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Cellular Responses of *Pseudomonas* sp. KK1 to Two-Ring Polycyclic Aromatic Hydrocarbon, Naphthalene

Hyung-Yeel Kahng[1,2]*

[1]*Research Institute for Basic Sciences,*
[2]*Department of Life Science, Cheju National University, Jeju 690-756, Korea*

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The strain KK1 isolated from soil contaminated with polycyclic aromatic hydrocarbons was identified as *Pseudomonas* sp. based on analyses by MIDI and Biolog Identification System. Cellular and physiological responses of strain KK1 to two-ring polycyclic aromatic hydrocarbon, naphthalene were evaluated using radiorespirometry, PLFAs and sequence analysis of Rieske-type iron sulfur center of dioxygenase. KK1 was found to be able to rapidly mineralize naphthalene. Notably, KK1 cells pregrown on phenanthrene were able to mineralize naphthalene much more rapidly than naphthalene-pregrown cells. The total cellular fatty acids of KK1 were comprised of eleven C-even and two C-odd fatty acids (fatty acids < 0.2% in abundance were not considered in this calculation). Lipids 12:0 2OH, 12:0 3OH, 16:0, 18:1 6c, 18:0 increased for naphthalene-exposed cells, while lipids 18:1 7c/15:0 iso 2OH, 17:0 cyclo, 18:1 7c, 19:0 cyclo decreased. Data from Northern hybridization using a naphthalene dixoygenase gene fragment cloned out from KK1 as a probe provided the information that naphthalene dioxygenase gene was more highly expressed in cells grown on phenanthrene than naphthalene.

Key words: naphthalene, PLFA, dioxygenase, *Pseudomonas*

The microbial degradation of polycyclic aromatic hydrocarbons (PAHs) including fluorene, naphthalene and phenanthrene has been extensively studied (Bae *et al.*, 2000; Boldrin *et al.*, 1993; Casellas *et al.*, 1997; Geiselbrecht *et al.*, 1998; Goyal and Zylstra, 1996; Lee *et al.*, 2001; Laurie and Lloyd-Jones, 1999; Manohar and Kim, 1999; Sanseverino *et al.*, 1993; Schneider *et al.*, 1996; Shin *et al.*, 1999). Many bacterial strains have been well characterized and the information is readily available. However, little information is known about the cellular and physiological responses of *Pseudomonas* strains capable of naphthalene mineralization. In an effort to understand cellular responses of *Pseudomonas* sp. to PAHs including naphthalene, strain KK1 isolated from PAH-contaminated soil owing to its ability to grow rapidly on a mixture of PAHs such as naphthalene and phenanthrene was tested for this study. We have determined the physiological changes in the microorganism following its exposure to naphthalene by using radiorespirometry, Northern hybridization, and Microbial Identification System (MIDI) analysis. Naphthalene dioxygenase gene from strain KK1 was also analyzed in this study.

* To whom correspondence should be addressed.
(Tel) 82-64-754-3998; (Fax) 82-64-756-3541
(E-mail) kahng@cheju.ac.kr

Materials and Methods

Isolation of the strain KK1

Five-gram samples of Manufactured Gas Plant (MGP) soil were incubated with a mixture of PAHs in 100 mL of inorganic salts solution (0.10 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3$, 0.10 g $\cdot MgSO_4 \cdot 7H_2O$, 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) at 30°C for two weeks. Naphthalene was dissolved in acetone in a final concentration of 10 mg/mL and used as the substrate for enrichment. After two weeks of incubation, a consortium capable of degrading naphthalene was obtained. Serial dilutions of the enrichment consortium (10^{-1} to 10^{-3}) were transferred to the solid media containing naphthalene, and then the fast-growing colonies were screened. Strain KK1 was selected from the colonies and used in this study.

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, USA). Cells harvested following 24 h of growth on Tryptic Soy Agar (TSA) were heated to 100°C with NaOH-methanol to saponify cellular lipids and the released fatty acids were methylated by heating with HCl-methanol at 80°C. Fatty acid methyl esters (FAMEs) were solvent-extracted, 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 shifted 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 naphthalene were analyzed as described above.

Evaluation of naphthalene mineralization using radiorespirometry

The catabolic potential of strain KK1 for various PAHs was determined by measuring the radioactivity of $^{14}CO_2$ evolved from mineralization of [^{14}C]-labeled PAHs. Cells were grown in TSB, naphthalene, and phenanthrene 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 was then inoculated to a mineral salts solution containing radiolabeled naphthalene as the sole carbon source and incubated at 30°C for 10 days with shaking (100 rpm). The 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 was suspended a small vial containing 1.0 mL of 0.5 N NaOH 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, USA).

Analysis of dioxygenases for naphthalene 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 (Cigolini, 2000). PCR amplification of the dioxygenase gene fragment from strain KK1 was performed in a total volume of 50 mL using Perkin Elmer reagents (Perkin Elmer, Branchburg, NJ). 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 10 min at 72°C. The PCR products were inserted into pGEM-T vector, and transformed into *E. coli* JM109. A portion of 200 *ng* 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. Sequence analysis was performed with Lasergene software (DNA STAR, Inc., Madison, WI) and BLAST searches of the databases.

Enzyme assay

Naphthalene dioxygenase was assayed with KK1 cells grown on naphthalene, or other substrates according to the method previously published (Ensley *et al.*, 1982). A colony of KK1 cells was grown on TSB, naphthalene, salicylate, or phenanthrene. 10^5/mL cells collected from one of the substrates were washed in potassium phosphate buffer two times. Washed cells were broken by a bead beater (bead 0.1 mm, Biospec) and the cell debris was removed for preparation of cell-free extract. Cell-free extract was recovered and used for assay of naphthalene transformation.

RNA preparation and 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). Cells 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 naphthalene and incubated for 12 h at 30°C. Total RNA was extracted from the KK1 cells using a Nucleospin RNA extraction kit according to the procedure provided by the manufacturer (Clontech Lab., Inc., Palo Alto, CA). The DNA fragment for a probe in Northern hybridization was labeled by the random priming method provided by Promega (Promega, Madison, WI). Five milligrams of total RNA were used for Northern hybridization with a naphthalene dioxygenase probe obtained from KK1.

Results

Naphthalene concentrations in soil and mineralization of naphthalene by strain KK1

Analysis of the MGP soil (0-2 m below surface) used in this study revealed that it was seriously contaminated with naphthalene. Six hundred and eighty five grams of naphthalene were recovered per gram of soil by solvent extraction. The strain KK1 grown on TSA was able to mineralize naphthalene within 10 days of incubation at 30°C, however it could not degrade benzo[a]pyrene, chrysene, and pyrene during the same incubation period. When 10^5 cells/mL were used, 13% of naphthalene was mineralized during the 10-day incubation (Fig. 1). KK1 cells pregrown on naphthalene or phenanthrene were evaluated for naphthalene mineralization. KK1 cells pre-grown on phenanthrene exhibited much quicker and stronger catabolic

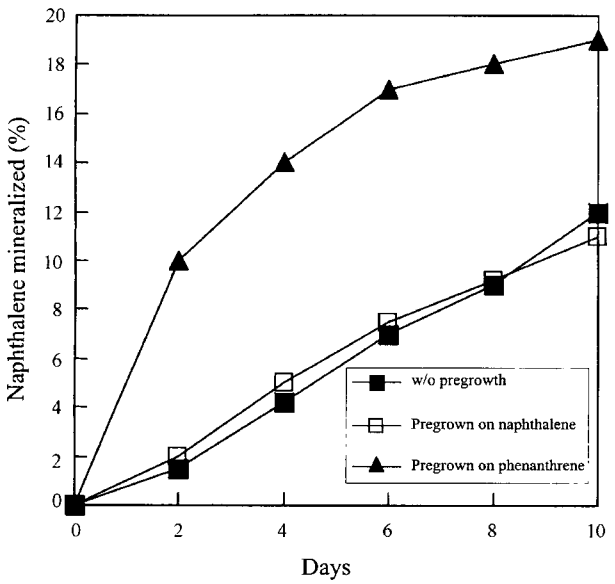

Fig. 1. Rates of naphthalene mineralization by KK1 cells pregrown with or without naphthalene or phenanthrene. Cells were grown on the media containing tryptic soy broth and harvested by centrifugation at 8,000 rpm for 10 min. 10^5 cells were transferred to naphthalene media and incubated for 10 days. During the incubation period, 1mL of KOH was used to analyze the amount of CO_2 evolved from mineralization of [^{14}C]-labeled naphthalene. The percentage of naphthalene mineralized by KK1 cells pregrown on naphthalene or phenanthrene was determined by calculating [^{14}C]-labeled CO_2 released from degradation of naphthalene during the same incubation period.

potential for the substrates naphthalene (Fig. 1). Enhancement of naphthalene mineralization was not observed in cells pre-grown on naphthalene, but phenanthrene-grown cells mineralized naphthalene approximately two times faster during the same incubation time.

Dioxygenases for naphthalene metabolism in strain KK1

To evaluate the catabolic potential of naphthalene by KK1, total DNA extracted from the strain KK1 was analyzed for the presence of dioxygenases capable of hydroxylating unactivated aromatic nuclei using a specific PCR primer set. PCR products were cloned and 50 randomly selected clones were sequenced. The clone selected for the study on naphthalene catabolism showed 100% homology with the *NdoB* of naphthalene dioxygenase from

Table 1. Naphthalene dioxygenase activities of KK1 cells grown on different substrate

Growth Substrate	Naphthalene dioxygenase activity* (U/mg protein)
Naphthalene	4.370
Salicylate	0.375
Phenanthrene	6.210

*Enzyme activities in the cell free extract are given in units per milligram of protein. 1U represents the conversion of 1 umol naphthalene per minute. KK1 cells pregrown on naphthalene, salicylate, or phenanthrene were transferred to 40 mM potassium phosphate buffer containing only naphthalene, and incubated for 1 day to analyze transformation rate of naphthalene.

Pseudomonas putida NCBI 9816 (Kurkela *et al.*, 1988) *or N12O* from *Pseudomonas* sp. strain C18 (Denome *et al.*, 1993), or *Chain A* from *Pseudomonas putida* KT2440 (Carredano *et al.*, 2000) (Fig. 2). Also, it shared extensive homology with PAH dioxygenase large subunit from *Comamonas testosterni* H (Moser and Stahl, 2001), *NdoB* iron sulfur protein from *Pseudomonas fluorescens* ATCC17483 (AAB61370), or *Pseudomonas putida* G7 (Simon *et al.*, 1993). Naphthalene dioxygenase was assayed with cell-free extract obtained from KK1 cells grown on naphthalene or other substrates. Phenanthrene-grown cells exhibited the highest naphthalene dioxygenase activity (Table 1). This result was consistent with that obtained from radiorespirometry.

Expression of naphthalene dioxygenases in transcriptional level

Northern hybridization was carried out to analyze the expression pattern of naphthalene dioxygenase in strain KK1 using the DNA fragment having 100% homology with another known naphthalene dioxygenase as a probe. It indicated that the naphthalene dioxygenase gave similar positive signals for naphthalene and phenanthrene, suggesting a close linkage between naphthalene and phenanthrene catabolism (Fig. 3). However, signal intensity was stronger in cells grown in phenanthrene than naphthalene.

Shift of cellular fatty acid composition in naphthalene-exposed cells

The total cellular fatty acids of KK1 were comprised of

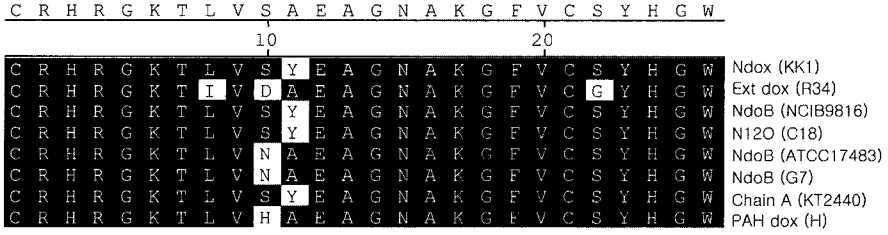

Fig. 2. Rieske iron-sulfur motif sequences of naphthalene dioxygenase in strain KK1. Twenty-six amino acid sequences of the Rieske iron-sulfur protein were aligned with other known dioxygenases responsible for catabolism of aromatic hydrocarbons using the Lasergene Multialign program.

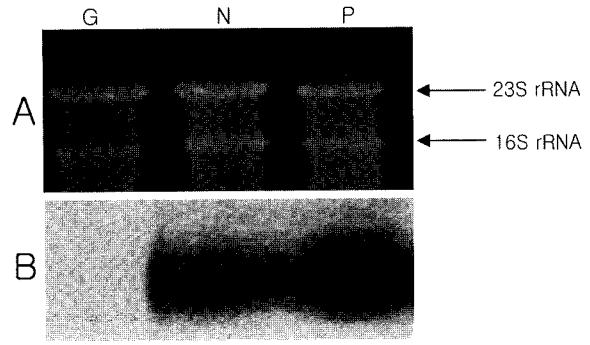

Fig. 3. Expression patterns of naphthalene dioxygenases in transcriptional level. The signal patterns obtained from hybridization between Rieske iron-sulfur motif sequences of the naphthalene dioxygenase and RNAs extracted from cells grown on glucose (G), naphthalene (N) or phenanthrene (P). The upper panel (A) shows an agarose gel profile of total RNA. The bottom panel (B) shows an autoradiogram of transcripts.

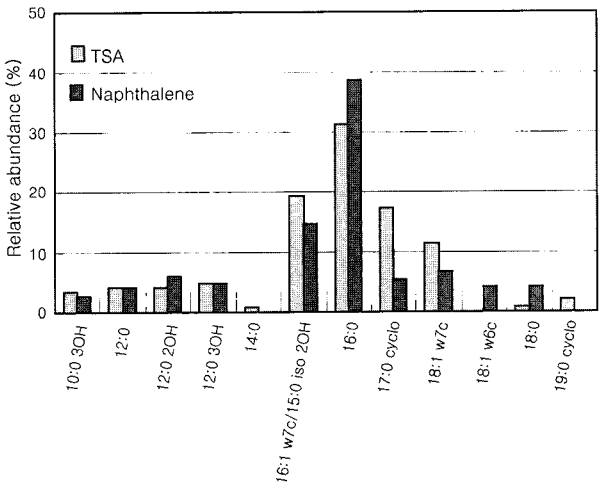

Fig. 4. Fatty acid profiles of strain KK1 cells exposed to naphthalene. Total cellular fatty acids were extracted from either TSA-, or naphthalene-grown cells, and comparatively analyzed by GC-FID.

eleven C-even and two C-odd fatty acids (fatty acids < 0.2% in abundance were not considered in this calculation). The predominant lipid 16:0 made up 32% of total cellular fatty acids for cells grown on complex medium (TSA), but increased slightly to 39% when cells were exposed to naphthalene (Fig. 4). Lipids 12:0 2OH, 12:0 3OH, 16:0, 18:1 ω6c, 18:0 increased for naphthalene-exposed cells, while lipids 18:1 ω7c/15:0 iso 2OH, 17:0 cyclo, 18:1 ω7c, 19:0 cyclo decreased, suggesting that the total cellular fatty acid composition of strain KK1 was greatly affected by exposure to naphthalene.

Discussion

Evaluation of PAH utilization by KK1 revealed that the strain was capable of mineralizing anthracene and phenanthrene, as well as naphthalene. In this respect, the result was consistent with some previous studies that naphthalene-degrading bacteria were capable of anthracene or phenanthrene metabolism through similar catabolic pathways. Unexpectedly, naphthalene mineralization was more stimulated by phenanthrene-grown cells than naphthalene-grown cells. The mineralization rate of naphthalene increased approximately 1.6 times in phenanthrene-grown cells (Fig. 1).

The PCR amplification of the Rieske iron-sulfur motif region of dioxygenases found in strain KK1 revealed that the strain has naphthalene dioxygenase genes for the catabolism of neutral aromatic hydrocarbons based on the deduced amino acid sequence as mentioned in Fig. 2. The iron sulfur motif sequence was also found to share significant homology with *PahAc* of PAH dioxygenase large subunit from *Pseudomonas putida* OUS82 (AB004059), *DntAc* of an extradiol dioxygenase for dinitrotoluene pathway in *Burkholderia cepacia* R34 (Johnson *et al.*, 2000), and *Pha2NT* of 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42 (Parales *et al.*, 1988) based on 26 amino acid sequences of the Rieske-iron center.

Northern hybridization was carried out to analyze the expression patterns of naphthalene dioxygenase at the transcriptional level in response to naphthalene (Fig. 3). Stronger signal intensity was observed in cells grown in phenanthrene than naphthalene, suggesting transcriptional expression of naphthalene dioxygenase in KK1 might be more stimulated by phenanthrene than naphthrene. This fact was consistent with the radiorespirometric results that naphthalene mineralization was much more rapidly enhanced by cells grown in phenanthrene than naphthalene as shown in Fig. 2. A combination of data mentioned above suggests that amino acid sequences of the probe (5-CRHRGKTLVSVEAGNAKGFVCCYHGW) might originate from the conserved region of dioxygenases for naphthalene catabolism in KK1.

Exposure of KK1 cells to naphthalene resulted in the changes of cellular fatty acid composition (Fig. 4). The lipids 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH, 17:0 cyclo, 18:1ω7C, 18:1ω6C, 18:0 occupy small parts of the total cellular fatty acids. It is noteworthy mentioning that the lipids 14:0 and 16:0 3OH, which were not detectable when the cells were grown on TSA, increased after exposure of the cells to naphthalene. Such changes in cellular fatty acid composition before and after exposure to naphthalene can be ascribed to the physiological adaptation of the cells in response to toxic chemicals, as well as to the utilizable substrates. The conversion of unsaturated fatty acids from *cis* to *trans* has been linked to prevention of membrane damage by decreasing membrane fluidity (Warth, 1991). Pinkart *et al.* reported that solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains were able to produce *trans*-unsaturated fatty acids following exposure to *o*-xylene.

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