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Polycyclic Aromatic Hydrocarbon-Degrading Capabilities of *Phanerochaete laevis* HHB-1625 and Its Extracellular Ligninolytic Enzymes

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The ability of *Phanerochaete laevis* HHB-1625 to transform polycyclic aromatic hydrocarbons (PAHs) in liquid culture was studied in relation to its complement of extracellular ligninolytic enzymes. In nitrogen-limited liquid medium, *P. laevis* produced high levels of manganese peroxidase (MnP). MnP activity was strongly regulated by the amount of Mn^{2+} in the culture medium, as has been previously shown for several other white rot species. Low levels of laccase were also detected. No lignin peroxidase (LiP) was found in the culture medium, either by spectrophotometric assay or by Western blotting (immunoblotting). Despite the apparent reliance of the strain primarily on MnP, liquid cultures of *P. laevis* were capable of extensive transformation of anthracene, phenanthrene, benz[*a*]anthracene, and benzo[*a*]pyrene. Crude extracellular peroxidases from *P. laevis* transformed all of the above PAHs, either in MnP-Mn^{2+} reactions or in MnP-based lipid peroxidation systems. In contrast to previously published studies with *Phanerochaete chrysosporium*, metabolism of each of the four PAHs yielded predominantly polar products, with no significant accumulation of quinones. Further studies with benz[*a*]anthracene and its 7,12-dione indicated that only small amounts of quinone products were ever present in *P. laevis* cultures and that quinone intermediates of PAH metabolism were degraded faster and more extensively by *P. laevis* than by *P. chrysosporium*.

Several developments in recent years have helped to make bioremediation—the use of live organisms to decontaminate polluted soil or water—an important pollution control tool. One key advance involves an ecologically distinct group of organisms known as white rot fungi. To date, most of the research concerning bioremediation with these fungi has centered on a single species, *Phanerochaete chrysosporium*, which is known to metabolize a wide range of xenobiotic compounds (for reviews, see references 3, 18, and 27). Recently, however, other *Phanerochaete* species, as well as members of other genera, have begun to be evaluated for their pollutant-degrading abilities. Notable differences among fungi with regard to the extent of pollutant mineralization and transformation ability, as well as the nature of pollutant-derived metabolites, have been demonstrated in these studies. Substantial variations in xenobiotic mineralization capacities have been observed; these are both intergeneric, such as those reported for dieldrin and dichloroaniline (23, 24), and intrageneric, as seen with different *Phanerochaete* species in the case of pentachlorophenol mineralization (20). The ability of white rot species to transform pollutants is similarly quite variable. Field et al. (10) have shown a wide range of anthracene- and benzo[*a*]pyrene-degrading abilities among 12 different white rot fungi. Similarly, a recent report (2) details extensive variability among basidiomycetes with respect to their capacity to degrade four polycyclic aromatic hydrocarbon (PAH) species (naphthalene, fluoranthene, chrysene, and benzo[*a*]pyrene). Finally, the nature of the transformation products formed during pollutant degradation differs among white rot species. This has been best demonstrated for anthracene degradation (10). Significant accumulations of 9,10-anthraquinone (up to 50% of input anthracene) were detected concomitant with depletion of anthracene from 1-, 2-, or 4-week-old liquid cultures of species from some genera (*Bjerkandera*, *Phanerochaete*, and *Ramaria*), with none or very little detected in others (*Trametes* and *Daedaleopsis*).

Undoubtedly, all three levels of variability described above are consequences of both differences in the enzymology of the various white rot species and differences in growth and enzyme production responses of various fungi to different culture media. Lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccases are all known to be produced by white rot fungi, although the specific enzyme complements of different species are highly variable (36). Each of these enzyme classes has been implicated in pollutant degradation by these fungi (11).

The objective of the present study was to characterize the PAH-degrading capacity of *Phanerochaete laevis* HHB-1625 and its extracellular enzymes. This strain had been previously identified in screening studies (6a) as having above-average pentachlorophenol-mineralizing ability relative to other *Phanerochaete* species. This paper details the results of this work.

MATERIALS AND METHODS

Chemicals. [5,6-^{14}C]benz[*a*]anthracene (7.9 mCi · mmol^{-1}) and [7,10-^{14}C]benzo[*a*]pyrene (9.6 mCi · mmol^{-1}) were obtained from California Bionuclear Corp., Los Angeles, Calif.; the radiochemical purity of both compounds was >97%. Radiolabeled phenanthrene ([9-^{14}C]phenanthrene; 10.9 mCi · mmol^{-1}, 99% pure) was purchased from Sigma, St. Louis, Mo. [1,4-^{14}C]Anthracene (3.0 mCi · mmol^{-1}, >98% pure) was generously provided by K. E. Hammel. The radiochemical purity of all four PAHs was verified by high-pressure liquid chromatography (HPLC); all four were used without further purification. Unlabeled phenanthrene and benzo[*a*]pyrene were obtained from Sigma. Benz[*a*]anthracene was from Supelco, Bellefonte, Pa. Benz[*a*]anthracene-7,12-dione and 9,10-phenanthrenequinone were purchased from Aldrich, Milwaukee, Wis. [5,6-^{14}C]benz[*a*]anthracene-7,12-dione was prepared from [^{14}C]benz[*a*]anthracene by the ceric ammonium sulfate method of Periasamy and Bhatt (29) and was purified by HPLC with the gradient described below. The identity and purity of the product thus isolated were confirmed by gas chromatography-mass

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spectrometry. Benzo[*a*]pyrene quinones were produced by LiP-catalyzed oxidation of benzo[*a*]pyrene. All solvents used were HPLC grade, and all other chemicals were the highest commercially available grade.

Fungus. Master cultures of *P. laevis* HHB-1625 were obtained from the collection of the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. These cultures were subcultured onto yeast extract-malt extract-peptone-glucose agar slants (20). Following 1 week of growth at room temperature, the slants were maintained at 4°C until use. Mycelium scraped from slants was used to inoculate 20 ml of 1.5% malt extract broth in stoppered flasks. After 7 days, the resultant mycelial mats were removed and homogenized in a Waring blender for 15 s at low speed; this mycelial suspension was used as the inoculum for cultures in chemically defined BIII medium (see below). Typically, 10 to 20 malt extract-grown mats were homogenized in 100 ml of H_2O and 1-ml aliquots (ca. 2 mg [dry weight] of mycelium) were used for inoculation of 20-ml BIII cultures.

Enzyme assays. Activities of MnP, LiP, and laccase were assayed spectrophotometrically in extracellular fluid of basal Mn (200 μM) cultures set up as described above. MnP activity was determined by measuring vanillylacetone oxidation in the presence of 100 μM Mn^{2+} (28); oxidation of vanillylacetone in the absence of Mn^{2+} or H_2O_2 was measured to correct for potential laccase interference. MnP assays were also conducted on cultures grown in low-Mn^{2+} BIII medium (4 μM Mn^{2+}, in contrast to the standard 200 μM) to assess the role of Mn^{2+} in regulating enzyme production. Laccase was assayed via the H_2O_2-independent oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (pH 3.0) (26). Prior to LiP assays, culture fluid was dialyzed against 5 mM sodium acetate (pH 6.0). The buffer/sample ratio during dialysis was 50:1, and buffer was changed three times over 24 h. Lignin peroxidase determinations were based on oxidation of veratryl alcohol (35). All enzyme activities are expressed in units per milliliter, with 1 unit equal to 1 nmol of substrate oxidized per min.

PAH transformation studies. For all mass balance, mineralization, and metabolite profile studies, *P. laevis* HHB-1625 was inoculated as described above into 20 ml of *trans*-aconitic acid-buffered BIII medium with glucose (56 mM) and ammonium tartrate (1.1 mM) as sources of carbon and nitrogen, respectively (17), with Tween 20 omitted, in 125-ml Erlenmeyer flasks fitted with gas exchange ports. Cultures were maintained without agitation at room temperature (ca. 25 to 27°C) and flushed with O_2 every 3 to 5 days.

PAHs (1 to 1.5 μM) were added, after establishment of mats (3 days), in 10 μl of *N*,*N*-dimethylformamide, along with 50,000 to 200,000 dpm of radiolabeled compound. To measure the production of $^{14}CO_2$, headspaces of triplicate cultures were periodically flushed with O_2 through traps containing 10 ml of a toluene-ethanolamine-based scintillation cocktail (19). $^{14}CO_2$ thus trapped was measured by liquid scintillation counting in a 1214 RackBeta liquid scintillation counter (Wallac Oy, Turku, Finland). Volatilization of parent compound and/or metabolites was assessed by flushing three parallel cultures through Orbo-32LG activated charcoal traps (Supelco) positioned in line prior to the CO_2 traps. The difference in total apparent $^{14}CO_2$ evolution (i.e., mineralization) between these cultures and those without charcoal traps was attributed to the production of volatile products.

Following 4-week incubations, PAH-supplemented cultures were acidified to pH 1.5 with concentrated H_2SO_4, homogenized in a Waring blender, and extracted three times with 20-ml volumes of ethyl acetate. Fungal mycelium was separated from the extracted culture fluid by vacuum filtration and combusted in an OX-600 biological oxidizer (R. J. Harvey Instrument Corp., Hillsdale, N.J.). The resultant $^{14}CO_2$ was trapped and counted, as were 1-ml aliquots of the organic extract and the remaining aqueous phase. The organic extracts were then dehydrated with anhydrous Na_2SO_4, evaporated to dryness under N_2, and redissolved in acetonitrile for HPLC analysis as described below.

In vitro PAH transformation. Crude *P. laevis* peroxidase was prepared as previously described (4). Extracellular fluid from ca. 60 7-day-old stationary cultures was collected and concentrated 20-fold. Polysaccharide slime was removed via freezing-thawing-centrifugation; the crude peroxidase was then dialyzed against 5 mM sodium acetate (pH 6.0), further concentrated (final volume, 5 ml), and sterilized with low-protein-binding filters. The final MnP activity of this preparation was ca. 12,000 units · ml^{-1}, and there was no LiP activity. Degradation of PAHs by *P. laevis* extracellular enzymes was studied by using two reaction systems. MnP reaction mixtures (1.0 ml) contained 0.1 M sodium tartrate (pH 5.0), 100 μM $MnSO_4$, 20% dimethylformamide, 4 μM PAH, and 50,000 to 100,000 dpm of [^{14}C]PAH. Crude enzyme (5 μl) and H_2O_2 (50 μM) were added four times during the 30-h course of the reaction. MnP-dependent lipid peroxidation reactions (in 1.0-ml volumes) were set up as previously described (4, 22), with 4 μM PAH and 50,000 to 100,000 dpm of [^{14}C]PAH. Crude enzyme was added daily (for a total of 7 days) in 20-μl aliquots. All reactions were terminated by addition of 1 ml of acetonitrile, and the reaction products were filtered prior to HPLC analysis.

HPLC. All HPLC analyses described herein were performed with a Vydac 201TP54 (25- by 0.46-cm) C_{18} reverse-phase column (Nest Group, Southboro, Mass.). The gradient used consisted of water-acetonitrile (each containing 1% glacial acetic acid) as follows: 0 to 2 min, 80:20; 2 to 23 min, ramp to 0:100; 23 to 28 min, isocratic at 0:100. The flow rate throughout was 1 ml · min^{-1}. Radioactivity profiles were generated by passing column eluent through a Flo-One radiochromatography detector (Packard Instruments Co., Downers Grove, Ill.), which was operated in TR-LSC mode with Flo-Scint V (5 ml · min^{-1}) as the scintillation cocktail.

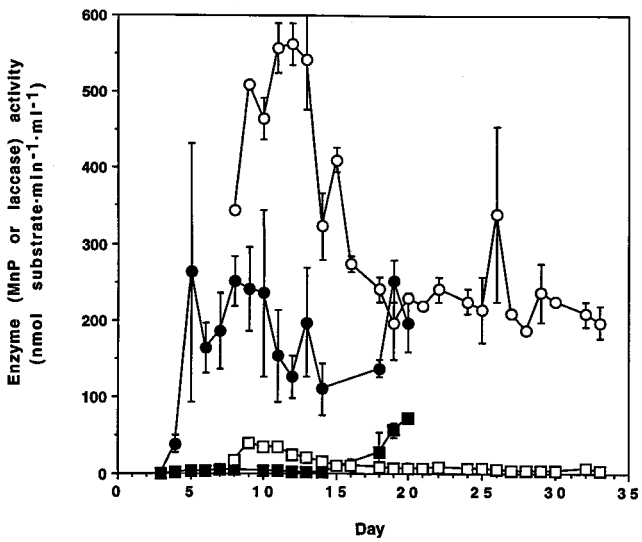

FIG. 1. Extracellular MnP (● and ○) and laccase (■ and □) activities in two sets of *P. laevis* HHB-1625 liquid cultures, grown in N-limited BIII medium. One set of cultures is represented by solid symbols, and one is represented by open symbols. Each datum point represents the average and standard deviation of assays of two to three cultures.

RESULTS

Enzyme activities. LiP activity was measured spectrophotometrically in dialyzed and undialyzed *P. laevis* culture medium. Dialysis proved to be necessary to avoid anomalous results in LiP assays. Specifically, LiP assays on undialyzed culture fluid from various time points gave identical results (linear decreases in absorbance or transient linear increases in absorbance followed by leveling off) in the presence or absence of veratryl alcohol (data not shown). This phenomenon most probably represents one of the potential interferences of the veratryl alcohol assay put forth by Archibald (1), probably the presence of one or more low-molecular-weight species with absorbances at this wavelength. Following dialysis of culture fluid, which eliminated this behavior, no LiP activity was detectable, even after concentration. Activities of MnP and laccase were, in contrast, unaffected by dialysis, and were therefore assayed in undialyzed culture fluid.

Figure 1 shows time courses of MnP and laccase activity in two sets of *P. laevis* liquid cultures; MnP was clearly the dominant enzyme under these conditions. MnP activity, although highly variable, appeared on day 3, increased rapidly on day 5, and persisted, with some fluctuation, throughout the 5-week duration of the experiment. The level of MnP activity was strongly dependent on the concentration of Mn(II) in the culture medium (Fig. 2). Laccase activity was consistently detected in the cultures at low levels (≤5 units · ml^{-1}).

Mineralization and transformation of PAHs. Table 1 shows the distribution of radioactivity from cultures of *P. laevis* 4 weeks after supplementation with [^{14}C]anthracene, [^{14}C]phenanthrene, [^{14}C]benz[*a*]anthracene, or [^{14}C]benzo[*a*]pyrene. Mass balances for the four PAHs were similar, with the largest portion (≈50%) of input radioactivity being present in the organic-extractable fraction. Total recovery of radioactivity from the four PAHs ranged from 66.8% ± 9.2% (anthracene) to 82.2 ± 1.2% (benzo[*a*]pyrene).

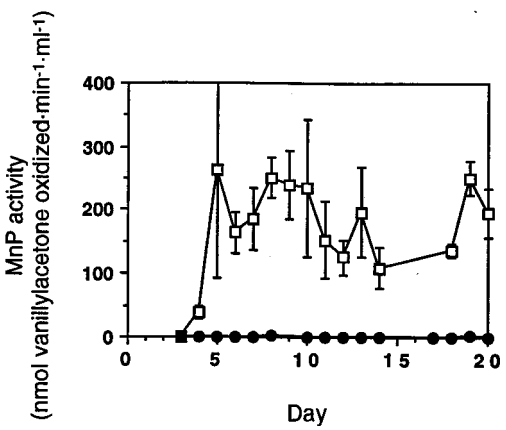

FIG. 2. MnP activity in standard-Mn^{2+} (200 μM, □) and low-Mn^{2+} (4 μM, ●) cultures of *P. laevis*. Levels of MnP in low-Mn^{2+} cultures averaged approximately 0.5 to 1.0 unit · ml^{-1}.

The low rates of mineralization of three of the four PAHs in this study, coupled with the relatively slight (10% or less of input ^{14}C) accumulations of water-soluble products, raised the possibility that little transformation of these compounds had actually occurred. However, dehydration and evaporation of the organic extracts resulted in losses (most probably by volatilization) of 35 to 50% of the extracted ^{14}C, indicating the formation of volatile products. Furthermore, HPLC analysis of the ethyl acetate-soluble products proved that significant PAH transformation had occurred. Figure 3 shows reverse-phase HPLC chromatograms of the organic-soluble ^{14}C disintegrations per minute from PAH-supplemented *P. laevis* cultures. In each case, significant transformation to polar products was observed, with only a small fraction remaining as the parent PAH. Approximately 25% of recovered ethyl acetate-soluble benz[*a*]anthracene-derived radioactivity (ca. 12% of input ^{14}C) was unmetabolized. This value is similar to that for benzo[*a*]pyrene (19% of recovered, 9.5% of input), whereas no unchanged anthracene or phenanthrene remained in cultures after 4 weeks. The chromatograms in Fig. 3 also show the approximate retention times of quinones derived from the respective PAHs; in each case, *P. laevis* cultures showed no accumulation of these compounds at the end of the 4-week incubations.

In vitro PAH transformation. As shown in Table 2, MnP/Mn^{2+} reactions produced small amounts of quinone products from anthracene and benzo[*a*]pyrene; formation of diones was abolished by omission of Mn^{2+} or H_2O_2 from the reaction mixture (data not shown). No transformation of benz[*a*]anthracene or phenanthrene was observed. In contrast, MnP-dependent lipid peroxidation reactions supported transformation of all four PAHs (Fig. 4). Extremely polar products, presumably ring-cleavage products analogous to diphenic acid (22), were seen in the cases of phenanthrene, benz[*a*]anthracene, and benzo[*a*]pyrene. Anthracene oxidation resulted in quantitative accumulation of anthraquinone.

TABLE 1. Percent distribution of radioactivity in fractions of 4-week stationary-phase liquid cultures of *P. laevis* HHB-1625 spiked with 1 to 1.5 μM PAHs

Fraction	% Radioactivity after addition of[a]:			
	ANT	PHE	BAA	BAP
Organic soluble	40.9 (5.4)	48.6 (0.0)	50.6 (0.5)	50.5 (0.2)
H_2O soluble	10.7 (2.6)	6.1 (0.3)	5.3 (0.0)	9.3 (0.4)
Mineralized	5.8 (0.2)	0.2 (0.0)	3.1 (0.7)	1.5 (0.3)
Volatilized	4.9 (1.0)	1.9 (0.7)	2.1 (0.2)	0.6 (0.3)
Mycelial mat	4.5	14.9	12.2	20.3
Total recovery	66.8 (9.2)	71.7 (1.0)	73.3 (1.4)	82.2 (1.2)

[a] ANT, anthracene; PHE, phenanthrene; BAA, benz[*a*]anthracene; BAP, benzo[*a*]pyrene. Data are given as means (n = 3), with standard deviations in parentheses.

DISCUSSION

The enzymology of *P. laevis* differs considerably from that of the most widely studied *Phanerochaete* species, *P. chrysosporium*. Both commonly used strains of the latter species, BKM-1767 and ME446, produce large quantities of MnPs and LiPs under liquid culture conditions; although very low levels of laccase activity have been reported from *P. chrysosporium* ME446 grown in slurries of pulp (16), this enzyme is not observed in liquid culture (11). *P. laevis*, in contrast, produced high levels of only MnP. The observed laccase activity was very low, and LiP activity, as determined by veratryl alcohol oxidation, was absent. Further, no LiP protein was detectable in Western blots (immunoblots) of concentrated *P. laevis* culture fluid or of an extract of homogenized mycelia when these were probed with polyclonal antibodies to *P. chrysosporium* LiP (9). MnP was readily detected with antibodies raised against *P. chrysosporium* MnP (9) (data not shown). White rot fungi from a variety of genera other than *Phanerochaete* produce MnP and laccase in the absence of LiP: *Ceriporiopsis subvermispora* (31, 33), *Phlebia brevispora* (31), *Stereum hirsutum* (25), *Panus tigrinus* (21), *Rigidoporus lignosus* (12), and *Ganoderma valesiacum* (25) have all been reported to exhibit this pattern. The laccase levels produced by *P. laevis* under the conditions studied here, however, were quite low. For example, *C. subvermispora* cultured in the same medium yielded 50- to 100-fold-higher levels (33). The enzymology of *P. laevis* is therefore perhaps closer to that of another recently studied species, *P. sordida*. This species produces only MnP, with no detectable LiP or laccase, under standard liquid culture conditions (32), although very low levels of laccase have been detected in cultures grown under certain solid-state conditions (31a).

MnP levels in *P. laevis*, like those in *P. chrysosporium* (5), *P. sordida* (32), *Phlebia radiata* (5), *Phlebia subserialis* (5), *Lentinus edodes* (5, 7), and *Dichomitus squalens* (30), are strongly influenced by the concentration of Mn(II) in the culture medium. This is most probably due to regulation at the level of mRNA transcription, a phenomenon which has been demonstrated with the *mnp* genes of *P. chrysosporium* (6).

Recent studies with *C. subvermispora* indicated that significant transformation and mineralization of lignins could occur in the absence of detectable LiP activity (34), i.e., that MnP and laccase activities are sufficient to initiate lignin degradation. The work described in this paper demonstrates the ability of *P. laevis*, a species whose ligninolytic system is dominated by MnP, to cause extensive transformation of PAHs in vivo. MnP produces Mn^{3+}, which is reportedly capable of oxidizing PAHs with ionization potentials at or below 7.7 eV (8). In addition, lipid peroxidation driven by MnP from *P. chrysosporium* catalyzes the ring fission of phenanthrene in vitro (22) and supports the oxidation of a wide range of PAHs (4). It therefore seemed likely that these two mechanisms would be pivotal in the degradation of PAHs by this organism. Indeed, the results of in vitro work with MnP/Mn^{2+} (Table 2) or MnP-based lipid peroxidation systems (Fig. 4) show that the action of *P. laevis* extracellular enzymes is sufficient to account for the initial

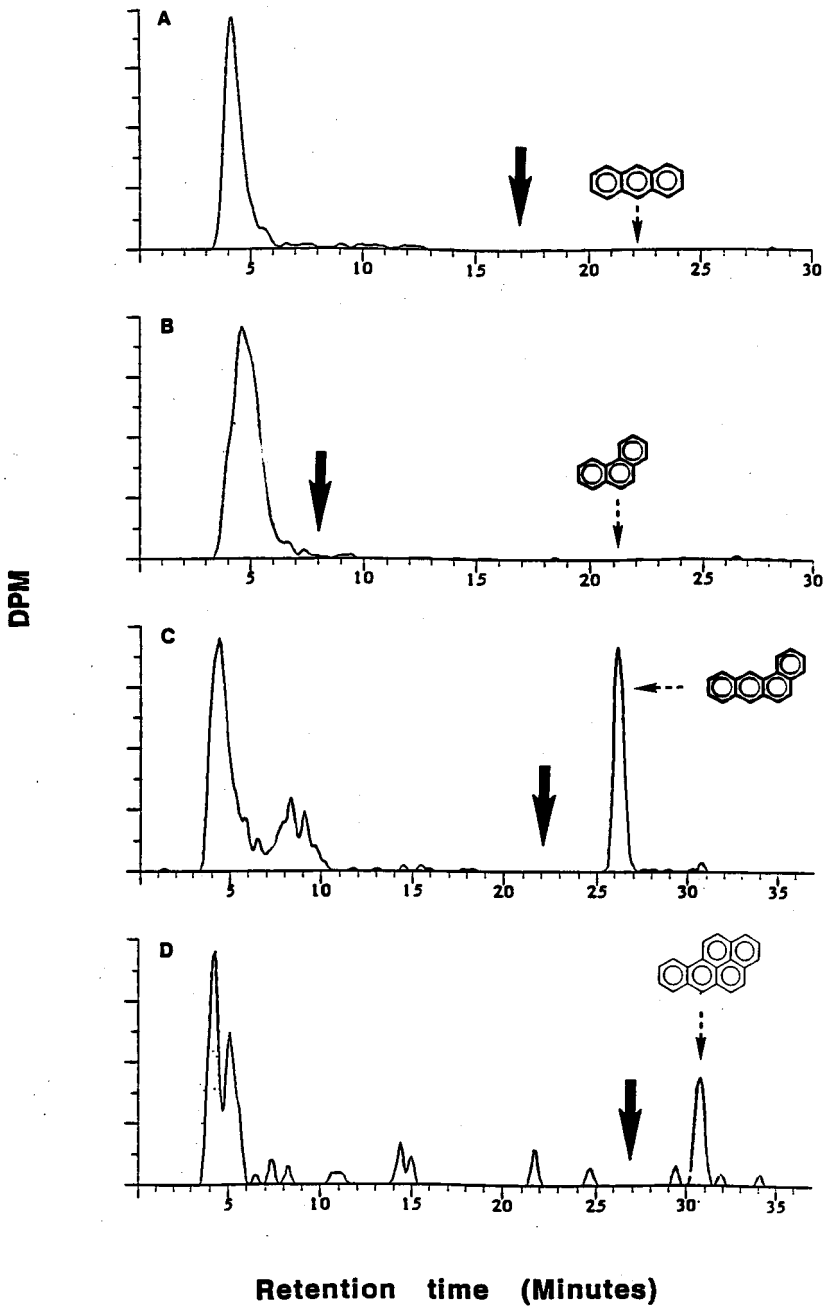

FIG. 3. Reverse-phase HPLC chromatograms of organic-soluble metabolites of PAHs from *P. laevis* liquid cultures. [^{14}C]anthracene (A), [^{14}C]phenanthrene (B), [^{14}C]benz[*a*]anthracene (C), and [^{14}C]benzo[*a*]pyrene (D) were added (initial concentration, 1 to 1.5 μM) to cultures, and metabolites were extracted after 28 days. Retention times of parent PAHs are shown, as are approximate retention times for quinone metabolites (bold arrows).

oxidation of all four PAHs in this study and for the ring cleavage of all but anthracene.

It is of interest that the observed products of PAH degradation in vivo in *P. laevis* differ from those seen with *P. chrysosporium*. In the latter species, transformation of PAHs is frequently characterized by accumulation of quinonoid intermediates. Quinones have been identified in various studies as major products in the degradation of anthracene (10, 14), pyrene (15), benz[*a*]anthracene (3a), and benzo[*a*]pyrene (13) in liquid culture. These products most probably arise via one-electron oxidative pathways. LiP catalyzes two successive elec-

TABLE 2. Formation of quinone products from PAHs in MnP/Mn^{2+} reactions (see text for conditions)

PAH[a]	% ^{14}C recovered as:	
	Parent PAH	Quinones
Anthracene	94	6
Benzo[*a*]pyrene	91	9
Benz[*a*]anthracene	100	0
Phenanthrene	ND[b]	ND

[a] The initial PAH concentration was 4 μM.
[b] ND, not determined.

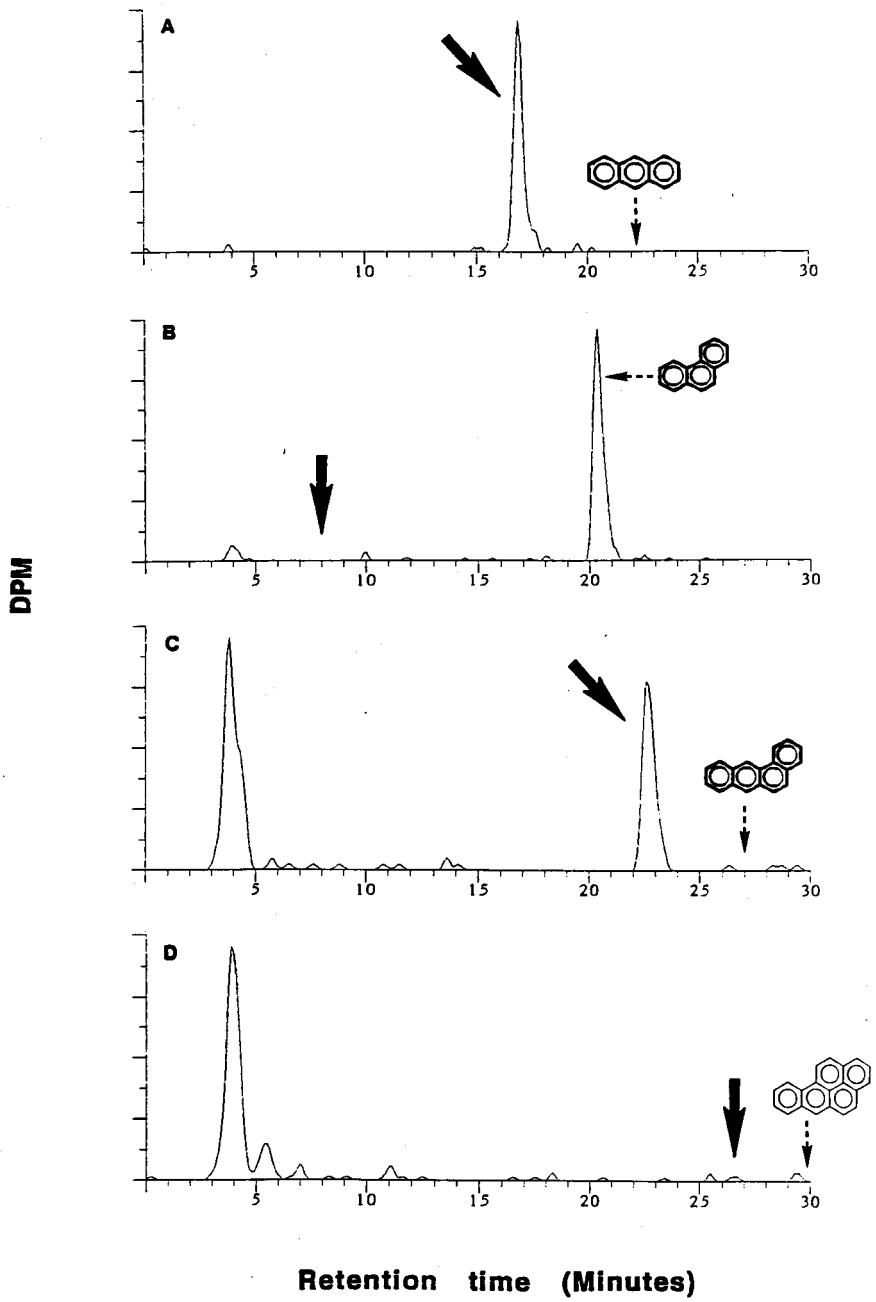

FIG. 4. Transformation of PAHs during lipid peroxidation reactions with crude *P. laevis* MnP. As in Fig. 3, retention times of parent PAHs and quinones (bold arrows) are shown. Traces are labeled as in Fig. 3.

tron abstractions from PAHs with ionization potentials below 7.55 eV (15), leading to quinone formation (13, 15). MnP-dependent lipid peroxidation (22) oxidizes PAHs above this threshold, apparently by similar mechanisms (4). In some cases, quinones are genuine degradative intermediates in *P. chrysosporium*. For example, although benzo[*a*]pyrene cometabolism by *P. chrysosporium* is known to proceed via quinone intermediates (13), such products apparently do not build up to any appreciable level in liquid culture (10). Anthraquinone is cleaved to phthalic acid by ligninolytic cultures of *P. chrysosporium* and is mineralized at the same rate as anthracene (14). However, substantial accumulations of anthraquinone are observed after 2 or 4 weeks during liquid culture degradation of anthracene by *P. chrysosporium* (10), indicating that degradation of anthraquinone may be a rate-limiting step in this species. Similar accumulations of benz[*a*]anthracene-7,12-dione are observed during *P. chrysosporium* degradation of benz[*a*]anthracene in liquid culture (3a). *P. laevis*, in contrast, caused extensive degradation of all four PAHs in the present study (anthracene, phenanthrene, benz[*a*]anthracene, and benzo[*a*]pyrene) in 4 weeks, with no significant accumulation of quinone intermediates (Fig. 3). Indeed, the data in Fig. 5 demonstrate that benz[*a*]anthracene-7,12-dione, although it was produced, was never present at levels above approximately

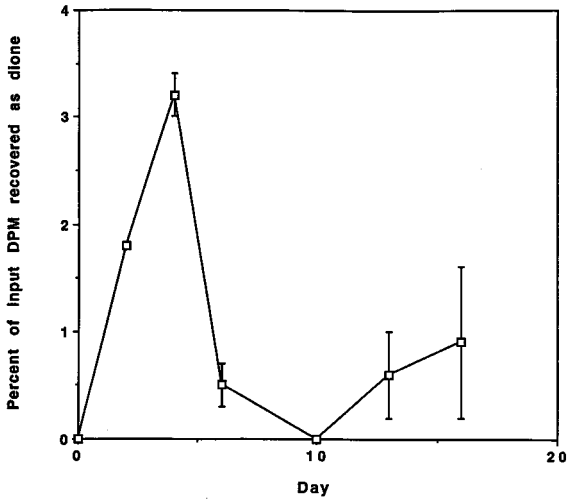

FIG. 5. Levels of benz[*a*]anthracene-7,12-dione in liquid cultures of *P. laevis* spiked with benz[*a*]anthracene (1.5 μM).

3% of input ^{14}C, indicating that it is apparently metabolized very rapidly in this fungus. This conclusion is supported by the fact that [^{14}C]benz[*a*]anthracene-7,12-dione is mineralized much more extensively and rapidly in cultures of *P. laevis* than of *P. chrysosporium* (Fig. 6). This is most probably also true for anthraquinone, which, although it is apparently a dead-end product of the lipid peroxidation system (Fig. 4), does not accumulate in vivo. There is a marked contrast between the results of this study and those of Field et al. (10), particularly with regard to anthracene transformation. These authors observe significant accumulation of quinones during the metabolism of at least some PAHs (e.g., anthracene) by *P. chrysosporium*. Clearly, therefore, it will not be possible to broadly define the xenobiotic-degrading capabilities of white rot fungi at the genus level on the basis of studies of individual species. Rather, screening and evaluation of individual species will most probably be required for selection of the most appropriate fungus during design of bioremediation programs.

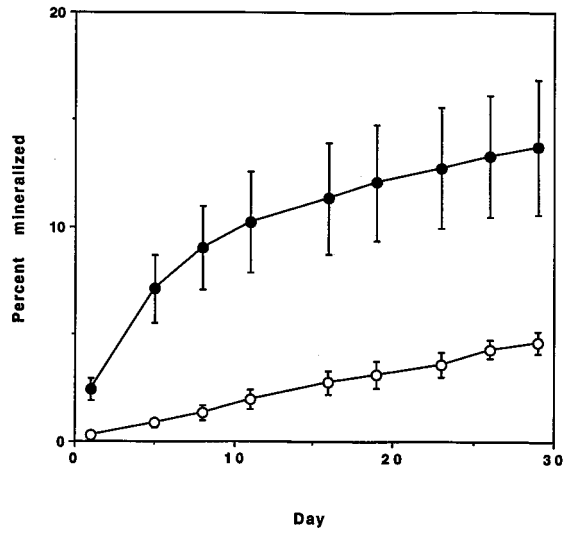

FIG. 6. Mineralization of [5,6-^{14}C]benz[*a*]anthracene-7,12-dione in N-limited liquid cultures of *P. laevis* HHB-1625 (●) and *P. chrysosporium* BKM-1767 (○).

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