Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 40 (2006) 71–78
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Evaluating the genetic diversity of dioxygenases for initial catabolism of aromatic hydrocarbons in *Pseudomonas rhodesiae* KK1

Seung-Il Kim [a], Jerome J. Kukor [b], Kye-Heon Oh [c], Hyung-Yeel Kahng [d,*]

[a] *Proteome Analysis Team, The Korea Basic Science Institute, Daejon, Republic of Korea*
[b] *Biotechnology Center for Agriculture and the Environment, Rutgers University, New Brunswick, NJ 08901-8520, USA*
[c] *Department of Life Science, Soonchunhyang University, P.O. Box 97, Asan, Chung-Nam 336-600, Republic of Korea*
[d] *Department of Environmental Education, Sunchon National University, 315 Maegok-dong, Sunchon 540-742, Republic of Korea*

Received 18 March 2005; received in revised form 11 October 2005; accepted 14 October 2005

Abstract

Pseudomonas rhodesiae strain KK1 was found to be capable of utilizing a broad range of aromatic compounds as well as polycyclic aromatic hydrocarbons in this study. The Rieske-type iron–sulfur center sequences of dioxygenases obtained from KK1 through PCR process using a universal dioxygenase primer set were used to evaluate catabolic potential for aromatic compounds. Comparative analysis of predicted amino acid sequences from 50 randomly selected dioxygenase clones capable of hydroxylating inactivated aromatic nuclei indicated that the clones from strain KK1 could be divided into five groups. Amino acid sequences of each dioxygenase clone were a part of the genes encoding enzyme for initial catabolism of benzoate, carbazole, *p*-cumate, naphthalene, phenanthrene, biphenyl, or vanillate. Radiorespirometric analysis revealed that strain KK1 was able to mineralize polycyclic aromatic hydrocarbons such as anthracene, naphthalene and phenanthrene, as well as a nitrogen heterocyclic aromatic compound-like carbazole. Also, it has been found through HPLC analysis that benzoate, biphenyl, *p*-cumate, and vanillate also could be degraded by strain KK1. These results were consistent with data obtained through analyses of dioxygenase clones and substrate conversion assay, providing the evidence that strain KK1 has the capability to degrade a broad range of aromatic substrates including polycyclic aromatic hydrocarbons.

Keywords: Dioxygenases; Aromatic hydrocarbons; Radiorespirometry; *Pseudomonas rhodesiae* KK1

1. Introduction

Studies on the fate of aromatic hydrocarbons including polycyclic aromatic hydrocarbons (PAH) in the environment are of great human concern because many of these compounds have been found to be cytotoxic, mutagenic, and potentially carcinogenic [1,2]. Much effort to isolate pure bacterial strains capable of degrading aromatic hydrocarbons has been made to elucidate the degradation mechanisms as well as to make a strategy to decontaminate aromatic hydrocarbon (AH)-polluted environments. As a result, diverse dioxygenase genes for catabolism of aromatic hydrocarbons from one- to multi-ring aromatic compounds have been identified in microorganisms. Especially, dioxygenase genes for catabolism of aniline, carbazole, dioxin, phenanthrene, or phthalate have been identified and characterized in a few microorganisms [3–13]. A few recent studies on genetic diversity have been mainly conducted with microbial populations or many bacteria. This study focuses on analyzing diversity of dioxygenase genes of a bacterium, *Pseudomonas rhodesiae* KK1 capable of utilizing a broad range of monoaromatic compounds including polycyclic and heterocyclic aromatic hydrocarbons such as carbazole and naphthalene previously mentioned [14]. For the analysis of dioxygenase genes, the Rieske-type iron–sulfur center sequences of dioxygenases were selected, because every large subunit of a dioxygenase enzyme contains a Rieske-type iron–sulfur center, as well as the iron–sulfur center has two characteristic amino acid sequence motifs surrounding a region of amino acids whose sequence varies from enzyme to enzyme [15]. Radiorespirometry, enzyme assay and molecular techniques have been used for the comparative study of dioxygenase genes in this bacterial strain.

* Corresponding author. Tel.: +82 61 750 3385; fax: +82 61 750 3308.
E-mail address: kahng@sunchon.ac.kr (H.-Y. Kahng).

0141-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.10.041

2. Materials and methods

2.1. Analysis of soil texture and extraction of polycyclic aromatic hydrocarbons

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 (MGP) site in New Jersey. The soil was classified as loamy sand, consisting of 78% sand, 11% silt, and 11% clay. The soil slurry used in this study 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 for 48 h at 30 °C for the extraction of PAH. 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 PAH-containing solvent to remove residual water completely from solvent. The concentration of PAH in the water layer was less than the detection limit of the analytical procedure used in this study. The extract was then concentrated to 1–2 ml using an evaporator (Buchi Rotavapor, Buchler Instruments Inc., Fort Lee, NJ, U.S.A.) for further analysis. By this procedure, PAH in the 0–2 m layer were recovered. The extract was passed through a 0.45-μm Teflon syringe filter to remove any particulates 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 cross-bound with 5% diphenyl and 95% dimethylpolysiloxane (30 m × 0.53 mm interior diameter; 0.25-μm film thickness, Restek Corporation, Bellefonte, PA). The oven temperature was programmed at 40 °C for 6 min, followed by a linear increase of 10 °C/min to 300 °C, and then the temperature was held for 15 min. Injector and detector temperatures were maintained at 300 °C. Two microliters of the extract were injected and nitrogen was used as a carrier gas.

2.2. Molecular analysis of bacterial communities in the depth of 0–2 m below PAH-contaminated surface soil

PAH-contaminated soil samples were used to analyze the compositions of the microbial communities from 16S rDNA clonal library. For structural analyses of microbial communities, DNA was extracted directly from soil samples and 16S rDNA genes were amplified using 27f and 1522r primers as mentioned previously [16]. PCR products were cloned and 100 randomly selected clones were sequenced. Sequence results obtained from PAH-contaminated soils were analyzed based on database homology searches using the Blast algorithm, and a phylogenetic tree was generated with the 100 partial 16 rDNA sequences.

2.3. Preparation of culture media and isolation of PAH-degrading bacteria

Enrichment culture technique was used to isolate pure bacterial strains capable of utilizing PAH. Five-gram samples of MGP soil were incubated with a mixture of PAH 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 °C for 2 weeks. PAH including anthracene, naphthalene, or phenanthrene dissolved in acetone at the concentration of 10 mg/ml was used as substrates for the enrichment. After 2 weeks of incubation, 10 ml of the supernatant were collected and incubated for 2 more weeks as described above. By this procedure, a consortium capable of degrading a variety of PAH was obtained and used for isolating pure bacterial strains capable of degrading PAH. The substrate utilization test was also performed on solid media containing the same inorganic salts mentioned above and different substrate. The final concentration of the substrates used for preparation of solid media was 1 mg/ml. The petroleum chemicals, benzene and toluene were supplied with vapor using Durham tube. All chemicals were purchased from Sigma Chemical Company. Ten milliliters of stock solution were made in a 20-ml amber vial with a Teflon-lined cap by dissolving 10 mg each of anthracene, naphthalene, phenanthrene, and pyrene and 1 mg each of benzo[a]pyrene and chrysene in 1 ml of dichloromethane.

2.4. Determination of PAH mineralization using radiorespirometry

The catabolic potential of strain KK1 for PAHs was determined by measuring radioactivity of CO_2 evolved from mineralization of [^{14}C]-labeled PAHs. Cells were pregrown on TSB (tryptic soy broth) to late-exponential phase, harvested by membrane filtering, and washed with inorganic salts medium twice. Approximately 10^5 cells were inoculated to anthracene, carbazole, naphthalene, phenanthrene, or pyrene liquid media and incubated for 10 days. During the incubation period, 1 ml of the culture was taken out, and used for measurement of the amount of CO_2 evolved from PAH mineralization. Radiolabeled anthracene ([1,2,3,4,4A,9A-^{14}C]; specific activity 20.6 mCi/mmol), benzopyrene ([7-^{14}C]; specific activity 26.6 mCi/mmol), carbazole ([7-^{14}C]; specific activity, 28.2 mCi/mmol), chrysene ([4,5,9,10-^{14}C]; specific activity 14.0 mCi/mmol), naphthalene ([UL-^{14}C]; specific activity, 31.3 mCi/mmol), phenanthrene ([9-^{14}C]; specific activity 14.0 mCi/mmol), and pyrene ([4,5,9,10-^{14}C]; specific activity 14.0 mCi/mmol) were used for this experiment. Approximately 10^5 dpm of radiolabeled compound was added to 10 ml inorganic salts solution. Each flask was then sealed with a Teflon-wrapped silicon stopper, in which was placed an 18-gauge needle and 16-gauge steel canula. From the canula was suspended a small vial containing 1.5 ml of 0.5N NaOH to trap $^{14}CO_2$ released from mineralization. The NaOH solution was periodically removed and replaced with fresh solution, and the amount of evolved $^{14}CO_2$ was determined in a liquid scintillation counter (Model LS 5000 TD, Beckman Instruments Inc., Irvine, CA, U.S.A.).

2.5. Substrate conversion assay

The catabolic potential of strain KK1 for benzoate, biphenyl, *p*-cumate, or vanillate was evaluated by HPLC analysis. A single colony on TSA (tryptic soy broth agar) was inoculated into to the media containing either benzoate, biphenyl, *p*-cumate, or vanillate, and incubated for 48 h. During the incubation period, 1 ml of the culture was taken out periodically, and used for measurement of substrate conversion rate by reverse phase high performance liquid chromatography (HPLC). Reverse-phase HPLC was performed with a PhaSep H4726 column (4.6 mm × 250 mm) filled with Spherisorb ODS2 (particle diameter, 5 μm) proceeded by a Whatman CSKI guard column (6.5 mm × 65 mm) coupled to a Shimadzu SCL-6B solvent delivery system and a CR501 chromatopac computing integrator. A methanol–water (90:10) solvent was used at a flow rate 1 ml/min. The analytes were detected by monitoring at A_{280} and the concentration was calculated by a comparison with a standard curve.

2.6. Analysis of dioxygenases for catabolism of aromatic hydrocarbons

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. PCR amplification of dioxygenase gene fragments from strain KK1 was performed in a total volume of 50 μl using Perkin-Elmer reagents (Perkin-Elmer, Branchburg, NJ, U.S.A.). PCR reactions were performed for 1 min at 95 °C, cycled 33 times (1 min at 95 °C, 1 min at 55 °C, 1 min 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. Two hundred nanograms 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, U.S.A.) and BLAST searches of the databases. Total RNA isolated from each of the substrates was used for hybridization with two dioxygenase gene fragments to estimate the PAH-degrading potential of strain KK1.

2.7. RNA preparation and northern hybridization

In order to analyze expression pattern in the transcriptional level, cells were grown overnight in TSB media to the mid-log phase (o.d. 0.8–1.0). Cells were harvested and washed twice with the inorganic salts solution. Approx-