rRNA genes, respectively. Plasmids containing inserts of DGGE-band-derived fragments were used to create an internal six-point standard curve ranging from 10^3 to 10^8 gene copies per reaction.

Construction of predicted metagenomes

The method applied for constructing predicted metagenomes was according to Okuda *et al.* (2012) and Langille *et al.* (2013). All 16S rRNA gene sequences of prokaryotes with complete genome sequences in Genbank have been collected as a reference sequence for a universal tree. Subsequently, we mapped 16S rRNA gene sequences from DGGE bands to the universal tree of the reference sequences through computing the multiple sequence alignment with MAFFT and constructing the phylogenetic tree with MEGA by the neighbour-joining method. Evolutionary distances between query sequence against the reference RNA set were computed with the closest 16S rRNA sequences. The genome of query 16S rRNA was by the closely related genomes determined by the above mapping process. COGs in all closely related genomes were extracted, and a profile of the presence or absence across the genomes was determined. On the basis of the profile, the functional content of the genomes was expressed as a vector of existence probabilities of COGs based on the ratio of the number of closely related

genomes possessing the COGs to the total number of the closely related genomes. Each band in the DGGE gel corresponds to a reconstructed genome. All the unidentified bands were treated as identified bands located on the same rows because a band at the same row position was assumed to represent the same 16S rRNA sequence. Finally, the metagenomes were reconstructed based on the relative existence probabilities of COGs in each reference genome, the relative abundance of each sequences calculated based on band density, and the proportion of bacterial and archaeal 16S rRNA gene copies. The proportions of bacteria and archaea were based on the copy numbers of 16S rRNA genes quantified with the qPCR assay (Figs S2 and S3).

Statistics

All the statistics were performed in R software (http://www.R-project.org). The rda function in the *vegan* package for redundancy analysis (RDA) was used for community constrained ordination since the values of the first axis lengths in the detrended correspondence analysis (DCA) were less than three. The constraints in RDA were the distance from root surface (dis), PAHs spike concentration (conc) and culturing time (time). The *varpart* function in the *vegan* package was used for partitioning the variations in the microbial community to three environmental factors. The *envfit* function in the *vegan* package was used for fitting environmental factors onto the ordination results of RDA. The dendrogram in heatmap for functional genes was based on the Hierarchical cluster analysis.

Correlation networks are useful for showing the composition of, and interactions between, multiple elements in communities (Haruta *et al.* 2009). An adjacency matrix of microbial species was constructed based on the pairwise Spearman's rank correlations of relative abundances of 16s rRNA genes among all the samples. If two different species were significantly positively correlated ($r^2 > 0$, $P < 0.001$) or negative correlated ($r^2 < 0$, $P < 0.001$), then their adjacency relationship was set to 1 or −1, or otherwise set to zero. A correlation network was then generated based on the adjacency matrix with the *igraph* package. The network plot was visualized with *Gephi*.

Results

The response of the microbial community to PAH degradation in the rice rhizosphere

The DGGE profiles showed the structure of the bacterial community in the rice rhizosphere soils with different PAHs concentration, distances from the root surface and culture time (Fig. S1a). There were four bands, including

bands 4 and 9 related to *Symbiobacterium thermophilum*, band 5 related to *Nocardioides furvisabuli*, and band 10 related to *Anaerovorax odorimutans*, consistently dominant in all samples, indicating the widespread distribution of these organisms in rice rhizosphere soils. The band number in the L-PAHs soil was greater than in the H-PAHs soil, in which bands 1, 21, 24 and 25 were absent. Bacterial bands 1 and 24 were related to *Alkaliflexus imshenetskii*. Bands 21 and 25 were related to *Clostridium drakei* and *Ignavibacterium album*, respectively (Fig. S1c). In the L-PAHs rhizosphere soils, band 11–20 were present in all rhizosphere soil layers, while band 21–27 were only present in rhizosphere soils at a distance of 2–3 mm from the root surface. However, in H-PAHs rhizosphere soils, band 22, 23, 26 and 27 were present in the rhizosphere soils at a distance of 1 mm from the root surface.

Archaeal communities were influenced by PAHs concentration and distance from the root surface, but not the culture time (Fig. S1b). Archaeal bands 1–15 were present in the rhizosphere soils with low PAHs concentrations. However, only band 1–3 and band 6 were found in H-PAHs rhizosphere soils. In the L-PAHs rhizosphere soils, archaeal band 1–3, band 5 and band 6 consistently dominated all samples. Bands 8–15 were only present in the rhizosphere soils at 1–2 mm from the root surface. These results indicated that band 1–3 and band 5 related to uncultured archaea, and band 6 related to *Methanocella arvoryzae*, were not affected by rhizosphere processes, but band 8–15, related to *Nitrososphaera gargensis* and *Methanocella paludicola*, were colonized near root surface. Archaeal diversity in rhizosphere soils near the root surface was higher than in the rhizosphere soils more than 2 mm from the root surface. With H-PAHs soils, archaeal diversity was extremely low in rhizosphere soils near the root surface, where only band 6 was always present.

The effects of constrained environmental factors, including PAHs concentration, distance from the root surface, and culture time, on the bacterial and archaeal communities in the rhizosphere during PAH degradation were analysed with RDA (Fig. 1). These constrained environmental factors were only responsible for 10·7% of the variation of the prokaryotic communities in the rice rhizosphere during PAHs degradation, with 4·5% for PAHs concentration, 4·0% for distance from root surface and 2·2% for culture time. The results for fitting environmental factors onto an ordination showed that PAHs concentration ($r^2 = 0.57$, $P = 0.001$) and distance from the root surface ($r^2 = 0.44$, $P = 0.001$) influenced the microbial communities, but the effect of culture time ($r^2 = 0.05$, $P = 0.54$) could be ignored.

The relationship of different species in communities was given in the correlation network (Fig. 2). All bacterial and archaeal species were involved in the correlation
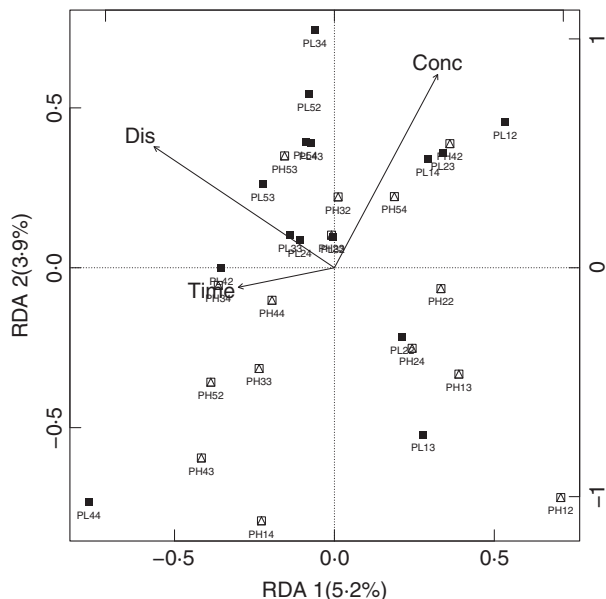
Figure 1 Ordination plot of redundancy analysis (RDA) for the bacterial and archaeal communities in rice rhizosphere during PAHs degradation. The dots labelled with PL and PH denotes the communities in rhizosphere soils spiked with low and high PAHs concentrations, respectively. The first number after PL and PH denotes the distance from root surface. The second number after PL and PH denotes the sampling time. Three constrained variations *conc, dis* and *time* denote spiking concentration, distance from root surface and culture time, respectively. (▲) PH and (■) PL

Figure 2 Correlation network plot showing the correlations between archaea and bacteria from communities in rice rhizosphere during PAHs degradation. Black and white nodes labelled with A and B denotes archaeal and bacteria, respectively. The following numbers denote the band numbers. The black and grey lines indicate significant negative and positive correlations ($P < 0.05$) between two nodes, respectively. The node size denotes the number of connecting line of nodes.

network, except the bacterial band 19 related to *Al. imshenetskii* and band 26 related to *I. album*. Hub nodes bacterial bands 10 and 27, and archaeal bands 2–4 and 6, which were the nodes with the maximum number of edges in the network, played an important role in rhizosphere communities. Bacterial band 10 and archaeal bands 2, 3 and 6 were the species constantly present in the rhizosphere. Bacterial band 27 was an important component in L-PAHs rhizosphere soils at 2–3 mm from the root surface and in H-PAHs rhizosphere soils at 1 mm from the root surface. Archaeal band 4 was an important component in L-PAHs rhizosphere soils at 1 mm from the root surface. The negative correlation in the network suggested competition between species. Most of the negative correlation in the present network was related to the nodes with high edge numbers. This implied that these hub node species competed with each other, and occupied similar ecological niches.

Predicted metagenome in the rice rhizosphere

A predicted metagenome was reconstructed based on the distance between 16S rRNA genes of fully sequenced genomes and the sequence of sliced bands from DGGE
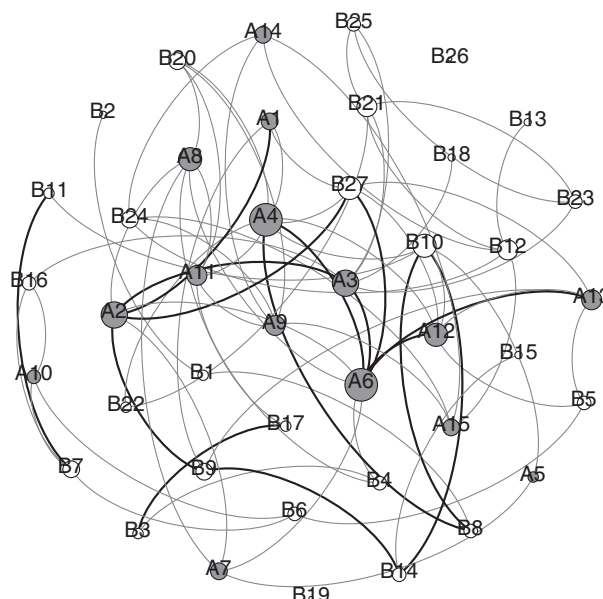
gel. Abundances of functional gene classes, including defense mechanism, replication and recombination and repair, and hypothesis protein, were high in H-PAHs rhizosphere soils at 1 mm from the root surface and L-PAHs rhizosphere soils at a distance of 2 mm from the root surface (Fig. 3). The opposite trend in abundance occurred with the gene classes for RNA processing and modification, inorganic ion transport and metabolism, energy production and conversion, and intracellular trafficking, secretion and vesicular transport.

Thirty metagenomes were hierarchically clustered based on fifty dominant functional genes, each contributing more than 0·1% to the reconstructed metagenomes (Fig. 4). Thirty metagenomes in the rhizosphere soils were clustered into two groups, which were dominated with metagenomes in the rhizosphere soils with high and low PAHs concentrations, respectively. However, some metagenomes in the L-PAHs rhizosphere soils were clustered with metagenomes in soil with high PAHs concentrations, including the metagenomes in L-PAHs rhizosphere soils at 2 mm from the root after 15 days cultivation and at 1, 2 and 4 mm from the root after 30 days cultivation. Reconstructed metagenomes in the H-PAHs rhizosphere soils at 3–5 mm from the root after 45 days of cultivation were clustered with the metagenome in soil with low
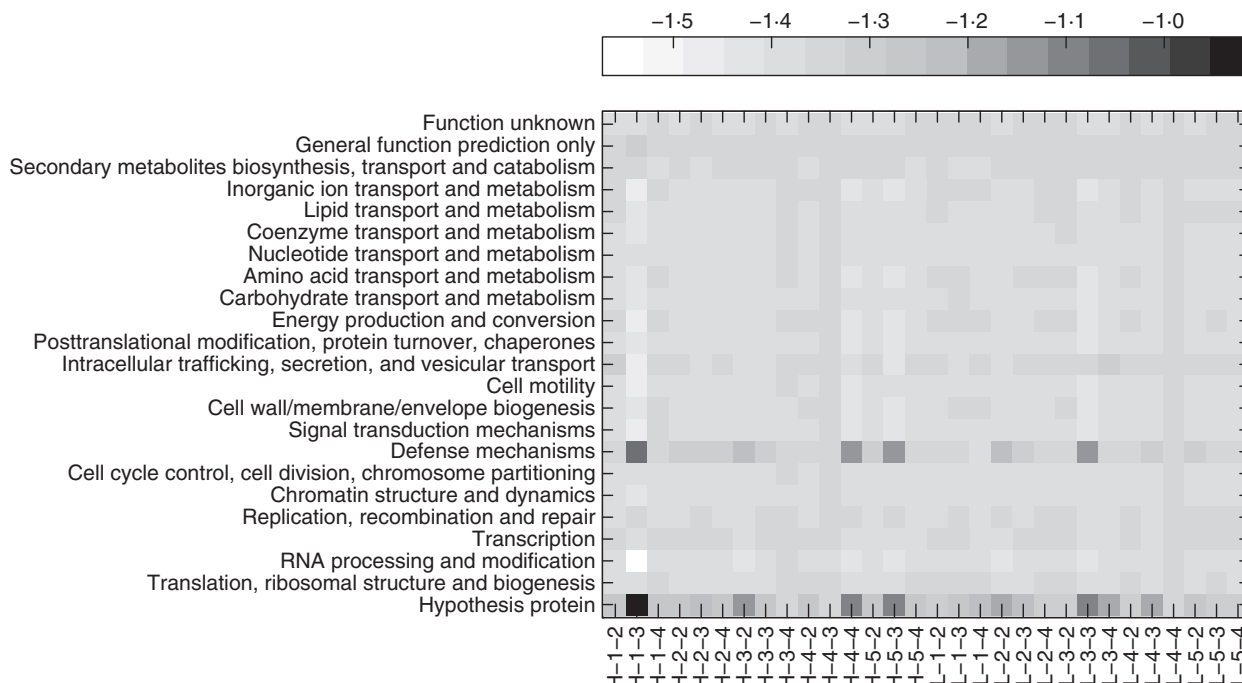
Figure 3 Heat map indicating difference in relative abundances of gene classes among predicted metagenomes from rice rhizosphere soils during PAHs degradation. The colour indicated the logarithmic percentage values for each gene class. The metagenomes labelled with L and H denotes the communities in rhizosphere soils spiked with low and high PAHs concentrations, respectively. The first number after L and H ranging from 1 to 5 denote the distance from root surface. The second number after L and H ranging from 2 to 4 denotes the sampling time at 15, 30 and 45 days after planting, respectively. The horizon and vertical side bar denotes different clusters in samples and genes, respectively.

PAHs concentrations. Our previous study showed that the PAHs degradation rate in the former cluster was greater than in the latter (Okuda *et al.* 2012). The highest degradation rate was detected in L-PAHs rhizosphere soil at 2 mm and in the H PAHs soils at 1 mm from the root. Therefore, the high abundance functional genes in these metagenomes were involved. In metagenomes of low rate PAHs in rhizosphere soil at 2 mm from root surface, the abundance of the functional genes for diaminopimelate epimerase, D-glucarate permease and oxalate/formate antiproter protein was greater than the other metagenomes. In metagenomes of high rate PAHs at 1 mm from the root surface, the abundance of functional genes for diaminopimelate epimerase, chemotaxis protein methyltransfersase, 4-oxalocrotonate decarboxylase, phospholipase, PP-loop superfamily ATPase, biopolymer transport ExbD-related transmembrance protein, nuclease subunit of the excinuclease compex protein and P pilus assembly protein/porin PapC protein were clearly greater than in other metagenomes.

The biodegradation of PAHs catalysed by microbial enzymes is the most important approach for the remediation of PAHs contamination in the rhizosphere. The pathway of PAHs degradation included three important

enzyme categories, dioxygenase, dehydrogenase and aldolase. The abundance of the dioxygenase gene was greater in both H- and L-PAHs rhizosphere soils at 1 mm from the root surface (Fig. 5). The abundance of the aldolase gene was great in both H- and L-PAHs rhizosphere soils at 1–2 mm from the root surface, and also in the H-PAHs rhizosphere soils at 4 and 5 mm from the root surface. The abundance of the dehydrogenase gene was similar in all rhizosphere soils.

Discussion

Changes of microbial communities in the rice rhizosphere during PAH degradation

Microbial communities in the rice rhizosphere were influenced by both PAHs concentration and distance from the root surface. Archaeal bands related to *N. gargensis* and *M. paludicola* and bacterial bands related to *Al. imshenetskii*, *Cl. drakei* and *I. album*, were eliminated in the rice rhizosphere with high PAHs concentrations. It indicated that PAHs in soil might toxic to micro-organisms, which were in intimate contact with the soil environment and are considered to be the best indicators of soil pollution.
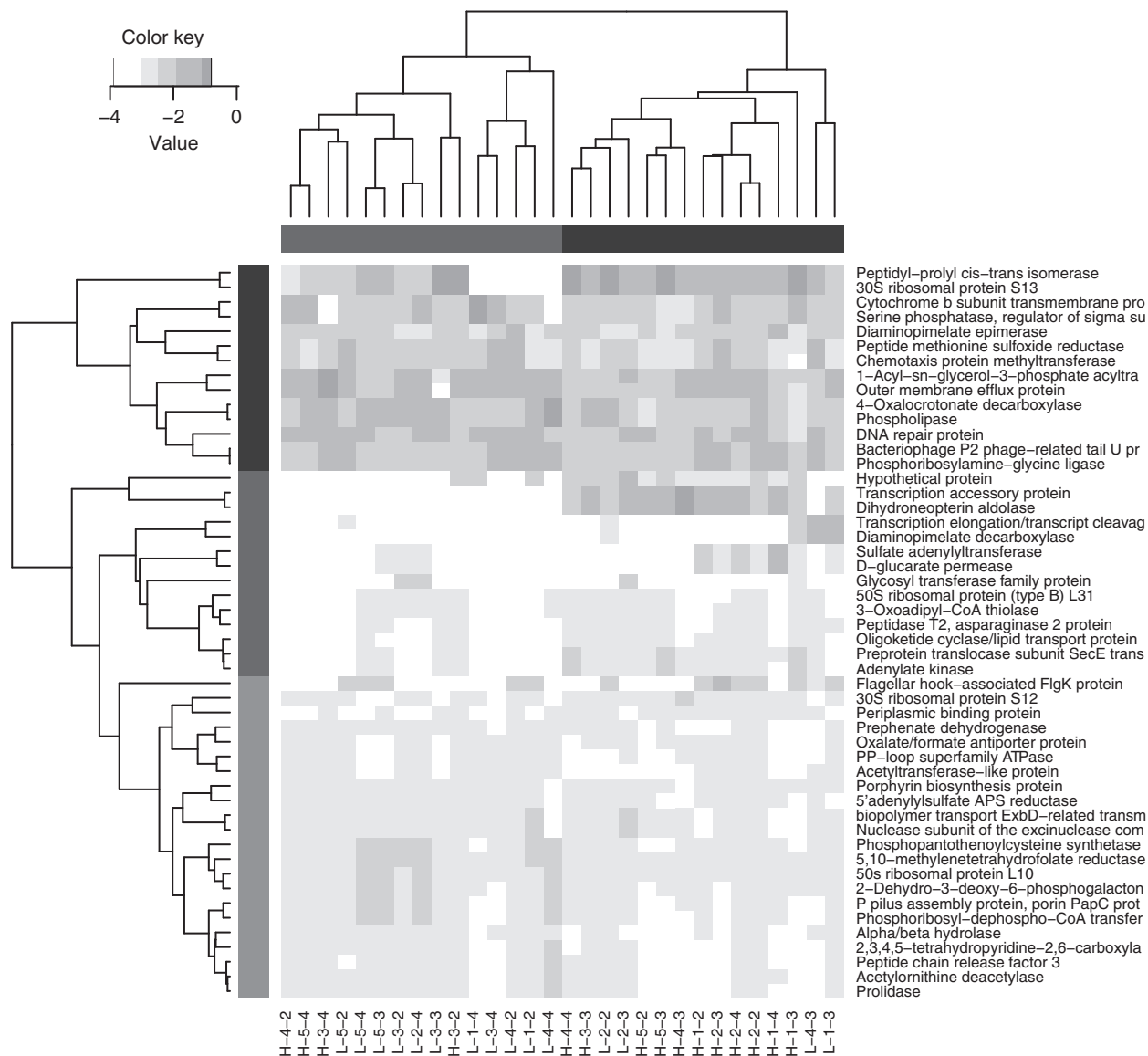
Figure 4 Heat map indicating difference in relative abundances of 50 dominant genes among predicted metagenomes from rice rhizosphere soils during PAHs degradation. The colour indicated the logarithmic percentage values for each gene. The metagenomes labelled with L and H denotes the communities in rhizosphere soils spiked with low and high PAHs concentrations, respectively. The first number after L and H ranging from 1 to 5 denotes the distance from root surface. The second number after L and H ranging from 2 to 4 denotes the sampling time at 15, 30 and 45 days after planting, respectively.

However, our previously study showed that high PAHs degradation rates presented in L-PAHs rhizosphere soil at 2 mm from the root surface and in H-PAHs rhizosphere soil at 1 mm from the root surface (Okuda *et al.* 2012). A high PAHs concentration would facilitate PAHs degrading micro-organisms which are capable of degrading PAHs into less toxic metabolites, and so aid the remediation of PAH-polluted soils (Peng *et al.* 2008). Therefore, the bacteria associated with high PAHs degradation rates may be potential PAHs degrading

species, including those with archaeal bands related to *M. paludicola* and bacterial bands related to *Clostridium tyrobutyricum*, *Kibdelosporangium aridum*, *I. album,* and *Prolixibacter bellariivorans*. *Clostridium* sp. has the greatest degradation potential among bacterial strains isolated from river sediments (Yuan and Chang 2007), *K. aridum* is involved in phenol degradation via an algal-bacterial consortium (Safonova *et al.* 2004), *Ignavibacterium* is one of the most important genera involved in anthranenedegradation in alkaline soils (Castro-Silva *et al.* 2013),
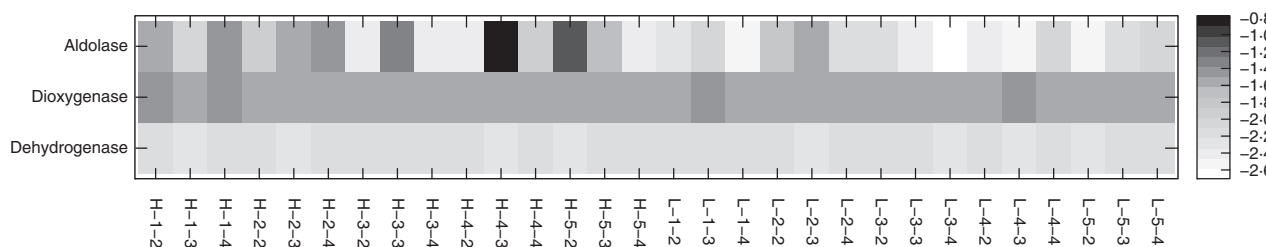
Figure 5 Heat map indicating difference in relative abundances of aldolase, dioxygenase and dehydrogenase from rice rhizosphere soils during PAHs degradation. The colour indicated the logarithmic percentage values for each gene. The metagenomes labelled with L and H denotes the communities in rhizosphere soils spiked with low and high PAHs concentrations, respectively. The first number after L and H ranging from 1 to 5 denotes the distance from root surface. The second number after L and H ranging from 2 to 4 denotes the sampling time at 15, 30 and 45 days after planting, respectively.

and *Prolixibacter* sp. is the dominant genus involved in the degradation of aromatic pollutants (Li *et al.* 2012). These results prove the differential response of archaeal and bacterial communities in soils (Nicol *et al.* 2004).

Correlation networks showed that archaeal nodes were important hub nodes for the interactive relationship network in microbial communities. Network analysis indicated that some archaea was hub species during PAHs degradation in the rice rhizosphere. The archaeal band highly involved in networks was closely related to methanogenic archaeal strains, which couple with the degradation of PAHs under anaerobic conditions (Chang *et al.* 2006). In soils without PAHs, the methanogenic archaea population is too small to be amplified with PCR. However, the population of methanogenic archaea significantly increased in soils spiked with PAHs (Chang *et al.* 2006). The degradation of PAHs is inhibited after adding bromine ethanesulfonic acid (BESA) which interrupts methanogenesis in soil (Chang *et al.* 2003).

Changes of reconstructed metagenomes in rice rhizosphere during PAHs degradation

Degradation rates in the rice rhizosphere reported in our previously study (Okuda *et al.* 2012) were related to the abundance of three functional gene classes, including defense mechanism genes, replication, recombination and repair genes, and hypothesis protein genes. The main toxic effect of PAHs is the formation of reactive metabolites, which are electrophilic species, which bind covalently to macromolecules such as proteins and DNA, and affect their functions (El-Alawi *et al.* 2002). Defense mechanism gene and replication, recombination, and repair genes, which are central to the proliferation, evolution and maintenance of life, are capable of repairing PAHs damage caused by PAHs (Braithwaite *et al.* 1998). The greater abundance of defense mechanism gene and

replication, recombination and repair genes in rhizosphere soils with high degradation rates suggest that micro-organisms in communities with high degradation potential can effectively eliminate the adverse effects of PAHs in soil. The high abundance of hypothesis protein genes indicates that currently unknown degradation mechanisms play an important role in PAHs degradation.

Based on the profiles of 50 dominate functional genes, all metagenomes were assigned into one of two clusters dominated by metagenomes from soils with low and high PAHs degradation rates, respectively. The proportions of metabolism genes and information storage and processing genes was greater in the metagenomes with high PAHs degradation potential. The higher proportion of metabolism genes in metagenomes was corresponding with the higher PAHs degradation potential. In such PAHs metagenomes, derived from soils with low PAHs concentration, the relative abundance was the greatest for the functional genes for diaminopimelate epimerase, D-glucarate permease and oxalate/formate antiporter protein. These genes may play an important role in the pathway of PAHs degradation. There is no evidence that diaminopimelate epimerase takes part in PAHs degradation, but diaminopimelate decarboxylase contributes to pyrene degradation in *Mycobacterium gilvum* PYR-GCK (Badejo *et al.* 2013). D-glucarate, which is able to enter the cells via D-glucarate permease (Monterrubio *et al.* 2000), can promote PAHs degradation through the Cu/glucaric acid/ H_2O_2 radical generating system (Gabriel *et al.* 2004). Oxalate/formate antiporter protein is a carrier protein catalysing the exchange of extracellular oxalate and formate (Ruan *et al.* 1992), and which is reported to increase bioavailability of PAHs in soils, and facilitate PAHs degradation (Sun *et al.* 2012). Moreover, there was a high relative abundance of diaminopimelate epimerase in reconstructed metagenomes with high PAHs degrading potential. The 4-oxalocrotonate decarboxylase gene was more abundant in the reconstructed

metagenome with high PAHs degradation potential. This gene is observed in metagenome of hydrocarbon contaminated environments (Abbai *et al.* 2012). However, in contrast with Sabirova *et al.* (2011) who reported that pilus assembly protein genes were down-regulated during oil spill degradation, we found that these genes were more abundant in metagenomes with high PAHs degrading potential, as derived from soils with high PAHs concentrations.

In addition to the 50 genes with relative abundances of more than 0·1%, we analysed the abundance of dioxygenase, dehydrogenase and aldolase genes, which regulate the most important enzyme classes in the PAH degradation pathway (Peng *et al.* 2008). The abundance of dioxygenase genes was greatest near the root surface of rice. Sipila *et al.* (2008) showed that birch increased extradiol dioxygenase diversity in the rhizosphere, showing a significant rhizosphere effect. Radial oxygen losses from rice roots could increase the activity of aerobic micro-organisms, which will lead to increased dioxygenase genes near the root surface. The diffusion distance of radial oxygen losses from rice roots in soil, which leads to a high abundance of dioxygenase genes, can extend to 1–2 mm from the root surface. The rhizosphere soils at a distance of more than 2 mm from the root surface were anaerobic, where dioxygenase is inhibited. The abundance of aldolase is promoted near the root surface, coincident with the distribution of the dioxygenase gene, suggesting that aldolase plays an active part in PAHs degradation in rice rhizosphere. However, there was also a high abundance of aldolase in the rhizosphere soils at 4–5 mm from the root surface where the soil is anaerobic. Lao *et al.* (2013) reported that aldolase induced under these conditions, and its activity rapidly decreased following the transition of plants to aerobic conditions. The anaerobic biodegradation of PAHs is a slow process, and the biochemical mechanisms involved have not yet been fully elucidated (Haritash and Kaushik 2009). Our results indicate that aldolase is actively involved in both aerobic and anaerobic PAHs degradation. Similarly, the high activity of dehydrogenase under both aerobic and anaerobic condition results in a relatively constant dehydrogenase abundance in rhizosphere soils at increasing distances from root surface. These results suggest that the dioxygenase gene class will be found to be a reliable indicator of PAHs degradation in the rhizosphere.

Though the usability of reconstructed metagenomes was demonstrated in plenty of studies (Langille *et al.* 2013), there are few shortcomings to what we present here. One aspect of reconstruction metagenomes that we cannot test is the transfer of plasmids. Dioxygenase, one of the vital PAH degradation genes, was located on plasmids and could be transferred between phylogenetically distant strains or loss. However, the influence of gene transfer is relatively low since the gene library is not alerted while plasmids transfer into other strains. Also, the genomes would be altered greatly while microbiota survive at various niches in rhizosphere. The environmental gradients in rhizosphere could maintain complex niches, where the accessory genomes of microbial strains have been altered greatly (Fraser *et al.* 2009).

In summary, PAH concentrations and distance from the root surface influenced the bacterial and archaeal communities during PAHs degradation in the rice rhizosphere. Correlation network analysis showed that archaea plays an important role in PAHs degradation. The metagenome reconstructed from 16S rRNA gene mirrored the changes of functional profiling during PAHs degradation in the rice rhizosphere soils with different distance from root surface. Defense mechanism genes and replication, recombination and repair genes were critical gene classes for PAHs degradation, because of the relative high proportion in rhizosphere soils with high PAH degradataion potential. The abundance of the dioxygenase gene varied with distance from root surface, and represented the PAH degradation potential in soils. The reconstructed metagenomes based on 16S rRNA genes can link microbial functional profiles with the PAHs degrading processes, and mining functional genes involved in PAHs degradation mechanisms. This study suggests that the reconstructed metagenomic approach could provide useful insights into the changes of functional profiling during PAHs degradation.

Acknowledgements

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Bacterial and archaeal communities in rice rhizosphere during PAHs degradation.

Figure S2 The copy numbers of bacterial 16S rRNA gene in rice rhizosphere.

Figure S3 The copy numbers of archaeal 16S rRNA gene in rice rhizosphere.

Table S1 Bacterial clones of bands from DGGE.

Table S2 Archaeal clones of bands from DGGE.