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Molecular Characteristics of *Pseudomonas rhodesiae* Strain KK1 in Response to Phenanthrene

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Abstract Radiorespirometric analysis revealed that *Pseudomonas* sp. strain KK1 isolated from a soil contaminated with petroleum hydrocarbons was able to catabolize polycyclic aromatic hydrocarbons such as phenanthrene and naphthalene. The rate and extent of phenanthrene mineralization was markedly enhanced when the cells were pregrown on either naphthalene or phenanthrene, compared to the cells grown on universal carbon sources (i.e., TSA medium). Deduced amino acid sequence of the Rieske-type iron-sulfur center of a putative phenanthrene dioxygenase (*PhnA1*) obtained from the strain KK1 shared significant homology with *DxnA1* (dioxin dioxygenase) from *Spingomonas* sp. RW1, *BphA1b* (biphenyl dioxygenase) from *Spingomonas aromaticivorans* F199, and *PhnAc* (phenanthrene dioxygenase) from *Burkholderia* sp. RP007 or *Alcaligenes faecalis* AFK2. Northern hybridization using the dioxygenase gene fragment cloned from KK1 showed that the expression of the putative *phn* dioxygenase gene reached the highest level in cells grown in the minimal medium containing phenanthrene and KNO_3, and the expression of the *phn* gene was repressed in cells grown with glucose. In addition to the metabolic change, phospholipid ester-linked fatty acids (PLFA) analysis revealed that the total cellular fatty acid composition of KK1 was significantly changed in response to phenanthrene. Fatty acids such as 14:0, 16:0 3OH, 17:0 cyclo, 18:1ω7*c*, 19:0 cyclo increased in phenanthrene-exposed cells, while fatty acids such as 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH, 16:1ω7*c*, 15:0 iso 2OH, 16:0, 18:1ω6*c*, 18:0 decreased.

Key words: Phenanthrene, PLFA, dioxygenase, *Pseudomonas rhodesiae*, radiorespirometric analysis

Polycyclic aromatic hydrocarbons (PAHs) such as anthracene, chrysene, naphthalene, phenanthrene, and pyrene are considered to be serious environmental contaminants, because they are not readily degradable and hence persistent in the environment [4, 8, 25, 26]. Microbial biodegradation of some PAHs has been extensively studied. Degradative mechanisms or metabolic pathways of PAHs such as anthracene, naphthalene, phenanthrene, and pyrene have been elucidated or proposed [3, 5, 10, 17, 18]. Recently, the dioxygenase genes for phenanthrene catabolism have been studied and well characterized in a few bacterial strains, even though only limited information is available [18, 24]. To the best of our knowledge, there is no information on phenanthrene catabolism by *Pseudomonas rhodesiae*. Thus, it is significant to analyze genes for catabolism of phenanthrene in *Pseudomonas rhodesiae* KK1 in a hope that more information can be obtained on the genetic diversity for phenanthrene degradation. *Pseudomonas rhodesiae* KK1, the strain used for this study, is known to have the capability to degrade a two-ring polycyclic aromatic hydrocarbon naphthalene [13]. The present study was carried out as a continued work to evaluate the catabolic potential of strain KK1 for a three-ring polycyclic aromatic hydrocarbon, phenanthrene, as well as to analyze the cellular responses of KK1 cells to the hydrocarbon. The cellular responses of the strain KK1 following its exposure to phenanthrene have been determined by Northern hybridization and change of phospholipid composition.

MATERIALS AND METHODS

Evaluation of Phenanthrene Mineralization by Radiorespirometry

The catabolic potential of strain KK1 for phenanthrene was determined by measuring the radioactivity of $^{14}CO_2$ evolved from mineralization of [9-^{14}C]-labeled phenanthrene (specific activity, 14.0 mCi/mmol; Sigma Chemical Company). Thus, cells were grown in 100 ml of TSB supplemented

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with either phenanthrene (100 μg) or naphthalene (1,000 μg) to the late exponential phase, harvested by membrane filtration, and washed twice with mineral salts solution (0.10 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3$, 0.10 g $MgSO_4 \cdot 7H_2O$, 0.10 g NH_4NO_3, 0.20 g KH_2PO_4, and 0.80 g K_2HPO_4/l of dH_2O; pH 7.0). Approximately 10^5 cells from each of the substrates were then inoculated to 20 ml of mineral salts solution containing phenanthrene (20 μg) supplemented with 10^5 dpm of radiolabeled phenanthrene as a sole carbon source. A 50-ml flask used for the mineralization experiment was sealed with a Teflon-wrapped silicone stopper through which was placed an 18-gauge hypodermic needle and a 16-gauge steel cannula. From the cannula, a small vial containing 1.0 ml of 0.5 N NaOH was suspended to trap $^{14}CO_2$ released by mineralization. The flask was then incubated at 30°C with shaking (150 rpm) and $^{14}CO_2$ formation was determined for 10 days by periodically removing the NaOH and replacing it with fresh solution. The radioactivity was measured by a liquid scintillation counter (LS 5000 TD; Beckman Instruments, Inc., Fullerton, CA, U.S.A.).

Analysis of Dioxygenases for Phenanthrene Catabolism in Strain KK1

To detect and amplify the dioxygenase genes from the total genomic DNA of KK1, we used degenerate oligonucleotide primers that were designed for the conserved Rieske iron-sulfur motif of dioxygenases found in many bacterial species capable of degrading neutral aromatic hydrocarbons [6, 22]. 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. Ten ng of genomic DNA were used as a template. PCR amplification of the dioxygenase gene fragment from strain KK1 was performed in a total volume of 50 μl using Perkin Elmer reagents (Perkin Elmer, Branchburg, NJ, U.S.A.). PCR reactions were performed for 1 min at 95°C, cycled 33 times (1 min at 95°C, 1 min at 55°C, 1 min at 72°C), and then extended for 10 min at 72°C. The PCR products were inserted into pGEM-T vector, and transformed into *E. coli* JM109. A 200 ng portion of the double stranded DNA was used as a template for sequencing together with both the T7 and SP6 primers. Nucleotide sequencing was carried out using an ABI 373A automated sequencer (Perkin Elmer, Branchburg, NJ, U.S.A.). Sequence analysis was performed with Lasergene software (DNA STAR, Inc., Madison, WI, U.S.A.) and BLAST searches of the databases. 16S rRNA of *Pseudomonas rhodesiae* KK1 has been deposited to the GenBank data library under accession no. AY043360.

RNA Preparation for Northern Hybridization

In order to analyze the expression pattern of dioxygenases at the transcriptional level, cells were grown overnight in TSB to the mid-log phase (O.D. 0.8–1.0), were harvested, and washed twice with the mineral salts solution. Approximately 10^5 cells/ml were transferred to the medium containing 5 mg/ml of phenanthrene and incubated for 12 h at 30°C. Total RNA was extracted from the KK1 cells using a Nucleospin RNA extraction kit (Clontech Lab., Inc., Palo Alto, CA, U.S.A.) according to the procedure provided by the manufacturer. The DNA fragment for a probe in Northern hybridization was labeled by the random priming method provided by Promega (Promega, Madison, WI, U.S.A.). Five milligrams of total RNA were used for Northern hybridization with a putative phenanthrene dioxygenase probe obtained from KK1.

Analysis of Phopholipid Ester-Linked Fatty Acids (PLFAs)

PLFAs that exist in strain KK1 were analyzed in the form of fatty acids methyl ester (FAMEs) using the MIDI system (Microbial Insights, Inc., Newark, DE, U.S.A.). Cells harvested following 24 h of growth on Tryptic Soy Agar (TSA) were heated to 100°C with NaOH-methanol (NaOH 45 g and methanol 150 ml in 150 ml of deionized distilled water) to saponify cellular lipids, and the released fatty acids were methylated by heating with HCl-methanol (325 ml of 6.0 N HCl in 275 ml of methanol) at 80°C. Fatty acid methyl esters (FAMEs) were extracted by the mixture of hexane and methyl-*tert* butyl ether (1:1), and analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). FAMEs were identified by comparing their retention times and mass spectra with those of authentic standards provided by the MIDI database. To examine the fatty acids changed in response to naphthalene exposure, cells grown on Tryptic Soy Broth (TSB) were collected and washed twice in potassium phosphate buffer (pH 7.0). The washed cells were incubated in mineral salts media containing 5 mg/ml of naphthalene. After 24 h of incubation at 30°C, changes in the composition of FAMEs before and after exposure to phenanthrene were analyzed by the MIDI protocols. Other procedures not mentioned above were performed as previously described [16].

RESULTS AND DISCUSSION

Mineralization of Phenanthrene by Strain KK1

When 10^5 cells/ml of strain KK1 grown on TSA were used for degradation of phenanthrene, 22% of the compound was mineralized during the 10-day incubation (Fig. 1). To study the effect of preexposure of the cells to hydrocarbons on the mineralization patterns, KK1 cells pregrown on either naphthalene or phenanthrene were evaluated for phenanthrene mineralization. KK1 cells pregrown on phenanthrene exhibited a much faster rate and higher extent of phenanthrene mineralization (Fig. 1). Similarly, naphthalene-pregrown cells demonstrated the similar rate and extent of phenanthrene

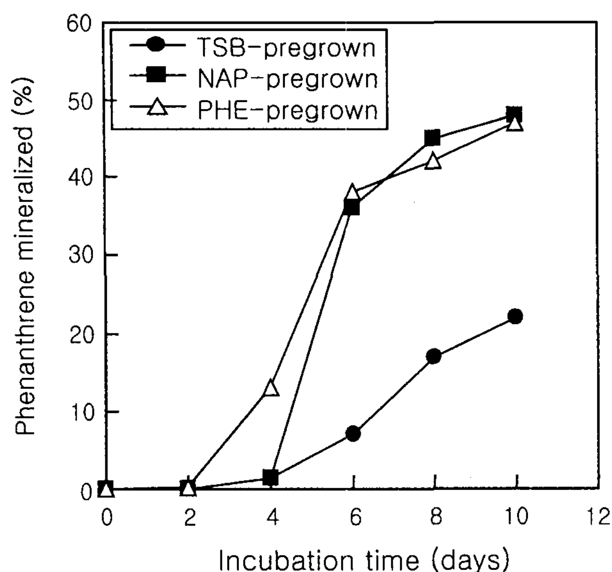

Fig. 1. Rates of phenanthrene mineralization by KK1 cells pregrown with or without naphthalene or phenanthrene.

Cells were grown on the media containing TSB (tryptic soy broth) and harvested by centrifugation at 8,000 rpm for 10 min. 10^5 cells were transferred to phenanthrene media and incubated for 10 days. During the incubation period, 1 ml of KOH was used to analyze the amount of CO_2 evolved from mineralization of [^{14}C]-labeled phenanthrene. The percentage of phenanthrene mineralized by KK1 cells pregrown on naphthalene (NAP), phenanthrene (PHE), or TSB was determined by calculating [^{14}C]-labeled CO_2 released from phenanthrene degradation during the same incubation period.

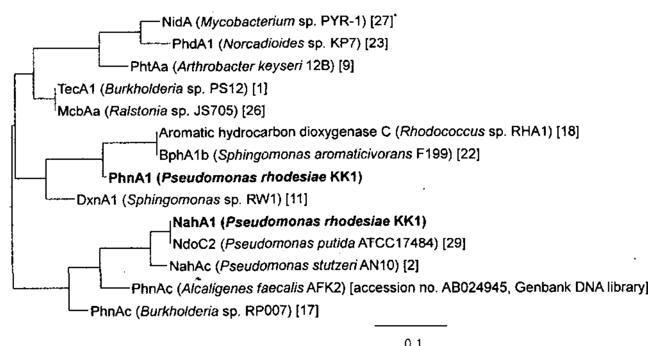

Fig. 2. Phylogenetic tree of Rieske iron-sulfur motif sequences of putative dioxygenases in strain KK1.

Twenty-six amino acid sequences were aligned with other known dioxygenases responsible for catabolism of aromatic hydrocarbons by using the Lasergene Megalign program. The bar scale represents 10 nucleotide substitutions per 100 nucleotides. The asterisk [*] indicates references or sources of dioxygenase amino acid sequences.

mineralization, suggesting a close linkage between phenanthrene and naphthalene metabolism pathways. A previous study [13] found that naphthalene mineralization by KK1 cells was more stimulated when the cells were pregrown on phenanthrene than the cells pregrown on naphthalene. Phenanthrene can be catabolized to produce the key intermediate, 1-hydroxy-2-naphthoic acid *via* several metabolic steps, and the intermediate can be further metabolized to 1,2-dihydroxynaphthalene or 2-carboxybenzaldehyde under aerobic conditions [7, 10]. The compound 1,2-dihydroxynaphthalene can be mineralized using the naphthalene catabolic pathway. In this regard, the fact that even naphthalene catabolism can be further stimulated by phenanthrene than naphthalene appeared to be reasonable. Kahng *et al.* [15] reported that expression of genes in the *tbc* operons in *Burkholderia* sp. strain JS150 can be similarly stimulated by either benzene, toluene, ethylbenzene, or xylene (BTEX). Accordingly, it was assumed that similar compounds might chemically affect the gene expression, and degradation of some compounds having similar catabolic pathways might be enhanced by the gene in an operon.

Dioxygenase Detection for Phenanthrene Metabolism in Strain KK1

Total DNA extracted from the strain KK1 was analyzed for the presence of phenanthrene dioxygenases capable of hydroxylating unactivated aromatic nuclei using a specific PCR primer set. PCR products were cloned and 50 randomly selected clones were sequenced. Amino aicd sequence analysis of the Rieske-type iron-sulfur center of a putative phenanthrene dioxygenase (*PhnA1*), obtained through a PCR process using KK1 genomic DNA as a template revealed that the deduced amino acid sequence of *PhnA1* shared significant similarity with *DxnA1* (dioxin dioxygenase) from *Spingomonas* sp. RW1 [11], the aromatic hydrocarbon dioxygenase C from *Rhodococcus* sp. RHA1 [17], or *BphA1b* (biphenyl dioxygenase) from *Spingomonas aromaticivorans* F199 [23], and *PhnAc* (phenanthrene dioxygenase) from *Burkholderia* sp. RP007 [18] and *Alcaligenes faecalis* AFK2 [accession no. AB024945, Genbank data library] (Fig. 2). Our previous study [13] revealed that the deduced amino acid sequence of the Rieske-type iron-sulfur protein of naphthalene dioxygenase in strain KK1 shared 100% similarity with that of *NdoC2* (naphthalene dioxygenase) from *Pseudomonas putida* ATCC17484 [30]. The naphthalene dioxygenase was different in the deduced amino acid sequence from the putative phenanthrene dioxygenase obtained in this study (Fig. 3).

Expression of Phenanthrene Dioxygenases at the Transcriptional Level

The expression patterns of naphthalene dioxygenase at the transcriptional level in response to phenanthrene were analyzed using Northern hybridization. It indicated that the phenanthrene dioxygenase gave similar positive signals for naphthalene and phenanthrene. This result might have resulted from significant similarity between dioxygenase sequences for catabolism of naphthalene and phenanthrene (Fig. 4). Analysis of deduced amino acid sequences revealed that the putative *PhnA1* (5-CRHRGNKVCFAEAGNAR-GFICSYHGW) shared approximately 70% homology with

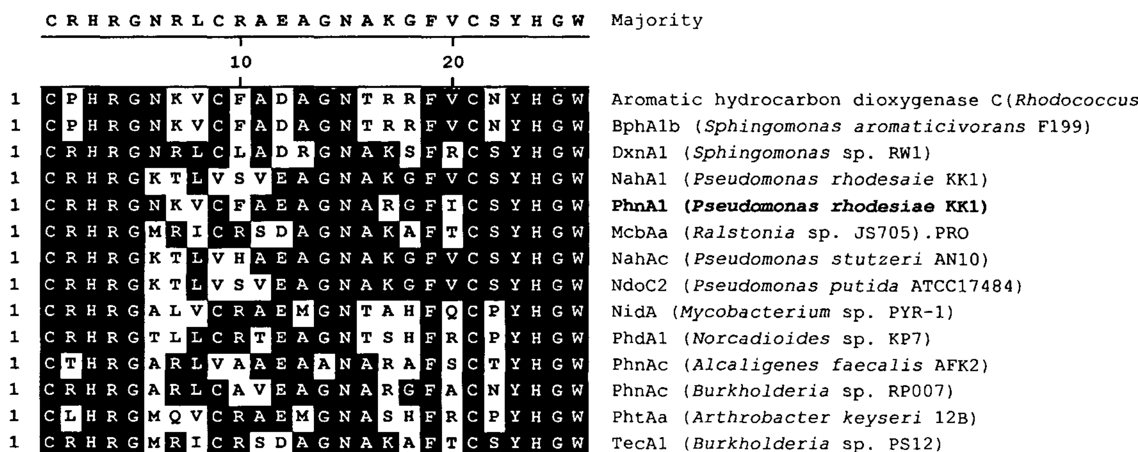

Fig. 3. Comparative analysis of amino acid sequences of the putative phenanthrene dioxygenase (*PhnA1*) from strain KK1 and other aromatic hydrocarbon dioxygenases. Multialignment was drawn using the Jotun Hein method of the Lasergene Megalign program. Dioxygenase genes from *Pseudomonas rhodesiae* KK1 are marked in bold.

NahA1 (naphthalene dioxygenase) in KK1. A little stronger signal intensity was observed in cells grown in phenanthrene than naphthalene. However, it was quite possible that the transcriptional expression of phenanthrene dioxygenase (*PhnA1*) in KK1 might be effectively stimulated by either phenanthrene or naphthalene, in that the expression of *PhnA1* at the transcriptional level was also activated by naphthalene. Our previous study demonstrated that naphthalene was much more rapidly mineralized by cells grown in phenanthrene than naphthalene [13]. These findings suggested a linkage of dioxygenase genes to initial catabolism of naphthalene and phenanthrene, warranting further intensive study.

Shift in Cellular Fatty Acid Composition in Response to Phenanthrene

The total cellular fatty acids of KK1 comprised of eleven C-even and two C-odd fatty acids (fatty acids less than 0.2% in abundance were not considered in this calculation). The predominant lipid 16:0 accounted for 32% of total cellular fatty acids for cells grown on a complex medium (i.e., TSA), but the amount increased slightly to 39% when cells were exposed to phenanthrene (Fig. 5). The percentages of other fatty acids including 14:0, 16:0 3OH, 17:0 cyclo, 18:1ω7*c*, 18:0, and 19:0 cyclo also increased in phenanthrene-exposed cells while the abundance of 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH, 16:1ω7*c*, 15:0 iso 2OH, 16:0, 18:1ω6*c*, and 18:0 decreased. It is worthy to mention that the lipids 14:0 and 16:0 3OH, which were not

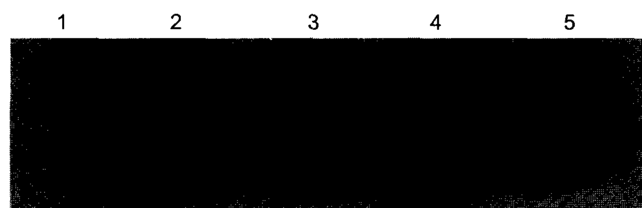

Fig. 4. Expression patterns of the putative phenanthrene dioxygenases at the transcriptional level.

The signal patterns obtained from hybridization between Rieske iron-sulfur motif sequences of *PhnA1* and RNAs extracted from KK1 cells grown on glucose (Lane 1), naphthalene (Lane 2), phenanthrene (Lane 3), phenanthrene plus KNO_3 (Lane 4), and naphthalene plus KNO_3 (Lane 5).

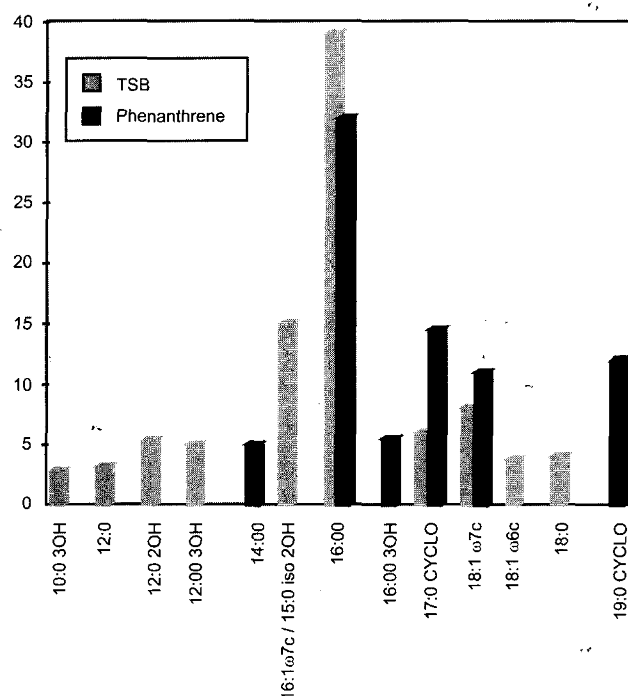

Fig. 5. Fatty acid profiles of strain KK1 cells exposed to phenanthrene.

Total cellular fatty acids were extracted from either TSB- or phenanthrene-grown cells, and analyzed by GC-FID.

detectable when the cells were grown on TSA, increased following the exposure of the cells to phenanthrene. Such changes in cellular fatty acids composition before and after exposure to phenanthrene can be ascribed to transport-related physiological adaptation of the cells in response to available carbon sources or toxic chemicals. Warth [29] reported that the conversion of unsaturated fatty acids from *cis* to *trans* has been linked to prevention of membrane damage by decreasing membrane fluidity. A *Pseudomonas putida* strain that was solvent-tolerant and solvent-sensitive was found to be able to produce *trans*-unsaturated fatty acids following exposure to *o*-xylene [21]. Shifts to utilizable fatty acids have been extensively analyzed in *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Ochrobacterium*, and *Pseudomonas* [14, 20]. As for *Burkholderia* sp. KP 3, capable of mineralizing PAHs including phenanthrene, lipids 17:0 cyclo and 19:0 cyclo ω8*c* increased in cells grown in phenanthrene, whereas 16:0, 16:1ω7*c*, and 18:1ω7*c* decreased. A similar shift of fatty acids composition was also observed in KK1 cells.

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