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Biodegradation of Polycyclic Aromatic Hydrocarbons by New Isolates of White Rot Fungi

JIM A. FIELD,* ED DE JONG, GUMERSINDO FEIJOO COSTA,† AND JAN A. M. DE BONT

Division of Industrial Microbiology, Department of Food Science, Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

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Eight rapid Poly R-478 dye-decolorizing isolates from The Netherlands were screened in this study for the biodegradation of polycyclic aromatic hydrocarbons (PAH) supplied at 10 mg liter^{-1}. Several well-known ligninolytic culture collection strains, *Phanerochaete chrysosporium* BKM-F-1767, *Trametes versicolor* Paprican 52, and *Bjerkandera adusta* CBS 595.78 were tested in parallel. All of the strains significantly removed anthracene, and nine of the strains significantly removed benzo[*a*]pyrene beyond the limited losses observed in sterile liquid and $HgCl_2$-poisoned fungus controls. One of the new isolates, *Bjerkandera* sp. strain Bos 55, was the best degrader of both anthracene and benzo[*a*]pyrene, removing 99.2 and 83.1% of these compounds after 28 days, respectively. Half of the strains, exemplified by strains of the genera *Bjerkandera* and *Phanerochaete*, converted anthracene to anthraquinone, which was found to be a dead-end metabolite, in high yields. The extracellular fluids of selected strains were shown to be implicated in this conversion. In contrast, four *Trametes* strains removed anthracene without significant accumulation of the quinone. The ability of *Trametes* strains to degrade anthraquinone was confirmed in this study. None of the strains accumulated PAH quinones during benzo[*a*]pyrene degradation. Biodegradation of PAH by the various strains was highly correlated to the rate by which they decolorized Poly R-478 dye, demonstrating that ligninolytic indicators are useful in screening for promising PAH-degrading white rot fungal strains.

Polycyclic aromatic hydrocarbons (PAH) are important pollutants found in soil and sediments. To date, most of the biological approaches considered for the restoration of PAH-contaminated sites depend on the activity of bacteria. Whereas low-molecular-weight PAH are usually readily degraded, high-molecular-weight PAH of five or more rings resist extensive bacterial degradation in soil and sediment media (2–4, 28, 29, 44). The recalcitrant behavior can be attributed to the limited bioavailability of PAH strongly adsorbed onto soil organic matter (40, 41, 58, 61).

White rot fungi would be expected to have greater access to poorly bioavailable substrates, since they secrete extracellular enzymes involved in the oxidation of complex aromatic compounds like lignin. Although the exact mechanism by which lignin polymers are depolymerized and mineralized is not fully understood (32, 50), a number of extracellular components that participate in the ligninolytic system have been identified (33). Extracellular peroxidases such as lignin peroxidase (LiP) and manganese peroxidase (MnP) are able to directly degrade dimeric lignin model compounds (35, 37, 39, 51, 53, 54, 56), and in some studies their involvement in the initial oxidation of lignin polymers has been demonstrated (21, 27, 34, 36, 37, 39, 53, 60). Since lignin itself is a random polymer, the system involved in its decomposition must also function nonspecifically. Thus, it is not surprising that the ligninolytic system of white rot fungi also attacks a large variety of aromatic pollutants (1, 6, 7).

Biodegradation of PAH by the white rot fungus *Phanerochaete chrysosporium* has clearly been verified in several studies that report mineralization of ^{14}C-labeled PAH (5, 7, 25, 42, 49). Although the highest level of $^{14}CO_2$ recovery ever measured is only 19%, much greater yields of the label are usually found as unidentified metabolites in the aqueous and organic phases (5, 25, 42, 49). Two studies have shown that at least one extracellular ligninolytic enzyme, LiP, is directly involved in the oxidation of some PAH to their respective quinones (24, 26). These quinones correspond to products anticipated from one-electron oxidation of aromatic compounds; this mechanism is like that postulated for the LiP-catalyzed oxidation of lignin model compounds (51). Biphenyl substructures in lignin, which have a certain analogy with PAH molecules, are also known to be attacked by ligninolytic fungi (30).

Extensive biodegradation of PAH, e.g., fluorene and benzo[*a*]pyrene (B[*a*]P) by *P. chrysosporium* in soil media was reported in two studies (16, 48). These results indicate that at least one white rot fungus offers real potential for the biorestoration of PAH polluted soils and sediments. Aside from *P. chrysosporium*, only two other strains, *Trametes versicolor* TV1 and *Chrysosporium lignorum* CL1, have been shown to degrade PAH (42). The PAH-degrading capability of other white rot fungal strains has been overlooked.

The main objective of this study was to screen white rot fungi for strains that would make good candidates for PAH biodegradation during soil and sediment bioremediation. Newly isolated white rot strains were tested for PAH biodegradation in parallel with well-known lignin-degrading culture collection strains. Additionally, we were able to study the validity of utilizing ligninolytic indicators like the polymeric dye Poly R (22) in screening for promising PAH degraders.

MATERIALS AND METHODS

Isolation and screening methods. A modification of method of Nishida et al. (45) was used for isolating basidiomycetes.

* Corresponding author.
† Permanent address: Department of Chemical Engineering, University of Santiago de Compostela, Santiago de Compostela, Spain.

The isolation medium was composed of powdered lignocellulosic substrate (hemp stem wood, 0.2%), guaiacol (0.01%), benomyl (15 mg liter^{-1}), and agar (1.5%). A total of 127 guaiacol browning isolates were obtained from numerous samples of rotted wood, litter, or soil from forest habitats in the Netherlands. The isolates capable of decolorizing Poly R-478 (0.02%) agar plates (with the hemp stem wood substrate) were then screened for the rate of Poly R decolorization in liquid medium as described by Gold et al. (22), except that Poly R was added before inoculation. Taxonomic determinations were carried out by the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands).

Microorganisms. *P. chrysosporium* BKM-F-1767 (ATCC 24725) was a gift from T. K. Kirk, Forest Products Laboratory, Madison, Wis. *T. versicolor* Paprican 52 (ATCC 20869) was obtained from the American Type Culture Collections (Rockville, Md.). *Bjerkandera adusta* CBS 595.78 and *Polyporus pinsitus* CBS 678.70 were obtained from the Centraal Bureau voor Schimmelcultures. *Ramaria* sp. strain 158, a promising ligninolytic isolate (15), was a kind gift from E. Agosin, Department of Chemical Engineering, Catholic University of Chile (Santiago, Chile). Some of the following new isolates used in this study were at least partially determined: *Trametes* sp. strain Naald 11; *Trametes* sp. strain Eik 39; *Trametes* sp. strain Berk 41; an unidentified strain of the order *Agaricales*, strain Beuk 47; *Bjerkandera* sp. strain Bos 55; *Daedaleopsis confragosa* GM 2; and *Stereum* sp. strain Schim 22. These strains were originally isolated from rotting pine needles, rotting oak wood, rotting birch wood, rotting beech wood, forest litter, forest soil, and forest litter, respectively. Unidentified strain Hen 37 was isolated from hemp stems, and unidentified strain Par 30 was isolated from a thermomechanical pulping effluent (Parenco Corp., Renkum, The Netherlands). New isolates and culture collection strains were maintained at 4°C on hemp stem wood (0.2%)–BIII (55) medium, from which they were transferred to malt extract agar plates (1.5% agar, 0.35% malt extract, 0.5% glucose) and incubated at 30°C for 7 to 10 days before use in the experiments of this study. In all cases, single 6-mm-diameter mycelium-agar plugs (obtained along a uniform circumference) from the plates were used as the inocula.

Culture conditions. The standard basal medium used in the experiments was N-limited liquid BIII medium (55) with 10 g of glucose liter^{-1} as the primary substrate in a 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. The medium was autoclaved, and then a filter-sterilized thiamine solution (200 mg liter^{-1}) was added (10 ml liter^{-1}). Aliquots (5 ml) of BIII medium were placed in 30-ml loosely capped serum bottles, and experiments were incubated statically under an air atmosphere at 30°C in complete darkness.

Biodegradation assays. Immediately before inoculation, a 0.05-ml aliquot of acetone containing 1 g of PAH liter^{-1} was introduced into the medium, providing final concentrations of acetone and PAH of 10 ml liter^{-1} and 10 mg liter^{-1}, respectively. The entire culture was utilized for the extraction procedure. Consequently, separate cultures were prepared and then destroyed for sampling on days 0, 7, 14, and 28 of the incubation. Typically, experiments were conducted in quadruplicate except the abiotic controls, which were replicated 18 times (6 per day, 3 offset days of analysis). Controls containing fungi only were run in duplicate to ensure that suspected degradation intermediates were not formed de novo. PAH were also incubated for 28 days with killed-fungus controls (replicated fourfold), which consisted of 7-day-old cultures that were poisoned with 7 g of $HgCl_2$ liter^{-1}.

Incubation of extracellular culture fluids. Extracellular culture fluids from triplicate 10-day-old cultures of Bos 55 and Beuk 47 grown on BIII medium were centrifuged for 10 min at 5,000 × *g*. Then 2-ml samples of the centrifuged fluid were transferred with a sterile pipette to previously autoclaved 30-ml serum bottles. Anthracene (112 nmol) was added in 0.02 ml of acetone, and the cultures were incubated for 48 h without any extraneous H_2O_2.

Enzyme assays. LiP activity was assayed with veratryl alcohol as described by Tien and Kirk (55) but was corrected for veratryl alcohol oxidase activity (i.e., activity in the absence of H_2O_2). MnP activity was measured by a method modified from that of Paszczynski et al. (47). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4$, and up to 650 μl of culture broth in a total volume of 1 ml. The reaction was started by adding 0.4 mM H_2O_2 and was corrected for background oxidase activity.

Toxicity assays. Two types of toxicity assays were conducted. The first toxicity test was based on measuring the CO_2 produced from cultures in 5 ml of BIII medium placed in 1,150-ml rubber septum-sealed serum bottles. These were initially filled with air and incubated statically at 30°C for 2 weeks. The second toxicity test was based on monitoring Poly R-478 dye (0.2 g liter^{-1}) decolorization (22) in 15 ml of BIII medium placed in 115-ml loosely capped serum bottles. These were incubated statically at 30°C under air. In both assays, acetone and PAH (in acetone) were added to the medium at rates of 10 ml liter^{-1} and 10 mg liter^{-1}, respectively.

Extraction and analysis. The culture bottles were weighed before and after the experiment to correct for concentration effects resulting from evaporation of water. Acetonitrile (10 ml) was added to each bottle, providing an extraction medium that contained 1:2 H_2O-CH_3CN. The bottles were then sealed with a Teflon-lined silicone septum, and the extraction was carried out for 1 h in the dark at room temperature (22 to 25°C) on a shake table providing 300 strokes (2 cm long) per min. The average extraction efficiencies after 1 h in BIII medium were 90.7 ± 3.2, 100.6 ± 5.4, 102.5 ± 5.7, 91.3 ± 4.0, and 111.2 ± 3.4 for 10 mg of anthracene, B[*a*]P, pyrenediones, and fluorenone, respectively, per liter. A 2-ml sample of the extract was centrifuged at 5,000 × *g* for 10 min. The supernatant (50 μl) was then analyzed on a Hewlett-Packard HPLC ChemStation chromatograph (Pascal Series) (Hewlett-Packard, Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector, and a HP9000-300 data processor. The column (200 by 3 mm) filled with ChromSpher C18-PAH (5-μm particles) was from Chrompack (Middelburg, The Netherlands). The flow rate was 0.4 ml min^{-1}, and the column temperature was 30°C. For the analysis of anthracene, isocratic conditions were used with 20% water and 80% acetonitrile. Fluorenone and anthraquinone were also analyzed under isocratic conditions but with 30% water and 70% acetonitrile. B[*a*]P and pyrenediones were analyzed with the following gradient: 80:20, 0:100, and 0:100 H_2O-CH_3CN at 0, 10, and 25 min, respectively. The UV and visible-light absorbances were monitored at 2-nm wavelength intervals from 210 to 360 nm or from 210 to 600 nm. The wavelengths used for integrating peak areas were 250, 250, 265, 240, and 250 nm for experiments with anthracene, anthraquinone, B[*a*]P, pyrenediones, and fluorenone, respectively.

CO_2 evolution was determined in the headspace by gas chromatography with a Packard 427 (Palo Alto, Calif.)

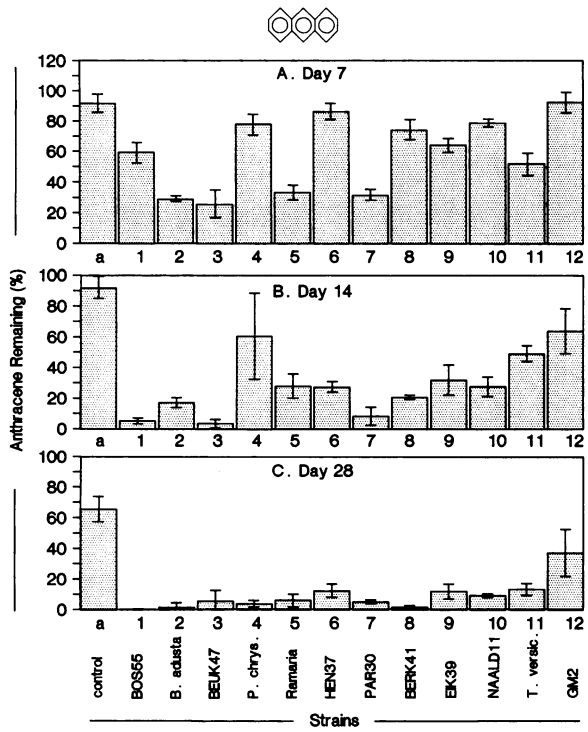

FIG. 1. Recovery of anthracene from liquid medium after 7, 14, and 28 days of incubation with 12 different strains of white rot fungi.

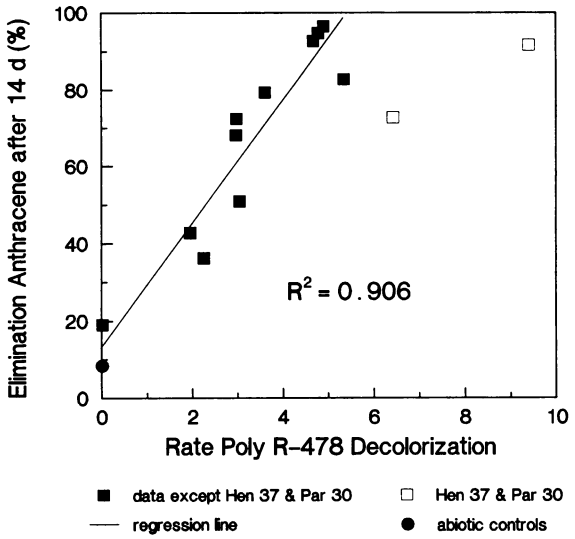

FIG. 2. Correlation of anthracene biodegradation with Poly R-478 dye decolorization (Hen 37 and Par 30 were excluded from regression; see the explanation in the text). The rate of decolorization was measured as the change in the A_{520}/A_{350} ratio per hour and multiplied by 10^3.

apparatus fitted with a thermal conductivity detector (140°C). The column (Hayesep Q; Chrompack) was maintained at 110°C, and helium was used as carrier gas (30 ml min^{-1}). The injection port was maintained at 110°C. The injection volume was 100 μl.

A_{350} and A_{520} readings for the Poly R-478 dye decolorization test were conducted with a Perkin-Elmer (Norwalk, Conn.) 550A UV-Vis spectrophotometer at 30°C with a 1-cm cuvette. Before the absorbance readings, 0.1 ml of centrifuged sample was diluted with 0.9 ml of demineralized water. The same spectrophotometer was used for the enzyme assays.

Chemicals. Anthracene, B[*a*]P, 9,10-anthraquinone, 9-fluorenone, guaiacol, veratryl alcohol, and 2,6-dimethoxyphenol were obtained from Janssen Chimica (Tilburg, The Netherlands) or Aldrich (Steinheim, Germany). These compounds were claimed to have purities ranging from 97 to 99%. A mixture of pyrenediones (mostly 1,6- and 1,8-diones) was a kind gift of J. Luchtenbrug from The Gorlaeus Laboratory (Leiden, The Netherlands). Poly R-478 was obtained from Sigma (St. Louis, Mo.). Benomyl was obtained from Du Pont Chemical Co. (Wilmington, Del.). Hemp (*Cannabis sativa* Fibrimon 56) stem wood was kindly supplied by the Agrotechnological Research Institute (Wageningen, The Netherlands).

RESULTS

Anthracene biodegradation. The amounts of anthracene remaining after 7, 14, and 28 days of incubation at 30°C with the 12 strains tested are shown in Fig. 1 together with the results from an uninoculated control. After 28 days, all of the strains showed significant elimination of anthracene that was well beyond that found for the abiotic control. The highest level of anthracene elimination (99.2% relative to the abiotic control concentration on day 28) was observed in cultures inoculated with *Bjerkandera* sp. strain Bos 55. *B. adusta* and *Trametes* sp. strain Berk 41 could also be grouped among the better degraders of anthracene. In a separate experiment, *P. chrysosporium* was incubated at 37°C with anthracene for 2 weeks; 92.6% elimination of anthracene was observed.

The validity of utilizing Poly R-478 for screening efficient anthracene-degrading strains of white rot fungi was examined by plotting the anthracene elimination data with Poly R-478 decolorization rates obtained (6 months previously) during the screening program. The linear regression shown in Fig. 2 demonstrates that anthracene degradation was highly correlated with the Poly R-478 decolorization rate. Data points belonging to the unidentified Hen 37 and Par 30 strains were excluded from the correlation because those two strains lost their Poly R-478-decolorizing activity after repeated transfers. *P. pinsitus* and *Stereum* sp. strain Schim 22, which are strains with low dye-decolorizing activity, were also tested for anthracene degradation in a separate experiment and included in the graph (42.7 and 19.0% anthracene elimination, respectively).

During biodegradation of anthracene, one intermediate was detected by the high-performance liquid chromatography method utilized. This compound accumulated significantly in many of the cultures. An example of the high-performance liquid chromatography plots is shown in Fig. 3 for the cultures inoculated with *Bjerkandera* sp. strain Bos 55. One peak at 4.4 min was evident in cultures supplied with anthracene but was not present in the abiotic anthracene control or in the fungal control. The degradation intermediate at 4.4 min of retention time could be identified as 9,10-anthraquinone based on matching the retention time and UV spectrum with those of an anthraquinone standard (peaks at 251, 272, and 327 nm; dips at 220, 268, and 287 nm).

Figure 4 illustrates the large differences among the 12 strains in the accumulation of anthraquinone during the experiment. Strains belonging to the genus *Trametes* (*T.*

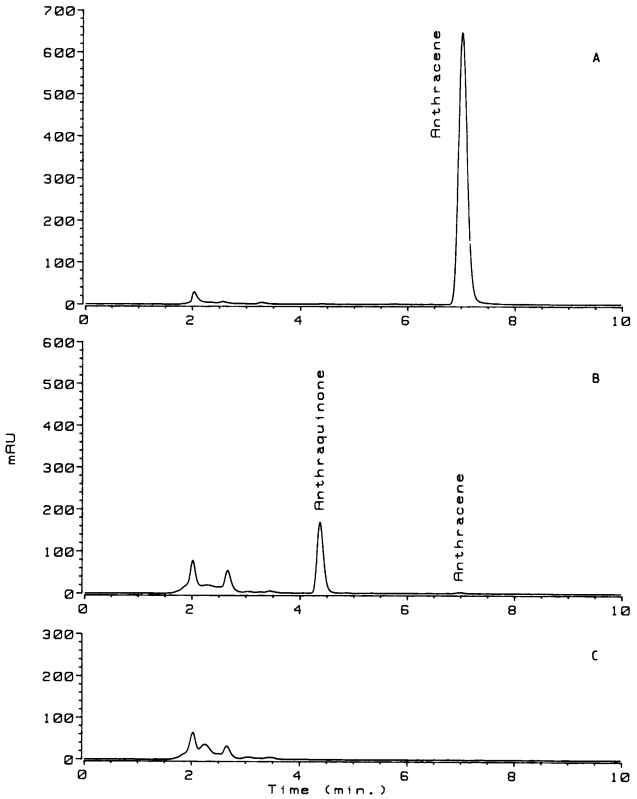

FIG. 3. High-performance liquid chromatograms after 28 days of anthracene biodegradation: (A) sterile medium control with anthracene; (B) medium inoculated with *Bjerkandera* sp. strain Bos 55, which initially contained anthracene; (C) medium without anthracene inoculated with Bos 55.

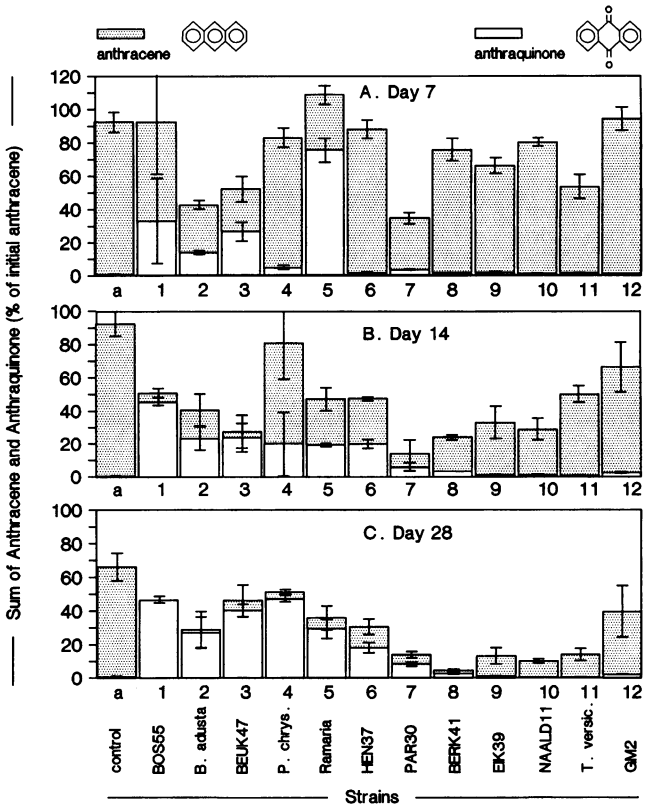

FIG. 4. Accumulation of anthraquinone during anthracene biodegradation by 12 different strains of white rot fungi.

versicolor; *Trametes* sp. strains Naald 11, Eik 39, and Berk 41) eliminated anthracene without accumulating anthraquinone. In contrast to the *Trametes* group, two *Bjerkandera* strains (*B. adusta* and *Bjerkandera* sp. strain Bos 55), *P. chrysosporium*, *Ramaria* sp. strain 158, and strain Beuk 47 were all notable accumulators of anthraquinone. *Ramaria* sp. strain 158 was the only strain of the accumulator group that was able to decrease the level of anthraquinone that had previously amassed. *Trametes* sp. strain Berk 41 and *Bjerkandera* sp. strain Bos 55 were taken as examples of anthraquinone-nonaccumulating and -accumulating strains, respectively, and examined for their ability to degrade various PAH quinones (Table 1). *Trametes* sp. strain Berk 41 was able to metabolize anthraquinone as well as other PAH quinones. *Bjerkandera* sp. strain Bos 55 was able to metabolize some PAH quinones but not anthraquinone.

To ensure that in vivo elimination of anthracene was not caused by reduced extraction efficiency resulting from anthracene adsorption on fungal mycelium, 7-day-old cultures (fluid and mycelium) were poisoned with $HgCl_2$ and incubated for an additional 28 days with anthracene (added directly after poisoning). The results shown in Fig. 5 clearly demonstrate that the extraction technique employed was able to adequately recover anthracene from the mycelium in killed cultures.

A surprising result was that four strains (Bos 55, Beuk 47, *B. adusta*, *Ramaria* sp. strain 158) oxidized anthracene to anthraquinone after $HgCl_2$ poisoning. A possible explanation for this conversion is that $HgCl_2$ killed the cells without completely inhibiting extracellular enzymes involved in the oxidation. The four strains displaying anthraquinone formation in killed 7-day-old cultures were also the only four strains that produced significant levels of anthraquinone by day 7 in vivo (Fig. 4). This indicates that the enzymes involved in anthraquinone formation were present in killed 7-day-old cultures.

To test the involvement of extracellular enzymes in the oxidation of anthracene to its quinone, centrifuged extracel-

TABLE 1. Elimination of PAH quinones by two strains of white rot fungi

Strain or medium	% Elimination (SD) (n = 4) of:		
	Anthraquinone[a]	Fluorenone[b]	Pyrenedione[c]
Trametes sp. strain Berk 41	82.5 (2.0)	100.0 (0.0)	98.4 (2.1)
$HgCl_2$-killed Berk 41	−2.1 (5.3)	ND[d]	11.1 (3.0)
Bjerkandera sp. strain Bos 55	2.5 (1.7)	100.0 (0.0)	97.7 (2.2)
$HgCl_2$-killed Bos 55	−0.6 (1.4)	ND	10.5 (1.4)
Sterile medium	3.1 (2.7)	55.7 (1.9)	8.9 (10)

[a] Anthraquinone (35.7 μM) was incubated for 28 days.
[b] Fluorenone (55.5 μM) was incubated for 14 days.
[c] The pyrenedione mixture (10 mg liter^{-1}) was incubated for 28 days.
[d] ND, not determined after 14 days. However, fluorenone elimination values in killed Berk 41, killed Bos 55, and sterile medium after 28 days were 74.2 ± 0.6, 74.0 ± 1.7, and 82.0 ± 0.8, respectively.

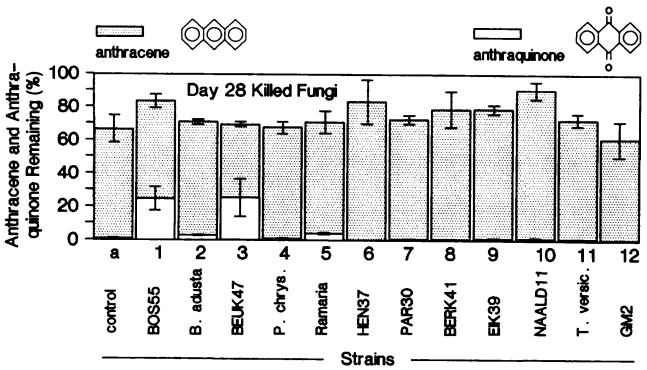

FIG. 5. Recovery of anthracene after 28 days of incubation with $HgCl_2$-killed fungus controls.

lular fluids from 10-day-old cultures of *Bjerkandera* sp. strain Bos 55 and strain Beuk 47 were incubated for 2 days with anthracene. The results in Table 2 confirm that this oxidation step does, indeed, occur extracellularly. LiP activity was not present in the centrifuged extracellular fluids; thus, LiP had no role in the oxidation. MnP activity was present in the fluids of both strains. $HgCl_2$ did not completely inhibit the extracellular anthracene-oxidizing activity. It is plausible that the available $HgCl_2$ concentration was much lower (and thus less inhibiting) in the dead fungus controls, since high levels of heavy metal sorption onto fungal biomass are frequently reported (14, 38).

B[*a*]P biodegradation. The 12 strains of fungi used in the anthracene experiment were also tested for biodegradation of the high-molecular-weight PAH compound B[*a*]P. The amounts of B[*a*]P remaining after 7, 14, and 28 days of incubation with the fungi are shown in Fig. 6. After 28 days, most of the strains eliminated significantly more B[*a*]P than did the slowest Poly R-478-decolorizing strain (GM 2) and the abiotic control. The recovery of B[*a*]P after 28 days of incubation with $HgCl_2$-poisoned 7-day-old cultures is shown in Fig. 6C; 9 of the 12 strains tested eliminated B[*a*]P to an extent statistically superior to that observed for the killed fungi. The best anthracene degrader, *Bjerkandera* sp. strain Bos 55, was also the most efficient degrader of B[*a*]P. This strain eliminated 83.1% of the B[*a*]P when normalized to the amount still present in the abiotic control. Bos 55 proved to be a statistically significantly better than almost all of the strains tested, including the commonly studied culture collection strains, at degrading B[*a*]P. Beuk 47 and *B. adusta* were also good degraders of B[*a*]P. A graph illustrating the good correlation of B[*a*]P degradation with Poly R-478 decolorization rates is shown in Fig. 7.

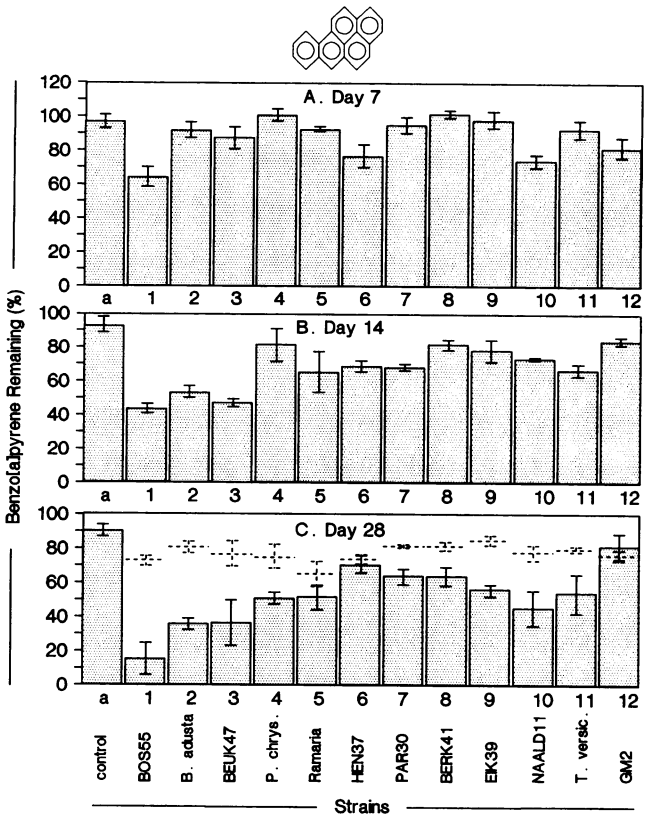

FIG. 6. Recovery of B[*a*]P from liquid medium after 7, 14, and 28 days of incubation with 12 different strains of white rot fungi. The dashed lines in graph C show the recovery of B[*a*]P from $HgCl_2$-killed fungus controls.

TABLE 2. Anthraquinone formation by centrifuged extracellular liquid of 10-day-old cultures

Source of culture fluid[a]	$HgCl_2$ concn (g liter^{-1})	Anthraquinone formed after 48 h,[b] nmol (SD)
Bos 55	0	4.6 (2.6)
Bos 55	7	0.5 (0.4)
Bos 55 (boiled)[c]	0	0.0 (0.0)
Beuk 47	0	6.4 (2.5)
Beuk 47	7	0.3 (0.4)
Beuk 47 (boiled)[c]	0	0.0 (0.0)
Sterile medium	0	0.0 (0.0)
Sterile medium	7	0.0 (0.0)

[a] Extracellular culture fluids were from *Bjerkandera* sp. strain Bos 55, an unidentified strain of the order *Agaricales*, strain Beuk 47, or sterile medium.
[b] Anthracene (112 nmol) was incubated in 2 ml of extracellular fluid for 48 h under air at 30°C with no extraneous H_2O_2 addition. Extracellular fluids were obtained from three separate cultures.
[c] Extracellular culture fluids were boiled for 20 min.

During the experiment, no intermediates of B[*a*]P biodegradation were detected in any of the strains, even though the high-performance liquid chromatography method used was suitable for separating and detecting B[*a*]P quinones. Thus, apparently, during in vivo degradation under the culture conditions of this study, the quinones were either rapidly metabolized further or they were never intermediates of B[*a*]P biodegradation.

Toxicity of acetone and PAH compounds. The PAH were introduced into the medium of the biodegradation assays in acetone, resulting in a final solvent concentration of 10 ml liter^{-1}. Because of concerns over the possibility for inhibition, two strains were tested in triplicate for 2 weeks for growth on glucose in the presence of 0, 10, 50, 100, and 200 ml of acetone liter^{-1}. No growth inhibition was visible with 10 ml of acetone liter^{-1}. Complete growth inhibition was only evident with 100 and 200 ml of acetone liter^{-1} for Berk 41 and Bos 55, respectively. Inhibition could also result from the PAH compounds. This possibility was checked by monitoring both the CO_2 production and the Poly R-478 decolorization rates in the presence of the PAH compounds. The results listed in Table 3 illustrate that generally no significant

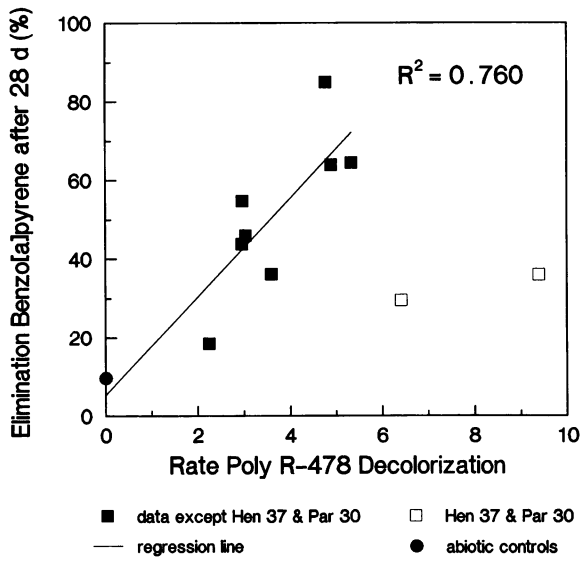

FIG. 7. Correlation of B[*a*]P biodegradation with Poly R-478 dye decolorization (Hen 37 and Par 30 were excluded from regression; see explanation in text). The rate of decolorization was measured as the change in the A_{520}/A_{350} ratio per hour and multiplied by 10^3.

TABLE 3. Effects of acetone and PAH on CO_2 production and Poly R-478 dye decolorization rates of two white rot strains[a]

Strain and addition to culture	Mean (SD) CO_2 production rate (n = 3)	Mean (SD) dye decolorization rate[b] (n = 3)
Bjerkandera sp. strain Bos 55		
Control	0.163 (0.029)	−0.214 (0.007)
Acetone (10 ml liter^{-1})	0.278 (0.023)	−0.222 (0.009)
B[*a*]P[c]	0.293 (0.010)	−0.165 (0.020)
Anthracene	0.276 (0.032)	−0.224 (0.007)
Anthraquinone	0.255 (0.024)	−0.208 (0.015)
Trametes sp. strain Berk 41		
Control	0.297 (0.012)	−0.160 (0.025)
Acetone (10 ml liter^{-1})	0.223 (0.016)	−0.154 (0.015)
B[*a*]P[c]	0.263 (0.009)	−0.147 (0.025)
Anthracene	0.214 (0.027)	−0.163 (0.002)
Anthraquinone	0.236 (0.003)	−0.185 (0.009)

[a] For strain Bos 55, the mean CO_2 production rate per day was measured between days 2 and 13 and the mean dye decolorization rate per day was measured between days 5 and 7. For strain Berk 41, the mean CO_2 production rate per day was measured between days 5 and 13 and the mean dye decolorization rate per day was measured between days 5 and 7.
[b] Measured as the change in A_{520}/A_{350} ratio per day.
[c] PAH (10 mg liter^{-1}) were introduced with acetone (10 ml liter^{-1}).

inhibition was observed. In only one case, B[*a*]P caused slight inhibition (26%) of the decolorizing activity. A curious result was the fact that acetone markedly stimulated both the yield and rate of CO_2 production by *Bjerkandera* sp. strain Bos 55. In a separate experiment, we were able to demonstrate that this strain did not utilize acetone as a sole C source for growth.

DISCUSSION

Previously, several research groups demonstrated that the white rot fungus *P. chrysosporium* is able to degrade PAH (5, 7, 25, 26, 42, 49). In this study, we found that PAH biodegradation is a ubiquitous phenomenon among white rot fungi. All 12 strains tested degraded anthracene, and 9 of the strains degraded B[*a*]P, significantly beyond the level in the poisoned mycelium controls. Although PAH biodegradation was found to be a universal characteristic of the white rot fungi tested, two distinct patterns of PAH biodegradation could be distinguished.

Strains from the genera *Bjerkandera*, *Phanerochaete*, and *Ramaria* and one undetermined strain of the order *Agaricales* (Beuk 47) efficiently converted anthracene to anthraquinone, which was found to be a dead-end metabolite. This result was similar to those obtained previously when N-limited cultures of *P. chrysosporium* were incubated with anthracene (23). The accumulation of anthraquinone by these fungi should not necessarily be considered as an unfortunate fate of the PAH pollutant, because anthraquinone is readily degraded by bacteria (13, 43).

The accumulation of anthraquinone in high yields indicates the involvement of the extracellular ligninolytic system in PAH biodegradation. The extracellular nature of the conversion was confirmed by observing anthraquinone formation in the centrifuged culture fluids of selected strains. The oxidation of anthracene to anthraquinone is a known reaction of LiP (23). However, no LiP activity was detected in the extracellular fluids that were utilized in the experiment for confirming the extracellular conversion of anthracene to anthraquinone. The possibility that other peroxidases that oxidize PAH compounds were present certainly deserves consideration. For example, MnP might be worth investigating, since it is excreted by all of the strains tested. Additionally, a peroxidase activity that cannot be ascribed to LiP nor MnP has been detected in the extracellular fluids of *Bjerkandera* strains (12, 59).

Strains belonging to the genus *Trametes* degraded anthracene without any accumulation of anthraquinone. In a similar fashion, no quinone intermediates were formed by any of the strains during the biodegradation of B[*a*]P. These results indicate either that the PAH quinones were never formed or that they were rapidly metabolized. White rot fungi are certainly able to degrade at least some types of PAH quinones. George and Neufeld (16) reported the biodegradation of fluorenone by *P. chrysosporium*, and Hammel et al. (26) found that pyrenediones were fleeting intermediates during in vivo degradation of pyrene by *P. chrysosporium*. In this study, we were able to confirm that a *Trametes* sp. degraded anthraquinone. In contrast, *Bjerkandera* sp. strain Bos 55 did not convert anthraquinone. *Ramaria* sp. strain 158 had a transient capacity to metabolize anthraquinone that was lost during prolonged incubations. Thus there are distinct differences among white rot fungi in their ability to metabolize anthraquinone. Interestingly, *Bjerkandera* sp. strain Bos 55 metabolized fluorenone and pyrenediones, indicating that at least some PAH quinones would not behave as dead-end metabolites in anthraquinone-accumulating strains. 10-Hydrohydroxyl-anthracene-9-one (hydroxyanthrone), a postulated key intermediate of anthracene metabolism by *P. chrysosporium*, is suspected to be degraded further under low O_2 concentrations (23). Under pure O_2, hydroxyanthrone is converted abiotically to the dead-end metabolite anthraquinone. High yields of anthraquinone were observed in this study under an air atmosphere. In contrast to the results of our study and that of Haemmerli (23), a recent report demonstrated that ligninolytic cultures of *P. chrysosporium* metabolized anthraquinone (25). This finding indicates that even anthraquinone-

accumulating strains degrade anthraquinone under certain conditions.

A number of nonligninolytic fungi have intracellular mechanisms for PAH degradation that lead to dihydrodiol and hydroxy PAH metabolites (8–10, 17, 62). These products are suspected to originate from the activity of cytochrome P-450 monooxygenases in conjunction with epoxide hydrolase (10). The nonligninolytic fungi can also convert certain PAH compounds to PAH quinones, but the yields are relatively low compared with those of other metabolites (8). Even *P. chrysosporium* has been shown to metabolize phenanthrene under nonligninolytic conditions to dihydrodiol and hydroxylated PAH metabolites (52). Such metabolites were not detected in this study.

Another possible fate of PAH that still needs to be investigated is the polymerization of the PAH compounds to humic polymers. In one study, the copolymerization of benzo[*a*]pyrenediones with coniferyl alcohol into lignin polymers was demonstrated with horseradish peroxidase (57).

The exact mechanism of PAH degradation could not be determined in this study because we only monitored PAH elimination and quinone formation. However, we did observe that both anthracene and B[*a*]P biodegradations were highly correlated to the strain's ability to decolorize the polymeric dye Poly R-478. This observation suggests that primarily a lignin metabolism-mediated attack of PAH compounds was occurring in this study. Polymeric dyes are considered to be good indicators of ligninolytic activity because dye decolorization in vivo coincides with the onset of lignin metabolism in white rot fungal cultures (19, 22). The peroxidases that perform such an important function in lignin model compound and PAH biodegradation are also the enzymes directly involved in the decolorization of polymeric dyes (11, 12, 18, 20, 35, 46). The decolorization observed in vivo is indicative of global peroxidase activity, since it reflects the combined effect of peroxidases and the H_2O_2-generating oxidases. Mutants that lack oxidase do not decolorize the dye in vivo (31).

The eight new strains evaluated in this study were selected from 127 isolates based on dye decolorization rates. The results from the screening test with PAH revealed that one of the eight strains, *Bjerkandera* sp. strain Bos 55, was an outstanding PAH degrader. This strain removed B[*a*]P to an extent that was statistically superior to those of the three most commonly studied culture collection strains with high ligninolytic activity (*P. chrysosporium*, *T. versicolor*, and *B. adusta*). Therefore, it can be concluded that the preliminary screening with Poly R-478 was a useful tool in selecting a promising PAH-degrading fungus. *Bjerkandera* sp. strain Bos 55 has been chosen for further studies that encompass optimizing the culture conditions.

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