Journal of Bioscience and Bioengineering
VOL. 113 No. 5, 624–630, 2012

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Case study of the relationship between fungi and bacteria associated with high-molecular-weight polycyclic aromatic hydrocarbon degradation

Shuozhi Wang, Nobuhiko Nomura, Toshiaki Nakajima, and Hiroo Uchiyama*

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305–8572, Japan

Received 24 October 2011; accepted 5 January 2012
Available online 2 February 2012

Although bacteria play dominant roles in microbial bioremediation, few of them have been reported that were capable of utilizing high-molecular-weight (HMW) organic pollutants as their sole sources of carbon and energy. However, many soil fungi can metabolize those of pollutants, although they rarely complete mineralization. In this paper, we investigated the dynamic relationship between fungi and bacteria associated with degradation of HMW-polycyclic aromatic hydrocarbons (PAHs). Artificial fungal-bacterial mixed cultures were constructed to simulate the environment of actual polluted sites. Four bacterial strains and seven fungal strains were isolated that related to the removal of phenanthrene, fluoranthene and pyrene in the soil. Furthermore, these strains were used to create mixed culture of bacteria (Bact-mix), mixed culture of fungi (Fung-mix), fungal–bacterial co-cultures (Fung-Bact), respectively. The maximal pyrene removal rate (67%, 28 days) was observed in the Fung-Bact, compared with cultures of Fung-mix (39%) and Bact-mix (56%). The same tendency was also indicated in the degradation of phenanthrene and fluoranthene. In addition, a dynamic relationship during the degradation process between fungi and bacteria was monitored through using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method.

[**Key words:** Polycyclic aromatic hydrocarbons (PAHs); Soil fungi; Soil bacteria; Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE); Degradation]

Bacteria play dominant roles in bioremediation even though there are a large amount of other microbes existing in the polluted site, e.g. fungi, algae, protozoa (1). In recent years, the fungal ability to degrade high-molecular-weight (HMW) xenobiotics had attracted attention due to the enzymatic predominance and multiplex pathways. Fungi are more likely than bacterial intracellular enzymes to initiate attack on HMW-pollutants in the soil because of their ability to diffuse in the soil and release extracellular enzymes (2).

Terrestrial ecosystems can be contaminated with large amounts of polycyclic aromatic hydrocarbons (PAHs). This contamination may occur through the incomplete combustion of fossil fuels and biomass, improper disposal of waste incineration and accidental oil leakage from underground storage tanks and pipelines. Microbial degradation is an expected approach for the removal of PAHs, and its application has been extensively studied. In general, PAHs which contain two or three aromatic rings are readily degradable by indigenous bacteria. Several bacteria have been demonstrated to utilize these low molecular weight PAHs as their sole sources of carbon and energy (2,3). However, PAHs containing four or more aromatic rings (HMW-PAHs) are recalcitrant for bacterial degradation because they are thermodynamically stable, hydrophobic and strongly adsorb onto solid particles (4). On the other hand, many soil fungi can remove HMW-PAHs, although they rarely complete mineralization. Two groups, including ligninolytic and non-ligninolytic fungi, have been reported to remove HMW-PAHs (5). The ligninolytic group is mostly composed of white rot fungi, e.g., *Pleurotus ostreatus* (5), *Phanerochaete chrysosporium* (2) and *Irpex lacteus* (6). Ligninolytic fungi are capable of cleaving the aromatic rings of PAHs by using extracellular ligninolytic enzymes, and this can lead to mineralization. In contrast, non-ligninolytic fungi, such as *Cunninghamella elegans* (7), *Penicillium janthinellum* (8,9) and *Syncephalastrum* sp. (9), can only oxidize PAHs into hydrophilic or detoxified products. Hence, mineralization is not detected.

The aim of this study is to investigate the dynamic relationship between fungi and bacteria in soil associated with degradation of HMW-PAHs. Conventional cultivation techniques and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) were utilized to monitor changes in the microbial communities.

MATERIALS AND METHODS

Chemicals, media and soils Phenanthrene (98% purity) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fluoranthene (>98% purity) and pyrene (>98% purity) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Microbial nutrient media, including nutrient broth (NB) and potato dextrose broth (PDB), were purchased from Difco Laboratory (Detroit, MI, USA). Agar was purchased from Nacalai Tesque.

The Bushnell Haas minimal medium (BH) (10), had a pH of 6.5 and consisted of the following components (per liter of distilled water): $(NH_4)_2HPO_4$, 1 g; KH_2PO_4, 1 g; KNO_3, 1 g; $MgSO_4$, 0.2 g; $FeCl_3$, 0.05 g; and $CaCl_2$, 0.02 g.

The soil was obtained from the surface horizon of a forest, which has no history of hydrocarbon contamination, at Tsukuba University, Japan. Before the experiments, the moist soil was screened through a 2-mm sieve and pre-incubated at 28°C for 2 days to acclimatize the microbial communities to the experimental conditions.

* Corresponding author. Tel./fax: +81 29 853 6626.
E-mail address: uchiyama.hiroo.fw@u.tsukuba.ac.jp (H. Uchiyama).

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doi:10.1016/j.jbiosc.2012.01.005

Isolation of PAHs-degrading fungi and bacteria in the soil To isolate PAHs-degrading bacteria and fungi, 100 g soil was placed into 250-ml flasks. A mixture of phenanthrene, fluoranthene and pyrene, dissolved in acetone, was then added. The acetone was evaporated in the clean bench for 12 h. The final concentration was 200 mg kg^{-1} for each category of PAHs. The soil was incubated at 28°C with a moisture content of 30% for 30 days. BH dilutions of the soil subsamples were spread on BH-agar plates (BH medium including 1.5 g l^{-1} agar and 200 mg l^{-1} of PAHs) and nutrient agar plates (NB and PDB media including 1.5 g l^{-1} agar), respectively. Colonies were selected based on the growth capabilities and abundance that were compared between the BH and nutrient plates.

DNA sequencing and phylogenetic analysis To analyze the isolated PAHs-degrading strains, the bacterial 16S rRNA gene and the D1-D2 region of the fungal 26S rRNA gene were selected as the sequencing targets. All of the PCR amplifications in this study were performed in a thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). All PCR mixtures were prepared using the TaKaRa Ex™ Taq kit (Takara Bio Inc., Japan) which included 10 mM (2.5 mM each) deoxynucleoside triphosphates (dNTPs), 10× Ex Taq Buffer (1 ml), and 250 U TaKaRa Ex Taq polymerase. Each PCR reaction mixture contained 1 × Ex Taq buffer, 0.8 mM total concentration of dNTPs mixture, 0.5 U TaKaRa Ex Taq polymerase. Primers were added at 0.25 μM each, and approximately 20 to 50 ng of genomic DNA was added into each mixture. The primers used for PCR amplification of the 16S rRNA gene were 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1494r (5′-TGACTGACTGAGGYTACCTTGTTAC-3′). Briefly, the 16S rRNA gene fragments were amplified by PCR at 95°C for 30 s, 55°C for 1 min and 72°C for 2 min for a total of 35 cycles (11). The primers for the D1-D2 region of the fungal 26S rRNA gene were NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (12,13). PCR was conducted at 95°C for 50 s, 55°C for 50 s and 72°C for 1 min for a total of 28 cycles. The DNA sequencing reactions were performed using a BigDye™ Terminator Cycle Sequence Kit (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA). The purified PCR products were used directly in 16S rRNA gene sequencing reactions with the primers 27f, 357f (5′-CCTACGGGAGGCAGCAG-3′), 518r (5′-GTATTACCGCGGCTGCTGG-3′), 907r (5′-CCGTCAATTCCTTTGAGTTT-3′), and 1494r. All products were analyzed with an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). The sequences were used to query the GenBank database, and phylogenetic trees were constructed using the neighbor-joining method in MEGA 5.04 (14).

PAHs degradation experiment All of the PAHs stock solutions were as previously described. The uncontaminated sterile sand soil (7.3 g kg^{-1} of total organic carbon and 0.9 g kg^{-1} of total nitrogen; autoclaved at 121°C for 20 min, repeated every 24 h three times; 36.7 g of dry soil per 100 ml flask) was treated to obtain a final concentration of 75 mg kg^{-1} for each of the PAHs (phenanthrene, fluoranthene and pyrene) in the soil. The acetone in the soil completely evaporated in the clean bench for 12 h.

Bacterial inocula were grown at 30°C and 150 rpm in NB medium (8 g l^{-1}, 300 ml in 500 ml flasks) until growth reached the late exponential phase. Fungal inocula were treated in the same manner as the bacteria with the exception that PDB (24 g l^{-1}) was used as the growth medium. Cells or mycelia were harvested by centrifugation, washed twice and resuspended with sterile BH as the inocula. Sterile inocula were treated by autoclaving (121°C, 20 min).

Four isolated strains of soil bacteria were used to construct a mixed culture of bacteria (Bact-mix), and seven strains of soil fungi were used to construct a mixed culture of fungi (Fung-mix). A fungal–bacterial co-culture (Fung-Bact) was constructed using all of the strains above. Equal amounts of the individual fungal inocula were added into the PAHs-mixed sand soil to create the Fung-mix (1×10^6 colonies g^{-1} soil). The Bact-mix was made in the same manner (1×10^7 colonies g^{-1} soil). Half quantity of fungi and bacteria (in contrast to the Fung-mix and Bact-mix) were mixed to obtain the Fung-Bact. The negative control was made with the same quantity of sterile inocula. All of the cultures were conducted in triplicate.

The cultures were incubated in the dark at 28°C for 28 days with a moisture content of 16% (adjusted with BH). Soil subsamples were collected at weeks 0, 1, 2, 3 and 4 for quantification of the PAHs, extraction of DNA and biomass determination.

Quantification of PAHs To determine the remaining concentrations of the PAHs, 1 g of soil (in triplicate) from each subsample was extracted twice with 3 ml of ethyl acetate (15). The extracts were dried under nitrogen gas flow. The residue was dissolved in 1-ml mixture of methanol/water (90:10, v/v) and analyzed by high performance liquid chromatography (HPLC; Shimadzu Co., Japan) with an ultraviolet (UV) detector (SPD-M20A). A Symmetry C18 column (150 mm × 2.1 mm, I.D. 3.5 μm; Waters) was used in the analysis. The mobile phase consisted of methanol/water (90:10, v/v) and had a flow rate of 0.2 ml min^{-1}. The oven temperature was 30°C. Phenanthrene (254 nm, 5.0 min), fluoranthene (235 nm, 6.2 min) and pyrene (235 nm, 6.9 min) were identified by their retention times in comparison with the standard and were quantified using calibration curves obtained with 10, 50 and 100 ppm.

DNA extraction The DNA was extracted from 0.5 g of soil using a FastDNA SPIN kit for soil (BIO 101 Inc.) with an operating condition of speed 5.5 for 30 s. Further procedures followed the manufacturer's instructions. To extract the DNA from each isolated single strain, the chloroform method was used (16). The extracted chromosomal DNA was confirmed using agarose gel electrophoresis (1.2% gel, 100 V, 30 min).

PCR-DGGE conditions To analyze the microbial communities in the soil, the respective primer sets were used for PCR-DGGE of the bacteria and fungi.

The first set targeting the V3 region of bacterial 16S rRNA gene consisted of GC-357f (5′-GC clamp-CCTACGGGAGGCAGCAG-3′) (GC clamp = CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG) and 518r (5′-GTATTACCGCGGCTGCTGG-3′). The PCR condition was set as follows: initially 94°C for 5 min; 20 cycles of 94°C for 30 s, 65°C (touchdown −0.5°C/cycle to 55°C) for 30 s and 72°C for 15 s; 8 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 15 s; and finally 72°C for 2 min. The second set, targeting a segment of the fungal 18S rRNA gene, consisted of Euk1A (5′-CTGGTTGATCCTGCCAG-3′) and Euk516r-GC (5′-ACCAGACTTGCCCTCC-GC clamp-3′) (GC clamp = CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG) (17). The PCR cycling protocol was set as follows: initially 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 2 min; and finally 72°C for 7 min. All PCR mixtures were prepared same to section 2.3.

All of the PCR amplicons were electrophoresed on an agarose gel to ascertain the sizes, as described above and purified using the UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc.).

The DGGE analysis was conducted using a D-Code universal mutation detection system (Bio-Rad Laboratories, Inc.). The condition for bacterial separation was conducted as follows: 8% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide; Nacalai Tesque) with a denaturing gradient from 30% to 60% (100% denaturant defined as 7 M urea and 40% formamide, v/v), 36 V and 60°C for 18 h. The fungal separation was performed in a 6% polyacrylamide gel with a denaturing gradient from 10% to 35%, which was operated at 60 V for 16 h at 60°C. The gels were stained with SYBR GOLD Nucleic Acid Gel Strain (Eugene, OR, USA), and the bands were visualized with an UV transilluminator (ATTO Corporation, Japan).

Biomass determination The colony-forming units (CFUs) were used to estimate the bacterial and fungal biomasses (18). The soil was suspended in BH medium and mixed by vortexing. All of the subsamples were 10-fold serially diluted in BH. A volume of 100 μl from each dilution was inoculated into each of three replicate solid-medium plates (NB for bacteria; Rose Bengal-PDB medium for fungi) (9). All of the cultures were incubated at 30°C for 2 to 4 days, and the colonies arising from the growth were scored. The CFUs-estimate was conducted relative to sterile inocula soil as negative controls.

Statistical analysis A two-tailed Student *t* test was used for statistical analysis. Differences were considered significant if the resultant *P* value was <0.1, and extremely significant if *P* value was <0.05. The results at the end of the degradation experiment were applied to analyze the difference of PAHs removal rate between two groups (the Fung-Bact and Fung-mix, the Fung-Bact and Bact-mix).

Accession numbers The 16S rRNA gene sequences of strains TKB 1–4 were deposited in the DDBJ, GenBank, and EMBL nucleotide sequence databases under accession numbers AB647347, AB647348, AB647349, and AB647350, respectively. The partial fragments of the fungal 26S rRNA gene sequences of strains TKF 1–7 were deposited under accession numbers AB647351, AB647352, AB647353, AB647354, AB647355, AB647356, and AB647357.

RESULTS

Isolation and characterization of the PAHs-degrading microorganisms PAHs-degrading microbial strains were isolated from the acclimated PAHs-contaminated soil. Seven strains of fungi and four strains of bacteria were obtained, respectively. The isolated strains were designated as follows: TKF 1–7 (fungi) and TKB 1–4 (bacteria). All of the strains were individually verified for their abilities to remove PAHs in the soil cultures. The nucleotide sequence of the bacterial 16S rRNA gene and the partial fragments of the fungal 26S rRNA gene were analyzed (Table 1).

Phylogenetic positions of the obtained strains Phylogenetic trees of the obtained fungi and bacteria were constructed and are shown in Figs. 1 and 2, respectively. The nucleotide sequence data were derived from the sequences mentioned above and those obtained from GenBank.

The tree for the bacterial 16S rRNA genes showed that the isolated strains belonged to four genera. TKB 4 belonged to phylum *Actinobacteria*, and the other three strains belonged to phylum *Proteobacteria*. The tree of the fungal 26S rRNA partial genes indicated that all of the obtained strains mainly belonged to *Hyphomycetes* except TKF 1 which belonged to *Ascomycetes*.

PCR-DGGE analyses The DGGE profiles of the soil dosed with PAHs showed band changes clearly throughout the 28-day incubation (Fig. 3). In the profile of the bacterial 16S rRNA V3 region fragments, bands corresponding to all of the inoculated strains were detected. Several bands intensified in both the Bact-mix and Fung-Bact. For example, the bands of TKB 1, 3 and 4 intensified during the incubation from which the bacterial growth could be conferred. The profiles of the Fung-Bact showed very similar patterns in comparison with the Bact-