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PAH utilization by *Pseudomonas rhodesiae* KK1 isolated from a former manufactured-gas plant site

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Abstract *Pseudomonas rhodesiae* KK1 was isolated from a former manufactured-gas plant site, due to its ability to grow rapidly in a mixture of polycyclic aromatic hydrocarbons (PAHs). Radiorespirometric analysis revealed that strain KK1 was found to be able to mineralize anthracene, naphthalene and phenanthrene. Notably, phenanthrene-grown cells were able to mineralize anthracene much more rapidly than naphthalene-grown cells. Comparative analysis of amino acid sequences from 17 randomly selected dioxygenases capable of hydroxylating unactivated aromatic nuclei indicated that the enzymes for catabolism of PAHs, such as naphthalene and phenanthrene, might exist redundantly in strain KK1. Northern hybridization for cells grown on naphthalene or phenanthrene, using the putative naphthalene or phenanthrene dioxygenase gene fragment as a probe, suggested that the enzyme for naphthalene catabolism might share some homology in deduced amino acid sequences with phenanthrene dioxygenases. Also, it was found that three lipids (17:0 cyclo, 18:1 ω7c, 19:0 cyclo) increased in response to both naphthalene and phenanthrene, while the shift of other lipids varied from substrate to substrate.

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Introduction

The manufactured-gas plant (MGP) site used in this study has a history of over 100 years of contamination with coal tar. It is well known that coal tar is composed of many polycyclic aromatic hydrocarbons (PAHs) including anthracene, benzo(a)pyrene, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, and pyrene (Arvin and Williams 1992; Prince and Drake 1999). These PAHs are considered among the major contaminants in soil and water environments, because 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 PAHs, including fluorene, naphthalene, and phenanthrene, has been extensively characterized (Menn et al. 1993; Sanseverino et al. 1993a, b; Serdar and Gibson 1989; Simon et al. 1993; Takizawa et al. 1994; Yang et al. 1994). Several reports suggested that the enzymes involved in naphthalene degradation also have the ability to degrade phenanthrene and anthracene through similar catabolic steps (Menn et al. 1993; Sanseverino et al. 1993a, b; Yang et al. 1994; Zylstra et al. 1994). However, Cigolini (2000) reported that the pyrene- and phenanthrene-degrading bacterium, *Mycobacterium* sp. strain PYO1, could not degrade naphthalene. The genes for the initial steps in the degradation of naphthalene and phenanthrene have been cloned from *Pseudomonas* strains and their nucleotide sequences have been determined. However, little is known about dioxygenase genes for the initial catabolism of PAHs (except for naphthalene and phenanthrene), or their functions. In our efforts to understand the mechanisms for utilization of PAHs, strain KK1 was isolated

from the coal tar-contaminated soil at a former manufacturing facility, owing to its ability to grow rapidly on PAHs, such as naphthalene (Kahng 2002). This study was undertaken not only to evaluate the catabolic potential of KK1 strains for PAHs, but also to analyze their diversity and the possible role of dioxygenase for PAH degradation.

Materials and methods

Media, soil samples, and isolation of pure bacterial strains capable of degrading PAHs

The PAH-contaminated soil used in this study was collected at a depth of 0–2 m below the surface at a former MGP site in New Jersey. The soil was classified as loamy sand, consisting of 78% sand, 11% silt, and 11% clay. PAH-degrading bacteria were isolated from PAH-contaminated soil at the MGP site through enrichment culture technique. Samples of MGP soil (5 g) 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 $MgSO_4 \cdot 7H_2O$, 0.10 g NH_4NO_3, 0.20 g KH_2PO_4, 0.80 g K_2HPO_4 in 1 l of dH_2O; pH 7.0) at 30 °C for 2 weeks. PAHs, including anthracene, naphthalene, or phenanthrene, dissolved in acetone at the concentration of 10 mg/ml were used as substrates for the enrichment. After 2 weeks of incubation, 10 ml of the supernatant were collected and incubated for a further 2 weeks, as described above. By this procedure, a consortium capable of degrading a variety of PAHs was obtained and used for the isolation of pure bacterial strains that were able to degrade PAHs.

Determination of PAH mineralization using radiorespirometry

The catabolic potential of strain KK1 for PAHs was determined by measuring the radioactivity of CO_2 evolved from the mineralization of [^{14}C]-labeled PAHs. For this purpose, radiolabeled PAHs, including naphthalene ([UL-^{14}C], 31.3 mCi/mmol), phenanthrene ([9-^{14}C], 14.0 mCi/mmol), and anthracene ([1,2,3,4,4A,9A-^{14}C], 20.6 mCi/mmol), were purchased from Sigma Chemical Company (St. Louis, Mo.). Cells were pregrown on tryptic soy broth (TSB), naphthalene, or phenanthrene to late-exponential phase, harvested by centrifugation, and washed twice with inorganic salts medium. Approximately 10^5 cells from each of the substrates were inoculated into 100 ml of inorganic salts solution containing anthracene, naphthalene, or phenanthrene, respectively, and were then incubated for 10 days for mineralization experiments. The concentration of nonradiolabeled PAH used for the mineralization experiments was 10 µg/ml and 10^5 disintegrations/min of radiolabeled PAH were used. During the incubation period, the $^{14}CO_2$ evolved from PAH mineralization was trapped at intervals by 1 ml of 0.5 N NaOH solution and the amount was determined by liquid scintillation counting.

Analysis of dioxygenases for PAH catabolism in strain KK1

To detect and amplify dioxygenase genes from KK1 total genomic DNA, we used degenerate oligonucleotide primers that were designed for the conserved Rieske iron–sulfur motif from dioxygenases found in many bacterial species capable of degrading neutral aromatic hydrocarbons (Cigolini 2000). Genomic DNA was purified by use of a Clonetech tissue kit. For PCR amplification of the dioxygenase Rieske-type iron–sulfur motif sequences from strain KK1, 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. The PCR reaction, other molecular techniques for DNA sequencing, and sequence analysis were performed as described by Kahng et al. (2000).

RNA preparation and Northern hybridization

In order to analyze expression patterns at the transcriptional level, cells were grown overnight in TSB media to the mid-log phase (optical density = 0.8–1.0). Cells were harvested and washed twice with the inorganic salts solution. Approximately 10^5 cells/ml were transferred to the PAH medium containing 5 mg/ml of either naphthalene or phenanthrene and were incubated for 12 h. Total RNA was extracted from the KK1 cells using a Nucleospin RNA extraction kit, according to the procedure provided (Clontech, Palo Alto, Calif.). DNA fragments for probes in Northern hybridization, diox14 (5'-tgtcggcatcgcggcaacaaggtgtgctttgcccggcaacgcccgcggcttatctgctcgtaccacggctgggg-3') and diox40 (5'-tgcagtcatagcggcaagacgctggtgagcgtggaagccggcaatgccaaaggttttgtttgttgctaccacgggtggg-3'), were dissolved in sterile distilled water at the concentration of 50 µg/ml and were labeled after denaturation by heating them in screw-capped microcentrifuge tubes at 95–100 °C, according to the random-priming method provided (Promega, Madison, Wis.). Five milligrams of total RNA were used as template for Northern hybridization with the representative probes of two dioxygenase groups (the putative naphthalene or phenanthrene dioxygenase groups) obtained from KK1 to estimate the PAH-degrading potential of strain KK1. Northern hybridization was carried out as described by Kahng et al. (2001).

Analysis of total cellular fatty acids in strain KK1

Cells harvested following 24 h growth on tryptic soy agar (TSA) were heated with NaOH–methanol to saponify cellular lipids; and the released fatty acids were methylated by heating with HCl–methanol. Fatty acid methyl esters (FAMEs) were solvent-extracted and analyzed by gas chromatography with flame ionization detection and gas chromatography–mass spectrometry. FAMEs were identified by comparing their retention times and mass spectra with those of authentic standards provided in the MIDI database. To examine fatty acids shifted in response to PAH exposure, cells grown on TSB were collected and washed twice in potassium phosphate buffer (pH 7.0). Washed cells were incubated in 5 mg/ml of anthracene, naphthalene, or phenanthrene mineral salts media for 24 h. Cells were extracted after 24 h and lipids extracted from those cells were used for fatty acid analysis, as described above.

Physiological characterization of strain KK1

A GN2 MicroPlate (Biolog, Hayward, Calif.) was used to characterize strain KK1, based on substrate-utilization profiling. A single KK1 colony grown on a TSA plate was streaked onto Biolog universal growth agar medium containing 5% sheep blood and incubated overnight at 30 °C. Cells were suspended in normal saline (0.15% NaCl) and inoculated into the GN2 MicroPlate. After incubation for 24 h, the resulting pattern was read, using the Biolog automated Micro-Station. The result obtained through Biolog substrate-utilization analysis indicated that strain KK1 was a member of the *Pseudomonas* spp cluster, with 96% matching similarity (data not shown). Analysis of a 1,500-bp fragment of the amplified 16S rDNA sequence from strain KK1 indicated that strain KK1 was closest to the γ-proteobacterium, *P. rhodesiae* (GenBank accession no. AY043360).

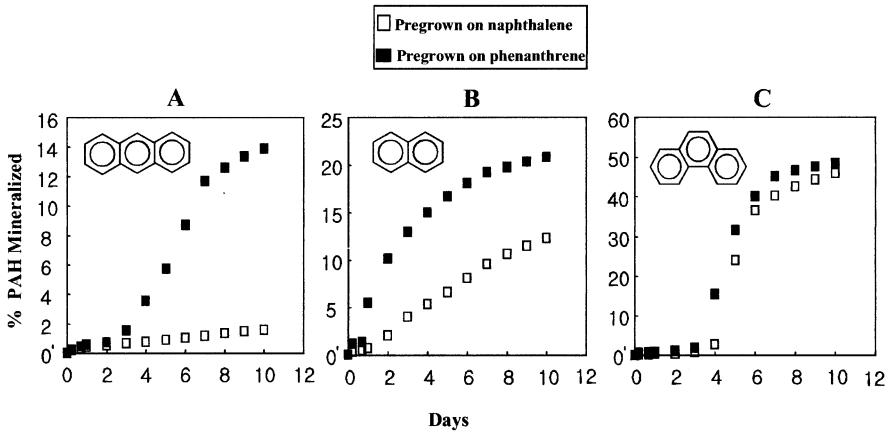

Fig. 1A–C Percentage of polycyclic aromatic hydrocarbon (*PAH*) mineralized by KK1 cells pregrown on naphthalene or phenanthrene. Cells were grown on naphthalene or phenanthrene and were harvested by centrifugation at 8,000 rpm for 10 min. Then, 10^5 cells were transferred to PAH liquid media containing 10 μg/ml of anthracene, naphthalene, or phenanthrene radiolabeled with [C^{14}] and were incubated for 10 days. During the incubation period, 1 ml of NaOH was used to analyze the amount of CO_2 evolved from the mineralization of [C^{14}]-labeled PAHs. The amount of PAH mineralized was determined by calculating the [C^{14}]-labeled CO_2 liberated from the degradation of anthracene (**A**), naphthalene (**B**), or phenanthrene (**C**) during the 10-day incubation period

Results

PAH mineralization by KK1 cells

A single colony of KK1 cells grown on TSA was able to mineralize 10 μg/ml of anthracene, naphthalene, or phenanthrene within 10 days of incubation. However, it could not degrade benzo[a]pyrene, chrysene, or pyrene during an equivalent incubation. After 10 days of incubation, approximately 1.8% of anthracene was mineralized, while 13% of naphthalene and 22% of phenanthrene were mineralized, respectively (data not shown). KK1 cells pre-grown on naphthalene or phenanthrene were evaluated for PAH mineralization. There was no change in the substrate spectrum of PAHs utilizable by KK1 cells pregrown on naphthalene or phenanthrene. However, KK1 cells pregrown on phenanthrene exhibited much quicker and stronger catabolic potential for the three substrates, anthracene, naphthalene, and phenanthrene. A much quicker rate of anthracene mineralization was observed in phenanthrene-grown cells, while naphthalene-grown cells had no effect on anthracene degradation (Fig. 1A). Naphthalene mineralization was not enhanced by cells pregrown on naphthalene, but phenanthrene-grown cells mineralized naphthalene approximately twice as quickly at the same incubation time-point (Fig. 1B). It was also found that phenanthrene mineralization was greatly enhanced by cells pregrown on either naphthalene or phenanthrene (Fig. 1C).

Dioxygenases for PAH metabolism in strain KK1

To investigate the catabolic potential for initial catabolism of PAHs in KK1 cells, we analyzed total DNA extracted from strain KK1 for the presence of dioxygenases capable of hydroxylating unactivated aromatic nuclei, using a specific PCR primer set. PCR products were cloned and 17 randomly selected dioxygenases were sequenced. Comparative analysis of amino acid sequences indicated that 26 clones might originate from PAH dioxygenases in strain KK1. The PAH dioxygenase clones could be divided into three groups, suggesting that strain KK1 has the diverse genes for the catabolism of PAHs, such as naphthalene and phenanthrene (Fig. 2, Table 1). This finding is consistent with radiorespirometric data showing that strain KK1 has the physiological and catabolic ability for PAH mineralization. Subfamily 1 shared significant similarity in deduced amino acid sequences with biphenyl or phenanthrene dioxygenases (55–68% similarity). Subfamily 2 was closest to naphthalene dioxygenase, with 90–100% similarity. Subfam-

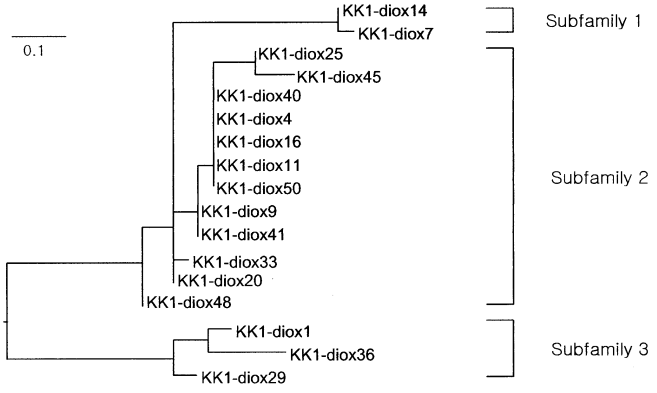

Fig. 2 Phylogenetic tree based on the deduced amino acid sequences of the putative PAH dioxygenase clones. Bootstrap values at the nodes of the dendrogram indicate the percentage of occurrence of the branching order in 500 bootstrapped trees (only values of 50 or above are shown). The *bar scale* represents 10 nucleotide substitutions in 100 nucleotides. The *brackets* indicate the three dioxygenase subfamilies that are discussed in the text

Table 1 Diversity of the putative polycyclic aromatic hydrocarbon dioxygenases in strain KK1. *Numbers in brackets* indicate the dioxygenase clone names obtained through cloning of the PCR product

Dioxygenase group	Amino acid sequence	Possible functions
Subfamily 1 (two clones)	5'-CRHRGNKVCFAEAGNARGFICSYHGW-3' (14) 5'-CRHSGNKVCFAEAGNARGFICSYHGW-3' (7)	Biphenyl or phenanthrene hydroxylation
Subfamily 2 (twelve clones)	5'-CRHRGKTLVSVEAGNAKGFVCSYHGW-3' (20) 5'-CRHRGKTLVSVEAGNAKGFVCCYHGW-3' (9, 41) 5'-CRHSGKTLVSVEAGNAKGFVCCYHGW-3' (4, 11, 16, 40, 50) 5'-CIHRGKTLVSVEAGNAKGFVCCYHGW-3' (25) 5'-CXHRGKTLVSVEAGNAKGFVCCYHGW-3' (45) 5'-CRHRGKTLVSVEAGNAKGFVCYHGW-3' (33) 5'-CRHSGKTLVSVEAGNAKGFVCSYHGR-3' (48)	Naphthalene hydroxylation
Subfamily 3 (three clones)	5'-CRHRGKTLVSVEPAMPKVLFVLITAG-3' (1) 5'-FRHRGKTMVSVEPAMPKVLFVAITAG-3' (36) 5'-CRHSGKTLVSVEPAMPKVLFAATMDG-3' (29)	Unknown

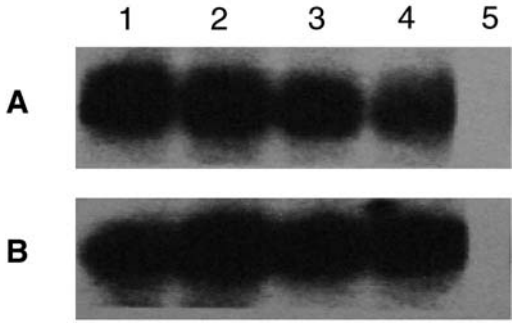

Fig. 3A, B Northern hybridization using two representative dioxygenase probes. Probes Diox14 (**A**) and Diox40 (**B**) were obtained from subfamily 1 and subfamily 2, respectively. Cells were pregrown on tryptic soy broth (TSB) with either naphthalene, phenanthrene, or glucose and were then collected. Approximately 10^5 cells were transferred to 40 mM potassium phosphate containing naphthalene (*lane 1*), naphthalene plus KNO_3 (*lane 2*), phenanthrene (*lane 3*), phenanthrene plus KNO_3 (*lane 4*), or glucose (*lane 5*) and were incubated at 160 rpm at 30 °C. Total RNA (5 μg) isolated from cells following 20 h incubation was used for Northern hybridization

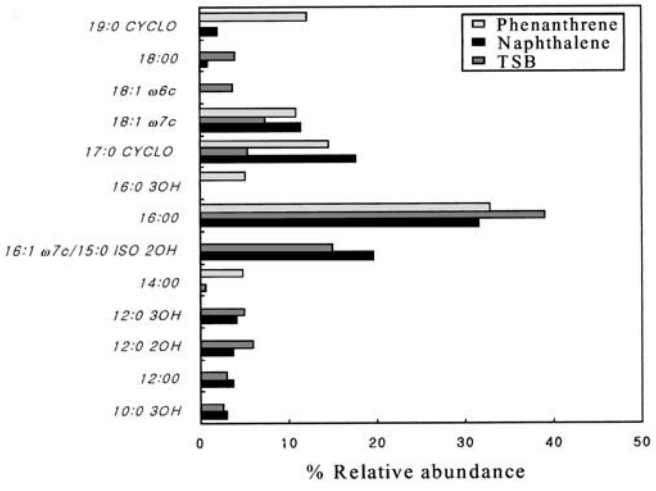

Fig. 4 Comparative analysis of fatty acid profiles of strain KK1 cells exposed to naphthalene and phenanthrene. Total cellular fatty acids were extracted from either TSB-, naphthalene-, or phenanthrene-grown cells and analyzed by gas chromatography with a flame ionization detection

ily 3 was close to naphthalene dioxygenase, but demonstrated high divergence in the amino acid sequence.

Transcriptional expression of the *nah* and *phn* dioxygenase genes

Northern hybridization was carried out to analyze the expression pattern of dioxygenases in response to different PAHs, using one representative from subfamily 1 [assumed to be phenanthrene (*phn*) dioxygenase], or subfamily 2 [naphthalene (*nah*) dioxygenase] as a probe. The amino acid sequences of the probes were 5'-CRHRGNKVCFAEAGNARGFICSYHGW-3' for subfamily 1 and 5'-CSHSGKTLVSVEAGNAKGFVCCYHGW-3' for subfamily 2. The two representative probes gave strong positive signals for naphthalene and phenanthrene, whereas they gave negative signals for glucose (Fig. 3). The putative naphthalene or phenanthrene dioxygenase gene was expressed at a similar level in cells grown on naphthalene or phenanthrene.

Shift in cellular fatty acid composition for PAH-exposed cells

The total cellular fatty acids of KK1 were composed of 11 C-even and 2 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 the total cellular fatty acids for cells grown on complex medium (TSA), but this increased slightly to 38% when cells were exposed to naphthalene and to 33% when exposed to phenanthrene (Fig. 4). Lipid 18:1ω7c/15:0 iso 2OH made up 19% of the total cellular fatty acids for TSA-grown cells. This lipid decreased to 15% for naphthalene-exposed cells and disappeared for phenanthrene-exposed cells. Lipid 16:0 3OH, which was not detectable in TSA-grown cells, increased to a 5% abundance for phenan-

threne. Lipid 9:0 cyclo ω8c dramatically increased from 2% for TSA-grown cells to a 12% abundance for phenanthrene-exposed cells. When KK1 cells were exposed to naphthalene or phenanthrene, 16:0 3OH and 18:1ω6c were no longer detectable, suggesting that the total cellular fatty acid composition of strain KK1 was greatly affected by exposure to naphthalene or phenanthrene.

Discussion

The MGP site used in this study was found to be seriously contaminated with PAHs, such as anthracene, bezo[a]pyrene, chrysene, fluorene, naphthalene, phenanthrene, and pyrene. A pure strain, KK1, was isolated from the highly active consortium obtained from the MGP site. Evaluation of PAH utilization by KK1 revealed that the strain was capable of mineralizing anthracene, naphthalene, and phenanthrene (Fig. 1). In this respect, the result was consistent with previous studies that naphthalene-degrading bacteria were capable of anthracene or phenanthrene degradation, through similar catabolic steps. However, there is no evidence that the three PAHs can be degraded by a catabolic enzyme. *Mycobacterium* sp. strain PYO1 is capable of mineralizing phenanthrene and can also catabolize the four-ring PAH, pyrene. But, this organism cannot metabolize naphthalene. This fact suggests that the enzyme for the initial catabolism of phenanthrene might exist independently from the naphthalene dioxygenase.

KK1 cells pregrown on phenanthrene made a more dramatic effect on the mineralization of anthracene and phenanthrene than naphthalene-grown cells (Fig. 1). Even naphthalene mineralization was much more stimulated by phenanthrene-grown cells. The mineralization rates of anthracene, naphthalene, and phenanthrene were increased approximately 10.0-, 1.6-, and 2.2-fold, respectively, in phenanthrene-grown cells. It is well known that pre-growth on the same substrate stimulates substrate degradation. The result obtained in this study was different from previous findings, in that quicker mineralization of anthracene or naphthalene was performed by phenanthrene-pregrown cells. This result suggested the possibility that some metabolites which are produced during the degradation of phenanthrene are needed for the induction of the enzymes for the degradation of anthracene or naphthalene. The most interesting thing is that approximately 4 days of lag-time was observed for the degradation of anthracene or phenanthrene, whereas there was no lag-time for the degradation of naphthalene. It was assumed that the difference in lag-time between the naphthalene and anthracene (or phenanthrene) degradations might result from differences in chemical structure. Three-ring PAHs, such as anthracene and phenanthrene, probably need a longer time for mineralization than the two-ring PAH, naphthalene. However, the finding that a much longer lag-time than expected was observed in the degradation of phenanthrene warrants further study. Mineralization of other PAHs, such as benzo(a)pyrene, chrysene, and pyrene, were not observed by the naphthalene- or phenanthrene-grown cells. It is generally considered that the degradation rate of any substrate can be enhanced by cells pregrown on the same substrate. However, the data we obtained are different from the general consideration. 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 (Cerniglia and Heitkamp 1989; Goylal and Zylstra 1996). The compound 1,2-dihydroxynaphthalene can be mineralized, using the naphthalene catabolic pathway. These findings suggest that even naphthalene catabolism can be stimulated by phenanthrene in strain KK1.

The PCR amplification of the Rieske iron–sulfur motif region from dioxygenases found in strain KK1 revealed that strain KK1 has diverse dioxygenase genes for the catabolism of neutral aromatic hydrocarbons (Fig. 2). 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 comprising a short electron-transport chain and terminal oxygenase (Cerniglia et al. 1994; Mason and Cammack 1992; Rieske et al. 1964). Typically, the terminal dioxygenase is composed of two dissimilar subunits: large (alpha) and small (beta) subunits. Every large subunit of a dioxygenase enzyme contains a Reiske-type iron–sulfur center (Batie et al. 1987; Geary et al. 1984; Gurbiel et al. 1989; Mason 1988). 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 strain KK1 was of significance, considering that we have very limited information about the role of dioxygenase for the degradation of PAHs. Figure 3 shows the diversity of the putative PAH dioxygenases in strain KK1. A variety of dioxygenase amino acid sequences in a family might result from a PCR reaction using degenerate primers. It is noticeable that subfamily 3 demonstrated highly divergent amino acid sequences, even though close to naphthalene dioxygenases.

Expression patterns of PAH dioxygenases at the transcriptional level were analyzed using Northern hybridization with two representative probes (Fig. 3). The two probes gave strong positive signals for naphthalene and phenanthrene, while they gave negative signals for glucose. These findings were consistent with our expectation that hybridized signals might be obtained in response to naphthalene and phenanthrene. Any indistinguishableness among transcriptional signals in response to different PAH substrates might result from the fact that there is some similarity between the two dioxygenase probes used for Northern blotting. In fact, there is approximately 62% similarity in deduced amino acid

sequences between the two probes used for Northern hybridization.

Exposure of KK1 cells to naphthalene or phenanthrene resulted in changes in the total cellular fatty acid composition (Fig. 4). It is notable that lipids 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH, 17:0 cyclo, 18:1 ω7C, 18:1 ω6C, and 18:0 occupy a small proportion of the total cellular fatty acids, but only lipids 14:0 and 16:0 3OH (which were not detectable on TSA) increased, following exposure to naphthalene or phenanthrene. It was considered that changes in these fatty acids in response to different substrates might affect the cells' survival tolerance, or enhance the cells' ability to utilize the substrate. This finding was consistent with our previous report that several *cis*-unsaturated fatty acids in *Bukholderia* sp. HY1 increased in response to aniline, along with an increase in some *trans*-fatty acids (Kahng et al. 2000). These facts suggest that shifts from *cis*- to *trans*-fatty acids (or vice versa) in KK1 cells might result from the cells' response for both survival and use of substrate in the presence of naphthalene and phenanthrene. Interestingly, lipids 17:0 cyclo, 18:1 ω7c, and 19:0 cyclo increased in response to both naphthalene and phenanthrene. This fact suggested that lipids 17:0 cyclo, 18:1 ω7c, and 19:0 cyclo might play a key role in the cells' tolerance or adaptation to PAHs, warranting further intensive study.

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