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NOTE

Molecular Detection of Catabolic Genes for Polycyclic Aromatic Hydrocarbons in the Reed Rhizosphere of Sunchon Bay

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This study focused on detecting catabolic genes for polycyclic aromatic hydrocarbons (PAHs) distributed in the reed rhizosphere of Sunchon Bay, Korea. These marsh and mud environments were severely affected by human activities, including agriculture and fisheries. Our previous study on microbial roles in natural decontamination displayed the possibility that PAH-degrading bacteria, such as *Achromobacter* sp., *Alcaligenes* sp., *Burkholderia* sp. and *Pseudomonas* sp. play an important decontamination role in a reed rhizosphere. In order to gain further fundamental knowledge on the natural decontamination process, catabolic genes for PAH metabolism were investigated through PCR amplification of dioxygenase genes using soil genomic DNA and sequencing. Comparative analysis of predicted amino acid sequences from 50 randomly selected dioxygenase clones capable of hydroxylating inactivated aromatic nuclei indicated that these were divided into three groups, two of which might be originated from PAH-degrading bacteria. Amino acid sequences of each dioxygenase clone were a part of the genes encoding enzymes for initial catabolism of naphthalene, phenanthrene, or pyrene that might be originated from bacteria in the reed rhizosphere of Sunchon Bay.

Key words: catabolic genes, PAH degradation, reed rhizosphere, Sunchon Bay

The reed bed of the bay is well known to play the role of the bridge between the coastal and terrestrial ecosystems that can frequently link both marine and inland environments, as well as the role of the buffering zone to reduce the sudden changes from environmental stress. Sunchon Bay is surrounded by areas of Yeosu and Goheung, the most southern part of Korean Peninsula, which has abundant fisheries, education, and biological diversity. The reed bed area widely developed at the size of 5.4 km^2 is reported to be extending every year. Such environmental features allow diverse life forms to thrive, resulting in high productivity and diverse biological functions such as natural decontamination. Therefore, the reed bed system has been used for the treatment of sewage or waters contaminated with organic compounds or heavy metals (Gersberg *et al*., 1986; Cooper *et al.*, 1989; Kern and Idler, 1999; Obarska-Pempkowoak and Klimkowska, 1999; Begg *et al*., 2001). Farrell *et al.* (2000) reported that aquatic plants including reeds play a role in the phytoremediation of petroleum hydrocarbons.

Natural decontamination contains microbial degradation of several pollutants, including polycyclic aromatic hydrocarbon(PAHs). PAHs, such as anthracene, naphthalene, phenanthrene and pyrene, have been considered the major contaminants in soil and water. Many of these compounds have been found to be cytotoxic, mutagenic, and potentially carcinogenic (Cerniglia *et al*., 1994; van Agteren *et al*., 1998). The microbial degradation of polycyclic aromatic hydrocarbons including fluorene, naphthalene and phenanthrene has been extensively characterized (Menn *et al*., 1993; Sanseverino *et al*., 1993; Simon *et al*., 1993; Yang *et al*., 1994; Han *et al*., 2004). As shown in Fig. 1, several reports suggested that the degradation of PAH begins with initial attacks of iron-sulfur large subunit of dioxygenases involved in PAH degradation (Menn *et al*., 1993; Sanseverino *et al*., 1993; Yang *et al.* 1994; Zylstra *et al.*, 1994). However, little is known about dioxygenase genes for initial catabolism of PAHs, except for naphthalene and phenanthrene, as well as about their functions (Kahng *et al*., 2002). For our understanding of the mechanisms for decontamination at the reed rhizosphere, this

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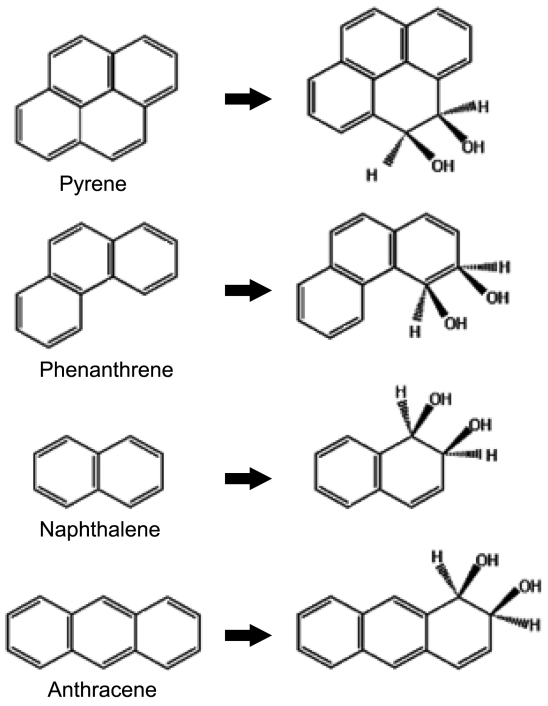

Fig. 1. Chemical structures of some polycyclic aromatic hydrocarbons (PAH) targeted in this study and modes of initial attacks by iron sulfur large subunit of dioxygenase from PAH-degrading bacteria.

study initially focused on the catabolic genes for initial catabolism for polycyclic aromatic hydrocarbons, which might originate from bacteria in the reed rhizosphere of Sunchon Bay.

The soil samples used in this study were collected from a depth of approx 30-50cm at eight sites of the reed rhizosphere in Sunchon Bay. The collected soil was transferred to amber bottle and stored at 4°C until needed. The media used in this study are LB broth and solid ones containing ampicillin (50 μg/ml) for selection of transformants as well as for culturing *Escherichia coli* cells. DNA was directly extracted from 1g of the soil sample. The soil sample was placed into an Eppendorf tube, and 75 μl of 500 mM EDTA (pH 9.4) were added and gently mixed by tapping. The tubes containing soil samples were frozen in liquid nitrogen and thawed quickly by placing them in a water bath that was heated for 1 min 20 sec in a microwave. This step was performed four times, and then the sample was resuspended in 225 μl of miniprep solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) and 100 μl of a lysozyme solution (4 ml of miniprep soilution I, 10-100 μg/ml of lysozyme). 50 μl of 10% SDS was added to it, and followed quickly by 800 μl of phenol-chloroform in the hood. It was vortexed for 1 min to form an emulsion. Then, it was centrifuged at 14,000 × g for 3 min.

The top phase (aqueous, pink) was transferred to a new tube, and 800 μl of phenol-chloroform was added it. It was vortexed and spun for 3 min, and then the top layer was transferred to a new tube. Soil DNA was precipitated with a miniprep solution II (2 ml glycogen. 30 ml 3M sodium acetate, 1 ml 100% ethanol). It was spun at a maximum speed at 4°C for approximately 15 min, and the supernatant was decanted by pouring off. Excess supernatant was pulled off with a pipet following pulse centrifugation for a few seconds, and dried in the speed vac for 5 min. The pellet was resuspended in 50 μl sterile miliQ water, and visualized by gel electrophoresis. The DNA solution was adjusted to 1 ml before 1 g of cesium chloride was added and it was ultra-centrifuged overnight. DNA band was extracted from the microcentrifuge tubes and dialyzed for 40 min by placing a 0.025 mm filter on the surface of a petri dish containing sterile water and placing an aliquot of the DNA sample on the filter. DNA was recovered from the membrane filter, and used for PCR amplification. The recovered DNA was stored -70°C until it was used. For PCR amplification of dioxygenase based on the Rieske iron-sulfur motif sequences from rhizosphere soil, two universal degenerate oligonucleotides, 5'-AGG GAT CCC CAN CCR TGR TAN SWR CA-3' and 5'-GGA ATT CTG YMG NCA YMG NGG-3' were used as sense and antisense primers, respectively (Cigolini, 2000). The PCR reaction, other molecular techniques for DNA sequencing, and sequence analysis were performed as described in the previously published paper (Kahng *et al*., 2002).

This work was conducted to evaluate catabolic potential for initial catabolism of polycyclic aromatic hydrocarbons, such as anthracene, naphthalene, phenanthrene, and pyrene, in the reed rhizosphere of Sunchon Bay. The microbial degradation of mono- and polycyclic aromatic hydrocarbons is often initiated by ring hydroxylating dioxygenase enzymes. The ring hydroxylating dioxygenases thus far identified are soluble multicomponent enzymatic systems comprised of a short electron transport chain and terminal oxygenase (Reiske *et al*., 1964; Mason and Cammack, 1992; Cerniglia *et al*., 1994; Yun *et al*., 2004). Typically the terminal dioxygenase is composed of two dissimilar subunits, large (or alpha) and small (or beta) subunits. Every large subunit of a dioxygenase enzyme contains a Reiske-type iron-sulfur center (Geary *et al*., 1984; Batie *et al*., 1987; Mason, 1988; Gurbiel *et al*., 1989). The iron-sulfur center has two peculiar amino acid sequence motifs surrounding a region of amino acids whose sequence varies from enzyme to enzyme. Accordingly, identifying amino acid sequences of the Reiske-type iron-sulfur motif regions in the reed rhizosphere was of significance, considering that we have very limited information about the role of dioxygenase for degradation of PAHs in the reed rhizosphere.

Dioxygenases capable of hydroxylating inactivated aromatic nuclei were analyzed through a PCR technique using a specific PCR primer set and total genomic DNA

extracted from the reed rhizosphere soils. Approximately 80-bp PCR products were obtained and dioxygenase clonal libraries were constructed using the recovered PCR products (Fig. 2). Fifty clones randomly selected from the clonal library were used for detection of dioxygenases through DNA sequencing. Comparative analysis of amino acid sequences indicated that fifty dioxygenase clones were divided into three groups, two of which might originate from PAH-degrading bacteria in the reed rhizosphere of Sunchon Bay. One group was designated as SCB DOG1, the other one, SCB DOG2 while the other non-PAH dioxygenase group were excluded from this study (Fig. 3). Amino acid sequence analysis showed that SCB DOG1 was found to have sequence similarity of 92% with BphA1 of biphenol dioxygenase from *Pseudomonas pseudoalkaligenes* KF707. The PAH dioxygenase clones in SCB DOG2 could be divided into 3 subgroups, suggesting that the diverse genes for catabolism of PAHs, such as naphthalene, phenanthrene and pyrene, were present in the reed rhizosphere of Sunchon Bay. Representatives of dioxygenase Subgroup I (SCB DOG2-1, -4, -6, -7, -8) shared extensive similarity with naphthalene dioxygenases from *Pseudomonas* strains, phenanthrene dioxygenases from *Mycobacterium* sp. PYO1, *Alcaligenes faecalis* AFK2, *Burkholderia* sp. RP007 and *Norcaioides* sp. KP7, DxnA1 of dioxin dioxygenase from *Sphingomonas* sp. RW1, or PyrAc2 of pyrene dioxygenase from *Mycobacterium* sp. PYO1 with 60~77% similarity (Fig. 4). Representatives of dioxygenase Subgroup II (SCB DOG2-2, and SCB DOG2-5) and Subgroup III (SCB DOG2-3) exhibited divergence in their amino acid sequences from known dioxygenase

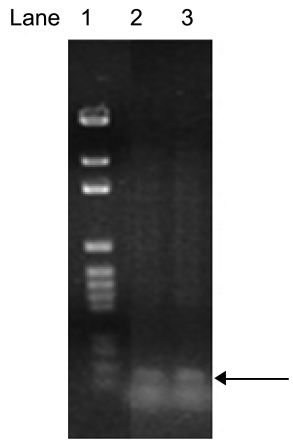

Fig. 2. PCR products of genes specifying iron-sulfur large subunit dioxygenases from the reed rhizosphere of Sunchon Bay. Degenerate universal primers were used for PCR. The arrow indicated the PCR products which are approximately 80-bp in size. Lanes 1, λ-*Hin*dIII marker, 2, 3, PCR products from different sites.

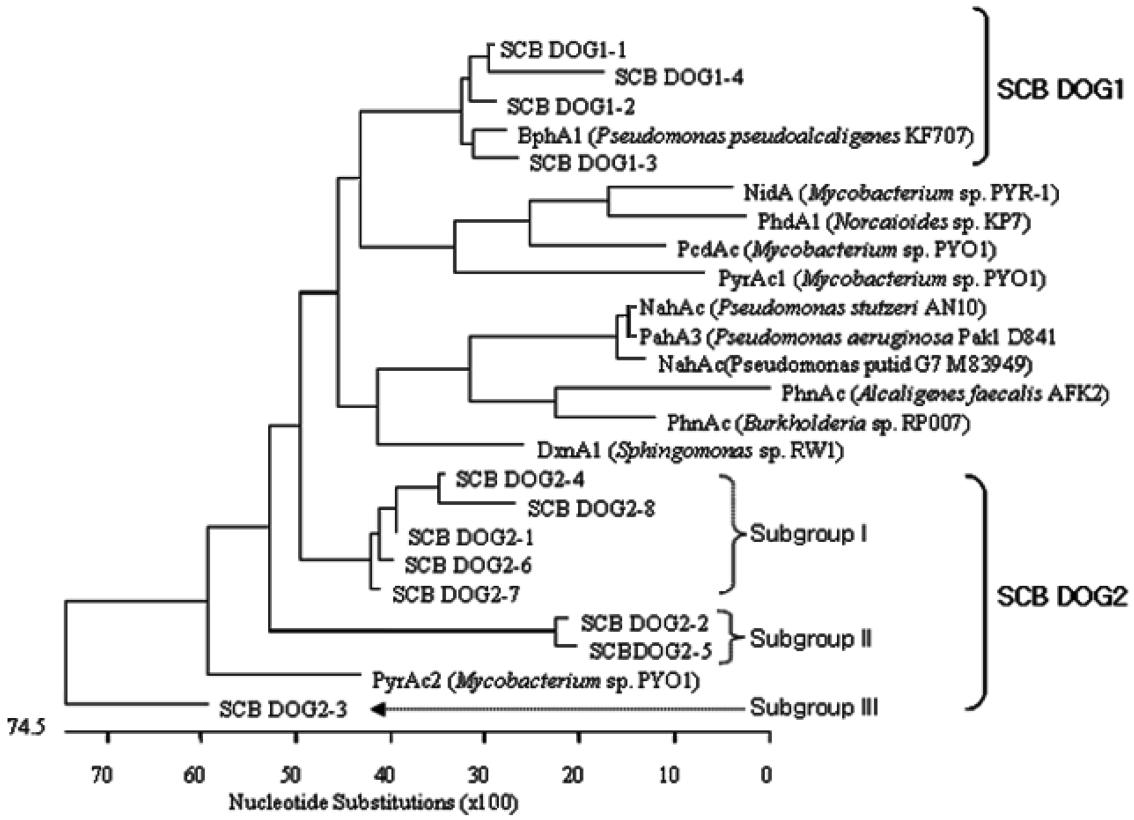

Fig. 3. Phylogenetic tree based on the deduced amino acid sequences of two group dioxygenases identified in this study. The other groups considered as non-PAH dioxygenases were excluded for this dendrogram.

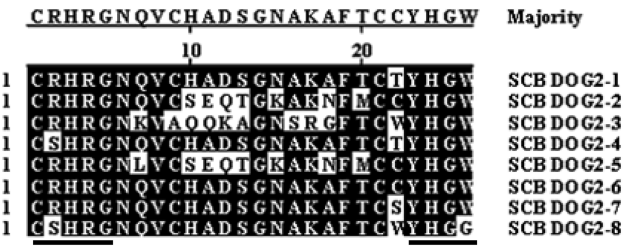

Fig. 4. Multiple alignment of deduced amino acid sequences from the reed rhizosphere, which were assumed to be putative dioxygenases for PAH catabolism such as naphthalene, phenanthrene, and pyrene. The two lines below amino acid sequences indicate the parts of primer sequences.

groups, resulting in its position in the phylogenetic tree with 65% similarity with PyrAc2 of pyrene dioxygenase from *Mycobacterium* sp. PYO1. The known amino acid sequences for PAH metabolisms, such as naphthalene or phenanthrene dioxygenases, are diverged from bacterial strains to strain in the level of species (or genus). Most enzyme information on PAH metabolism is restricted to naphthalene or phenanthrene dioxygenases from bacteria such as genera *Alcaligenes* (Weissenfels *et al*., 1990), *Burkholderia* (Laurei and Lloyd-Jones, 1999), *Comamonas* (Daane *et al*., 2001), *Mycobacterium*(Wang *et al*., 2001), *Norcaioides* (Saito *et al*., 2000), and *Pseudomonas* (Simon *et al*., 1993).

Our previous study on the Rieske iron-sulfur motif region from dioxygenases of *Pseudomonas rhodesiae* KK1 revealed that naphthalene dioxygenase which was placed into the group G1 exhibited 100% sequence identity with NdoC2 of naphthalene dioxygenase from *Pseudomonas putida* (Kahng *et al*., 2002). The representative amino acid sequence (5'-CRHRGKTLVSVEAGNAKGFVCSYHGW) of naphthalene dioxygenase in strain KK1 was not found in this study, even though there were several clones close to the naphthalene dioxygenases of G1 with approximately 60% similarity. Besides, the advanced study on Sunchon Bay revealed that PAH-degrading bacteria such as *Achromobacter* sp., *Alcaligenes* sp., *Burkholderia* sp. and *Pseudomonas* sp. were isolated from the reed rhizosphere of Sunchon Bay (Kim et al., 2005). These facts suggested that the highly divergent amino acid sequences of dioxygenases might originate from several bacteria including the bacteria described above living in the reed rhzosphere. In particular, SCB DOG2-2 (5'-CRHRGNQVCHADSGNAKAFTCTYHGW) or SCB DOG2-5(5'-CRHRGNLVCHADSGNAKAFTCTYHGW), and SCB DOG2-3(5'-CRHRGNKVAQQKAGNSRGFTCVYHGW) have been found to be different from known dioxygenase groups even though they are similar with pyrene dioxygenases (Fig. 3 and Fig. 4). There is still little Information available on the amino acid sequences of dioxygenase sequences for metabolisms of other PAH, except for naphthalane, phenanthrene, and pyrene is sparse at best. Therefore, those sequences first identified in this study which were designated SCB DOG2-2, SCB DOG2-3, and SCB DOG2-5 might be a part of unknown dioxygenase sequences for other PAH metabolism, such as anthracene or benzo(a)pyrene, warranting further intensive study, even though a variety of dioxygenase amino acid sequences in a family might result from the PCR reaction using degenerate primers.

Bacterial community and PAH-degrading bacteria from the reed rhizosphere have been reported (Daane *et al*., 2001; Lee *et al*., 2001). However, to the best of our knowledge, the dioxygenase genes for catabolism of PAH from the reed rhizosphere have been yet to be identified. It is expected that data and DNA fragments for PAH catabolism obtained in this study may stimulate further research on unknown PAH metabolism.

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