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Degrading Ability of Oligocyclic Aromates by *Phanerochaete sordida* Selected *via* Screening of White Rot Fungi

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ABSTRACT. Seventy-nine white rot strains were screened to determine if they had the potential for use in the degradation of oligocyclic aromates (PAHs) by measuring their dye-decoloration rate. Fourteen strains that were selected based on their dye-decoloration rate were then evaluated for the ability to tolerate various levels of PAHs spiked in agar medium. The ability of white rot fungi to degrade 3- or 4-ring PAHs (anthracene, phenanthrene, fluoranthene, pyrene) was determined. Two strains of *Phanerochaete sordida* (KUC8369, KUC8370) were possible PAHs degraders, degrading a significantly greater amount of phenanthrene and fluoranthene than the culture collection strain *P. chrysosporium* (a known PAHs degrader). The production of manganese peroxidase, the only extracellular ligninolytic enzyme detected during the cultivation, was evaluated.

Abbreviations

ANT	anthracene	FLT	fluoranthene
PHE	phenanthrene	PYR	pyrene
ELM(s)	extracellular ligninolytic enzyme(s)	LAC	laccase (phenol oxidase)
LiP	lignin peroxidase	MnP	Mn-dependent peroxidase
GC	gas chromatography	PAH(s)	oligocyclic aromate(s) ('polycyclic aromatic hydrocarbons')
MEA	malt extract agar medium	RBBR	Remazol brilliant blue R
MS	mass spectrometry	WRF	white rot fungus(i)

It is generally accepted that PAHs are ubiquitous pollutants that are commonly found in soil at wood preservation plants and gasworks (Potin *et al*. 2004). PAH contamination has recently attracted public and academic attention because some PAHs are known or suspected mutagens or carcinogens (Lei *et al*. 2007). Due to their low water solubility, as well as the resonance energy of their structures, 3- and 4-ring PAHs are very recalcitrant.

Many papers have been devoted to evaluating the ability of white rot fungi to remove PAH compounds from contaminated environments. The results have demonstrated that *Phanerochaete chrysosporium* is capable of degrading PAHs. WRFs have the ability to degrade lignin, a biopolymer in wood and woody plants that is resistant to attack by most microorganisms. Additionally, these fungi have the ability to degrade a wide variety of recalcitrant pollutants, such as PAHs (Suhara *et al*. 2003). However, not all fungi are able to degrade PAHs, and the ability of a species to remove PAHs can vary significantly between strains (Morgan *et al*. 1991). Therefore, screening of a wide range of WRFs for their ability to degrade PAHs is needed.

We used here two general methods to select fungi capable of degrading PAHs. ANT, PHE, FLT and PYR were chosen as model PAHs due to their high concentration in contaminated environments and their potential mutagenicity (Lei *et al*. 2002; Haritash and Kaushik 2009). Therefore, they should be studied in an attempt to develop a method of degrading PAHs and reducing the hazards associated with them.

Overall, our aim was to screen a number of newly isolated WRF to determine if they were capable of degrading organic pollutants by measuring their rates of RBBR dye-decolorization and their tolerance to

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several levels of PAHs in agar medium. We evaluated the ability of selected white rot strains to degrade four PAHs.

MATERIALS AND METHODS

Fungi used and molecular identification of newly isolated Phanerochaete *species.* A total of 79 species of WRFs including three recently isolated *Phanerochaete* species were used. The fungi were isolated from various wood products in service and pine logs exposed in the forest (Kim *et al*. 2005, 2009). *P. chrysosporium* (a well-known PAH degrader) was used for comparison. Prior to screening, fungi were maintained on 2 % MEA Petri plates at room temperature.

To identify selected isolates, the sequences of the ITS regions were used to conduct a BLAST search of the *GenBank* database (Altschul *et al*. 1994), and then aligned with the DNA sequences of related fungi using the Clustal X algorithm (Thompson *et al*. 1997) and PAUP*4.0b10 (Swofford 2002). *P. calotricha* KUC8003 previously identified based on the microscopic features of the fruiting body was also sequenced. Fungal DNA extraction and PCR were performed using the techniques of Lim *et al*. (2005). To amplify the ITS regions, PCR was conducted using the fungal universal primers, ITS5 and ITS4 (White *et al*. 1990; Gardes and Bruns 1993). Sequencing was then performed on an ABI 3700 automated sequencer (*Perkin-Elmer*, USA) at the *Macrogen DNA Synthesis and Sequencing Facility* (Seoul, Korea). The nucleotide sequences determined were deposited in the *GenBank* DNA sequence database under acc. no. EU047803–EU047806 (Fig. 1).

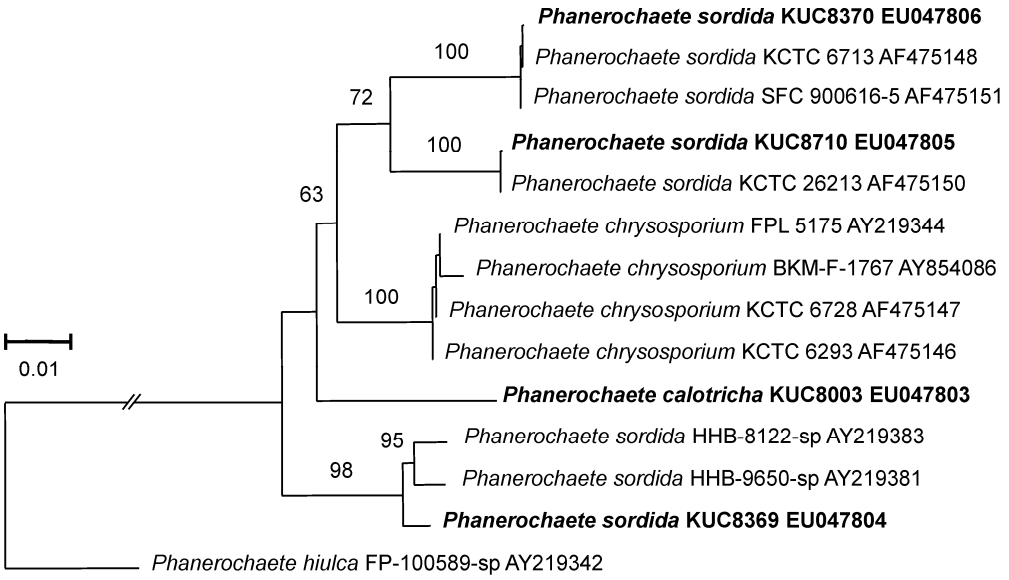

Fig. 1. Neighbor joining tree based the ITS sequences of *P. sordida* and its closely related species; *bold* – species newly identified; *numbers above the lines* – the value of 1000 bootstrap replicates.

Screening experiments. Dye-decolorization test: A dye-decolorization method was used as an initial screening method to determine if fungi were capable of degrading PAHs. RBBR (*Sigma Chemical*, USA), an ANT derivative, is dramatically decolorized by lignin-degrading fungi (Pasti and Crawford 1991). Therefore, 79 strains of fungi were inoculated onto MEA containing (per L) 20 g malt extract (*Difco Laboratories*, USA), 15 g agar and 100 mg RBBR, and incubated at their optimum growth temperature (25 or 30 °C). Each isolate that was capable of decolorizing the entire Petri dish within 1 week was selected for additional testing to determine its tolerance to PAHs (Table I).

Tolerance test: All 14 WRFs selected as a result of screening using RBBR media (Table I) were tested by culture on 2 % MEA supplemented with 200 ppm each of ANT, FLT, PHE and PYR. The PAHs (*Sigma-Aldrich*^{TM},) were of analytical grade. They are almost completely insoluble in water; therefore, acetone (>99 %; *Junsei Chemical*, Japan) was used to dissolve them in the MEA medium. One L of MEA was autoclaved and then cooled to ≈45 °C, after which the PAHs were added in 10 mL acetone solution. The agar plates were stored at room temperature for 5 d on a clean bench to allow the acetone to evaporate prior

to inoculation with the fungus. A total of 15 WRFs, including the *P. chrysosporium* isolate, were then inoculated onto Petri dishes spiked with each PAH and incubated at 25 or 30 °C (Table I). After 3 d of incubation, the growth inhibition rate

$$GIR = GRC - FGR/GRC \times 100$$

(where GRC is growth rate of control culture and FGRs fungal growth rate) was determined by measuring the diameter of the expanding colonies.

Table I. Decolorization and tolerance tests to select WRFs most effective at degrading PAHs

Fungus	Isolate (*GenBank* acc.no.)[a]	Days to decolorize the full dish	Fungal growth 3 d after incubation[b]			
			ANT	FLT	PHE	PYR
Bjerkandera adusta	KUC8072	6	+++	++	+	+++
Cerrena multicolor	KUC8808	6	+++	++	+	+++
Ceriporia lacerata	KUC8814	5	+++	++	++	++
Ceriporiopsis subvermispora	KUC8075	6	+++	++	++	++
Funalia trogii	KUC8607	6	+++	+++	+	+++
Irpex lacteus	KUC8605	5	+++	+++	++	+++
Peniophora sp.	KUC8837	7	+++	++	+	++
Phanerochaete calotricha	KUC8003	5	+++	++	+	++
P. chrysosporium	KCTC6293	4	ngi	+++	+	+++
P. sordida	KUC8370 (EU047806)	3	ngi	ngi	++	ngi
P. sordida	KUC8710 (EU047805)	4	ngi	+	+	++
P. sordida	KUC8369 (EU047804)	4	+++	+	+	++
Phlebia radiata	KUC8034	6	ngi	+++	+	+++
Polyporus arcularius	KUC8220	6	ngi	++	+	+++
Trametes versicolor	KUC8860	6	+++	++	+	+++

[a]KUC – *Korea University Culture Collection* (Seoul), KCTC – *Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology* (Daejeon).
[b]ngi – no growth inhibition; [+++]profuse growth (0 % < GIR < 20 %); [++]moderate growth (20 % < GIR < 40 %); [+]sparse growth (40 % < GIR).

Biodegradation of PAHs. Based on the screening experiments, the isolates of *P. sordida* sensu lato KUC8369, KUC8370, and KUC8710 were considered to be highly efficient PAH degraders. Therefore, the rate of degradation of the 4 PAHs by each *P. sordida* strain used was tested against that of a well-known lignin-degrading species, *P. chrysosporium* (Haritash and Kaushik 2009). The tolerance of the organisms to the PAHs used was evaluated, all samples being processed in triplicates.

Briefly, ten mycelium 1-cm^2 plugs were obtained from malt agar cultures of each isolate and then inoculated into 250-mL Erlenmeyer flasks containing 100 mL of 2 % malt extract liquid medium amended with 50 mg PAHs/L. The flasks were incubated for 14 d at 30 °C with shaking (orbital shaker, 150 rpm). Experiments were conducted in triplicates. The synthesis of ELMs was measured during incubation every other day.

The degradation efficiency of PAHs was evaluated by GC–MS. After the incubation period, the mycelial pellet was separated by centrifugation (12000 rpm, 1 h, 4 °C). The supernatant was then extracted 3× with 100 mL dichloromethane in a separating funnel on a shaker, after which the mycelial pellets were extracted 3× with 50 mL dichloromethane in an ultrasonic bath for 30 min. Each extracted sample was concentrated to 10 mL using a vacuum rotary evaporator. The samples were stored at 4 °C until GC analysis. The concentration of PAHs was determined by injecting 1 μL of extract into a *Shimadzu* high-resolution gas chromatograph equipped with a hydrogen flame ionization detector. Separation was achieved using a VB-1 capillary column (*Vici*) (diameter 0.32 mm, length 60 m, film thickness 0.25 μm). The carrier gas was Grade 5 helium at flow and split rates of 1.73 mL/min and 1⁄50, respectively. The injector and detector temperatures were 330 and 335 °C, respectively. The column temperature was set at 100 °C for 1 min, after which it was ramped at 10 K/min to 300 °C and then 20 K/min to 335 °C, where it was held for 10 min.

Enzyme assays. The ELM activities were investigated by spectrophotometric assays (Verdin *et al*. 2006). After filtration of mycelium using a syringe filter (0.45 μm) to remove the mycelium and spores, the enzymic activity of the crude supernatant was measured using a UV–VIS spectrophotometer. The LiP was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde based on the increase of absorbance A_{310} in pH 2.5 sodium tartrate buffer (Tien and Kirk 1988). The MnP was measured based on oxid-

ation of 2,6-dimethoxyphenol as indicated by the increase of absorbance A_{469} in pH 4.5 sodium malonate buffer (Wang *et al*. 2008). The LAC was estimated using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid as the substrate at 414 nm in pH 4.5 sodium acetate buffer (Novotný *et al*. 2004). One unit of enzyme activity produced 1 μmol of reaction product per min at room temperature; activity was expressed in U/L (Hadibarata *et al*. 2009).

RESULTS AND DISCUSSION

Molecular identification of Phanerochaete *isolates*. With the exception of 3 newly isolated *Phanerochaete* species, all white rot species used were identified based on their ITS region sequence and/or LSU rDNA sequence (Kim *et al*. 2005, 2009). The *Phanerochaete* isolates were identified through comparison of their ITS sequences to known sequences of other *Phanerochaete* species. Though three isolates were matched to *P. sordida* sensu lato in *GenBank*, they were separated into three distinct clades in the phylogenetic tree (Fig. 1), which is similar to the results of De Koker *et al.* (2003). They seemed to be different species but we named these isolates *P. sordida*. Determining whether they are the same species or different species should be the object of further work at morphological and molecular level.

Selection of fungi capable of degrading PAHs. The results of initial screening (Table I) allowed the selection of isolates with high degradation activity and rapid growth rates for further work. All selected fungi decolorized an entire MEA dish amended with 100 ppm RBBR within 1 week of incubation. *P. sordida* KUC8370 showed the greatest decolorization rate (within 3 d), being more rapid than that of *P. chrysosporium* KCTC6293. When grown on 2 % MEA with 200 ppm of each PAH, the fungi were generally most tolerant of the ANT-amended agar, followed by the PYR, FLT and PHE ones. *P. sordida* KUC8370 was most tolerant to the PAHs used. These results suggest that this fungus could effectively degrade PAHs in contaminated soils.

Degradation of PAHs. For the amount of removed PAHs after 14 d of incubation at 30 °C *see* Fig. 2. Elimination of each PAH differed among individual fungi. The highest level of ANT elimination

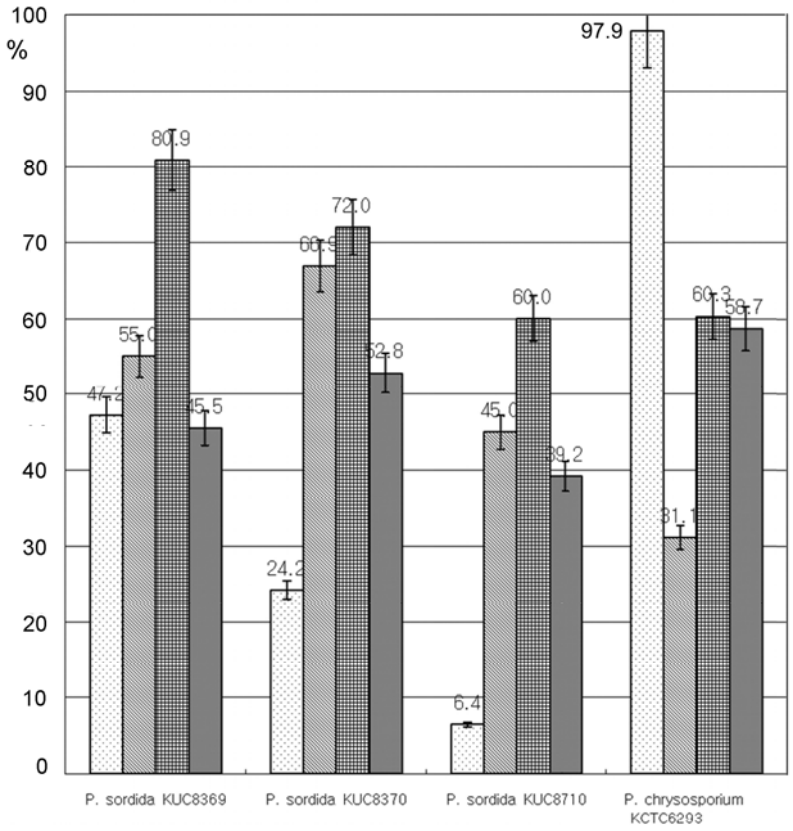

Fig. 2. Degradation rate (%) of four PAHs by selected *Phanerochaete* spp.; *1st columns* – ANT, *2st columns* – PHE, *3rd columns* – FLT, *4th columns* – PYR.

(97.9 %) was observed with *P. chrysosporium* KCTC6293 (known as the greatest degrader of PAHs). However, this species did not efficiently degrade PAHs. *P. sordida* KUC8369 had the highest degradation ability for FLT (80.9 %), followed by the strain KUC8370; its ability to degrade PHE was higher (66.9 %) than that of other *Phanerochate* spp. *P. chrysosporium* KCTC6293 had the highest efficiency (58.7 %) with PYR, but *P. sordida* KUC8370 also showed good degradation.

Several papers have demonstrated that the WRF *P. chrysosporium* is able to degrade PAHs including ANT, PHE, FLT and PYR (Bumpus 1989; Hammel *et al*. 1986; Morgan *et al*. 1991; Sanglard *et al*. 1986). However, it has been used almost exclusively in investigations of white-rot fungal degradation of hazardous compounds (Lamar *et al*. 1990); only a few other fungi, including the WRFs, *Irpex lacteus*, *P. chrysoporium* and *Pleurotus ostreatus*, have been reported to degrade PAHs (Hammel *et al*. 1986; Bezalel *et al*. 1996; Cerniglia *et al*. 1986; Hwang and Song 2000; Lambert *et al*. 1994; Lange *et al*. 1994). We found that our *P. sordida* isolates KUC8369 and KUC8370 are more efficient in the removal of PHE and FLT, respectively, than *P. chrysosporium*. For this reason, it is recommended that further effort should be devoted to optimizing the culture conditions of these two isolates.

Three major ELMs (LAC, MnP, LiP) can oxidize phenolic compounds by creating phenoxy radicals, while nonphenolic compounds are oxidized *via* cation radicals. MnP was the only one of these enzymes produced in this paper, LiP and LAC being not produced (*data not shown*). High MnP activity was observed in cultures containing all of the PAHs except for ANT. Moreover, higher MnP activity was observed in *P. sordida* KUC8369 cultures containing 50 ppm PHE than in the other strains. However, *P. chrysosporium* KCTC6293 produced the highest level of MnP in cultures containing the other three PAHs. *P. chrysosporium* likely did not produce LiP due to too low cultivation temperature: we incubated the cultures at 30 °C, but it has been reported (Tien and Kirk 1988; Vyas *et al*. 1994) that 39 °C is the optimum temperature for the growth of *P. chrysosporium* and production of spores.

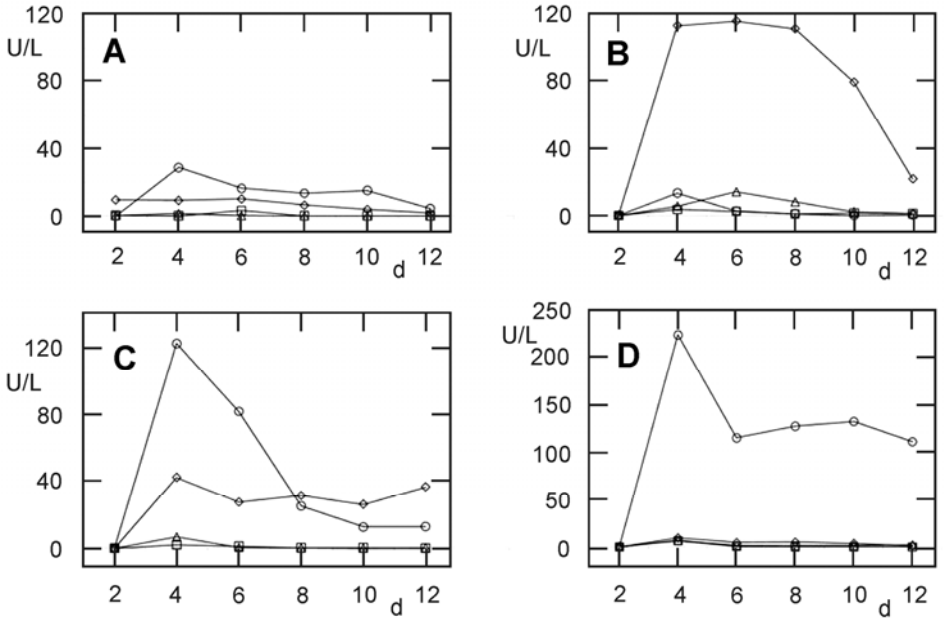

Fig. 3. MnP activity (U/L) in agitation cultures of selected *Phanerochaete* spp.; **A** – ANT, **B** – PHE, **C** – FLT, **D** – PYR; *diamonds* – *P. sordida* KUC8369, *squares* – *P. sordida* KUC8370, *triangles* – *P. sordida* KUC8710, *circles* – *P. chrysosporium* KCTC6293.

These results indicate that ELM might not be correlated with the degradation of PAHs (Juan *et al*. 2008). *P. chrysosporium* KCTC6293 eliminated ANT in the cultures with the highest rate of degradation, but MnP was not observed in high level. The MnP activity of *P. sordida* KUC8369 was not higher than that of *P. chrysosporium* KCTC6293, but this species showed the highest degradation rate of FLT. However, this does not mean that ELMs did not affect the degradation of lignin and PAHs as well. The mechanism of degradation is not only related to MnP, but also to enzymes such as LAC, LiP and Mn-independent peroxidase (Cajthaml *et al*. 2008), which are produced and complexly used for the degradation of recalcitrant materials such as lignin or PAHs. Accordingly, *P. chrysosporium* has the highest efficiency among known fungi because it produces the greatest amount of these enzymes (Singh and Chen 2008).

P. sordida and its related species as well as *P. chrysosporium* have a potential to tolerate and degrade four PAHs (Figs 1–3); *e.g.*, *P. sordida* KUC8370, having a faster growth rate on RBBR media and a higher tolerance to PYR than the other collected isolates and also higher PYR degrading ability than all other organisms, except for *P. chrysosporium*. These isolates obviously showed different ability to degrade PAHs (52.8 % PYR elimination), having the highest degradation rates of PHE. *P. sordida* KUC8710 also showed no growth inhibition by ANT, while KUC8369 was the greatest degrader of FLT, even though it produced less MnP than *P. chrysosporium*. It has therefore not been determined whether the three *Phanerochaete* isolates are the same or different species. More information regarding the intraspecific or intrageneric variations in the xenobiotic-degrading ability of WRFs is thus needed to fully understand the potential for these organisms to bioremediate soils contaminated with PAHs.

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