Biodegradation of Carbazole by *Pseudomonas rhodesiae* Strain KK1 Isolated from PAH-Contaminated Soil

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Suck Chan Koh

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Abstract

Pseudomonas rhodesiae KK1 was isolated from coal tar-contaminated soil and able to mineralize anthracene, naphthalene, and phenanthrene. In this study, strain KK1 was tested to evaluate carbazole-degrading potential using radiorespirometry and sequence analysis of Rieske-type iron sulfur center of dioxygenase. KK1 was found to be able to mineralize carbazole as a sole source of carbon and nitrogen. When KK1 was pre-grown on phenanthrene the cells were able to mineralize carbazole much more rapidly, suggesting a possible close linkage between the pathways for carbazole and phenanthrene catabolism. Such an increase in carbazole degradation was not appreciable when KK1 was pre-grown on naphthalene. Eleven dioxygenase genes cloned out from KK1 using a universal dioxygenase primer set were found to be the putative carbazole clones based on the similarity of their deduced amino acid sequences. The eleven clones could be categorized into four groups based on the similarity of their amino acid sequences. Data from Northern hybridization using the most possible carbazole dixoygenase gene as a probe confirmed that strain KK1 has car dioxygenase for catabolism of carbazole with other PAH dioxygenases. Analyses of 16S rRNA and PLFAs from strain KK1 revealed that KK1 was closest to *Pseudomonas rhodesiae* with 99% confidence, and designated *Pseudomonas rhodesiae* KK1. Analysis of PLFAs extracted from KK1 cells on carbazole medium revealed that lipids

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10:0 3OH, 17:0 cyclo, and 18:0 were representatives produced or significantly increased in response to carbazole. Tests for antibiotics provided the data that strain KK1 has resistance to antibiotic ampicillin, but susceptibility to chlorampenicol, gentamycin, kanamycine, streptomycin, and tetracycline. Strain KK1 demonstrated strong resistance to most heavy metals such as Ba, Cd, Fe, Hg, Pb used in this study.

제주대학교 중앙도서관
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TABLE OF CONTENTS

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제주대학교 중앙도서관

LIST OF TABLES

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

LIST OF FIGURES

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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INTRODUCTION

1. Environmental contamination by aromatic hydrocarbons and carbazole degradation by microorganisms

Recently, the global pollution of soil, rivers, lakes, marshes, and ocean by various aromatic hydrocarbons as shown in Figure 1. has been of great concern to environmental microbiologists. Particularly, much attention is being paid to polycyclic aromatic hydrocarbons (PAHs) such as anthracene, chrysene, fluorene, naphthalene, phenanthrene, and pyrene because PAHs are considered serious pollutants that are hard to be degraded in the environment. Bioremediation technology using microorganisms has been used to clean up the widely PAH-polluted environments (Jones *et al*., 1989; Pritchard and Costa, 1991). However, little success has been made, because many attempts have been tried without enough knowledge of the PAH-degrading mechanisms by microorganisms. In bioremediating polluted environment, it is imperative to isolate microorganisms that can degrade as well as such pollutants as PAHs, and to obtain information and knowledge about them.

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Carbazole is a nitrogen heterocyclic aromatic compound and considered one of the PAH pollutants. Unlike polyaromatic hydrocarbons, the fate of nitrogen heterocycles in the environment has received little attention, despite their toxicity (Santodonato and Howard, 1981; West *et al*., 1986). It is released into the environment with fossil fuels or their products such as creosote. Carbazole and their derivatives also have been used for the production of dyes, medicines, and plastics (Ouchiyama *et al*., 1993), and detected in diverse environments like

atomospheric samples (Santodonato and Howard, 1981), river sediment (West *et al*., 1986), and groundwater (Pereira *et al*., 1987).

Microorganisms play a primary role in the removal of many types of chemical pollutants from the environment (Sims and Overcash, 1983). Bacteria that can degrade N-heterocyclic aromatic compounds (NHA) such as carbazole, have been isolated (Finnerty *et al*., 1983; Foght and Westlake, 1988; Kobayashi *et al*., 1995; Shotbolt-Brown *et al*., 1996). Intermediates of carbazole degradation have been identified, including 3-hydroxy-carbazole (Resnick *et al*., 1993), anthranilic acid, and catechol (Ouchiyama *et al*., 1993; Hisatsuka and Sato, 1994).

2. Oxygenase Enzyme Systems in Metabolism of Aromatic Hydrocarbons

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In degradation of aromatic hydrocarbons, oxygenase enzymes are involved at several stages of metabolism. These enzymes differ in structure, function, coenzyme requirements, and substrate preference. Oxygenases can be classified into two groups: monooxygenase that incorporate one atom of oxygen into a substrate, and the dioxygenases that add both atoms of oxygen into a substrate.

In monooxygenase catalyzed reactions the other atom of oxygen is reduced to water. Thus, these enzymes are often referred to as mixed-function oxidases, acting as an oxygenase and an oxidase (Mason, 1988). The distinction between monooxygenase and dioxygenase is not always exclusive. Both toluene and naphthalene dioxygenases can catalyze reactions such as the oxidation of indene and indan to 1-indenol and 1-indanol (Wackett *et al*., 1988).

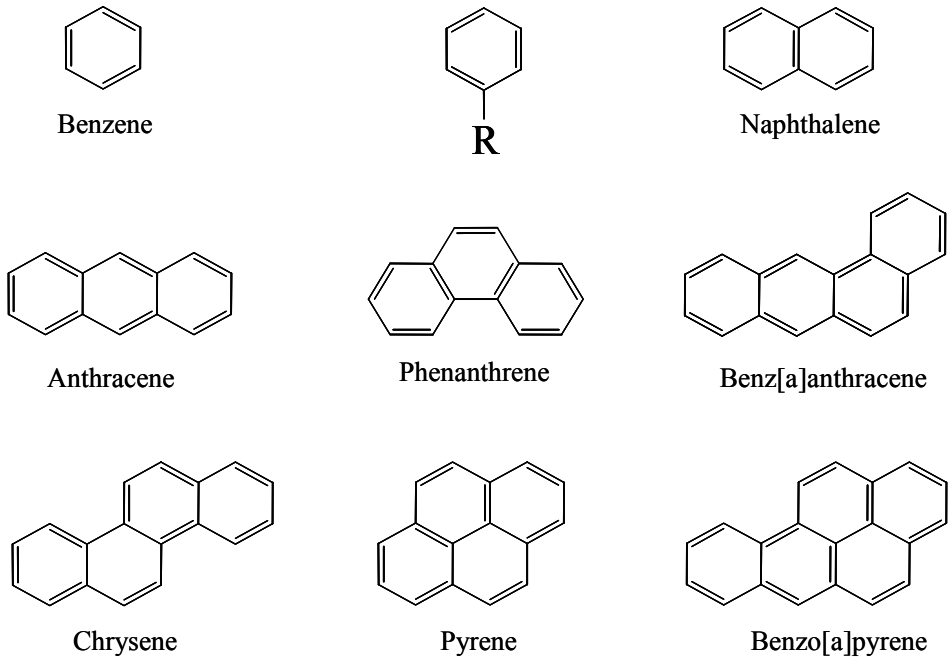

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Fig. 1. Chemical structures of aromatic hydrocarbons, major environmental contaminants.

Dioxygenases can further be divided into ring hydroxylating dioxygenases and ring fission dioxygenases. Ring hydroxylating dioxygenases all require reduced cofactors, either NADH or NADPH in addition to Fe(II) and molecular oxygen (Mason and Cammack, 1992). These enzymes dihydroxylate aromatic hydrocarbons producing *cis*-dihydrodiols. Dioxygenases involved in ring fission have no cofactor requirements (Mason and Cammack, 1992). These enzymes cleave the benzene ring of hydroxylated aromatic substrates.

Hydroxylating dioxygenases are further distinguished from each other by the distribution of redox components, and the number and size of protein subunits. The ring hydroxylating dioxygenases thus far investigated are soluble multicomponent enzymatic systems comprised of a short electron transport chain and terminal oxygenase (Fig. 2).

Three component dioxygenase systems have a flavoprotein act as oxidoreductases catalyzing the transfer of two electrons to the terminal dioxygenase. Flavoproteins of this type, such as those found in benzene dioxygenase and toluene dioxygenase, require an FAD prosthetic group (Axell *et al*., 1975; Subramanian *et al*., 1981). Not surprisingly in light of their cofactor requirements, these proteins typically have two binding sites for ADP moieties, one accommodating FAD and the other NAD.

Two component dioxygenase systems posses a functionally analogous flavoprotein. However, these proteins do not make use of separate ferredoxin proteins. Instead the ferredoxin type redox center is located within the flavoprotein itself. These iron-sulfur flavoprotein reductases require either FMN or FAD as prosthetic groups. Dioxygenases containing these types of flavoproteins include phthlate, benzoate, and toluate (Batie *et al*., 1991; Neidle *et al*., 1991).

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Ferredoxins are small electron transfer proteins containing iron-sulfur clusters (Brushi and Guerlesquin, 1988). Aromatic dioxygenase systems typically have ferredoxins containing an iron-sulfur cluster of the [2Fe-2S] type. The [2Fe-2S] type ferredoxins are also found in chloroplast membranes, in cytochrome P-450-dependent monooxygenase systems such as putidaredoxin from *Pseudomonas putida* camphor monooxygenase, as well as mammalian adrenal mitochondria (mason and Cammack. 1992)

Another class of proteins having a [2Fe-2S] cluster is the Rieske type proteins. These proteins are named after J. S. Rieske who isolated this type of protein from the cytochrome bc_1 complex of mitochondria (Rieske *et al*., 1964). These types of proteins are also found associated with $b6\text{-}_f$ complex of the thylakoid membrane in chloroplasts and the plasma membrane in various photosynthetic bacteria (Fee *et al*., 1984; Gabellini and Sebald 1986; Harnish *et al*., 1985; Riedel *et al*., 1991). One aspect of Rieske iron-sulfur clusters that differ from that of ferredoxin type iron-sulfur clusters is that coordination of the Rieske center iron atoms is accomplished by two sulfide ligands of cysteine moieties and two nitrogen ligands contributed by histidines, instead of the four sulfide ligands found in ferredoxin clusters (Fee *et al*., 1986; Gurbiel *et al*., 1989; Geary *et al*., 1990). Amino acid sequence analysis of Rieske-type proteins reveal two conserved cystein and two conserved histidine residues near the amino terminus of the proteins. These conserved residues make up the motif involved in binding to the Rieske-type [2Fe-2S] cluster (Mason and Cammack, 1992).

The catalytic component of oxygenase enzymes, the terminal oxygenase, also contains a Rieske-type [2Fe-2S] cluster (Batie *et al*., 1987; Geary *et al*., 1984; Gurbiel *et al*., 1989; Mason, 1988). In addition to the conserved cystein-histidine

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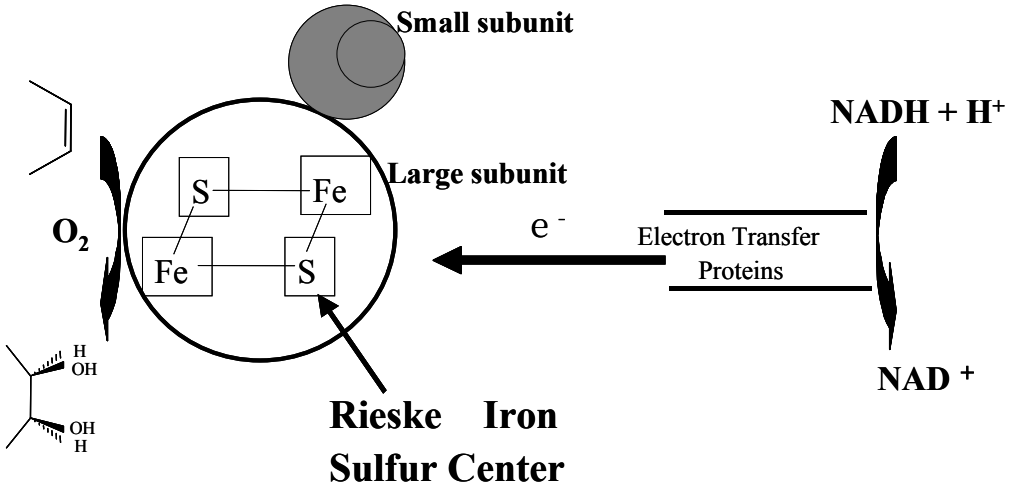

Fig. 2. Initial dioxygenase attack on an aromatic hydrocarbon substrate.

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tags cited above, these proteins also contain two conserved histidine and two conserved tyrosine residues near the center of the proteins (Ire *et al*., 1987; Kurkela *et al*., 1988; Neidle *et al*., 1991). These residues are believed to be responsible for coordinating the iron center of the enzyme where molecular oxygen is bound (Mason and Cammack, 1992; Neidle *et al*., 1991).

Most dioxygenase enzymes are made up of two dissimilar subunits. These subunits can be organized as $\alpha_2\beta_2$ dimmers, as in benzene, toluene, and naphthalene dioxygenases, or they can be organized in an $\alpha_3\beta_3$ arrangement like benzoate dioxygenase (Gibson and Subramanian, 1984; Sauber *et al*., 1973; Yamaguchi and Fujisawa, 1982; Zamanian and Mason, 1987). Exceptions to these arrangements exist, for example phthalate dioxygenase has four identical alpha monomers (Batie *et al*., 1987). The alpha subunit contains the catalytic core and is responsible for substrate recognition. The role of the beta subunit is as of yet unclear.

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3. Enzyme Systems in Carbazole Metabolism by Microorganisms

Ouchiyama *et al*., (1993) was the first to elucidate a carbazole biodegradation pathway, and it involved an angular dioxygenation. Gieg *et al*., (1996) identified several metabolites that were consistent with angular dioxygenation. Carbazole dioxygenation has been intensively studied by cloning and sequencing the genes involved with carbazole degradation in *Pseudomonas* sp. CA10 (Sato *et al*., 1997*a*, 1997*b*; Sheperd and Lloyd-Jones, 1998; Ouchiyama *et al*., 1998; Kasuga *et al*., 2001; Nojiri *et al*., 2001).

Carbazole 1,9a-dioxygenase has been identified as the enzyme responsible for the initial dioxygenation of carbazole in *Pseudomonas* sp. CA10 (Nojiri *et al*., 1999), and produced an unstable intermediate 2'-aminobiphenyl-2,3-diol from the carbazole degradation. This enzyme was found to consist of ferredoxin reductase (36 KDa), ferredoxin, and a monomeric terminal dioxygenase (43 KDa). Two dioxygenases and hydrolase were characterized to be involved with carbazole degradation in *Pseudomonas* sp. strain CA10.

Sequence analysis of the carbazole degradation genes of *Sphingomonas* sp. strain CB3 revealed that the angular dioxygense from strain CB3 was a three-component enzyme similar to biphenyl dioxygenase (Sheperd and Lloyd-Jones, 1998). Based on deduced amino acid sequences, they provided the evidence for a dehydrogenase, an extradiol dioxygenase, and a hydrolase responsible for carbazole degradation as shown in Figure 3. They demonstrated the extradiol dioxygenase activity by the formation of 2-hydroxymuconic semialdehyde from catechol. The enzymatic reactions in carbazole biodegradation studied by Sheperd and Lloyd-Jones(1998) was consistent with those observed by Sato *et al*.,(1997*a*, 1997*b*). *Ralstonia* sp. RJGII.123 that was isolated from soil of a former coal gasification plant was also demonstrated due to its ability to mineralize carbazole. (Schneider *et al*., 2000). However, the mechanism of carbazole degradation by microorganisms was not clear yet.

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In this study, we physiologically and biochemically characterized strain KK1 isolated from PAH-contaminated soil at a former gas plant site and tested KK1 for carbazole degradation potential using a dioxygenase universal primer based-PCR technique and radiorespirometry. The enzymes responsible for initial dioxygenation of carbazole in this bacterial strain are also described.

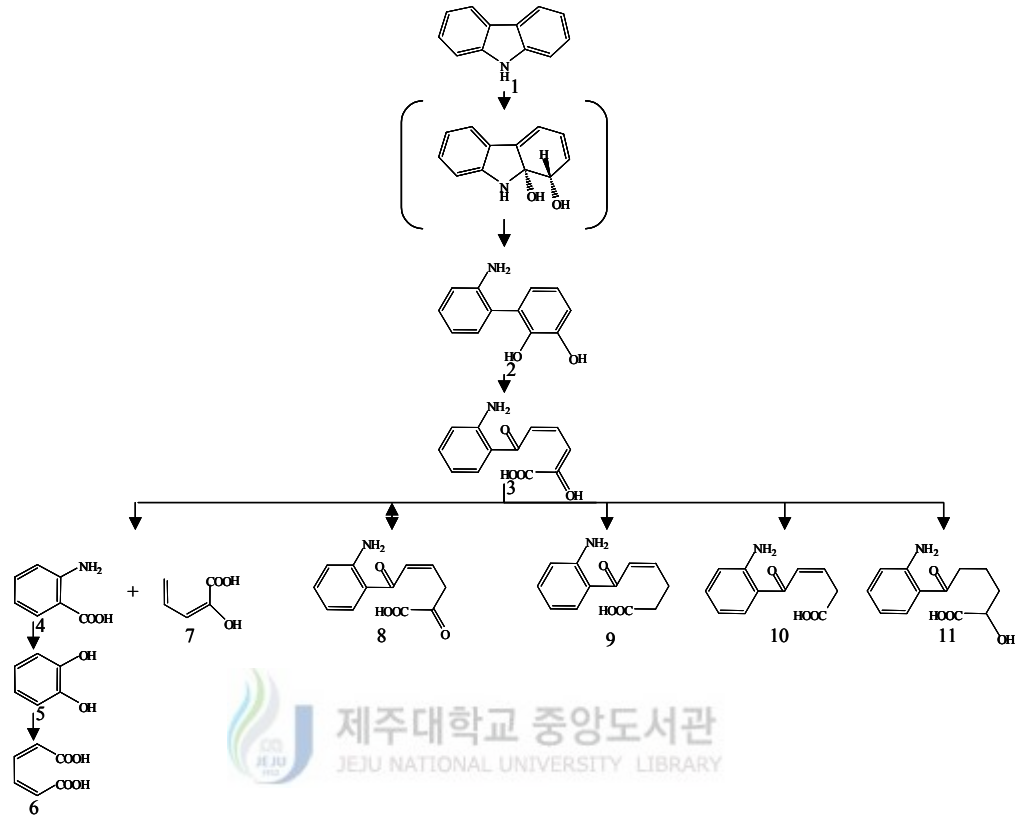

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Fig. 3. Proposed pathways of carbazole degradation by microorganisms.

1. Carbazole
2. 2'-aminobiphenol-2,3-diol
3. 2'-hydroxy-6-oxo-6-(2‘-aminophenyl)hexa-2,4-dionic acid
4. Anthranilic acid
5. Catechol
6. *cis*-muconate
7. 2-hydroxy-4-pentenoate
8. 6-dioxo-6-(2'-amniophenyl)hexa-4-enoic acid
9. 5-oxo-5-(2'-aminophenyl)hexa-4-enoic acid
10. 5-oxo-5-(2'-aminophenyl)penta-3-enoic acid
11. 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexanioc acid

MATERIALS AND METHODS

Overall experimental scheme to characterize strain KK1 and to evaluate its catabolic potential for the substrate carbazole was shown in Figure 4.

1. Isolation of the strain KK1

PAHs-degrading bacteria were isolated from PAH-contaminated soil at the MGP(Manufactured Gas Plant) site through enrichment culture technique. Five-gram samples of 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 $MgSO_4 \cdot 7H_2O$, 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℃ for two weeks. PAHs including anthracene, benzo[a]pyrene, chrysene, phenanthrene, and pyrene were dissolved in methanol (10 mg/mL for the first three compounds and 1 mg/mL for the others), and the PAHs were used as substrates for the enrichment. After two weeks of incubation, 10 mL of the supernatant were collected and incubated for two more weeks as described above. By this procedure, a consortium capable of degrading a variety of PAHs was obtained and used for isolation of pure bacterial strains that were able to degrade PAHs. Serial dilutions of the enrichment consortium (10^{-1} to 10^{-3}) were transferred to solid PAH media containing anthracene, naphthalene, phenanthrene, and then the fast-growing colonies of PAH-utilizing microorganisms were screened. Strain KK1 was selected from the colonies and tested for carbazole degradation potential in this study.

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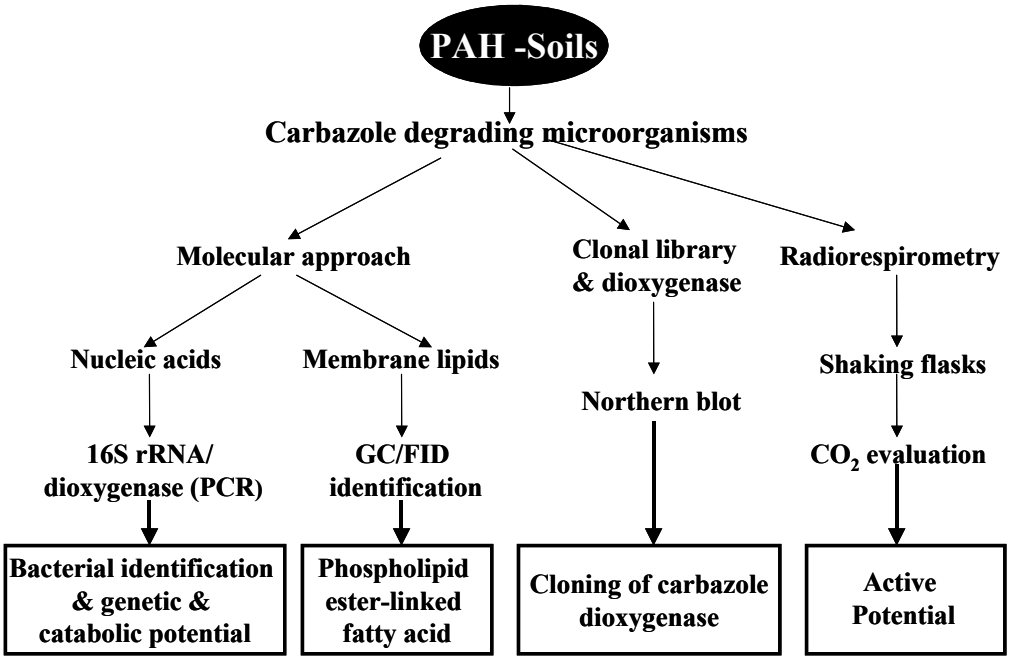

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Fig. 4. A scheme for analyses of carbazole-degrading bacteria and for evaluation of catabolic potential.

2. Resistance test of strain KK1 to antibiotics and heavy metals

Strain KK1 was tested for resistance to various antibiotics and heavy metals. For antibiotic test, LB (Luria-Bertani) media containing either ampicillin, chloramphenicol, gentamycin, kanamycin, streptomycin, or tetracycline were used. Ba, Cd, Co, Fe, Hg, or Pb was added to LB media to evaluate resistance of strain KK1 to heavy metals, and tested at several concentrations.

3. PCR amplification and sequence analysis of 16S rRNA

Genomic DNA of strain KK1 was purified by use of a Clonetech tissue kit (Clontech Lab., Inc., Palo Alto, CA). For PCR amplification of 16S rDNA from strain KK1, two universal primers, 27f (5' AGAG TTT GAT CCT GG CTC AG) and 1522r (5' AAG GAG GTG ATC CA(AG) CCG CA) were used as sense and antisense primers, respectively. The PCR reactions were run for 1 min at 95℃, cycled 25 times(1 min at 95℃, 1 min at 55℃, 1 min at 72℃), and then extended for 10min at 72℃. The PCR products were inserted into a pGEM-T vector and transformed into *E. coli* JM109(Promega Co., Madison, WI, U.S.A.). Two-hundred nanogram of double stranded DNA was used as a template for sequencing together with both 27f and 1522r primers. The nucleotide sequencing was carried out using an ABI 373A automated sequencer. The sequence analysis was performed using the Lasergene software(DNA STAR, Inc., Madison, U.S.A.), along with GCG and BLAST searches of the Genebank database.

4. Identification of strain KK1 using BIOLOG System

The pattern of carbon source utilization by strain KK1 was determined using the GN2 MicroPlate^{TM} (BIOLOG, Hayward, CA). A single KK1 colony grown on

a TSA plate was streaked onto BUG (Biolog Universal Growth) agar medium containing 5% sheep blood and incubated overnight at 30℃. Cells were suspended in normal saline (0.15% NaCl) and 150 uL of the suspension was inoculated into the GN2 MicroPlate™. After 24 hr of incubation the utilization pattern was read using the BIOLOG automated Micro-Station™ instrument.

5. Analysis of phopholipid ester-linked fatty acids (PLFAs)

PLFAs that exist in the strain KK1 were analyzed in the form of fatty acids methyl ester (FAMEs) using MIDI system (Microbial Insights, Inc., Newark, DE). The protocol provided by the manufacturer was used with some modification. Briefly, cells harvested following the 24 hr of growth on TSA were heated to 100℃ with NaOH-methanol to saponify cellular lipids and the released fatty acids were methylated by heating with HCl-methanol 80℃. 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 PAH exposure, cells grown on TSB were collected and washed twice in potassium phosphate buffer (pH 7.0). The washed cells were incubated in mineral salts media each containing 5 mg/mL of carbazole. After 24 hr of incubation at 30℃, changes in the composition of FAMEs before and after exposure to carbazole were analyzed as described above.

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6. Extraction of carbazole and other PAHs from MGP soil

The PAH-contaminated soil used in this study was collected from the depth of 0-2 m below surface at a former manufactured gas plant site in New Jersey, United States of America. The soil was classified as loamy sand, consisting of 78% sand, 11% silt, and 11% clay. The soil was transferred to a 50 mL Teflon centrifuge tube and centrifuged at 18,600 *g* for 15 min. After removing the supernatant, 10 mL each of dichloromethane and acetone were added to the soil and the soil-solvent suspension was shaken (200 rpm) for 48 hours at 30℃ for the extraction of PAHs. The tube was then centrifuged at 18,600 *g* for 15 min and the solvent mixture was transferred to a 50-mL test tube. After removing excess water (upper layer; ca. 2 mL) by pipetting, 4 g of anhydrous sodium sulfate were mixed with the PAHs-containing solvent to remove residual water completely from solvent. The concentration of PAHs in the water layer was less than the detection limit of the analytical procedure used in this study. The extracted was then concentrated to 1 to 2 mL using an evaporator (Buchi Rotavapor; Buchler Instruments Inc., Fort Lee, NJ) for further analysis. By this procedure, PAHs in the 0-2 m layer were recovered. The extract was passed through a 0.45 um polytetrafluoroethylene (PTFE) syringe filter to remove any particulate present and analyzed by a gas chromatography (GC) equipped with a flame ionization detector (Varian Star 3500; Varian Chromatography Systems, Walnut, CA). The GC was installed with a Rtx-5 silica column crossbounded with 5% diphenyl and 95% dimethylpolysiloxane (30 m × 0.53 mm interior diameter; Restek Corporation, Bellefonte, PA). The oven temperature was programmed at 40℃ for 6 min, followed by a linear increase of 10℃ per minute

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to 300℃, and then the temperature was held for 15 min. Injector and detector temperatures were maintained at 300℃. Two microliters of the extract were injected and nitrogen was used as a carrier gas.

7. Determination of carbazole mineralization using radiorespirometry

Catabolic potential of strain KK1 for carbazole was determined by measuring the radioactivity of $^{14}CO_2$ evolved from mineralization of [^{14}C]-labeled carbazole. Cells were grown in TSB and carbazole, respectively to 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 carbazole (10^5 dpm each) as a sole carbon source and incubated at 30℃ for 10 days with shaking (100 rpm). The 50-mL flask used for mineralization experiment was sealed with a Teflon-wrapped silicone stopper through which was placed an 18-guage hypodermic needle and a 16-guage 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℃ 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).

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8. Analysis of dioxygenases for PAH catabolism in strain KK1

To detect and amplify 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 dioxygenase gene fragment from the strain KK1 was performed in a total volume of 50 uL using the Perkin Elmer reagents (Perkin Elmer, Branchburg, NJ). PCR reactions were performed for 1 min at 95℃, cycled 33 times (1 min at 95℃, 1 min at 55℃, 1 min at 72℃), and then extended 10 min at 72℃. 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.

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9. 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 ($O.D._{600}$ = 0.8 ~ 1.0). At this time point cells were collected, and transferred to the medium containing either carbazole, or carbazole plus KNO_3, or carbazole plus glucose, or phenanthrene, or phenanthrene plus KNO_3, and incubated for 6 hours. Cells were then harvested, and washed twice with the mineral salts

solution. Approximately 10^5 cells/mL were transferred to the medium containing 5 mg/mL of carbazole, and incubated for 12 hr at 30℃. 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). DNA fragments for probes in Northern hybridization were labeled by the random priming method provided by Promega (Promega, Madison, WI). Five milligrams of total RNA were used for Northern hybridization with each representative probe from four dissimilar dioxygenase groups obtained from KK1. Other procedures were performed as described previously (Kahng *et al*., 2001)

10. Nucleotide sequence accession number

The 16S rRNA sequence data obtained through this study have been deposited in the GenBank data library under the accession number AY043360.

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11. Chemicals

All polycyclic aromatic hydrocarbons including carbazole used in this study were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.).

RESULTS

1. Carbazole and other PAH concentration of the MGP surface soil and mineralization of carbazole by KK1 strain

Analysis of the top layer (0-2 m below surface) at the MGP soil used in this study revealed that it was seriously contaminated with various PAHs such as anthracene, benzo(a)pyrene, chrysene, naphthalene, phenanthrene, and pyrene (Table 1). 472 g of anthracene, 91 g of benzo[a]pyrene, 162 g of chrysene, 223 g of fluorene, 1685 g of naphthalene, 539 g of phenanthrene, and 171 g of pyrene were extracted per gram soil. Also, seven hundred grams of carbazole were extracted from the surface soil. Strain KK1 was tested for its ability to degrade carbazole in mineral salts media under aerobic conditions. A single colony of KK1 cells grown on TSA was able to mineralize carbazole within 10 days of incubation, however it could not degrade benzo[a]pyrene, chrysene, and pyrene during an equivalent incubation. When 10^5 cells/mL were used, approximately 12% of carbazole was mineralized at the 10-day incubation time point (Fig. 5).

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2. Carbazole mineralization by naphthalene- or phenanthrene-induced cells

KK1 cells pre-grown on naphthalene or phenanthrene were evaluated for carbazole mineralization. There was no change in substrate specificity utilizable by KK1 cells pre-grown on naphthalene or phenanthrene. However, KK1 cells

pre-grown on phenanthrene exhibited much quicker and stronger catabolic potential for the substrate carbazole. Much quicker rate of carbazole mineralization was observed in phenanthrene-grown cells, while naphthalene-grown cells made no great effect on carbazole degradation (Fig. 5).

3. Dioxygenases for carbazole metabolism in KK1 strain

To investigate catabolic potential for initial catabolism of carbazole by 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 50 randomly selected clones were sequenced. Comparative analysis of deduced amino acid sequences indicated that the putative PAH dioxygenase clones from strain KK1 could be divided into 4 groups based on the similarity with known dioxygenase amino acid sequences, suggesting that KK1 strain has the multiple genes for catabolism of PAHs. One group that was assumed a possible carbazole dioxygenase was selected, and it was multialigned based on the deduced amino acid sequences(Fig. 6,7).

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Table. 1. Amount of PAHs extracted from the PAH-contaminated surface soil

PAHs	ANT	BaP	CAR	CHR	FLU	NAP	PHE	PYR*
Amount extracted (ug/g soil)	472	91	700	162	223	1685	539	171

The uppercase (*) indicates abbreviations of the following.

ANT, anthracene; BaP, benzo(a)pyrene; CAR, carbazole; CHR, chrysene; FLU, fluorene; NAP, naphthalene; PHE, phenanthrene; PYR, pyrene.

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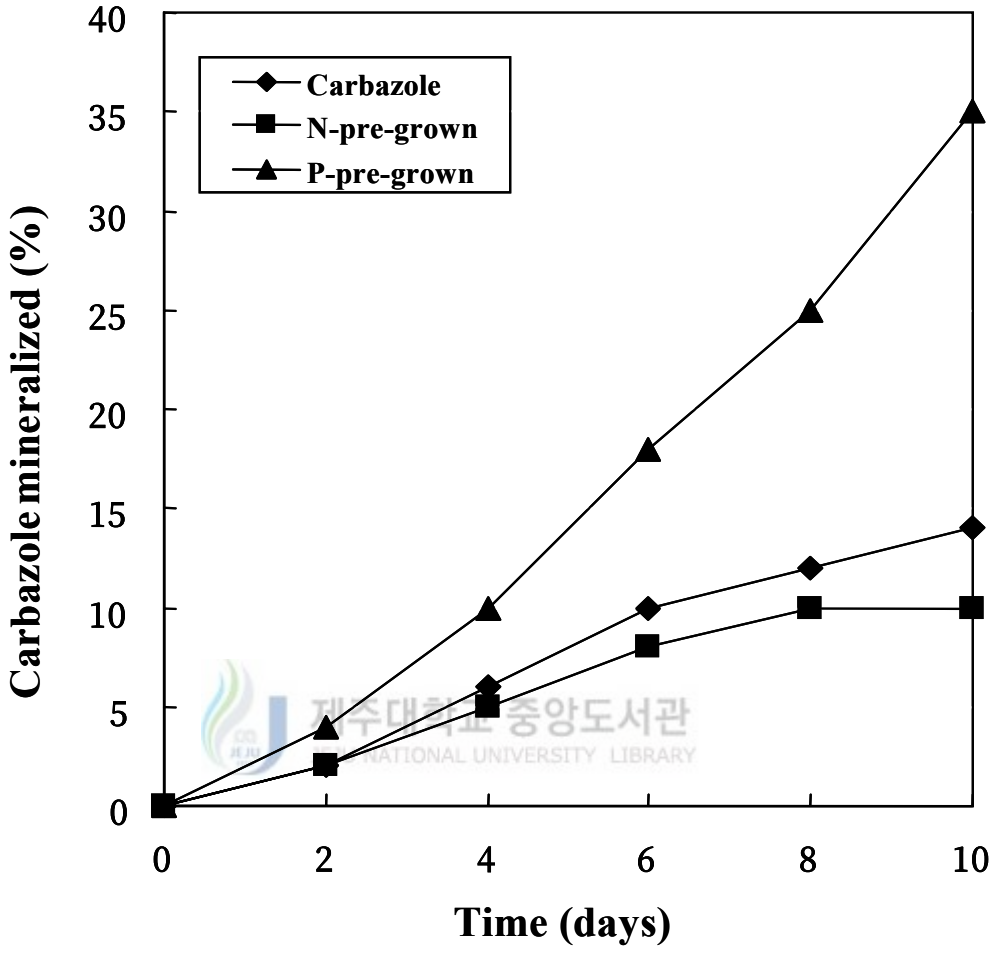

Fig. 5. Comparative analysis of rate of carbazole mineralization by KK1 cells pre-grown on naphthalene and phenanthrene.

tgcagtcata gcggcaagac gctggtgagc gtggaagccg	
gcaatgccaa aggttttgtt tgctgctatc acggctgggg	diox 4
tgtcgacatc gcggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgttgctatc acggctgggg	diox 5
tgcaggcaca gaggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgttcctacc atggctggg	diox 6
tgcaggcatc ggggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgctgttacc atggttggg	diox 8
tgccgacata gtggcaagac gctggtgagc gtggaagccg	
gcaatgccaa aggttttgtt tgttgctatc acggttgggg	diox 11
tgcagtcaca gcggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgctcctatc atggttgggg	diox 13
tgccgacatc gcggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgctcctatc acggatgggg	diox 15
tgcagccata gaggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgttgctatc atggctgggg	diox 17
tgccgtcatc gaggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgttgctatc acggatgggg	diox 24
tgtagtcata gcggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgcacttatc atggctgggg	diox 27
tgtagtcata gcggtgccac tgtttgcgag cacaagaagg	
gcaagaccaa cagcttcgtc tgcacttatc atggctgggg	diox 44

Fig. 6. Putative carbazole dioxygenase DNA sequences.

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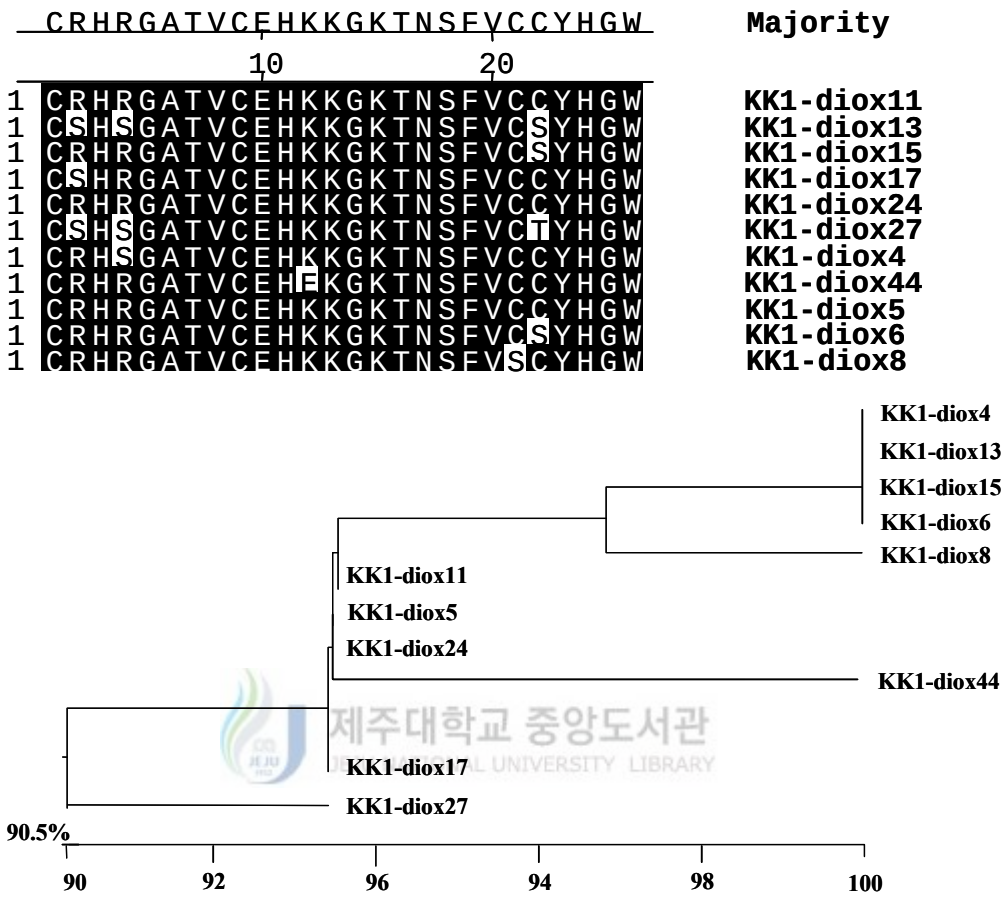

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Fig. 7. Putative carbazole dioxygenase amino acid sequences from strain KK1 and the phylogenetic tree.

4. Identification of dioxygenases for carbazole catabolism using northern blot

Northern hybridization was carried out to analyze the expression pattern of dioxygenases in response to carbazole under different conditions using one representative, KK1-diox24 as a probe. It indicated that carbazole dioxygenase gave stronger positive signals for carbazole containing KNO_3 than only carbazole (Fig. 8). However, glucose was found to repress expression of carbazole dioxygenase.

5. Shift in cellular fatty acid composition for carbazole-exposed cells

The total cellular fatty acids of KK1 were comprised 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 34% of total cellular fatty acids for cells grown on complex medium (TSA), but decreased slightly to 32% when cells were exposed to carbazole (Fig. 8). Lipids 10:0 3OH, 12:0 3OH, 17:0 cyclo, and 18:0 increased in response to carbazole, while lipids 12:00, 12:0 2OH, 19:0 cycloω8c were no longer detectable, suggesting that the total cellular fatty acid composition of strain KK1 was greatly affected by exposure to carbazole. Notably, lipid 18:0 that was not detectable on TSA-grown cells increased to 10% abundance for carbazole (Fig. 9).

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A. KK1-diox 24 nucleotide sequence used for a probe for northern blot

```
TGCCGTCATCGAGGTGCCACTGTTTGCGAGCACAAGAAGGGCAAGACCAACAGCTTCGTCTGTTGCTATCACGGATGGGG
                                                                              80
ACGGCAGTAGCTCCACGGTGACAAACGCTCGTGTTCTTCCCGTTCTGGTTGTCGAAGCAGACAACGATAGTGCCTACCCC
```

B. Northern hybridization with mRNAs extracted from cells exposed to carbazole

Lane 1 2 3 4 5

Fig. 8. Northern hybridization using KK1-diox 24 nucleotide sequence as a probe.

Lane 1: Phenanthrene + KNO_3

Lane 2: Phenanthrene

Lane 3: Carbzole + KNO_3

Lane 4: Carbazole

Lane 5: Carbazole + Glucose

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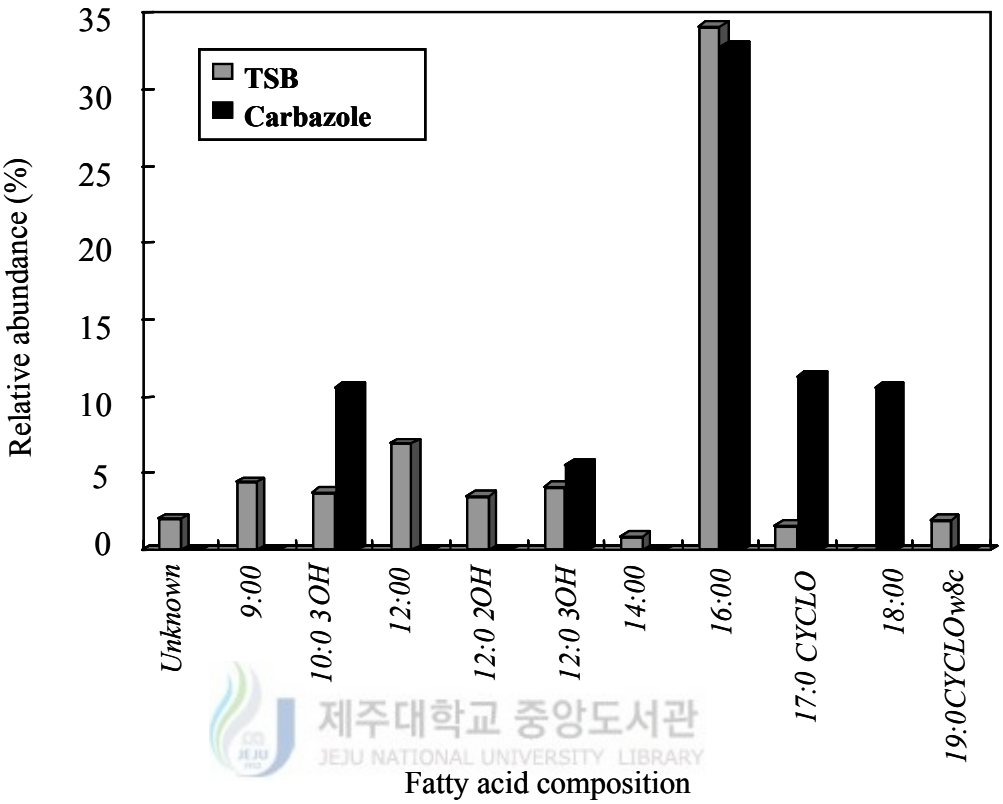

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Fig. 9. Comparative analysis of fatty acid compositions from strain KK1 cells grown on either TSB or carbazole.

6. Physiological and phylogenetic characterization of strain KK1

The result obtained through BIOLOG substrate utilization analysis indicated that strain KK1 was a member of the *Pseudomonas* species cluster, with 94% matching similarity(Table 2). Analysis of total cellular fatty acids by GC-FID also placed strain KK1 as a *Pseudomonas* species with over 90% confidence. Analysis of a 1521bp fragment of the amplified 16S rRNA sequence from strain KK1 and comparative multiple alignment of this sequence with others in the 16S rRNA database indicated that strain KK1 was placed among five major clonal types in the Proteobacteria, including *Pseudomonas rhodesiae*, *Pseudomonas grimontii*, *Pseudomonas marginalis,* and *Pseudomonas veronii, Pseudomonas fluorescens*. The greatest similarity was to *Pseudomonas rhodesiae*, with 99% similarity (Fig. 10). Based on combination of these analyses, we designate strain KK1 as *Pseudomonas rhodesiae* KK1. Complete 16S rRNA sequence of KK1 was demonstrated in Figure 11. Tests for antibiotics provided the fact that strain KK1 has resistance to antibiotic ampicillin, but susceptibility to chlorampenicol, gentamycin, kanamycine, streptomycin, and tetracycline. Also, strain KK1 demonstrated strong resistance to most of heavy metals such as Ba, Cd, Fe, Hg, Pb used in this study.(Table 3,4).

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Table 2. Physiological and biochemical characterization of strain KK1 using BIOLOG Analysis System

Physiological and biochemical tests		Physiological and biochemical tests	
Water	-	β-Hydroxybutyric acid	+
α-cyclodextrin	-	ρ-Hydroxyphenylacetic acid	-
Dextrin	ND*	γ-Hydroxybutyric acid	-
Glycogen	ND	α-Ketobutyric acid	+
Tween 40	+	α-Ketoglutaric acid	-
Tween 80	+	α-Ketovaleric acid	+
N-Acetyl-D-galactosamine	-	D,L-Lactic acid	+
N-Acetyl-D-glucosamine	-	Malonic acid	+
Adonitol	-	Propionic acid	+
L-Arabinose	+	Quinic acid	+
D-Arabitol	+	D-Saccharic acid	+
Cellobiose	-	Sebacic acid	-
i-Erythritol	ND	Succinic acid	+
D-Fructose	+	Bromo-succinic acid	+
L-Froctose	-	Suucinamic acid	+
D-Galactose	+	α-D-Glucose	+
meso-Inositol	+	α-D-Lactose	-
Lactulose	-	Maltose	-
Mannitol	+	Mannose	+
D-Mellibiose	-	β-Methyl-D-glucoside	-
D-Psicose	+	D-Raffinose	-
L-Rhamnose	-	D-Sorbitol	+
Sucrose	+	D-Trehalose	+
Turanose	ND	Xylitol	-
Methylpyruvate	+	Mono-methylsuccinate	+
Acetic acid	+	*cis*-Aconitic acid	+
Citric acid	+	Formic acid	+
D-Galactonic acid lactone	+	D-Galacturonic acid	+
D-Gluconic acid	+	D-Glucosaminic acid	+
D-Glucuronic acid	+	Glucuronamide	+

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Table 2. continued

Physiological and biochemical tests		Physiological and biochemical tests	
α-hydroxybutyric acid	+	Itaconic acid	+
Alanineamide	-	D-Alanine	+
L-Alanine	+	L-Alanyl-glycine	+
L-asparagine	+	L-Aspartic acid	+
L-Glutamic acid	+	Glycyl-L-aspartic acid	-
Gylcyl-L-glutamic acid	+	L-Histidine	+
Hydroxy L-proline	+	L-Leucine	+
L-Ornithine	ND	L-Phyenylalanine	-
L-Proline	+	L-Pyroglutamic acid	+
D-Serine	ND	L-Serine	+
L-Threonine	+	D,L-Carnitine	ND
γ-Aminobutyric acid	+	Urocanic acid	+
Inosine	+	Uridine	ND
Thymidine	-	Phenylethylamine	-
Putrescine	ND	2-Aminoethanol	+
2,3-Butanediol	ND	Glycerol	+
D,L-α-Glycerol phosphate	-	Glucose-1-phosphate	-
Glucose-6-phosphate	-	Gentiobiose	-

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*ND indicates 'Not Determined'.

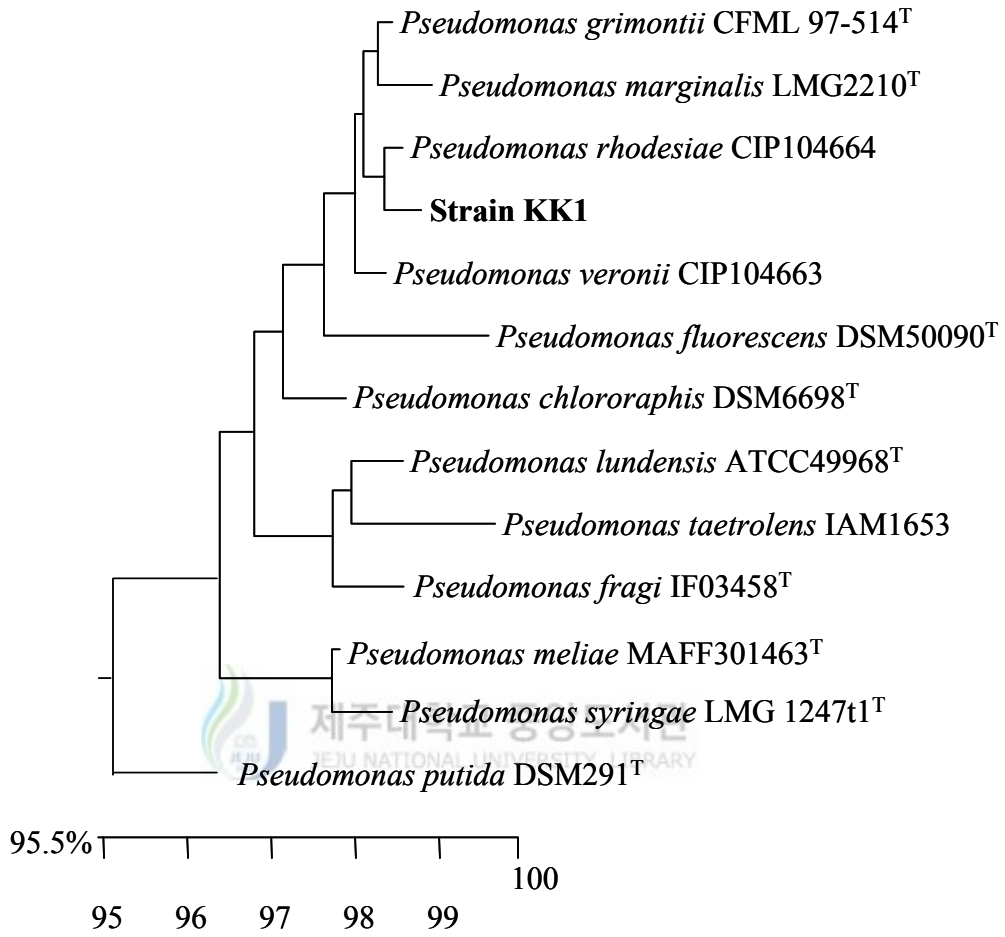

Fig. 10. Phylogenetic tree for *Pseudomonas* strains based on 16S rRNA sequences.

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5′-atcctggctc agattgaacg ctggcggcag gcctaacaca tgcaagtcga
gcggtagaga gaagcttgct tctcttgaga gcggcggacg ggtgagtaat
gcctaggaat ctgcctggta gtgggggata acgttcggaa acgaacgcta
ataccgcata cgtcctacgg gagaaagcag gggaccttcg ggccttgcgc
tatcagatga gcctaggtcg gattagctag ttggtggggt aatggctcac
caaggcgacg atccgtaact ggtctgagag gatgatcagt cacactggaa
ctgagacacg gtccagactc ctacgggagg cagcagtggg gaatattgga
caatgggcga aagcctgatc cagccatgcc gcgtgtgtga agaaggtctt
cggattgtaa agcgctttaa gttgggagga agggcagtta cctaatacgt
gattgttttg acgttaccga cagaataagc accggctaac tctgtgccag
cagccgcggt aatacagagg gtgcaagcgt taatcggaat tactgggcgt
aaagcgcgcg taggtggtta gttaagttgg atgtgaaatc cccgggctca
acctgggaac tgcattcaaa actgactgac tagagtatgg tagagggtgg
tggaatttcc tgtgtagcgg tgaaatgcgt agatatagga aggaacacca
gtggcgaagg cgaccacctg gactgatact gacactgagg tgcgaaagcg
tggggagcaa acaggattag ataccctggt agtccacgcc gtaaacgatg
tcaactagcc gttgggagcc ttgagctctt agtggcgcag ctaacgcatt
aagttgaccg cctggggagt acggccgcaa agttaaaact caaatgaatt
gacgggggcc cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg
cgaagaacct taccaggcct tgacatccaa tgaactttct agagatagat
tggtgccttc gggaacattg agacaggtgc tgcatggctg tcgtcagctc
gtgtcgtgag atgttgggtt aagtcccgta acgagcgcaa cccttgtcct
tagttaccag cacgtaatgg tgggcactct aaggagactg ccggtgacaa
accggaggaa ggtggggatg acgtcaagtc atcatggccc ttacggcctg
ggctacacac gtgctacaat ggtcggtaca gagggttgcc aagccgcgag
gtggagctaa tcccataaaa ccgatcgtag tccggatcgc agtctgcaac
tcgactgcgt gaagtcggaa tcgctagtaa tcgcgaatca gaatgtcgcg
gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccatgggagt
gggttgcacc agaagtagct agtctaacct tcgggggggac ggttaccacg
gtgtgattca tgactggggt gaagtcgtaa caaggtagcc gtaggggaac
ctgcggctgg atcacctcct t-3′ 1521 bp

Fig. 11. The nucleotide sequence of 16S rRNA gene from *Pseudomonas rodesiae* strain KK1.

Table 3. Resistance of *Pseudomonas rhodesiae* strain KK1 to antibiotics

Antibiotics (ug/mL)		*Pseudomonas rhodesiae* strain KK1
Ampicillin	100	+
	200	+
	400	+
Tetracycline	12.5	-
	25	-
	50	-
Kanamycin	12.5	-
	25	-
	50	-
Streptomycin	25	-
	50	-
	100	-
Chloramphenicol	50	-
	100	-
	200	-
Gentamycin	25	+
	50	-
	100	-

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Table 4. Resistance of *Pseudomonas rhodesiae* strain KK1 to heavy metals

Heavy metals(ug/mL)		*Pseudomonas rhodesiae* strain KK1
HgCl	5	+
	10	+
	20	+
	40	+
	80	+
$CdCl_2$	5	+
	10	+
	20	+
	40	+
	80	+
$PbCl_2$	5	+
	10	+
	20	+
	40	+
	80	+
$Co(NO)_3$	100	+
	200	-
	400	-
	800	-
	1600	-
$BaCl_2$	100	+
	200	+
	400	+
	800	-
	1600	-
$FeCl_2$	100	+
	200	+
	400	+
	800	+
	1600	-

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DISCUSSION

The MGP site used in this study was found to be seriously contaminated with polycyclic aromatic hydrocarbons (PAHs) such as anthracene, bezo[a]pyrene, carbazole, chrysene, fluorine, naphthalene, phenanthrene, and pyrene (Fig. 1). Previous studies revealed that PAHs have been found in wood preservation, petroleum, oily wastes, coal gasfication sites, tobacco smoke, automobile exhaust gas, incinerated waste, industrial effluents, and even in urban air (Sims and Overcash, 1983, Arvin *et al*., 1992, Pothuluri *et al*., 1993, Grifoll *et al*., 1994, van Agteren *et al*., 1998). These facts increase human concerns about the microbial degradation of PAHs. Many studies have been conducted to elucidate the microbial mechanism of PAH degradation as well as to decontaminate the PAH-polluted environments, but little is known about the microbial degradation of PAHs. Accordingly, in an effort to obtain more information about microbial degradation of PAHs, a consortium capable of PAH mixtures that consist of anthracene, bezo[a]pyrene, chrysene, fluorene, naphthalene, phenanthrene, and pyrene was obtained by an enrichment technique in this study.

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A pure strain, KK1 was isolated from the highly active consortium that had been obtained from the MGP site. Evaluation of PAH utilization by KK1 revealed that the strain was capable of mineralizing anthracene, carbazole, naphthalene, and phenanthrene. In this respect, the result was consistent with some previous studies that naphthalene-degrading bacteria were capable of anthracene or phenanthrene through similar catabolic steps. However, to our knowledge, there is no evidence that the three PAHs can be catabolized by a catabolic enzyme. *Mycobacterium* sp. strain PYO1 (Daane *et al*. 2001) is capable

of mineralizing phenanthrene, and also catabolize the four ring PAH pyrene. But, this organism cannot metabolize naphthalene. This fact suggests that the enzyme for initial catabolism of phenanthrene might exist independently from the naphthalene dioxygenase.

KK1 cells pre-grown on phenanthrene made a more dramatic effect on mineralization of carbazole than naphthalene-grown cells (Fig. 5). Mineralization rate of carbazole was increased approx. 2.8-times in phenanthrene-grown cells (Fig. 5). Mineralization of other PAHs such as benzo(a)pyrene, chrysene, and pyrene were never observed by the naphthalene- or phenanthrene-grown cells.

The PCR amplification of the Rieske iron-sulfur motif region from dioxygenases found in KK1 strain revealed that strain KK1 has diverse dioxygenase genes for catabolism of neutral aromatic hydrocarbons (Fig. 6). 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 (Cerniglia *et al*., 1994; Mason and Cammack, 1992). Typically the terminal dioxygenase is composed of two dissimilar subunits, large (or alpha) and small (or beta) subunits. It is considered that the large subunit is the catalytic core of the enzyme and is responsible for the recognition of aromatic hydrocarbons. However, the role of the small subunit in catabolism of aromatic hydrocarbons is yet unclear. Every large subunit of a dioxygenase enzyme contains a Rieske-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

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enzyme. Accordingly, identifying amino acid sequences of the Rieske-type iron-sulfur motif regions in strain KK1 was of significance, considering that we have very limited information about the role of dioxygenase for degradation of PAHs. As a part of finding dioxygenase for carbazole catabolism in strain KK1, 11 dioxygenase clones that were closer to known carbazole dioxygenase were phylogenetically analyzed (Fig. 7). One dioxygenase fragment of them was selected for northern hybridization with mRNA isolated from KK1 cells grown on carbazole, based on the similarity with known aromatic dioxygenase for catabolism of carbazole.

Northern hybridization was carried out with mRNAs extracted from five different media compositions (Fig. 8). Strongest positive signal was obtained for cells pre-grown on phenanthrene with KNO_3, while glucose-grown cells gave weakest signal, suggesting that the enzymes for carbazole catabolism might be activated very efficiently by phenanthrene. However, weaker signal in glucose-grown cells suggested that expression of carbazole dioxygenase might be repressed by glucose. Data obtained from northern hybridization provided that amino acid sequences of KK1-diox24 (5-CRHRGATVCEHKKGKTNSVCYHGW) might be originated from the dioxygenase for carbazole catabolism in KK1 strain, warranting further analysis of the full-length dioxygenase responsible for carbazole catabolism in the future.

Exposure of KK1 cells to carbazole resulted in changes of the total cellular fatty acid composition (Fig. 9). It is notable that lipids 17:0 cyclo, 18:0 significantly increased following exposure to carbazole. It was considered that changes of these fatty acids in response to different substrates might affect cells survival tolerance, or enhance cells ability to utilize the substrate. This finding

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was consistent with our previous study that several *cis*-unsaturated fatty acids in *Bukholderia* sp. HY1 increased in response to aniline, along with increase of some saturated fatty acids (Kahng *et al*., 2000). These facts suggest that shifts from *cis*- to *trans*-fatty acids (or *vice versa*), or from unsaturated to saturated fatty acids (or *vice versa*) in KK1 cells might result from cells response for both survival and use of substrate in the presence of naphthalene and phenanthrene. Many reports of a similar nature have suggested that 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*., (1996) reported that solvent- tolerant and solvent-sensitive *Pseudomonas putida* strains were able to produce *trans*-unsaturated fatty acids following exposure to *o*-xylene.

KK1 cells isolated from the MGP site were physiologically and genetically characterized using BIOLOG system and sequence analysis of 16S rRNA. KK1 strain was identified as *Pseudomonas* species with 94% confidence when Biolog system was applied, as *Pseudomonas* sp. with over 90% confidence by total cellular compositions of fatty acid, and as *Pseudomonas rhodesiae* with 99% confidence by 16S rRNA sequence (Fig. 11). Accordingly, strain KK1 was identified as *Pseudomonas rhodesiae* based on combination of these data, and designated *Pseudomonas rhodesiae* KK1. The phylogenetic tree based on 16S rRNA was shown in Figure 10., suggesting that strain KK1 was far away in the phylogenetic distance from the strains that can degrade polycyclic aromatic compounds.

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ABSTRACT IN KOREAN

안트라센 (anthracene), 나프탈렌 (naphthalene), 페난스렌 (phenanthrene)을 분해할 수 있는 *Pseudomonas rhodesiae* KK1을 콜타르 (coal tar)에 오염된 토양에서 분리하였다. 본 연구에서는 동위원소로 표지된 방향족 화합물 기질을 이용하여 물질대사의 결과 발생하는 이산화탄소를 측정하는 법 (radiorespirometry)과 Rieske Iron sulfur center의 dioxygenase의 염기서열을 이용하여 KK1 균주의 카바졸(carbazole) 분해능을 분석하였다. 그 결과, KK1균주는 단일 탄소원 및 질소원으로 카바졸을 이용할 수 있음을 밝혔다. 페난스렌에서 성장했을 때 KK1 세포는 카바졸을 훨씬 빠르게 이용하여 카바졸과 페난스렌 화합물을 분해하는데 필요한 물질대사의 경로가 상당한 연관성이 있음을 제시하였다. 반면, 나프탈렌에서 배양된 KK1은 카바졸 분해능의 증가가 뚜렸하지 않았다. Universal dioxygenase primer를 사용하여 카바졸 분해에 관련될 것으로 추정되는 11개의 dioxgenase 유전자를 KK1으로부터 분리하였고, 아미노산서열의 상동성에 의해 4개군으로 분류하였다. 11개의 클론중, 기존의 카바졸 dioxygenase와 가장 상동성이 높은 클론을 탐침자로 사용하여 Northern 혼성화반응을 실시한 바, KK1 세포는 카바졸을 분해하는데 필요한 dioxygenase를 가지고 있음을 밝혔다. 또한, 페난스렌과 KNO_3를 포함하는 배지에서 성장한 세포가 카바졸에 노출되었을 때, 가장 활발한 카바졸 dioxygenase의 발현양상을 보인 반면, 포도당에서 성장한 세포는 오히려 유전자의 발현이 억제되는 양상을 나타내었다. BIOLOG system을 이용한 생리 및 생화학적 실험, 16S rRNA 염기서열 분석 및 지방산 분석을 통하여 KK1은 99%의 신뢰도로 *Pseudomonas rhodesiae*으로 동정되어, KK1균주를 *Pseudomonas rhodesiae* KK1으로 명명하였다. KK1세포를 카바졸에 노출시켰을 때 10:0 3OH, 17:0 cyclo, 그리고 18:0 등의 지방산이 크게 증가하는 것으로

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나타났다. 항생제 및 중금속에 대한 저항성 실험의 결과, KK1세포는 암피실린에 대해서 내성을 보인 반면 클로람페니콜, 겐타마이신, 가나마이신, 스트렙토마이신, 테트라사이클린 에서는 감수성을 보였다. 본 실험에서 사용한 대부분의 중금속, 바륨, 카드뮴, 철, 수은, 납에 강한 저항성을 보였다.

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