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Coordination in Phenanthrene Biodegradation: Pyruvate as Microbial Demarcation

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Abstract The principle involved in the phenanthrene degradation is regarded as the key to unlock the mechanisms governing the pathway of other polycyclic aromatic hydrocarbons. Past studies have made some pathway proposals via metabolite analysis. In this study, two dominating species (*phn01* and *phn02*) were isolated from oil contaminated soil and were used in lab-scale experiment of phenanthrene degradation. The GC/MS results revealed the metabolites of pyruvate, phthalate, 1-hydroxy-2-naphthaldehyde and 9-phenanthrol at retention time of 12.01, 15.34, 16.82 and 18.16 min, respectively. A new proposal of pathway was derived. Selective degradation indicated the relationship of coordination between these two species, one of which was mainly responsible for pyruvate production and the other for pyruvate consumption. Pyruvate played a role of microbial demarcation which might be closely associated with invoking signal for microbial community during biodegradation.

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Polycyclic aromatic hydrocarbons (PAHs) are a class of recalcitrant organic compounds which are ubiquitous in the soil, groundwater and sediment (Bopp et al. 2005; Srogi 2007; Johnsen et al. 2005; Peng et al. 2008). Due to their potential risk to human health, PAHs are of serious concern (Smith 2008; Chen and Liao 2006; Sverdrup et al. 2002). Phenanthrene (PHN) is a tricyclic aromatic hydrocarbon. Since it is the smallest aromatic hydrocarbon to have a "bay-region" and a "K-region", PHN is often used as a model substrate for studies on metabolism of carcinogenic PAHs (Laor et al. 1999; Leglize et al. 2006).

Previous studies have been extensively carried out to explore the mechanisms governing the biodegradation process of PHN (Zhang et al. 2006; Keum et al. 2006; Prabhu and Phale 2003; Stingley et al. 2004; Mallick and Dutta 2008; Leneva et al. 2009). The metabolites are qualitatively detected and various degradation pathways are proposed. The discrepancy in these proposed pathways can be ascribed to the variety in enzyme systems produced by different microbial communities (Kasai et al. 2003; Kim et al. 2006; Corgie et al. 2006). The relationship involved in the microbial community is of great importance to guide the orientation of sequential reactions, which the qualitative result of metabolites is insufficient to explain. The potential coordination between the species may be of significance to deepen the understanding of PHN degradation pathway.

The objectives of this studies are to (1) identify the microbial species responsible for PHN degradation; (2) to explore the relationship involved in the microbial

community; and (3) to evaluate the degradation pathway by crucial metabolite.

Materials and Methods

PHN modified Luria–Bertani Broth plates were prepared for selecting PHN degrading bacteria. 1 mL of 3 mg L^{-1} PHN in acetone was spreading on the surface of every pre-dried plate and allow the acetone to evaporate under sterile condition. Oil contaminated soil was used as the microbial source. The soil sample was put into 37 g L^{-1} Brain Heart Infusion (Difco) solution and placed in bioshaker (100 rpm) at 30°C for 2 days. The mixture was then uptaken and plated on these plates.

16 s rDNA was extracted using DNA Purification Kit according to the quick protocol (Wizard, Promega Corporation). A 16 s rDNA analysis was performed by PCR-DGGE. The general primer pair (forward: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and reverse: 5′-GGT TAC CTT GTT ACG ACT T-3′) was employed to amplify the DNA fragments. The PCR program consisted of 35 cycles of 20 s at 95°C, 30 s at the annealing temperature at 55°C and 1 min at 72°C. Products were loaded on a 6% polyacrylamide gel with formamide-urea denaturing condition and run at 60 V on a Dcode system (Bio-Rad Laboratories, UK) for 10 h. The gel with bands was post-stained with ethidium bromide.

The sequence of 16s rDNA was determined by Genetic Analyzer 3130 (Applied Biosystems, US) with BigDye® Terminator Kit according to the protocol. Phylogenetic analysis was performed using a free software seaview (Galtier et al. 1996). Close relatives of the sequences were obtained by testing against public NCBI databases using Blastn (Altschul et al. 1990). All these related sequences were aligned in seaview by embedded clustalw (Thompson et al. 1994) and yielded phylogentic tree using neighbour joint distance method.

PHN (GC purity >95%) was purchased from Wako, Co. Ltd, Japan. 1 mg L^{-1} PHN solution was prepared and sterilized under ultraviolet before use. The species on the plates were inoculated into this solution. To avoid the inhibition caused by the lack of inorganic nutrient, the following chemicals were added to form 0.6 g/L K_2HPO_4 and Na_2HPO_4, 0.3 g/L NH_4Cl, 1.1 g/L $MgCl_2$, 8.0 g/L Na_2SO_4, and 0.006 g/L $CaCl_2$ and $FeCl_3$ (Allan et al. 2007).

Metabolites were detected and identified using GC/3-Dimensional Quadrupole MS (Hitachi High-technologies Corp., Japan). Helium was the carrier gas at a rate of 2 mL/min. The column temperature was held at 80°C (3 min), programmed to 340°C at a rate of 10°C/min, and held at 340°C for 5 min. Injector and analyzer temperatures were both 340°C. The mass spectrometer was operated in electron impact (EI) mode (70 eV).

Results and Discussions

After inoculated with species from the plates, the PHN solution was sampled at 0, 5 ,10, 15 and 20 days. Figure 1 showed the DGGE profile of microbial community. Two dominating species appeared during 20-day enrichment. Clear difference of band intensity was found between the early stage and late stage. *phn01* decreased while *phn02* increased. The alteration occured after 10 days. The metabolites produced might impact the microbial diversity and shatter the leading position of certain species.

Figure 2 showed the phylogenetic relationship of *phn01* and *phn 02* based on 16 s rDNA analysis. The result indicated their attribution to *Mycobacterium* sp. and *Rhodococcus* sp., respectively. The co-existence of these different species is of interest for further studying the microbial interactive relationship.

The GC/MS data was reorganized and presented in the form of 3D graphics. Figure 3 showed the metabolites produced during 10-day degradation of PHN. The metabolites were identified by comparing the MS patterns with NIST database. Five compounds (pyruvate, phthalate, 1-hydroxy-2-naphthaldehyde, PHN and 9-phenanthrol) were detected at retention time (RT) of 12.01, 15.34, 16.82, 17.17 and 18.16 min, respectively. The peak of PHN weakened while the other peaks performed various strength.

Based on the observation of metabolites, a new proposal of PHN degradation pathway was derived (Fig. 4). In the microbial mixture, PHN transformed to 9-phenanthrol and 3,4-dihydroxyphenanthrene under the function of mono-oxygenase and dioxygenase, respectively. Although dioxygenase was widely regarded to initiate the PHN pathway, some of the microorganisms might still use mono-oxygenanse. In this study, the peak of 9-phenanthrol implied the mono-oxygenase might be involved. The subsequent

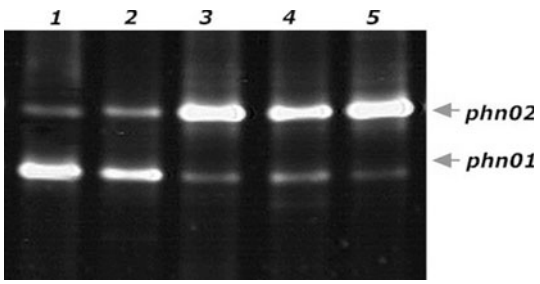

Fig. 1 DGGE profile of microbial community from PHN enrichment after 20 days of incubation. *Lane 1*: initial condition; *2*: after 5 days; *3*: after 10 days; *4*: after 15 days; *5*: after 20 days

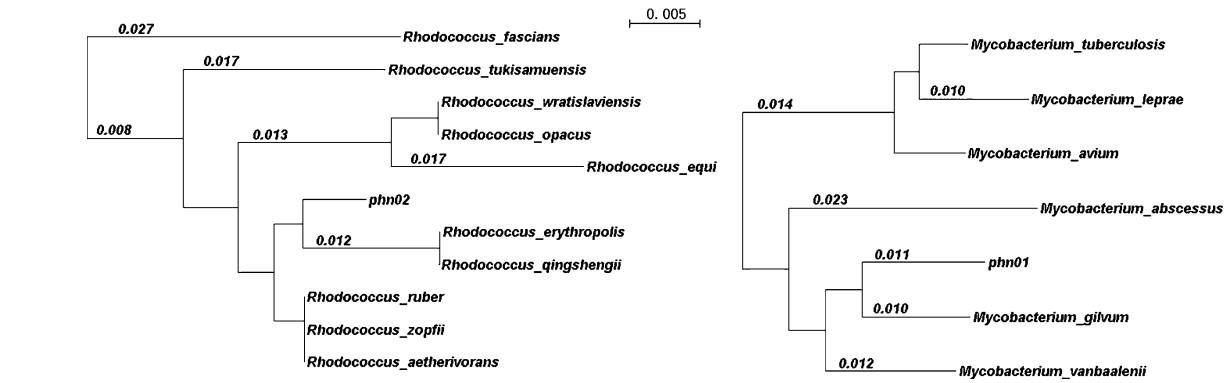

Fig. 2 Phylogenetic tree of *phn01* and *phn02* based on 16s rDNA analysis

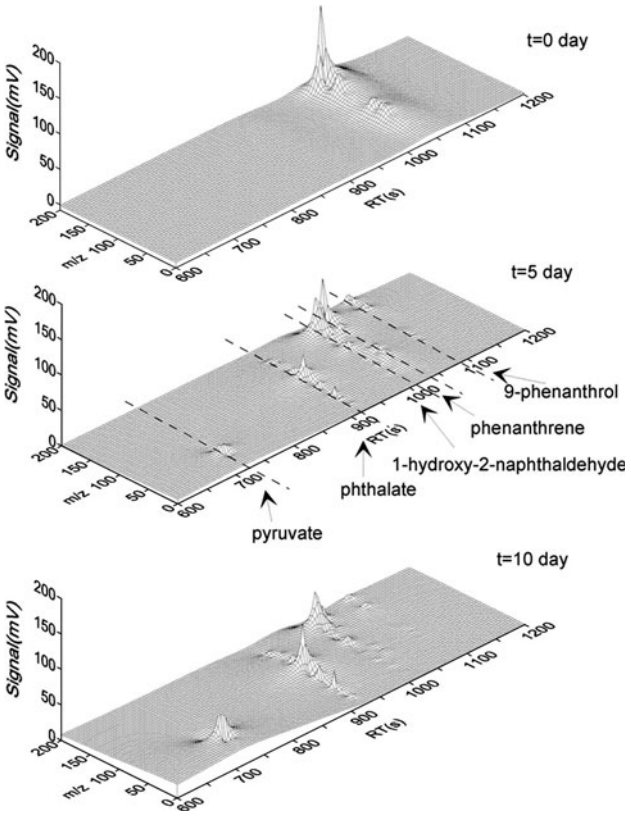

Fig. 3 Degradation of PHN by microbial mixture during 10 days

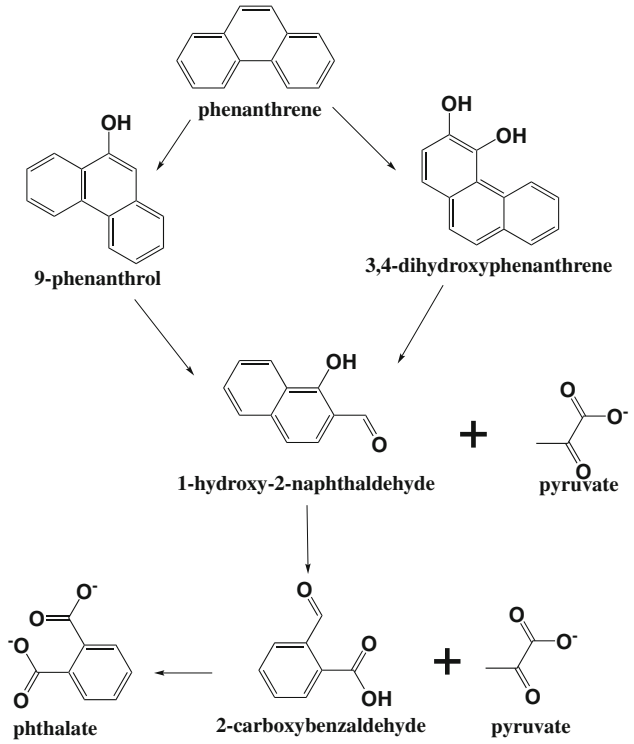

Fig. 4 Proposed PHN degradation pathway

reaction yielded 1-hydroxy-2-naphthaldehyde, which might explain the corresponding peak in Fig. 3. Pyruvate was accompanied by yielding of 1-hydroxy-2-naphthaldehyde and subsequential 2-carboxybenzaldehyde, which addressed its importance during PHN pathway. The detection of phthalate might be ascribed to following degradation leading to phthalate pathway.

Since the alteration of leading position between *phn01* and *phn02* was observed, the importance of metabolites was further studied by selective degradation. The experiment units were sterilized after 10 days and reinoculated with *phn01* + *phn02*, *phn01* and *phn02*, respectively. The results (Fig. 5) showed that the pyruvate accumulated in absence of *phn02*, indicating the function of degrading pyruvate by *phn02*.This may also be ascribed to the production of pyruvate in presence of *phn01*.

This phenomenon revealed the importance of pyruvate in the PHN degradation pathway. Pyruvate might act as the microbial demarcation. There was coordination between *phn01* and *phn02* that the assignment was segmentated by steps and allocated to different species, one of which was mainly responsible for pyruvate production and the other for pyruvate consumption. Pyruvate might be of close relationship with invoking signal for microbial community (Epstein 2009) and expected to be further identified.

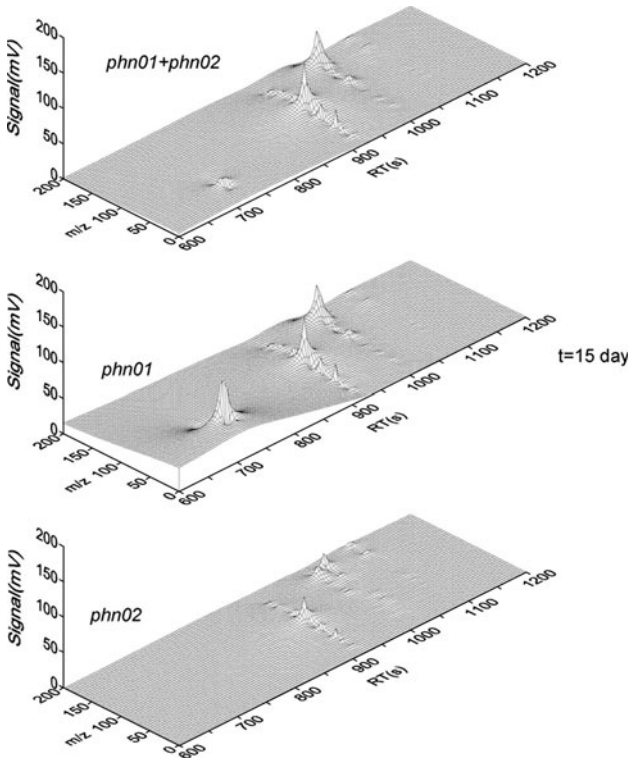

Fig. 5 Selective degradation by *phn01* + *phn02*, *phn01* and *phn02*, respectively at 15th day

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