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Metabolism of Pyrene by the Basidiomycete *Crinipellis stipitaria* and Identification of Pyrenequinones and Their Hydroxylated Precursors in Strain JK375†

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The metabolism of pyrene, a polycyclic aromatic hydrocarbon, by submerged cultures of the basidiomycete *Crinipellis stipitaria* was studied. After incubation for 68 h at 25°C in a 20-liter fermentor with complex medium and 20 mg of pyrene per liter, five metabolites were detected. The compounds were isolated by preparative high-performance liquid chromatography on RP18 and DIOL gels. By UV, infrared, and ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry, 1-hydroxypyrene, 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, 1,6-pyrenequinone, and 1,8-pyrenequinone were identified. 1,6- and 1,8-dihydroxypyrene were obtained from fungal cultures for the first time. The formation of these metabolites was confirmed by investigations with [4,5,9,10-^{14}C]pyrene.

Polycyclic aromatic hydrocarbons (PAHs) are classified as priority pollutants by the U.S. Environmental Protection Agency (13). They occur as natural constituents and combustion products of fossil fuels and are ubiquitous environmental contaminants (11, 12). Because of their toxic, mutagenic, and carcinogenic properties (19–21), the fate of these pollutants in nature is of environmental concern. Microbial degradation and transformation are thought to be important processes that result in the removal of PAHs from the natural environment (23). A wide variety of eukaryotic and prokaryotic organisms that are able to metabolize PAHs have been isolated (3, 5, 6, 14, 15, 22, 28).

In contrast to degradation of PAHs containing up to three aromatic rings, the microbial mineralization of aromatic hydrocarbons with four or more rings is considered to be difficult. Only a few bacterial cultures are able to mineralize these substances (8–10, 14, 28, 29). Pyrene, one of the most important compounds in PAH-polluted soils and aqueous samples (16), can be used by a *Mycobacterium* species (2) and a *Rhodococcus* species (27) as the sole source of carbon and energy.

The zygomycete *Cunninghamella elegans* metabolizes pyrene to 1-hydroxypyrene, 1-pyreneglucoside, 1-hydroxypyrene-6-glucoside, 1-hydroxypyrene-8-glucoside, 1,6-pyrenequinone, and 1,8-pyrenequinone (4). In cultures of *Phanerochaete chrysosporium*, 1,6- and 1,8-pyrenequinone generated by a lignin peroxidase were identified (7). Little is known about the role of plant-inhabiting and litter-decomposing basidiomycetes in organopollutant metabolism, although they are major constituents of the microbial biomass of grass lands and forest floors.

During a screening of 180 saprophytic and plant-inhabiting basidiomycetes for metabolization and degradation of PAHs, cultures of *Crinipellis stipitaria* JK375 and JK364 were found to metabolize pyrene. *C. stipitaria* belongs to the order *Agaricales*, family *Tricholomataceae*. The species occurs worldwide on grasses and rotting plant material (24). All strains of this species were found to produce crinipellins, antimicrobially active diterpenes with a tetraquinane skeleton bearing several keto, hydroxy, and epoxy groups (1, 17).

In this paper we report the isolation and identification of five metabolites formed by *C. stipitaria* JK375 from pyrene, including 1,6- and 1,8-dihydroxypyrenes, which have never before been obtained from fungal cultures, although they have been postulated as intermediates in reaction sequences leading to glucosylated transformation products (4). The metabolites formed by strain JK364 will be the subject of the accompanying paper (18).

MATERIALS AND METHODS

Chemicals. [4,5,9,10-^{14}C]pyrene (specific activity, 56 mCi/mmol), of 99% radiochemical purity, was obtained from Amersham-Buchler, Braunschweig, Germany. Pyrene was purchased from Aldrich Chemie, Steinheim, Germany. High-performance liquid chromatography (HPLC) grade solvents were purchased from E. Merck, Darmstadt, Germany. All other chemicals were of reagent grade and were the highest purity available.

Microorganism and culture conditions. Fruiting bodies of *C. stipitaria* (Fr.) Pat. JK375 were collected in 1977 in the vicinity of Deißlingen, Germany. The mycelial culture was obtained from a spore print.

The strain was maintained on YMG agar containing the following (in grams per liter): glucose, 4; malt extract, 10; yeast extract, 4; and agar, 10. The pH was adjusted to 5.5 before sterilization. Fermentations were carried out in a Biolafitte C-6 apparatus containing 20 liters of YMG medium with agitation (150 rpm) and aeration (3.33 liters/min) at 25°C. For the inoculum, 400 ml of a well-grown culture in the same medium was used. After 48 h of incubation, pyrene dissolved in methanol was added to a final concentration of 20 mg/liter. After an additional 68 h of incubation, the mycelia and culture broth were separated by filtration.

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† This paper is dedicated to H. Zähner on the occasion of his 65th birthday.

TABLE 1. TLC and HPLC properties of and mass-spectral data for metabolites formed from pyrene by *C. stipitaria* JK375[a]

Compound	TLC R_f	HPLC retention time (min)	MS (*m/z*) (%)
1-Hydroxypyrene	0.83	17.7	218 (100), 189 (59), 163 (4), 150 (1), 109 (7), 94 (10), 84 (8)
1,6-Dihydroxypyrene	0.35	12.9	234 (100), 205 (16), 188 (23), 176 (12), 151 (2), 117 (7), 94 (5)
1,8-Dihydroxypyrene	0.39	13.3	234 (100), 205 (17), 188 (25), 176 (12), 151 (4), 117 (6), 94 (7)
1,6-Pyrenequinone	0.37	13.5	232 (100), 204 (20), 176 (85), 150 (9), 88 (26)
1,8-Pyrenequinone	0.41	14.4	232 (100), 204 (28), 176 (89), 150 (11), 88 (27)

[a] TLC was performed with silica gel and toluene-acetone (7:3); HPLC was performed with a Nucleosil 5 C18 PAH column and an acetonitrile-water gradient as described in Materials and Methods.

Experiments with radioactively labeled pyrene were conducted with [4,5,9,10-^{14}C]pyrene (2 μCi) added to 5-day-old pregrown cultures of *C. stipitaria* JK375 in 100-ml flasks containing 30 ml of YMG medium. Unlabeled pyrene (10 mg/liter of medium) dissolved in methanol was added to each flask. After an incubation period of 3, 7, 14, or 21 days, the mycelia in one flask were filtered to separate them from the culture broth. The mycelia were extracted five times with 100 ml of acetone in a shaking water bath at 30°C for 15 min. The extracts were combined. The culture filtrate was extracted five times with equal volumes of ethyl acetate. The organic solvents were evaporated in vacuo, and the residues were dissolved in methanol and analyzed for metabolites by HPLC.

Physical and chemical analyses. A Merck-Hitachi high-performance liquid chromatograph equipped with a Merck-Hitachi photodiode array detector operated at 254 nm and fitted with a Nucleosil 5 C-18 PAH column (150 mm by 4 mm [inside diameter]; Macherey-Nagel, Düren, Germany) was used to analyze pyrene and the transformation products. The HPLC elution program consisted of a 20-min linear gradient of acetonitrile-water (10:90 to 100:0 [vol/vol]). During the next 5 min the concentration of acetonitrile was maintained at 100%. The flow rate was 1 ml/min. The retention time of pyrene under these conditions was 21.7 min.

In experiments with [4,5,9,10-^{14}C]pyrene, 0.5-ml fractions were collected every 0.5 min and added to scintillation vials containing 5 ml of scintillation fluid (Quickszint 501; Zinsser Analytik, Frankfurt/Main, Germany). Radioactivity was determined with a liquid scintillation counter (type Wallac 1410; Pharmacia, Wallac Oy, Turku, Finland).

For the isolation of pyrene metabolites, a high-performance liquid chromatograph (Gilson, Viliers de Bel, France) equipped with a variable UV detector (Knauer, Bad Homburg, Germany) operated at 210 nm and fitted with a LiChrosorb DIOL column (250 mm by 25 mm [inside diameter]; E. Merck) was used. Final separation of the pyrenequinones was achieved by using a high-performance liquid chromatograph with a Merck-Hitachi L photodiode array detector operated at 254 nm and equipped with a LiChrospher RP-18 end-capped column (250 mm by 10 mm [inside diameter]; E. Merck).

UV–visible-light absorption spectra of metabolites were recorded with a Hewlett-Packard HPLC 1090 instrument and equipped with a diode array detector. Thin-layer chromatography (TLC) was performed on silica gel 60 $WF_{254}S$ (E. Merck). The solvent used for TLC was toluene-acetone (7:3 [vol/vol]). Mass-spectral analyses were performed with a JEOL SX102 spectrometer. The spectra were recorded with electron impact ionization at 70 eV. The ionization chamber temperature was 250°C, and the electron multiplier voltage was 1,000 V. The ^{1}H nuclear magnetic resonance (NMR) measurements were carried out at 500 MHz in acetone-d_6 on a Bruker ARX500 spectrometer, with the solvent signal (at 2.05 ppm) as a reference.

RESULTS

Isolation of pyrene metabolites. For the isolation of the five metabolites formed from pyrene by *C. stipitaria* JK375, mycelia and culture broth were separated by filtration after a fermentation period of 68 h at 25°C. At this time, 7.5 mg of pyrene per liter remained in the mycelia and culture broth. The concentration of 1-hydroxypyrene was at its highest level of 2.5 mg/liter.

After all chromatographic steps, 10.1 mg of 1-hydroxypyrene, 1 mg of 1,6-dihydroxypyrene, 0.7 mg of 1,6-pyrenequinone, and 0.5 mg of 1,8-dihydroxypyrene were obtained. The last compound easily underwent autooxidation to 1,8-pyrenequinone.

Identification of pyrene metabolites. 1-Hydroxypyrene and 1,6- and 1,8-pyrenequinone were identified by comparison of their spectral data with those already published (4). There was no doubt about the identity of these three compounds. Summaries of the chemical and physical properties of the isolated compounds are given in Tables 1 and 2.

The mass spectra of the compounds identified as 1,6- and 1,8-dihydroxypyrene showed very similar fragmentation patterns. Each compound gave a molecular ion (M^+) at *m/z* 234 and fragment ions at 205 ($M^+ - COH$), *m/z* 188 ($M^+ - OH - COH$), and *m/z* 176 ($M^+ - 2COH$). The positions of the substituents were determined by NMR spectroscopy (Table 2). Because of symmetry, both compounds exhibited only four different aromatic resonances. The NMR spectrum of 1,6-dihydroxypyrene was consistent with a C-1 and C-6 dihydroxypyrene structure. This conclusion was drawn from the proton resonance at 7.56 ppm (2-H and 7-H) with $J_{2,3}$ and $J_{7,8}$ = 8.3 Hz, respectively, indicating coupling of a proton adjacent to a hydroxy carbon. The chemical shifts and coupling of the other pair of doublets are consistent with 4-H (9-H, respectively) and 5-H (10-H, respectively). The NMR spectrum of 1,8-dihydroxypyrene was consistent with a C-1- and C-8-substituted dihydroxypyrene. This conclusion was based on the pair of doublets at 7.57 ppm (2-H and 7-H) and 7.97 ppm (3-H and 6-H) with $J_{2,3}$ and $J_{6,7}$ = 8.3 Hz and the presence of two singlets at 7.76 ppm (4-H and 5-H) and 8.34 ppm (9-H and 10-H). The UV absorbance spectra of the dihydroxypyrenes are shown in Fig. 1. Maxima of 1,6-dihydroxypyrene (λ_{max}) were at 242, 265, 275, 333, 348, 376, and 397 nm. 1,8-Dihydroxypyrene showed UV absorbance maxima (λ_{max}) at 240, 265, 280, 351, 392, and 472 nm.

Experiment with radioactively labeled pyrene. The formation of the five pyrene metabolites by cultures of *C. stipitaria* JK375 was confirmed by investigations with [4,5,9,10-^{14}C]pyrene.

Figure 2A shows the HPLC elution profile of the metabolites formed by *C. stipitaria* upon incubation with [^{14}C]pyrene. The radioactivity elution profile of the mycelial crude extract obtained by HPLC on day 7 is presented in Fig. 2B. Data

TABLE 2. ^{1}H NMR (500-MHz) data for the pyrene metabolites obtained from pyrene metabolism by *C. stipitaria* JK375[a]

Position	δ [ppm]; multiplicity; *J* [HZ] for:				
	1-Hydroxypyrene	1,6-Dihydroxypyrene	1,8-Dihydroxypyrene	1,6-Pyrenequinone	1,8-Pyrenequinone
2-H	7.63;d;8.2	7.56;d;8.3	7.57;d;8.3	6.68;d;9.5	6.65;d;9.8
3-H	8.10;d;8.2	7.98;d;8.3	7.97;d;8.3	7.96;d;9.5	7.95;d;9.8
4-H	7.91;d;9.0	7.92;d;9.1	7.76;s	8.10;d;7.3	7.98;s
5-H	8.01;d;9.0	8.19;d;9.1	7.76;s	8.46;d;7.3	7.98;s
6-H	8.13;d;7.7		7.97;d;8.3		7.95;d;9.8
7-H	7.97;dd;7.7,7.7	7.56;d;8.3	7.57;d;8.3	6.68;d;9.5	6.65;d;9.8
8-H	8.14;d;7.7	7.98;d;8.3		7.96;d;9.5	
9-H	8.06;d;9.0	7.92;d;9.1	8.34;s	8.10;d;7.3	8.60;s
10-H	8.43;d;9.0	8.19;d;9.1	8.34;s	8.46;d;7.3	8.60;s

[a] The NMR spectra were recorded in acetone-d_6, and the solvent signal (2.05 ppm) was used as a reference. Chemical shifts are reported downfield from tetramethylsilane.

obtained with the culture filtrate crude extracts after day 21 are depicted in Fig. 2C. Because the fractions of the eluates were collected every 30 s, 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, and 1,6-pyrenequinone were not separated under these conditions.

The recovery rate of the labeled pyrene was higher than 90%, and no release of labeled carbon dioxide was observed during the incubation time. At the beginning of the fermentation, 70% of the labeled compounds were found in the mycelial extracts; after 7 days, only 54% of the recovered radioactivity was located in the mycelia and 46% was located in the culture filtrate. The mycelial crude extract contained considerable amounts of 1-hydroxypyrene, which accounted for 27.5% of the acetone-extractable radioactivity. In addition, 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, 1,6-pyrenequinone, and 1,8-pyrenequinone were detected. Together, these four compounds accounted for 20.3% of the acetone-extractable radioactivity. Of the radioactivity extractable with acetone after 7 days, 13.8% was due to pyrene (Fig. 2B). The distribution of metabolites in the culture broth was similar to that observed in the mycelia.

After 21 days, 52% of the recovered radioactivity was located in the culture filtrate and 48% was found in the mycelia. Most of the extractable radioactivity in the culture fluid was due to 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, and 1,6-pyrenequinone, which together accounted for 65.5%. Pyrene, 1-hydroxypyrene, and 1,8-pyrenequinone were present in only small amounts (4.5, 1.6, and 5.5%, respectively) in the culture broth (Fig. 2C). The distribution pattern of metabolites in the mycelia on day 21 was similar to that in Fig. 2C.

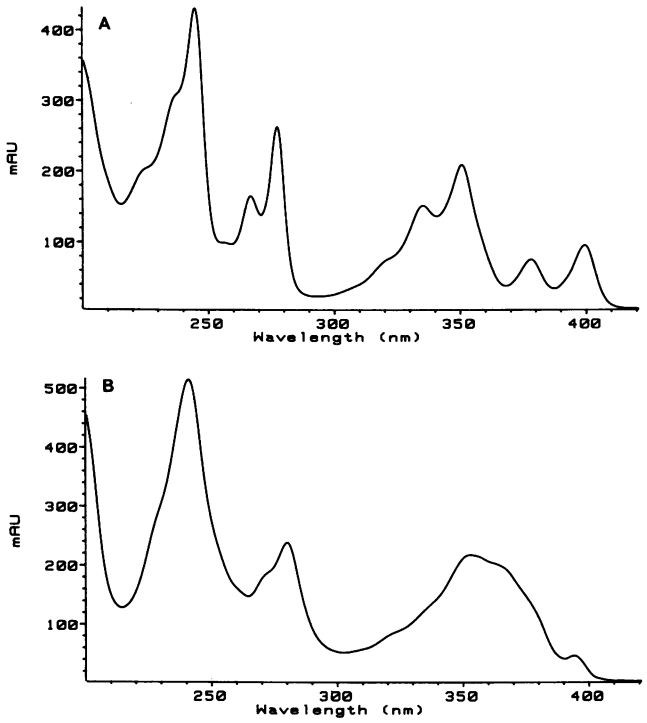

FIG. 1. UV-visible spectra of 1,6-dihydroxypyrene (A) and 1,8-dihydroxypyrene (B).

DISCUSSION

The present study is the first to be performed on metabolites of pyrene formed by the non-white-rot basidiomycete *C. stipitaria*. 1,6- and 1,8-dihydroxypyrene could be isolated from fungal cultures upon addition of pyrene, although these compounds are not stable for long. They were easily autooxidized to the corresponding quinones by air (25). Investigations of extracellular enzymes of *C. stipitaria* revealed that a laccase was excreted into the culture medium after 3 days of incubation in YMG medium as measured by using 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (data not shown). This enzyme might also be responsible for the formation of the pyrenequinones in the culture filtrate.

For *Cunninghamella elegans*, the dihydroxypyrenes were proposed as intermediates in reactions leading to glucosylated transformation products (4). Glucoside conjugate formation and simultaneous detoxification were not observed with our fungal strain. Also, no oxidation of the K region (position 4,5) of pyrene was noticed in this *C. stipitaria* strain. However, the second strain of this species, JK364, was found to transform pyrene to the 1-pyrenylsulfate (18), indicating that, like secondary metabolism (26), metabolization of PAHs may be strain specific.

Similar to metabolism of pyrene by *Cunninghamella elegans*, the initial oxidation of the molecule by *C. stipitaria* occurs at C-1 and is followed by the introduction of a second hydroxy group in position C-6 or C-8. This is shown by the fact that 1-hydroxypyrene appeared first during the course of incubation.

In white rot fungi, like *P. chrysosporium*, under N-limiting conditions, extracellular lignin peroxidases oxidize pyrene to the 1,6- and 1,8-quinones (7); however, in *C. stipitaria* the pyrene-hydroxylating enzyme seems to be located in the mycelium, as concluded from the distribution pattern of the radioactive 1-hydroxypyrene. Furthermore, no enzymatic oxi-

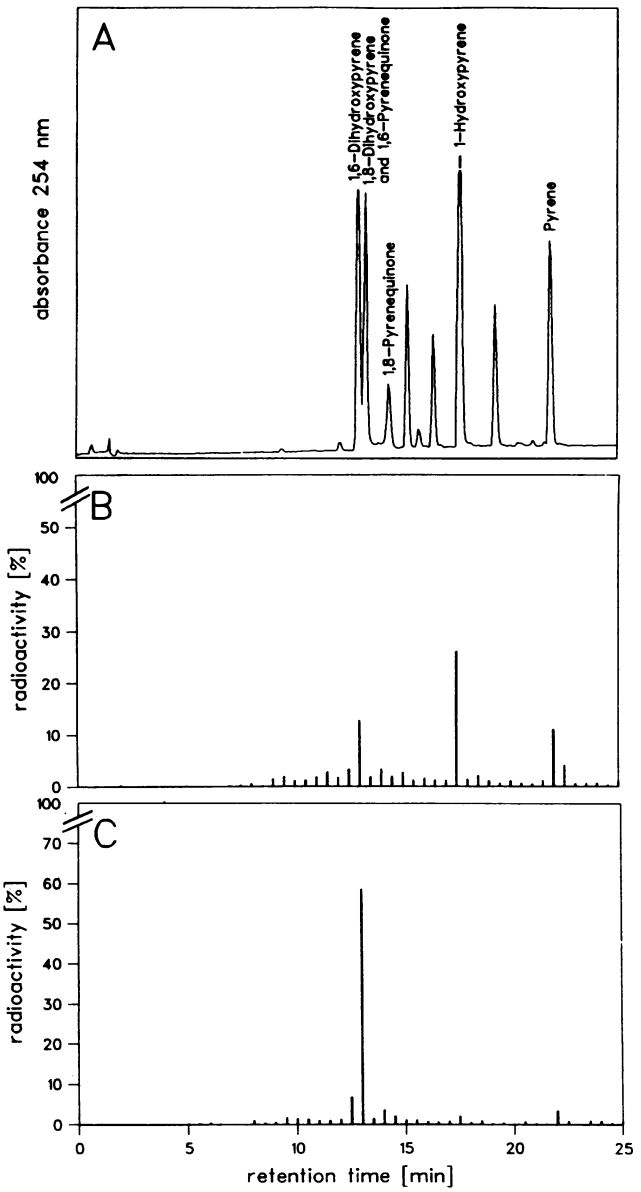

FIG. 2. (A) HPLC-chromatogram of a crude extract containing 1-hydroxypyrene, 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, 1,6-pyrenequinone, and 1,8-pyrenequinone formed by *C. stipitaria* JK375. (B and C) Radioactivity elution profile of the mycelia crude extract on day 7 (B) and of the culture filtrate crude extract on day 21 (C). Fractions eluting from the column were collected at 0.5-min intervals.

dation of pyrene was observed in the culture fluid. Whether a nonspecific fungal cytochrome P450-associated monooxygenase or possibly enzymes involved in biosynthesis of the crinipellins are responsible for the formation of 1-hydroxypyrene remains to be elucidated, because in terpenoid biosynthesis, the oxygenation usually takes place after synthesis of the carbon skeleton.

This study indicates that wood-inhabiting white rot fungi are not the only ones that contribute to the metabolization and degradation of PAHs but that plant- and litter-inhabiting basidiomycetes must also be taken into consideration when the fate of PAHs in the environment is discussed.

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