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Degradation of Benzo[*a*]pyrene by the Litter-Decomposing Basidiomycete *Stropharia coronilla*: Role of Manganese Peroxidase

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The litter-decomposing basidiomycete *Stropharia coronilla*, which preferably colonizes grasslands, was found to be capable of metabolizing and mineralizing benzo[*a*]pyrene (BaP) in liquid culture. Manganese(II) ions (Mn^{2+}) supplied at a concentration of 200 μM stimulated considerably both the conversion and the mineralization of BaP; the fungus metabolized and mineralized about four and twelve times, respectively, more of the BaP in the presence of supplemental Mn^{2+} than in the basal medium. This stimulating effect could be attributed to the ligninolytic enzyme manganese peroxidase (MnP), whose activity increased after the addition of Mn^{2+}. Crude and purified MnP from *S. coronilla* oxidized BaP efficiently in a cell-free reaction mixture (in vitro), a process which was enhanced by the surfactant Tween 80. Thus, 100 mg of BaP $liter^{-1}$ was converted in an in vitro reaction solution containing 1 U of MnP ml^{-1} within 24 h. A clear indication was found that BaP-1,6-quinone was formed as a transient metabolite, which disappeared over the further course of the reaction. The treatment of a mixture of 16 different polycyclic aromatic hydrocarbons (PAHs) selected by the U.S. Environmental Protection Agency as model standards for PAH analysis (total concentration, 320 mg $liter^{-1}$) with MnP resulted in concentration decreases of 10 to 100% for the individual compounds, and again the stimulating effect of Tween 80 was observed. Probably due to their lower ionization potentials, poorly bioavailable, high-molecular-mass PAHs such as BaP, benzo(*g,h,i*)perylene, and indeno(1,2,3-*c,d*)pyrene were converted to larger extents than low-molecular-mass ones (e.g., phenanthrene and fluoranthene).

Benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) consisting of five fused benzene rings, is known to be carcinogenic and mutagenic (10, 12). Together with other PAHs, BaP is commonly formed during the pyrolysis and incomplete combustion of biological material and organic compounds and is found in various concentrations in coal tar, petroleum, and oil-based fuels (42). Thus, soils from gasworks sites and carbochemical plants as well as refineries and filling stations are often contaminated with BaP. This causes an obvious health risk that has raised the public interest in the fate and the removal of BaP and similar compounds in and from the environment.

PAHs with more than four rings are considered highly recalcitrant and resistant to microbial degradation (10). In addition, the higher their molecular weight is, the lower is their water solubility and thus their bioavailability (54). While lower-molecular-mass PAHs—e.g., naphthalene, anthracene, and phenanthrene—are readily degraded by a number of aerobic bacteria, which can utilize these compounds as carbon sources (10, 12, 33), BaP and other PAHs with high molecular masses are cometabolically degraded by only a few bacterial species (*Mycobacterium* spp. and *Sphingomonas* spp.) and mixed microbial cultures (33).

In addition to certain bacteria, wood-colonizing basidiomycetes such as *Phanerochaete chrysosporium* and *Bjerkandera* sp. strain BOS55 have been shown to metabolize BaP in liquid culture and soil (9, 24, 36). These white-rot fungi produce extracellular ligninolytic oxidoreductases, namely, manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase, which attack aromatic substances, including PAHs, via the formation of free radicals (25, 26, 35). Since these fungi specialize in colonizing compact wood (timber and stumps) and cannot compete in soil for a prolonged time, their actual contribution to the removal of recalcitrant PAHs under natural conditions seems to be limited. There is, however, a second ecophysiological group of ligninolytic basidiomycetes—the litter-decomposing fungi—which have recently been shown to possess a ligninolytic enzyme system similar to that of white-rot fungi (48, 49). Furthermore, screening tests have demonstrated that litter-decomposing fungi are capable of metabolizing PAHs, including BaP, to some extent (21, 22, 47, 56).

In the present study, we report the degradation of BaP by the litter-decomposing fungus *Stropharia coronilla*, which preferably inhabits grasslands. The species is a typical grass dweller that colonizes pastures, meadows, and waysides and is found in both Europe and North America (8, 40). Particular attention is paid to the role of MnP, the predominant ligninolytic enzyme of this fungus, and the results indicate its crucial role in the degradation of PAH.

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MATERIALS AND METHODS

Organism, culture conditions, and enzyme preparation. The litter-decomposing fungus *S. coronilla* (Bull.: Fr.) Quél. strain TM 47-1 was obtained from the culture collection of the Institute of Microbiology, University of Jena (Jena,

Germany). It was maintained on malt extract agar (MEA) in culture slants and stored at 4°C in the dark.

The fungus was routinely precultured on 2% MEA agar plates for 2 weeks, and agar plugs (10 mm in diameter) were used to inoculate liquid cultures. The basal liquid medium contained 10 g of glucose liter^{-1}, 2 g of KH_2PO_4 liter^{-1}, 0.5 g of $MgSO_4 \cdot 7\ H_2O$ liter^{-1}, 0.1 g of $CaCl_2$ liter^{-1}, 0.5 g of ammonium tartrate liter^{-1}, 2.2 g of 2,2-dimethylsuccinate liter^{-1}, and 0.2 g of yeast extract liter^{-1} (48). The pH was adjusted to 5.0 with HCl prior to autoclaving. Certain liquid cultures were supplemented with 0.04 g of $MnCl_2 \cdot 4\ H_2O$ liter^{-1} (200 μM) to stimulate the production of MnP (48).

The medium used for the production of larger amounts of MnP contained 2.7 g of sodium succinate liter^{-1} instead of 2,2-dimethylsuccinate and Mn^{2+} as mentioned above. Cultivation was performed in 1-liter tissue culture flasks containing 200 ml of the medium, which was inoculated with 10 agar plugs. After 4 weeks of growth at 25°C in the dark, the culture liquid was harvested, filtered, concentrated, and dialyzed as described previously (49). The concentrated and dialyzed culture liquid is referred to as the crude enzyme. MnP 1 was purified by anion-exchange chromatography on a Mono Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) and characterized electrophoretically as described previously (46). The enzyme has a molecular mass of 41 kDa and a pI of 6.7 (49).

Enzyme assay. The activity of MnP was measured at 270 nm by following the formation of Mn^{3+}-malonate complexes (52). Laccase activity was determined by the oxidation of ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] and monitored at 420 nm (16). Enzyme activities were expressed in units, i.e., micromoles of product formed per minute.

Chemicals. BaP with a minimum purity of 98.0%, as determined by high-pressure liquid chromatography (HPLC) was obtained from Sigma, St. Louis, Mo. [7,10-^{14}C]BaP (50 mCi mmol^{-1}) with a radioactive purity of 97.1% was obtained from Amersham Buchler, Braunschweig, Germany. A quick-turn-around-method PAH mix containing 16 different PAHs regulated by the U.S. Environmental Protection Agency (concentration of each, 2,000 μg ml^{-1}) was purchased from Supelco, Bellefonte, Pa. All solvents were of HPLC grade, and all other chemicals were of the highest purity available and purchased from Merck, Darmstadt, Germany, or Fluka, Steinheim, Germany.

Conversion of BaP in liquid cultures of *S. coronilla*. Erlenmeyer flasks (50 ml) containing 15 ml of the basal or the Mn^{2+}-supplemented medium were inoculated with three agar plugs (10 mm in diameter) from *S. coronilla* MEA plates. Three days after inoculation, 100 μl of a 15% Tween 80 solution and 75 μg of BaP dissolved in *N,N*-dimethylformamide were added to give a final concentration of 50 mg liter^{-1} in each culture flask. Flask cultures were maintained as surface cultures at 23°C in the dark. Flasks (three per time point) were collected 7, 11, 14, 18, 21, and 28 days after the addition of BaP. After gentle shaking, 250-μl samples were taken from each flask to measure the enzyme activities as well as the concentration of oxalate and tartrate. To dissolve residual BaP and its conversion products, 25 ml of ethanol (98%) was added to the culture liquid in each flask (aqueous ethanolic solution). The flasks were sonicated in a sonication bath for 5 min and subsequently shaken at 150 rpm for 15 min. After filtration (Whatman no. 1 paper), aliquots of 1.2 ml were transferred into Eppendorf tubes and centrifuged for 5 min at 15,700 × *g*. The supernatant was transferred into HPLC vials and used for HPLC analysis. Control flasks without fungal inoculum or with autoclaved mycelium were treated in the same way.

HPLC. Organic acids (oxalate and tartrate) in the culture liquid were analyzed by using a model HP 1090 liquid chromatograph (Hewlett Packard, Waldbronn, Germany) equipped with an Aqua-C_{18} column (4.6 by 250 mm; Phenomenex, Torrance, Calif.). Phosphoric acid (10 mM) served as the solvent at a flow rate of 0.75 ml min^{-1} under isocratic conditions (detection wavelength, 210 nm).

Elution profiles of aqueous ethanolic BaP extracts from the liquid cultures were recorded by HPLC with a Merck LiChrospher 5-μm RP-18 reversed-phase column (4.6 by 125 mm) and the HPLC system mentioned above. Separations were run under isocratic conditions with a solvent consisting of acetonitrile and water (80/20, vol/vol) at a flow rate of 1 ml min^{-1}. Eluted substances were detected at a wavelength of 254 nm. BaP was identified by comparing its UV-visible spectrum and retention time with those of the authentic chemical.

HPLC elution profiles of the MnP-treated 16 PAHs selected by the U.S. Environmental Protection Agency as model standards for PAH analysis were obtained with a Merck Superspher 100 RP-18 (5-μm) reversed-phase column (4.0 by 250 mm) and the HPLC system mentioned above. Separations were run at a constant temperature of 40°C with a stepwise gradient of 85 to 100% methanol (0 and 7 min, 85%; 12 min, 90%; 20 min, 95%; 22 and 25 min, 100%; 27 min, 85%) in water.

Mineralization of [^{14}C]BaP in liquid culture. Mineralization studies using [7,10-^{14}C]BaP were carried out in 150-ml flasks containing 15 ml of the media mentioned above (i.e., with and without Mn^{2+} supplementation). [^{14}C]BaP and unlabeled BaP were added to each flask to give a final concentration of 30 mg liter^{-1} and a total activity of 484,000 dpm. Inoculated flasks (three in parallel) and respective controls without the fungus were sealed with rubber septa and aluminum caps. Incubation occurred at 23°C in the dark. ^{14}C-labeled volatile compounds and $^{14}CO_2$ were flushed out once a week with pure oxygen for 15 min and trapped by bubbling any gas released through two sequential flasks containing Opti-Fluor and Carbosorb/Opti-Fluor (Packard Instruments, Groningen, The Netherlands). Six weeks after inoculation, all culture flasks were sonicated (5 min) and shaken (15 min). Aliquots of 200 μl were taken from the flasks and centrifuged at 20,800 × *g* for 5 min, and 100-μl samples were used to determine the water-soluble radioactivity. Residual [^{14}C]BaP was dissolved by the addition of 25 ml of ethanol to each flask, followed by the extraction procedure described above. After filtration and centrifugation, aliquots of 100 μl were used to determine the radioactivity of the aqueous ethanolic extracts. The used filters including the fungal biomass were burned in a combustion chamber (Junitek, Turku, Finland) to determine the amount of bound ^{14}C as evolving $^{14}CO_2$.

The distribution of radioactivity in the ethanol extracts was analyzed by HPLC using the Merck RP-18 column and the conditions described above. Every 30 s, fractions (375 μl) were collected in scintillation vials for the subsequent determination of dissolved radioactivity.

A liquid scintillation counter model 1411 (Wallac, Turku, Finland) was used for all radioactivity measurements.

Cell-free experiments with MnP. *S. coronilla* MnP 1 was used in a cell-free experiment (in vitro) to convert BaP in a buffered reaction solution at 37°C in the dark. The reaction solution (200 ml in a 1-liter flask) consisted of sodium malonate (50 mM, pH 4.0), $MnCl_2$ (2 mM), Tween 80 (1%), glucose (10 mM), BaP (0.397 mM [100 mg liter^{-1}]) as well as purified MnP 1 (1 U ml^{-1}). The reaction was started by the addition of glucose oxidase (0.1 U ml^{-1}), and the mixture was stirred continuously at 475 rpm for 24 h. The first sample was taken just before the addition of glucose oxidase; afterwards, samples (320 μl) were taken after 0.5, 1, 2, 4, 6, 21, and 23 h of incubation. Samples were divided into three parts: 100 μl was used to determine the concentration of Mn^{3+} by the immediate oxidation of ABTS (29), 20 μl served for the monitoring of MnP activity, and 200 μl was mixed with 670 μl of ethanol for HPLC analysis.

Airtight Teflon-sealed reaction tubes (20 ml) were used for the in vitro conversion of [^{14}C]BaP. The reaction mixture (1 ml) was identical to that described above, except that a smaller amount of BaP (20 mg liter^{-1}) was applied; the final radioactivity was 43,000 dpm per reaction tube. In addition, Tween 20 was used in a separate experiment instead of Tween 80. After glucose oxidase (0.1 U ml^{-1}) was added, the reaction was performed under an oxygen atmosphere and continuous stirring at 37°C in the dark for 48 h. Controls did not contain MnP. The tubes were flushed after 17 and 41 h for 15 min with pure oxygen. Any gas released was trapped and analyzed for radioactivity as mentioned above. After 48 h, samples (50 μl) were taken to analyze the water-soluble compounds and radioactivity. Afterwards, the residual radioactivity (nonconverted [^{14}C]BaP and possible nonpolar conversion products) was dissolved by adding 1.65 ml of ethanol to the reaction mixture. After shaking and sonication, the solution was centrifuged and injected into the HPLC system, and the eluted radioactivity was analyzed by liquid scintillation counting.

Crude enzyme and MnP 1 from *S. coronilla* were used to convert a mixture of different PAHs in vitro. The reaction mixture and conditions were the same as in the radioactive in vitro experiment, except that BaP was replaced by a mixture of 16 different unlabeled PAHs (see Table 2). Their concentration was 20 μg ml^{-1} each, giving a total PAH amount of 320 μg ml^{-1}. After 48 h, PAHs were dissolved in ethanol and analyzed by HPLC.

RESULTS

Conversion of BaP by whole fungal cultures. *S. coronilla* converted 80% of the BaP in Mn^{2+}-supplemented cultures within 28 days, while only 20% disappeared in the nonsupplemented cultures (Fig. 1). In the presence of Mn^{2+}, the BaP concentration dropped rapidly in particular during the first week, whereas the slow decrease in the absence of additional Mn^{2+} was nearly linear throughout the test. The BaP amount was not diminished in controls without fungal inoculum, and only a negligible portion of the BaP (<1%) adsorbed to the autoclaved mycelium. UV-visible spectroscopic data indicated the transient formation of traces of BaP-1,6-quinone in Mn^{2+}-supplemented cultures (data not shown).

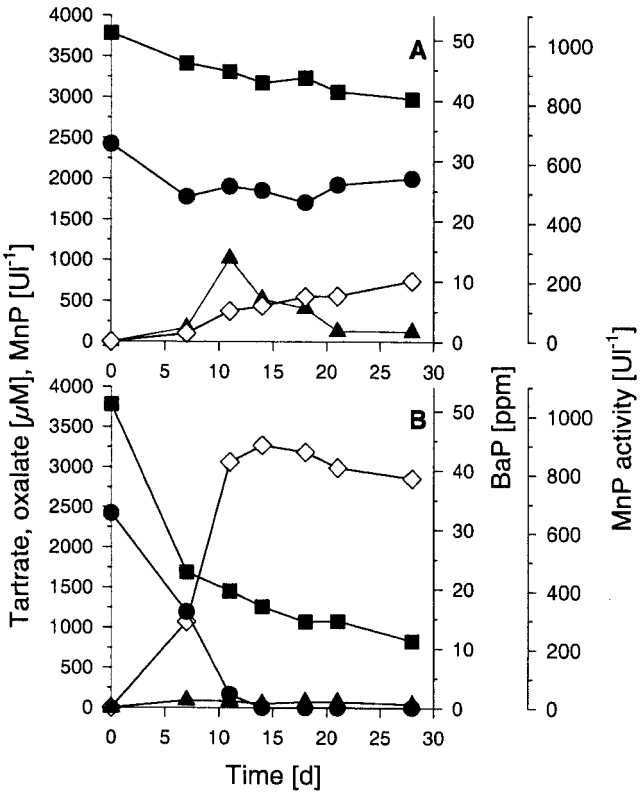

FIG. 1. Conversion of BaP (50 mg liter^{-1}, closed squares), production of MnP (open diamonds), and fate of tartrate (closed circles) and oxalate (closed triangles) in *S. coronilla* cultures supplemented with 200 μM Mn^{2+} (B) and nonsupplemented cultures (A). Data points are means for three parallel determinations; standard deviation was less than 5%.

MnP activity in Mn^{2+}-supplemented cultures, in which a constant level of 800 U liter^{-1} had been reached after 10 days, was about eight times as high as the maximum activity (100 U liter^{-1}) in nonsupplemented ones (Fig. 1). Throughout the experiment, laccase activities were relatively low and did not exceed 40 U liter^{-1} in both culture media (data not shown). Tartrate—added as the ammonium salt to supply nitrogen but also acting as an Mn chelator—readily disappeared in Mn^{2+}-containing cultures. Only negligible amounts of oxalate (< 10 μM), probably produced by the fungus or from tartrate, were detectable. In contrast, the tartrate concentration decreased by only 20% in nonsupplemented cultures and oxalate reached a temporary level of 1.1 mM (Fig. 1A).

The presence of additional Mn^{2+} caused a slight increase (about 10%) in the biomass (4.78 and 4.30 g [dry weight] liter^{-1}, respectively), but this did not strongly affected the mass balances.

Mineralization of [^{14}C] BaP. *S. coronilla* mineralized approximately 12% of the added [^{14}C]BaP in Mn^{2+}-supplemented cultures within 6 weeks, whereas only 1% was evolved as $^{14}CO_2$ in nonsupplemented ones (Fig. 2). About 40% of the label was detectable in the aqueous fraction and less than 4% was detectable in the ethanolic one when additional Mn^{2+} was added, indicating a substantial removal of BaP (Table 1). This finding was confirmed by the HPLC analyses, where BaP was not found in the elution profiles (Fig. 3C and F). Water-soluble radioactivity (25%) detected in the nonsupplemented cultures was less than that in the controls (30%), which may be explained by the presence of the nonionic surfactant Tween 80, preventing the complete separation of BaP from the aqueous phase by centrifugation. However, the actual BaP concentration in the aqueous ethanolic extracts of the controls was three times as high as that of nonsupplemented ones (Fig. 3A and B). This finding shows that BaP was also converted in the absence of additional Mn^{2+} (Table 1); the respective HPLC elution profiles support this assumption (Fig. 3A, B, D, and E). Furthermore, the distribution of soluble radioactivity demonstrates that, especially in the presence of Mn^{2+}, a substantial part of the [^{14}C]BaP (40%) was converted into polar products, which eluted within the first 2 min of the HPLC separation (Fig. 3D through F). Residual radioactivity associated with either the fungal mycelium or the filter material amounted to 40, 48, and 8% for Mn^{2+}-supplemented and nonsupplemented cultures as well as controls, respectively (Table 1).

Conversion of BaP by *S. coronilla* MnP. Purified MnP 1 from *S. coronilla* was able to convert a large amount of BaP (100 mg liter^{-1}) in vitro within 24 h (Fig. 4). In the beginning of the conversion process, the concentration of Mn^{3+} increased steeply, reaching a maximum level of 170 μM after 6 h. Afterwards, the concentration dropped to 30 μM at the end of the experiment. In the course of BaP conversion, we found clear evidence for the intermediary formation of BaP-1,6-quinone (Fig. 4, inset), which accumulated during the first 8 h but disappeared again afterwards (Fig. 4). The formation of BaP-1,6-quinone was concluded from the appearance of a novel peak in the HPLC elution profile, which showed the characteristic absorption behavior of this compound (maximum absorbance at around 450 nm) (11) (Fig. 4, inset). *S. coronilla* MnP proved to be a stable enzyme which lost about 40% of its activity during the first 4 h but recovered over the course of the reaction (to 85% of the initial activity after 24 h of incubation).

The effect of Tween 20 and Tween 80 on the MnP-catalyzed conversion was studied by using [^{14}C]BaP. Though *S. coronilla*

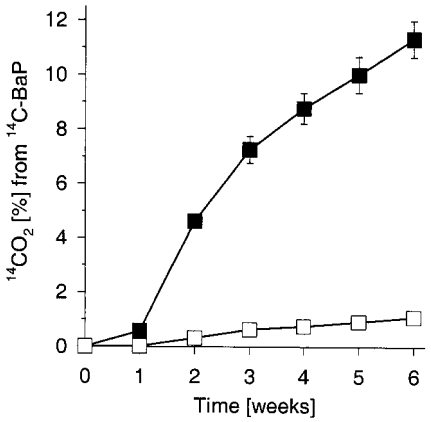

FIG. 2. Mineralization of [7,10-^{14}C]BaP (484,000 dpm; total BaP concentration, 30 mg liter^{-1}) by *S. coronilla* in liquid culture supplemented with 200 μM Mn^{2+} (closed squares) and nonsupplemented cultures (open squares). Mineralization in controls without fungal inoculum was below 0.1%. Data points are means of three parallel determinations, with standard deviations.

TABLE 1. Mass balance of radioactive carbon (^{14}C) from 7,10-^{14}C-labeled BaP (approximately 484,000 dpm) added to surface liquid cultures with *S. coronilla* after 6 weeks of growth

Culture	% of total added ^{14}C[a]					
	$^{14}CO_2$	^{14}C-labeled volatile organic compounds	Water-soluble compounds	Ethanol-soluble compounds	Residual ^{14}C	Recovery
Mn^{2+} free	1.0 ± 0.2	0.1 ± 0.1	25.0 ± 0.2	19.7 ± 6.2	48.4 ± 4.3	94.1 ± 2.1
Mn^{2+} supplemented	11.5 ± 0.4	0.2 ± 0.1	38.5 ± 0.6	3.3 ± 0.6	40.4 ± 0.6	93.8 ± 1.5
Uninoculated (control)	0.1 ± 0.1	0.1 ± 0.1	29.8 ± 1.9	56.2 ± 4.3	8.1 ± 3.9	94.2 ± 1.3

[a] Values are means ± standard deviations for three replicates.

MnP 1 oxidized the labeled BaP in the presence of both surfactants, considerable differences were observed depending on the particular type of Tween (Fig. 5B, D, F, and H). While only 25% of the BaP was converted in the presence of Tween 20, it disappeared completely in the Tween 80-containing samples. A small decrease of [^{14}C]BaP was also observed in the controls without MnP (~5%) (Fig. 5E and G).

The radioactivity distribution of Tween 80 samples resembles the pattern of a Mn^{2+}-supplemented fungal culture (Fig. 3F) and shows that MnP transformed all of the radioactive BaP into polar products (Fig. 5H). In addition to these products, a small part of the label was released as $^{14}CO_2$ (1.4% ± 0.1%). Significant mineralization occurred only in the presence of Tween 80; only traces of $^{14}CO_2$ were evolved from Tween 20-containing reaction mixtures (0.13% ± 0.1%; controls, <0.1%).

Finally, we tested the concurrent removal of 16 different PAHs in a cell-free reaction mixture with crude enzyme or purified MnP 1; also in this experiment, the influence of the surfactant was taken into consideration. In the presence of Tween 20, pronounced PAH removal was observed only for anthracene and BaP, whereas Tween 80 stimulated the conversion of all PAHs—except naphthalene, which volatilized completely—to some extent (reduction of 10 to 100%) (Table 2). Remarkably, some persistent five- and six-ring PAHs, e.g., benzo[*g,h,i*]perylene, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]-anthracene, were among the almost completely converted compounds (Table 2). The crude MnP was more efficient than the purified MnP 1, which could be attributed either to additional activities in the crude enzyme (other MnPs or laccase) or to its higher stability.

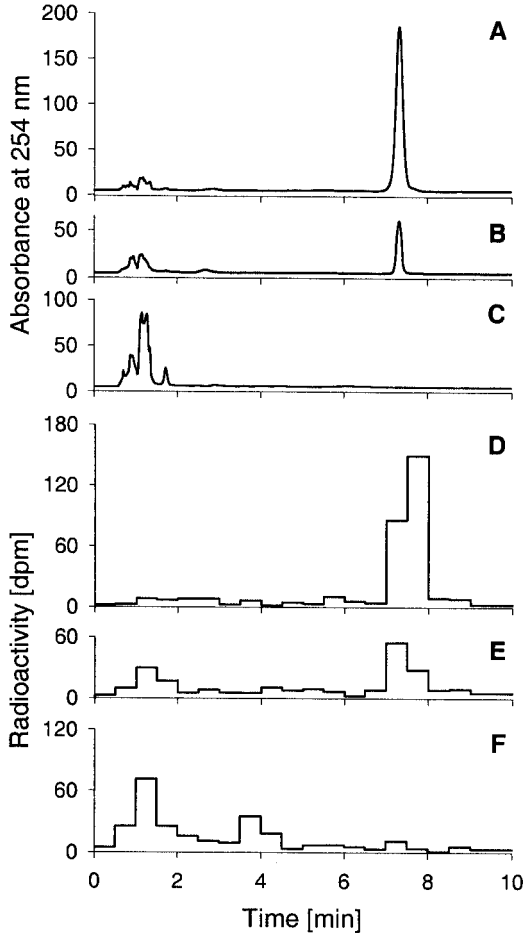

FIG. 3. Reversed-phase C_{18} HPLC elution profiles (A to C) and distribution of radioactivity in the eluted fractions (D to F) of aqueous ethanolic extracts from liquid cultures of *S. coronilla* amended with 30 mg of BaP liter^{-1} and [7,10-^{14}C]BaP (484,000 dpm) after 6 weeks of incubation. (A and D) control without fungus; (B and E) nonsupplemented fungal culture (C and F); fungal culture supplemented with 200 μM Mn^{2+} ($MnCl_2$).

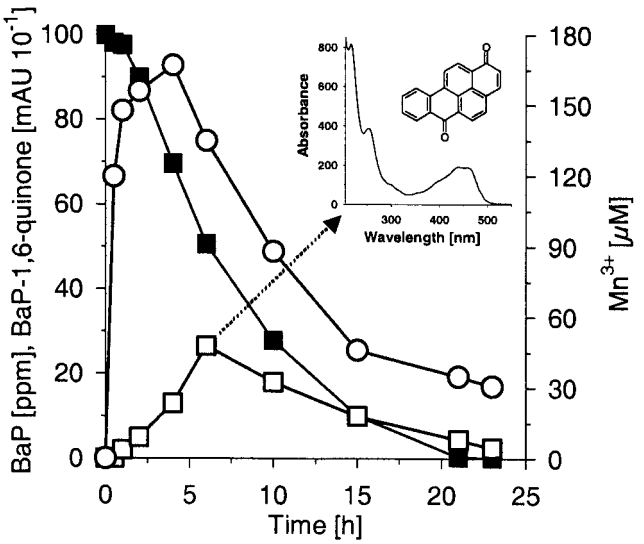

FIG. 4. Conversion of BaP (closed squares, 100 mg liter^{-1}) and formation of Mn^{3+} ions (circles) and BaP-1,6-quinone (open squares; the inset shows its UV spectrum) in a sodium malonate-buffered, cell-free reaction mixture (total volume, 200 ml) containing 1 U of purified MnP 1 from *S. coronilla* ml^{-1}. The enzymatic reaction was performed with continuous stirring at 37°C in the dark.

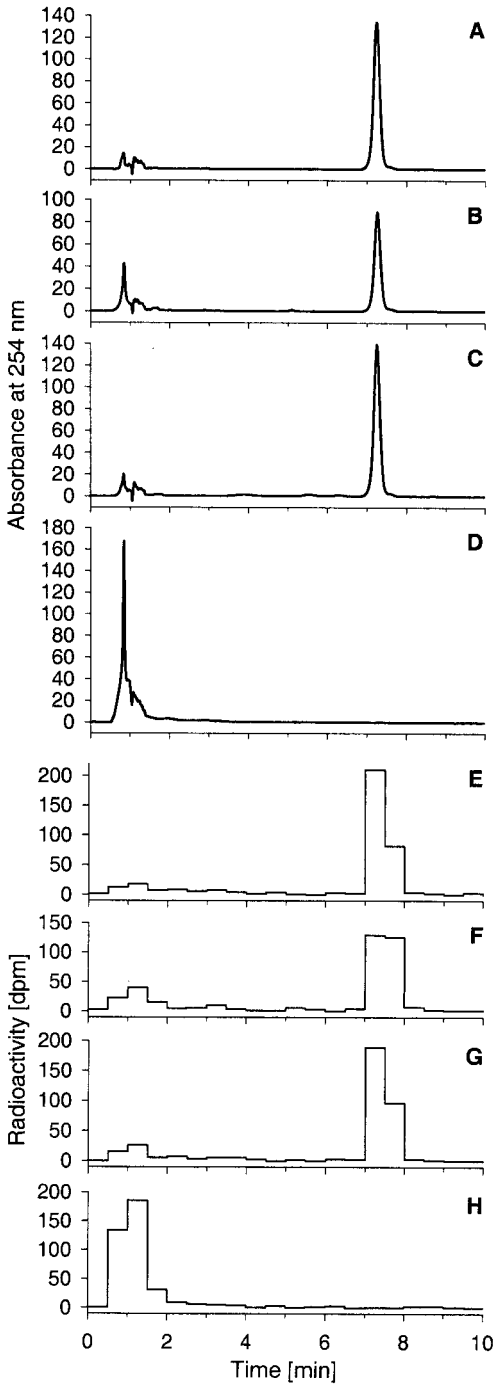

FIG. 5. Reversed-phase C_{18} HPLC elution profiles (A to D) and distribution of radioactivity in the eluted fractions (E to H) of ethanolic extracts from cell-free reaction mixtures containing 20 μg of BaP ml^{-1}, 43,000 dpm of [7,10-^{14}C]BaP, and 1 U of purified MnP 1 from *S. coronilla* ml^{-1} (reaction time, 48 h). (A and E) control plus Tween 20; (B and F) MnP plus Tween 20; (C and G) control containing Tween 80; (D and H) MnP plus Tween 80.

DISCUSSION

S. coronilla was found to be capable of degrading BaP in liquid culture. Mn^{2+} stimulated both the conversion and mineralization considerably. This effect can be attributed to MnP, whose activity increased in the presence of supplemental Mn^{2+}. Isolated *S. coronilla* MnP oxidized BaP and other PAHs in vitro, a process which was enhanced by the surfactant Tween 80.

Fungal metabolism of BaP has been shown for several molds (deuteromycetes and zygomycetes), among others *Aspergillus ochraceae* (14), *Cunninghamella elegans* (11), and a *Penicillium* sp. (32), which hydroxylate BaP via cytochrome P-450-dependent mono-oxygenases (1). The same enzymatic mechanism was demonstrated to be involved in the oxidation of pyrene and other PAHs by the white-rot basidiomycete *Pleurotus ostreatus* (4). Though, we did not find evidence (by HPLC) for the formation of monohydroxylated BaP derivatives in our in vivo studies, the involvement of intracellular reactions in the BaP metabolism cannot be ruled out. In particular, similar reactions could contribute to the BaP conversion in non-Mn^{2+}-supplemented cultures and explain its moderate decrease in the absence of additional Mn^{2+}. In Mn^{2+}-supplemented cultures, intra- and extracellular processes probably interlock and enable an efficient BaP degradation. This has already been proposed in earlier PAH degradation studies with different wood rot fungi (33, 44).

Various publications have shown the capability of wood-colonizing white-rot fungi to degrade different PAHs (33). First studies with BaP were carried out using *P. chrysosporium* and demonstrated its oxidation into quinones and the partial mineralization of [^{14}C]BaP (9, 18, 45). More recently, other fungi of this ecophysiological group, such as *Bjerkandera* sp. strain BOS55 (18, 36) and *P. ostreatus* (55, 57), were found to degrade BaP. A substantial conversion of BaP was even observed in soils which were spiked with BaP and amended with straw and white-rot fungi, e.g., *P. ostreatus* and *P. chrysosporium* (2, 15, 37, 39).

In all cases, the degradation of BaP has been attributed to the activity of ligninolytic enzymes (41). As the first of these enzymes, purified LiP from *P. chrysosporium* was shown to attack BaP via one electron abstractions leading to unstable BaP radicals that undergo further spontaneous reactions to hydroxylated metabolites and several BaP quinones (23, 50).

Because LiP is not produced by all white-rot fungi, more recent studies have focused on MnP, which is widespread among the basidiomycetes and found not only in wood decay fungi but also in litter-decomposing fungi (25, 27). The production of MnP can be selectively stimulated by Mn^{2+}, which is the actual substrate of the enzyme but also acts as an inducer on the genetic level (20, 52). This effect has been used to evaluate the role of MnP in the degradation of recalcitrant compounds. As in our experiments, a noticeable increase in the MnP activity and the concurrent stimulation of degradation was observed after Mn^{2+} supplementation in several studies using different fungal species and different substrates. Thus, lignin degradation in straw cultures of *P. ostreatus* was enhanced after addition of Mn^{2+} and the mineralization of humic acids in liquid cultures of the litter decomposer *Collybia dryophila* as well (34, 46); the manganese-enhanced biotransformation of the herbicide atrazine as well as of several PAHs has been reported for the white-rot fungi *P. chrysosporium*, *Pleurotus pulmonarius*, and *Trametes versicolor* (13, 38, 58).

In our study, Mn^{2+} stimulated not only the conversion of BaP but also the degradation of tartrate. The rapid disappear-

TABLE 2. Comparison of crude enzyme and purified MnP 1 from *S. coronilla* liquid cultures for their average conversion of 16 PAHs in cell-free reaction mixtures containing either the surfactant Tween 20 or Tween 80

PAH[b]	Remaining PAH concn (mg liter^{-1}) in reaction mixtures with[a]:					
	Tween 20			Tween 80		
	Control (no enzyme)	Crude enzyme	MnP 1	Control (no enzyme)	Crude enzyme	MnP 1
Naphthalene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Acenaphthylene	5.3	12.9 ± 3.7	<0.1	5.8	<0.1	<0.1
2-Bromonaphthalene	5.6	7.4 ± 0.2	9.2 ± 0.1	5.2	<0.1	<0.1
Acenaphthene	12.1	8.0 ± 0.2	9.3 ± 0.1	11.6	<0.1	<0.1
Fluorene	12.0	8.1 ± 0.3	9.4 ± 0.1	11.5	<0.1	<0.1
Phenanthrene	22.9	20.5 ± 1.1	21.6 ± 0.5	19.6	12.6 ± 1.0	18.3 ± 0.2
Anthracene	22.9	0.7 ± 0.2	1.7 ± 0.3	19.5	<0.1	<0.1
Fluoranthene	25.5	22.8 ± 1.5	24.4 ± 0.7	21.4	11.1 ± 1.0	17.4 ± 0.4
Pyrene	25.6	22.4 ± 1.5	24.3 ± 0.7	21.4	<0.1	2.2 ± 0.1
Chrysene	25.7	22.0 ± 1.7	24.1 ± 0.3	21.4	8.2 ± 0.8	13.3 ± 0.2
Benzo[*a*]anthracene	25.6	22.1 ± 1.6	24.0 ± 0.4	21.4	8.3 ± 0.9	13.4 ± 0.1
Benzo[*b*]fluoranthene	25.7	23.4 ± 1.5	25.0 ± 0.6	21.5	7.8 ± 0.9	13.4 ± 0.5
Benzo[*a*]pyrene	25.7	5.2 ± 0.5	7.7 ± 0.8	21.4	<0.1	<0.1
Dibenzo[*a*,*h*]anthracene	25.6	22.7 ± 1.9	24.4 ± 0.2	21.4	<0.1	2.7 ± 0.1
Indeno[1,2,3-*c*,*d*]pyrene	26.0	22.7 ± 1.4	24.4 ± 0.6	21.6	0.8 ± 0.2	2.1 ± 0.2
Benzo[*g*,*h*,*i*]perylene	25.7	23.0 ± 1.7	25.0 ± 0.2	21.6	<0.1	1.7 ± 0.1

[a] Values are means ± standard deviations for three replicates.
[b] Sixteen PAHs selected by the U.S. Environmental Protection Agency as model standards for PAH analysis.

ance of this chelating acid and the low level of oxalate formed in Mn^{2+}-supplemented cultures of *S. coronilla* can be explained by the MnP-catalyzed formation of high titers of Mn^{3+} complexes, which reacted with each other, leading to their own destruction. The resulting chelator deficiency could be responsible for the slowing down of the BaP conversion. A similar phenomenon has been observed for different organic acids during the in vitro conversion of milled pinewood by MnP from *Phlebia radiata* (28) and has been proposed as a source of H_2O_2 in cultures of *Ceriporiopsis subvermispora* (51).

Crude MnP from the agaric white-rot fungus *Nematoloma frowardii* oxidized several [^{14}C]PAHs, including pyrene and BaP, in vitro, leading to the formation of significant amounts of $^{14}CO_2$ ("enzymatic combustion") (43); mineralization increased 3- to 10-fold (up to 4% $^{14}CO_2$) when reduced glutathione (GSH) was present in the reaction solution (here we found an in vitro mineralization of 1.4%). The GSH effect, which was also observed during the MnP-catalyzed destruction and cleavage of lignin and nonphenolic lignin model compounds (19, 53), was attributed to the transient formation of particularly reactive thiyl radicals. However, it is rather unlikely that fungi secrete "valuable" substances such as GSH under natural conditions into their microhabitat. Therefore, alternative redox-mediators, enhancing the oxidative strength of the MnP system, have been sought.

Very promising compounds acting as such mediators were found among the unsaturated fatty acids (e.g., oleic and linoleic acids) and their derivatives (for example, Tween 80). These substances have been shown to act similarly to GSH (3) and were detected in liquid and solid fungal cultures (17, 30, 31). In the presence of Tween 80, MnP was able to oxidize fluorene, a PAH that cannot directly be oxidized by chelated Mn^{3+} due to its high ionization potential (8.2 eV), as well as a complex PAH mixture (creosote) (5, 7). Tween 80 enabled *S. coronilla* MnP to convert a large amount of BaP (100 mg liter^{-1}) into polar fragments, and BaP-1,6-quinone was detected as a transient metabolite, which was further broken down to unknown products. MnP activity was already previously shown to be responsible for the initial oxidation of BaP and the further degradation of formed BaP-1,6-quinone in cultures of *Phanerochaete laevis* and in an MnP-based lipid peroxidation system (6). However, a lag phase (about 20 h) prior to the onset of BaP disappearance as in the latter in vitro system was not observed during our tests due to the use of glucose and glucose oxidase for the generation of H_2O_2.

S. coronilla MnP oxidized the individual PAHs in a mixture of 16 different compounds according to their ionization potential and the presence of Tween 80. Only BaP and anthracene (ionization potential, <7.5 eV) were oxidized by the simple MnP system (i.e., with Tween 20), but the initiation of lipid peroxidation via unsaturated fatty acids components of Tween 80 resulted in the substantial decrease of all other nonvolatile PAHs, which is in agreement with earlier findings using MnPs from white-rot fungi (7). In addition, we report here for the first time that poorly bioavailable PAHs such as the six-ring compound benzo[*g*,*h*,*i*]perylene are also subject to MnP attack.

Based on our present results and previous findings, we propose that MnP is the key enzyme in the degradation of BaP and other PAHs by litter-decomposing basidiomycetes. The fact that litter-decomposing fungi are natural soil dwellers, carrying out the same type of decay as white-rot fungi in wood but in a more complex environment, makes them promising candidates for bioattenuation and bioremediation processes. White-rot fungi have already been shown to reduce the PAH content in soil, though their competitive potential was low (36). Therefore, our future studies will focus on the removal of PAHs by *S. coronilla* and other litter decomposers in soil environments.

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REFERENCES

1. **Atlas, R. M., and C. E. Cerniglia.** 1995. Bioremediation of petroleum pollutants. BioScience **45:**332–338.
2. **Baldrian, P., C. in der Wiesche, J. Gabriel, F. Nerud, and F. Zadrazil.** 2000. Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl. Environ. Microbiol. **66:**2471–2478.
3. **Bao, W., Y. Fukushima, K. A. Jensen, Jr., M. A. Moen, and K. E. Hammel.** 1994. Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett. **354:**297–300.
4. **Bezalel, L., Y. Hadar, P. P. Fu, J. P. Freeman, and C. E. Cerniglia.** 1996. Initial oxidation products in the metabolism of pyrene, anthracene, fluorene and dibenzothiophene by white-rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **62:**2554–2559.
5. **Bogan, B. W., and R. T. Lamar.** 1995. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **61:**2631–2635.
6. **Bogan, B. W., and R. T. Lamar.** 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597–1603.
7. **Bogan, B. W., R. T. Lamar, and K. E. Hammel.** 1996. Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl. Environ. Microbiol. **62:**1788–1792.
8. **Breitenbach, J., and F. Kränzlin.** 1995. Agaric fungi, part 2, vol. 4. Entolomataceae, Pluteaceae, Amanitaceae, Agaricaceae, Coprinaceae, Strophariaceae. Verlag Mykologia, Lucerne, Switzerland.
9. **Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by a white-rot fungus. Science **228:**1434–1436.
10. **Cerniglia, C. E.** 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351–368.
11. **Cerniglia, C. E., and D. T. Gibson.** 1979. Oxidation of benzo(a)pyrene by the filamentous fungus *Cunninghamella elegans*. J. Biol. Chem. **254:**12174–12180.
12. **Cerniglia, C. E., and M. A. Heitkamp.** 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41–68. *In* U. Varanasi (ed.), Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC Press, Inc., Boca Raton, Fla.
13. **Collins, P. J., and A. D. W. Dobson.** 1996. Oxidation of fluorene and phenanthrene by Mn(II) dependent peroxidase activity in whole cultures of *Trametes (Coriolus) versicolor*. Biotechnol. Lett. **18:**801–804.
14. **Datta, D., and T. B. Samanta.** 1988. Effect of inducers on metabolism of benzo(a)pyrene in vivo and in vitro: analysis by high pressure liquid chromatography. Biochem. Biophys. Res. Commun. **155:**493–502.
15. **Eggen, T., and A. Majcherczyk.** 1998. Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white-rot fungus *Pleurotus ostreatus*. Int. Biodeterior. Biodegrad. **41:**111–117.
16. **Eggert, C., U. Temp, J. F. Dean, and K. E. Eriksson.** 1996. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett. **391:**144–148.
17. **Enoki, M., T. Watanabe, S. Nakagame, K. Koller, K. Messner, Y. Honda, and M. Kuwahara.** 1999. Extracellular lipid peroxidation of selective white-rot fungus, *Ceriporiopsis subvermispora*. FEMS Microbiol. Lett. **180:**205–211.
18. **Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. de Bont.** 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219–2226.
19. **Forrester, I. T., A. C. Grabski, R. R. Burgess, and G. F. Leatham.** 1988. Manganese, Mn-dependent peroxidase, and the biodegradation of lignin. Biophys. Biochem. Res. Commun. **157:**992–999.
20. **Gettemy, J. M., B. Ma, M. Alic, and M. H. Gold.** 1998. Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. Appl. Environ. Microbiol. **64:**569–574.
21. **Gramss, G., B. Kirsche, K.-D. Voigt, T. Günther, and W. Fritsche.** 1999. Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures of fifty-eight fungi and the concomitant production of oxidative enzymes. Mycol. Res. **103:**1009–1018.
22. **Gramss, G., K. D. Voigt, and B. Kirsche.** 1999. Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. Biodegradation **10:**51–62.
23. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900–6903.
24. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **261:**16948–16952.
25. **Hatakka, A.** 2001. Biodegradation of lignin, p. 129–180. *In* M. Hofrichter and A. Steinbüchel (ed.), Lignin, humic substances and coal, vol. 1. Wiley-VCH, Weinheim, Germany.
26. **Hatakka, A.** 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:**125–135.
27. **Hofrichter, M.** 2002. Lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. **30:**454–466.
28. **Hofrichter, M., T. Lundell, and A. Hatakka.** 2001. Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. Appl. Environ. Microbiol. **67:**4588–4593.
29. **Hofrichter, M., D. Ziegenhagen, T. Vares, M. Friedrich, M. G. Jäger, W. Fritsche, and A. Hatakka.** 1998. Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide. FEBS Lett. **434:**362–366.
30. **Kapich, A., M. Hofrichter, T. Vares, and A. Hatakka.** 1999. Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of non-phenolic lignin model compounds and ^{14}C-labeled lignins. Biochem. Biophys. Res. Commun. **259:**212–219.
31. **Kapich, A., and L. N. Shishkina.** 1995. Lipid peroxidation and its regulation in the mycelium of xylotrophic basidiomycetes. Mikrobiologiya **64:**320–326.
32. **Kapoor, M., and W. S. Lin.** 1984. Studies on the induction of aryl hydrocarbon(benzo[a]pyrene) hydroxylase in *Neurospora crassa*, and its suppression by sodium selenite. Xenobiotica **14:**903–915.
33. **Kästner, M.** 2000. Degradation of aromatic and polyaromatic compounds, p. 211–239. *In* H.-J. Rehm and G. Reed (ed.), Biotechnology, vol. 11b. Wiley-VCH, Weinheim, Germany.
34. **Kerem, Z., and Y. Hadar.** 1995. Effect of manganese on preferential degradation of lignin by *Pleurotus ostreatus* during solid-state fermentation. Appl. Environ. Microbiol. **61:**3057–3062.
35. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
36. **Kotterman, M. J., E. H. Vis, and J. A. Field.** 1998. Successive mineralization and detoxification of benzo[a]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. Appl. Environ. Microbiol. **64:**2853–2858.
37. **Martens, R., M. Wolter, M. Bahadir, and F. Zadrazil.** 1999. Mineralization of ^{14}C-labelled highly-condensed polycyclic aromatic hydrocarbons in soils by *Pleurotus* sp. Florida. Soil Biol. Biochem. **31:**1893–1899.
38. **Masaphy, S., Y. Henis, and D. Levanon.** 1996. Manganese-enhanced biotransformation of atrazine by the white rot fungus *Pleurotus pulmonarius* and its correlation with oxidation activity. Appl. Environ. Microbiol. **62:**3587–3593.
39. **Morgan, P., S. A. Lee, S. T. Lewis, A. N. Sheppard, and R. J. Watkinson.** 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. **25:**279–287.
40. **Phillips, R.** 1991. Mushrooms of North America. Brown & Company Inc., New York, N.Y.
41. **Pointing, S. B.** 2001. Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol. **57:**20–33.
42. **Ramdahl, T.** 1985. PAH emissions from combustion of biomass, p. 61–85. *In* A. Bjørseth and T. Ramdahl (ed.), Handbook of polycyclic aromatic hydrocarbons. Marcel Dekker, New York, N.Y.
43. **Sack, U., T. M. Heinze, J. Deck, C. E. Cerniglia, R. Martens, F. Zadrazil, and W. Fritsche.** 1997. Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. Appl. Environ. Microbiol. **63:**3919–3925.
44. **Sack, U., M. Hofrichter, and W. Fritsche.** 1997. Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Microbiol. Lett. **152:**227–234.
45. **Sanglard, D., M. S. A. Leisola, and A. Fiechter.** 1986. Role of extracellular ligninases in biodegradation of benzo(a)pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209–212.
46. **Steffen, K. T., A. Hatakka, and M. Hofrichter.** 2002. Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. Appl. Environ. Microbiol. **68:**3442–3448.
47. **Steffen, K. T., A. Hatakka, and M. Hofrichter.** 2002. Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Appl. Microbiol. Biotechnol. **60:**212–217.
48. **Steffen, K. T., M. Hofrichter, and A. Hatakka.** 2000. Mineralisation of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. Appl. Microbiol. Biotechnol. **54:**819–825.
49. **Steffen, K. T., M. Hofrichter, and A. Hatakka.** 2002. Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. Enzyme Microb. Technol. **30:**550–555.
50. **Sutherland, J. B., F. Rafii, A. A. Khan, and C. E. Cerniglia.** 1995. Mechanisms of polycyclic aromatic hydrocarbon degradation, p. 269–306. *In* L. Young and C. E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss., New York, N.Y.
51. **Urzua, U., P. J. Kersten, and R. Vicuna.** 1998. Kinetics of Mn^{3+}-oxalate formation and decay in reactions catalyzed by manganese peroxidase of *Ceriporiopsis subvermispora*. Arch. Biochem. Biophys. **360:**215–222.
52. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688–23695.

53. **Wariishi, H., K. Valli, V. Renganathan, and M. H. Gold.** 1989. Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**14185–14191.
54. **Wilson, S. C., and K. C. Jones.** 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ. Pollut. **81:**229–249.
55. **Wolter, M., F. Zadrazil, R. Martens, and M. Bahadir.** 1997. Degradation of eight highly condensed polycyclic aromatic hydrocarbons by *Pleurotus* sp. Florida in solid wheat straw substrate. Appl. Microbiol. Biotechnol. **48:**398–404.
56. **Wunch, K. G., W. L. Alworth, and J. W. Bennett.** 1999. Mineralization of benzo[a]pyrene by *Marasmiellus troyanus*, a mushroom isolated from a toxic waste site. Microbiol. Res. **154:**75–79.
57. **Wunch, K. G., T. Feibelman, and J. W. Bennett.** 1997. Screening for fungi capable of removing benzo(a)pyrene in culture. Appl. Microbiol. Biotechnol. **47:**620–624.
58. **Zheng, Z. M., and J. P. Obbard.** 2002. Oxidation of polycyclic aromatic hydrocarbons (PAH) by the white rot fungus, *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **31:**3–9.