Journal of Basic Microbiology
Environment · Health · Techniques

Short Communication

White-rot fungus *Merulius tremellosus* KUC9161 identified as an effective degrader of polycyclic aromatic hydrocarbons

Hwanhwi Lee, Yeongseon Jang, Jong-Min Kim, Gyu-Hyeok Kim and Jae-Jin Kim

Division of Environmental Science & Ecological Engineering, Korea University, Seoul, Korea

Polycyclic aromatic hydrocarbons (PAHs) have a highly recalcitrant structure; however, they can be degraded by white-rot fungi which have the potential to biodegrade recalcitrant organic compounds. Four fungal isolates were selected from 23 newly isolated basidiomycetes, based on their dye decolorization rate, and they were evaluated for their ability to degrade 50 ppm of pyrene. The isolate phylogenetically affiliated to *Merulius tremellosus* KUC9161 demonstrated the highest degradation rate of pyrene, regardless of the production of ligninolytic enzyme activities. The selected isolates were tested for their ability to degrade pyrene and other PAHs in creosote-contaminated soil. The results of the degradation tests indicated that *M. tremellosus* KUC9161 degraded a larger variety of PAH compounds than *Phanerochaete chrysosporium*, a known PAH degrader. On the basis of our results, the isolate *M. tremellosus* KUC9161 has a high potential to be used in the large-scale biodegradation of PAHs, and the species may also be used to degrade recalcitrant materials in creosote-contaminated soil.

Keywords: Biodegradation / Creosote / Polycyclic aromatic hydrocarbons (PAHs) / Pyrene / White-rot fungi

Received: July 29, 2011; accepted: December 19, 2011

DOI 10.1002/jobm.201100368

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that are formed from the incomplete combustion or pyrolysis of organic materials, such as oil, petroleum gas, coal, and wood [1]. PAH contamination has attracted public and scientific attention due to their recalcitrant properties and mutagenic or carcinogenic effects [2], and many studies have been conducted to remove PAH compounds from contaminated environments [3]. In Korea and other countries, creosote-contaminated soil has received attention with regard to the degradation of PAHs due to the use of creosote-treated wood as railroad crossties and landscape facilities [4].

Creosote is a chemical mixture composed of approximately 85% PAHs, 10% phenolic compounds and 5% *N*-, *S*- and *O*-heterocyclic compounds [5]. The majority of the white-rot fungi (WRF) have the ability to degrade lignin, a biopolymer in wood and woody plants, and also a wide variety of unnatural recalcitrant pollutants, such as PAHs [6]. Among these WRF, *Phanerochaete chrysosporium* is well known as an effective degrader of PAHs [7]. Compared with *P. chrysosporium*, the potential for bioremediation of PAHs by new isolates of WRF collected from various forests in Korea was demonstrated [3, 4]. The objective of the study was to screen a number of isolated WRF to determine whether they were capable of degrading organic pollutants, by measuring their Remazol Brilliant Blue R (RBBR) dye decolorization rates and their pyrene degradation efficiencies. Pyrene is known as a recalcitrant PAH compound due to its chemical structure; it is composed of four fused benzene rings and therefore less biodegradable than other PAHs [8]. Thus, it might be suitable for removal/degradation tests of PAHs in creosote-contaminated soil by fungal species. In addition, focus should be placed on finding excellent fungal species for the biodegradation of PAHs in creosote-contaminated soil. In accordance with these issues, we present here the PAH biodegradation results in creosote-contaminated soil by a selected fungus, *Merulius tremellosus* KUC 9161, obtained by screening for RBBR decolorization and biodegradation of pyrene.

Correspondence: Jae-Jin Kim, Ph. D., Division of Environmental Science & Ecological Engineering, Korea University, 5-1 Anam-dong, Seongbuk-gu, Seoul, 136-701 Korea
E-mail: jae-jinkim@korea.ac.kr
Phone: +82-2-3290-3049
Fax: +82-2-953-0737

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Materials and methods

Identification and maintenance of fungal isolates

A total of 23 isolates of basidiomycetes were recently (between 2008 to 2009) collected from various areas such as national parks, mountains, several islands, and some local areas in Korea and were previously identified based on the taxonomy of the fruiting body [9]. For their isolation, small pieces of mushrooms or wood were put into 2% malt extract agar (MEA, malt extract 20 g, agar 15 g, distilled water ad 1000 ml) amended with benomyl (4 mg) to support only the growth of basidiomycetes [10]. To specifically identify WRF, fungal DNA extraction and polymerase chain reaction (PCR) amplification were performed using the techniques described by Lim *et al.* [11]. To amplify the partial large subunit (LSU) ribosomal DNA region, PCR was conducted using the fungal universal primers, LR0R/LR3, which were used to conduct a BLAST search of the GenBank database [12]. DNA sequencing of the PCR products was performed using the aforementioned primers and an ABI 3730XL system (Macrogen, Korea). The sequences obtained in this study were deposited under the GenBank accession nos. JF416672 to JF416693, and JF460009. The fungi were subcultivated every 5 d, and all isolates were deposited in sterile water at 4 °C in the Korea University Culture Collection (KUC), Korea University, Korea.

RBBR decolorization test

The RBBR decolorization method was used as an initial screening method in the study, to determine whether the WRF isolates were capable of degrading PAHs.

RBBR (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), an anthracene derivative, is dramatically decolorized by lignin-degrading fungi [13]. The fungi were inoculated onto 2% MEA containing 100 mg/l RBBR and then incubated at 27 °C. They showed strong decolorization activity, with the medium appearing whitish rather than pinkish after it was completely decolorized.

Biodegradation of pyrene

Erlenmeyer flasks (250 ml) containing 100 ml 2% ME liquid medium were aseptically inoculated with ten agar plugs (7 mm in diameter) of a fungal preculture. The cultures were incubated on a rotary shaker at 150 rpm (27 °C) after the addition of 50 mg/l pyrene (Sigma-Aldrich Chemie, Steinheim, Germany). Uninoculated flasks were treated similarly as an abiotic control, and *P. chrysosporium* KCTC 6293 was used as a positive control. The entire liquid and solid parts of each incubation sample were extracted with dichloromethane on the 15th d of incubation (Duksan, Korea), and each extracted sample was concentrated to 10 ml using a vacuum rotary evaporator (Eyela N-1000 series, Japan). The PAH concentrations in the extracts were then determined by injecting 1 μl of the extract into a gas chromatograph-mass spectrometer (GC-MS; Model 7890, Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using a DB-5MS column (0.25 mm in diameter by 30 m in length, film thickness 0.25 μm). The GC-MS analysis was conducted according to the method described by Lee *et al.* [3].

Biodegradation of PAHs in creosote-contaminated soil

The soil samples were collected from two mountains, Mt. Bukhan and Mt. Gaewoon, in Seoul, Korea, where the natural soil type is sandy loam. The samples, which required enhanced homogeneity, were passed through a 10-mesh screen. The soils were stored in the dark at 4 °C until use. These samples were mixed with creosote solution, including PAHs, and were dissolved in acetone at a ratio of 1:5 (v/v) before inoculation of the fungi. For the fungal degradation of the PAHs in the creosote-contaminated soils, microcosms were primarily cultivated on test tubes according to Byss *et al.* [14]. In a laboratory-scale experiment, glass tubes (17 mm inner diameter) were filled with 2 g of air-dried milled rice straw mixed with 6 ml water, sterilized at 120 °C and 103 kPa pressure for 15 min and inoculated with the selected fungi. After 1 week of fungal growth (when the straw was thoroughly colonized by the fungi), 20 g of creosote-contaminated soil and 4 ml nutrient solution (4 g glucose, 1.5 g malt extract, 0.3 g peptone, and 1 l distilled water) were added, and the water content was kept constant. Non-inoculated tubes were treated as abiotic controls, and *P. chrysosporium* KCTC 6293 was used as a positive control. At 14 d after the addition of the creosote-contaminated soil, the soil samples were dissolved in dichloromethane and ultrasonicated according to the EPA method 3550B [15]. Extraction was performed in three independent replicates. The extracted samples were filtered using a hydrophobic syringe filter (0.45 μm; Sartorius Stedim Biotech, Germany), and the removed PAHs were analyzed in comparison with standard PAHs (NSI Solutions, Raleigh, NC, USA) using a GC-MS, as described above. All data in terms of degradation were analyzed using the Statistical Analysis Systems (SAS 9.1; SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was used to assess the significance of degradation among the species in Tukey's test. The mean values were compared by using the least significant difference index at $p < 0.05$.

Results and discussion

The results of the initial screening allowed the selection of isolates with rapid growth rates and efficient

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

decolorization of RBBR for further investigation. Among the 23 isolated basidiomycetes, 19 isolates, including 14 species, were assessed for RBBR decolorization, and 6 isolates rapidly decolorized RBBR within 5 d (Table 1). From these results, four fungal species, which were phylogenetically affiliated to *Phlebia radiata*, *Porostereum crassum*, *Trametes versicolor*, and *M. tremellosus*, were selected for the degradation tests of pyrene. One strain was randomly selected from each of these species for screening. All selected fungi degraded pyrene, and no significant differences were found among all fungi ($p < 0.05$), except for *T. versicolor*. However, *M. tremellosus* KUC9161 demonstrated a higher rate (83.6%) of pyrene degradation than *P. chrysosporium* (68.5%) which is well known as a good degrader of PAHs (Fig. 1). The pyrene degradation rates of the other species were comparable to that of *P. chrysosporium*. These results suggest that *M. tremellosus* KUC9161can effectively degrade PAHs in contaminated soils. Accordingly, the degradation tests of PAHs were performed in creosote-contaminated soil using *M. tremellosus* KUC9161, which demonstrated the highest efficiency in pyrene degradation (Fig. 1), and the *M. tremellosus* KUC9161 PAH degradation efficiency was compared with that of *P. chrysosporium* KCTC6293. Among the 16 PAHs designated as major species by the

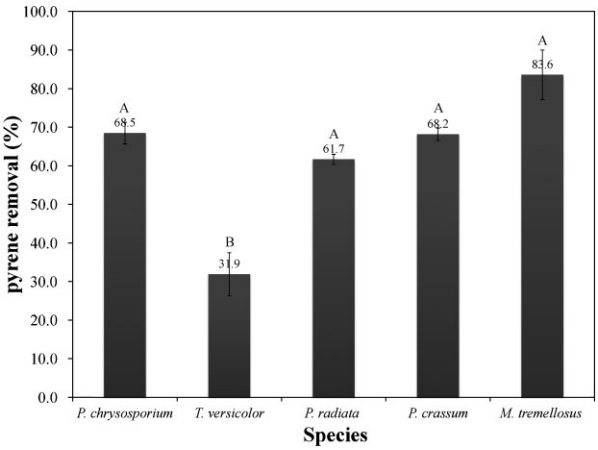

Figure 1. Degradation rates of pyrene by selected white-rot fungi after the 15th d of incubation. Error bars represent means ± standard deviation ($n = 3$). Different letters indicate statistically different means ($p < 0.05$).

Environmental Protection Agency (EPA) of the USA, 10 PAHs were detected in the soil used in this study; the concentration of the total PAHs was measured to be approximately 220 mg/kg in the original soil sample. At 2 weeks after the addition of the creosote-contaminated

Table 1. The identity of the fungal species according to the molecular analyses and their capacities in the RBBR decolorization test.

Isolate no.	Closest fungal match (acc. no.)	S[a] (%)	Fungal identity	Days[b]	Color[c]	Activity[d]
KUC9164	*Phlebia radiata* (AF287885)	99.55	*Phlebia radiata*	5	W	++
KUC9305	*Porostereum crassum* (GQ470634)	99.83	*Porostereum crassum*	5	W	++
KUC9161	*Merulius tremellosus* (AY293200)	99.89	*Merulius tremellosus*	5	W	++
KUC9303	*Trametes versicolor* (AY333793)	100.00	*Trametes versicolor*	5	W	++
KUC9196	*Trametes versicolor* (DQ208417)	100.00	*Trametes versicolor*	5	P	++
KUC9185	*Phlebia radiata* (AF287885)	99.53	*Phlebia radiata*	5	P	++
KUC9330	*Ceriporia lacerata* (FJ471540)	100.00	*Ceriporia lacerata*	6	P	++
KUC9183	*Tyromyces chioneus* (AF393080)	99.39	*Tyromyces chioneus*	6	P	++
KUC9162	*Porostereum crassum* (GQ470634)	99.83	*Porostereum crassum*	6	W	++
KUC9188	*Porostereum spadiceum* (DQ679918)	98.76	*Porostereum spadiceum*	6	W	++
KUC9302	*Porostereum spadiceum* (DQ679918)	99.09	*Porostereum spadiceum*	6	W	++
KUC9152	*Bjerkandera adusta* (EU918694)	99.00	*Bjerkandera adusta*	6	W	++
KUC9166	*Fomes fomentarius* (DQ208420)	99.09	*Fomes fomentarius*	8	P	++
KUC9158	*Perenniporia fraxinea* (AM269853)	98.05	*Perenniporia fraxinea*	8	P	++
KUC9190	*Phlebia radiata* (FJ644497)	99.34	*Phlebia radiata*	8	W	++
KUC9148	*Gloeocystidiellum luridum* (AF506421)	97.16	*Stereum peculiare*	9	P	++
KUC9326	*Gymnopilus junonius* (AF219598)	99.17	*Gymnopilus junonius*	14	W	+
KUC9309	*Dacryopinax spathularia* (AB299079)	99.26	*Dacryopinax spathularia*	20	W	+
KUC9348	*Gymnopus sepiiconicus* (AY639427)	98.38	*Gymnopus sepiiconicus*	20	W	+
KUC9160	*Polyporales* sp. (EU024961)	99.64	*Polyporales* sp.	–[e]	–	–
KUC9199	*Daedalea dickinsii* (EU024963)	100.00	*Daedalea dickinsii*	–	–	–
KUC9350	*Antrodia heteromorpha* (AY333840)	99.82	*Antrodia heteromorpha*	–	–	–
KUC9196	*Antrodia heteromorpha* (AY333840)	99.83	*Antrodia heteromorpha*	–	–	–

[a]Similarity score from closest BLAST match.
[b]Days to decolorize the full dish ($n = 3$).
[c]Color of the decolorized dish (W, whitish; P, pinkish).
[d]Activity of decoloriztion (++, strong; +, week; −, no activity).
[e]No activity for decolorization.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Concentrations of PAHs in creosote-contaminated soil before and after incubation with *M. tremellosus* and *P. chrysosporium* (mg/kg).

PAH name	Creosote-contaminated soil[a]		*M. tremellosus* KUC9161[b]		*P. chrysosporium* KCTC6293[c]	
Naphthalene	6.68	± 5.79[d]	1.38*	± 0.24	2.46	± 0.77
Acenaphthene	72.75	± 3.11	5.33*	± 1.54	15.57	± 5.90
Fluorene	34.57	± 3.75	2.05*	± 0.59	7.64	± 2.95
Phenanthrene	36.68	± 1.68	3.81*	± 1.12	14.74	± 6.02
Anthracene	8.51	± 7.37	0.67*	± 0.39	2.32	± 0.71
Fluoranthene	28.45	± 0.29	1.88*	± 0.43	7.96	± 3.27
Pyrene	23.00	± 0.61	1.23*	± 0.17	4.43	± 1.27
Benzo[a]anthracene	4.61	± 4.02	N.D.[e]	2.35	± 1.38	
Chrysene	4.38	± 3.81	N.D.		N.D.	
Benzo[k]fluoranthene	0.73	± 1.26	N.D.		N.D.	
Σ PAHs	220.34	± 57.28	16.35	± 4.49	57.47	± 22.26

[a]Original concentrations of PAHs.
[b]Concentration of PAHs in creosote-contaminated soil at the 14th d after inoculation with *M. tremellosus*.
[c]Concentration of PAHs in creosote-contaminated soil at the 14th d after inoculation with *P. crysosporium*.
[d]Mean ± standard deviation of a set of data ($n = 3$).
[e]N.D., Not detected.
*The asterisk indicates means that significantly differ between *M tremellosus* and *P. chrysosporium* in each PAH compound ($p < 0.05$).

soil, the levels of PAH removal were measured (Table 2). Regarding the degradation rates of all of the detected PAHs, *M. tremellosus* KUC9161 degraded higher concentrations of PAHs compared to *P. chrysosporium* KCTC6293 for each PAH compound, which are naphthalene (79.3%), acenaphthene (92.7%), fluorine (94.1%), phenanthrene (89.6%), anthracene (92.1%), fluoranthene (93.4%), and pyrene (94.7%). In addition, significant differences were found between the species for each PAH detected ($p < 0.05$). Therefore, we think that *M. tremellosus* KUC9161 has great potential as a degrader of various PAHs. Further biotechnological applications can be envisioned for the degradation of xenobiotic organic pollutants by indigenous fungal isolates found in Korea.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (KRF-2008-331-F00029) and was carried out with the support of the Forest Science & Technology Projects (Project No. S121010L110000) provided by the Korea Forest Service.

References

[1] Arun, A., Praveen, R.P., Arthi, R., Ananthi, M., *et al.*, 2008. Polycyclic aromatic hydrocarbons (PAHs) biodegradation by basidiomycetes fungi, *Pseudomonas* isolate, and their cocultures: Comparative *in vivo* and *in silico* approach. Appl. Biochem. Biotechnol., **151**, 132–142.

[2] Lei, A.P., Hu, Z.L., Wong, Y.S., Tam, N.F.Y. 2007. Removal of fluoranthene and pyrene by different microalgal species. Bioresour. Technol., **98**, 273–280.

[3] Lee, H., Choi, Y-S., Kim, M-J., Huh, N-Y. *et al.*, 2010. Degrading ability of oligocyclic aromates by *Phanerochaete sordida* selected via screening of white rot fungi. Folia Microbiol., **55**, 447–453.

[4] Kim, M-J., Lee, H., Choi, Y-S., Kim, G-H. *et al.*, 2010. Diversity of fungi in creosote-treated crosstie wastes and their resistance to polycyclic aromatic hydrocarbons. Antonie van Leeuwenhoek, **97**, 377–387.

[5] Mueller, J.G., Chapman, P.J., Pritchard, P.H. 1989. Creosote contaminated sites: Their potential for bioremediation. Environ. Sci. Technol., **23**(10), 197–1201.

[6] Suhara, H., Daikoku, C., Takata, H., Suzuki, S. *et al.*, 2003. Monitoring of white-rot fungus during bioremediation of polychlorinated dioxin contaminated fly ash. Appl. Microbiol. Biotechnol., **62**, 601–607.

[7] Bamforth, S.M., Sigleton, I. 2005. Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. J. Chem. Technol. Biotechnol., **80**, 723–736.

[8] Haritash, A.K., Kaushik, C.P. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. J. Hazard. Mater., **169**, 1–15.

[9] Lim, Y.W., Lee J.S., Kim, K.M., Jung, H.S. 2005. Wood-rotting fungal flora of Kanghwa island. Mycobiology, **33**(4), 167–172.

[10] Wang, C.J.K., Zabel, R.A. 1990. Identification Manual for Fungi from Utility Poles in the Eastern United States, American Type Culture Collection, Publisher, Rockville. 356 pp.

[11] Lim, Y.W., Kim, J-J., Chedgy, R., Morris, P.I., Breuil, C. 2005. Fungal diversity from western redcedar fences and their resistance to β-thujaplicin. Antonie van Leeuwenhoek, **87**, 109–117.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[12] Altschul, S.F., Boguski, M.S., Gish W., Wootton, J.C. 1994. Issues in searching molecular sequence databases. Nat. Genet., **6**, 119–129.

[13] Pasti, M.B., Crawford, D.L. 1991. Relationship between the abilities of streptomycetes to decolorize three anthron-type dyes and to degrade ligno-cellulose. Can. J. Microbiol., **37**, 902–907.

[14] Byss, M., Elhottová, D., Tříska, J., Baldrian, P. 2008. Fungal bioremediation of the creosote-contaminated soil: Influence of *Pleurotus ostreatus* and *Irpex lacteus* on polycyclic aromatic hydrocarbons removal and soil microbial community composition in the laboratory-scale study. Chemosphere, **73**, 1518–1523.

[15] EPA, 1996. Polycyclic aromatic hydrocarbons (PAHs): Ultrasonic Extraction, Method 3550B.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim