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Pyrene Metabolism in *Crinipellis stipitaria*: Identification of *trans*-4,5-Dihydro-4,5-Dihydroxypyrene and 1-Pyrenylsulfate in Strain JK364†

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The isolation and identification of two novel metabolites in the fungal metabolism of pyrene are described. The plant-inhabiting basidiomycete *Crinipellis stipitaria* JK364 metabolized pyrene, a polycyclic aromatic hydrocarbon containing four rings, when grown in submerged cultures in a medium containing malt extract, glucose, and yeast extract. In experiments with [^{14}C]pyrene, after 7 days of incubation 40% of the labeled substrate was converted into organic solvent-extractable metabolites. Metabolites isolated from cultures grown with pyrene were identified as 1-pyrenylsulfate and *trans*-4,5-dihydro-4,5-dihydroxypyrene. 1-Hydroxypyrene, the precursor of 1-pyrenylsulfate, was also detected. 1-Pyrenylsulfate was isolated from mycelial extracts, whereas *trans*-4,5-dihydro-4,5-dihydroxypyrene was recovered from the culture filtrate. Identification of the compounds was based on their UV spectra, mass spectra, and nuclear magnetic resonance spectra. This is the first report on the detoxification of a polycyclic aromatic hydrocarbon by a plant-inhabiting basidiomycete. The occurrence of 1-pyrenylsulfate and *trans*-4,5-dihydro-4,5-dihydroxypyrene among fungal metabolites of pyrene is also new.

The environmental and toxicological significance of higher-molecular-weight polycyclic aromatic hydrocarbons PAHs has caused an increasing interest in the fungal metabolism and detoxification of these compounds (9, 12, 27). Fungi are not able to utilize PAHs as sole sources of carbon and energy but, rather, transform PAHs under cometabolic conditions (3, 25).

The zygomycete *Cunninghamella elegans* oxidizes a wide variety of PAHs via cytochrome P450 monooxygenase- and epoxide hydrolase-catalyzed reactions to *trans*-dihydrodiols (11–13). In addition, the fungus forms glucoside, sulfate, and glucuronide conjugates of phenolic PAHs, which are important detoxification products of these primary metabolites (4, 6).

A different metabolic pathway is displayed by white rot fungi in response to nutrient-limited ligninolytic conditions (1, 15). *Phanerochaete chrysosporium*, a white rot basidiomycete, transforms the four-ring PAH pyrene in an unspecific lignin peroxidase-catalyzed oxidation to 1,6- and 1,8-pyrenequinone, and the compound is mineralized by the fungus (14, 15). 1,6- and 1,8-pyrenequinone are transient metabolites; however, they are potentially mutagenic and more toxic than the original compound (23). Besides white rot fungi and the zygomycete *Cunninghamella elegans*, only a few species of filamentous fungi and yeasts have been tested for their ability to transform PAHs (2, 5, 10, 17), and there is still a lack of knowledge about the metabolites produced and their toxicity (7, 8).

In the accompanying paper by Lambert et al., pyrene metabolism in *Crinipellis stipitaria* JK375 is described (21). The present investigation extends the study described in the accompanying paper by isolating and identifying novel pyrene metabolites formed by strain JK364 and demonstrates that different metabolic pathways occur in two strains of the same species.

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

MATERIALS AND METHODS

Chemicals. [4,5,9,10-^{14}C]pyrene with a specific activity of 56 mCi/mmol and a radiochemical purity of 95% was obtained from Amersham Buchler, Braunschweig, Germany. Unlabeled pyrene (purity, 99%) was purchased from Aldrich Chemie, Steinhofen, Germany. High-pressure liquid chromatography (HPLC)-grade solvents, acetonitrile, and methanol were purchased from Zinsser Analytic GmbH, Frankfurt, Germany, and from Riedel-de-Haen AG, Seelze, Germany. Arylsulfatase and D-saccharic acid 1,4-lactone were obtained from Sigma-Chemie, Deisenhofen, Germany. All other chemicals were of reagent grade and the highest purity available.

Microorganism and culture conditions. Fruiting bodies of *C. stipitaria* (Fr.) Pat. JK364 were collected in 1977 in Eningen, Baden-Württemberg, Germany. The mycelial culture was obtained from a spore print. Cultivation of the fungus was the same as in the accompanying paper (21).

For metabolization experiments with [4,5,9,10-^{14}C]pyrene, 250-ml cultures were preincubated for 3 days, before 5 mg of unlabeled pyrene and 5 μCi of [^{14}C]pyrene were added. Incubation with radioactively labeled pyrene was carried out for 7 days.

Fermentation. Fermentations were carried out in a Biolafitte apparatus containing 18 liters of YMG medium. After inoculation with 250 ml of seed cultures, the fermentation was conducted for 14 days at 21°C with agitation at 120 rpm and aeration at 3.3 liters/min. After 60 h, pyrene was added to the culture at 20 mg/liter (400 mg dissolved in 200 ml of dimethyl sulfoxide). During the fermentation, samples from the culture (50 ml) were taken to monitor the decrease in concentration of pyrene and the appearance of pyrene metabolites. The samples were extracted and analyzed as described below.

Isolation, detection, and identification of pyrene metabolites. Samples from the fermentation were filtered to separate

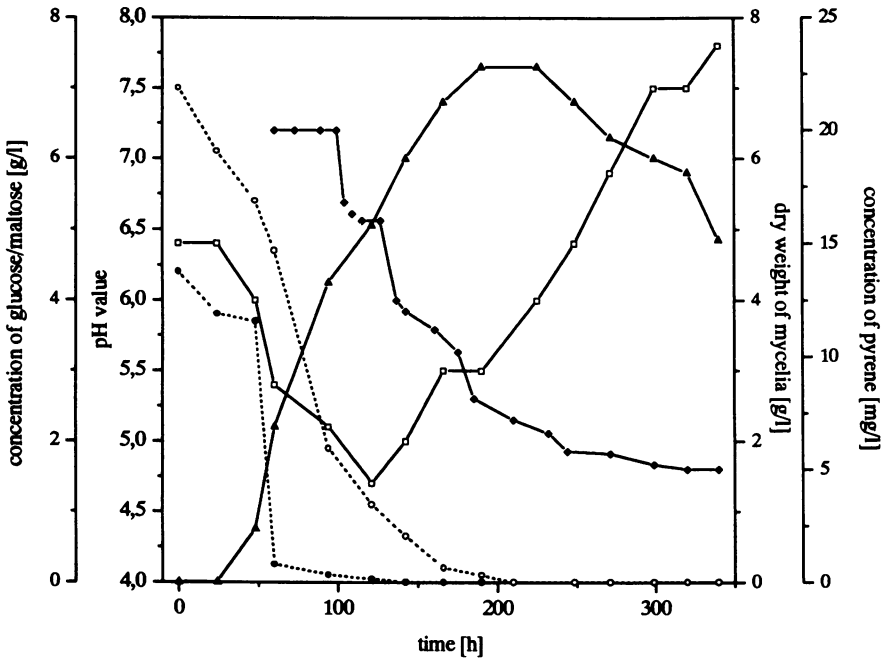

FIG. 1. Growth of *C. stipitaria* and disappearance of pyrene on a 20-liter scale. The fungus was pregrown in YMG medium for 60 h, and 20 mg of pyrene per liter was added. The pyrene concentration reflects the sum of mycelium and culture filtrate. Symbols: ○, concentration of maltose; ●, concentration of glucose; ▲, dry weight of mycelia; ◆, concentration of pyrene; □, pH value.

mycelia from the culture filtrate. The filtrate was extracted with 2 equal volumes of ethyl acetate, and the extract was dried with anhydrous sodium sulfate. The mycelia were extracted twice with acetone by stirring for 20 min each. The extracts were combined and evaporated under reduced pressure at 45°C, and the residues were dissolved in methanol and analyzed by HPLC as described in the accompanying paper (21).

After 7 days of incubation, cultures containing [^{14}C]pyrene were harvested and extracted as described above. Aliquots were removed from each extract for liquid scintillation counting. Scintillation counting and HPLC analyses of the radioactively labeled extracts were performed as described in the accompanying paper (21).

For the isolation of pyrene metabolites, the fermentation was interrupted after 10 days when the largest amount of metabolites was detected. The culture fluid and the mycelia were extracted as described above. The crude extracts were applied to a silica gel column (Merck silica gel 60; 40 to 63 μm), which was eluted with a cyclohexane-ethyl acetate gradient (9:1 to 5:5) followed by ethyl acetate and methanol. Final separation of 1-pyrenylsulfate was achieved by HPLC on an RP18 semipreparative column (inner dimensions, 250 by 10 mm; particle size, 10 μm) with a 20-min linear gradient of H_2O-methanol (80:20 to 0:100). Pyrene *trans*-4,5-dihydrodiol was separated on the same column by isocratic elution with H_2O-methanol (40:60) for 16 min.

UV-visible absorption spectra were determined in methanol with a CARY 17 UV spectrometer (Cary Instruments, Peckroad/Monrovia, Calif.). Mass-spectral analyses and ^{1}H nuclear magnetic resonance (NMR) measurements were carried out as described in the accompanying paper (21). Because of the instability of the sulfate ester at the high temperature used for electron impact ionization, the mass of 1-pyrenylsulfate was determined by thermospray ionization with a Finnigan MAT SSQ 70 mass spectrometer.

For the deconjugation of 1-pyrenylsulfate, 500 μg of the metabolite was dissolved in 5 ml of H_2O and diluted 1:1 with 0.2 M sodium acetate buffer (pH 4.5). The sample was incubated with 10 U of arylsulfatase (type V; Sigma-Chemie) and 10 μmol of D-saccharic acid 1,4-lactone. A control mixture was incubated without enzyme. The reaction mixtures were incubated for 24 h at 37°C on a rotary shaker operating at 150 rpm. Samples were then extracted with 5 equal volumes of ethyl acetate, dried under reduced pressure at 45°C, and analyzed by HPLC as described above.

RESULTS

Pyrene metabolism. The basidiomycete *C. stipitaria* JK364 transformed pyrene cometabolically when grown in YMG medium in submerged cultures. Pyrene as the sole source of carbon and energy could not support growth of the fungus. Figure 1 shows the growth of *C. stipitaria* JK364 in YMG medium and the disappearance of pyrene on a 20-liter scale. The strain was pregrown for 60 h before 20 mg of pyrene per liter was added. HPLC analyses of the mycelium and culture filtrate revealed that the concentration of pyrene in the culture decreased from 20 to 5 mg/liter within 11 days. Even if the incubation time was prolonged, no further decrease in the pyrene concentration could be observed. Mycelial dry weight increased as long as glucose and maltose were present in the culture broth. Shortly after complete degradation of the sugars, the decrease in the pyrene concentration slowed and stopped, which is in accordance with a cometabolic transformation of pyrene.

At the beginning of the incubation, 80% of the pyrene were found in the mycelium and 20% was extracted from the culture filtrate. During the incubation period, the percentage of pyrene remaining in the culture decreased successively, and after 320 h, pyrene was no longer detectable in the filtrate.

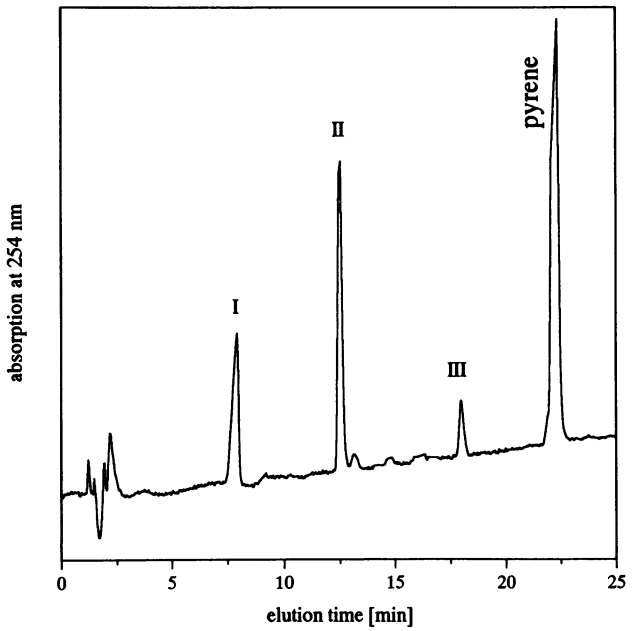

FIG. 2. HPLC elution profile of pyrene and pyrene metabolites formed by *C. stipitaria*. Metabolite I, 1-pyrenylsulfate; metabolite II, pyrene *trans*-4,5-dihydrodiol; metabolite III, 1-hydroxypyrene.

Detection and isolation of pyrene metabolites. After 10 days of incubation, the fermentation was stopped. Extraction of the culture resulted in 2.3 g of crude extract from the mycelium and 2.4 g of crude extract from the culture filtrate. Samples of the extracts from the mycelia and culture filtrate were analyzed by HPLC, and the pyrene metabolites were detected via their UV-visible spectra (see Fig. 3). Figure 2 shows the HPLC elution profile of pyrene metabolites formed by *C. stipitaria* JK364. Metabolites I, II, and III eluted at 7.9, 12.5, and 18.0 min, respectively. Isolation of the metabolites by silica gel column chromatography and reverse-phase HPLC gave 7 mg of metabolite I and 3 mg of metabolite II, which were used to perform the structural elucidation. Metabolite I was isolated from the extract of mycelium, whereas metabolite II was recovered from the extract of culture filtrate. To elucidate the structure of the isolated metabolites, mass-spectral and NMR spectral parameters were determined. Mass-spectral data are shown in Table 1, and ^{1}H-NMR spectral parameters are shown in Table 2. Metabolite I has a UV absorption spectrum (Fig. 3A) with absorption maxima at 232, 241, 254, 264, 274, 310, 324, and 339 nm. The UV spectrum shows a strong resemblance to that of pyrene, indicating that the metabolite still contains the four-ring structure. The mass spectrum of metabolite I shows a molecular ion [M-H$^-$] at *m/z* 297 and a large-fragment ion at *m/z* 217 when analyzed by thermospray ionization (Table 1). The mass spectrum recorded by electron impact ionization shows no molecular ion but contains fragments at *m/z* 218, 189, 187, 95, and 73, a pattern which is indicative of a pyrenol (20). The mass difference between the molecular ion at *m/z* 297 and the largest fragment at *m/z* 217 (or 218, when protonized) indicates a sulfate group as the substituent.

TABLE 1. Mass-spectral data for metabolites obtained from pyrene metabolism by *C. stipitaria*

Compound	Method[a]	MS (*m/z*) (% relative intensity)
I	TSP	297 (11.5), 217 (100)
I	EI	218 (93), 189 (50), 187 (11), 95 (14), 73 (32), 28 (100)
II	EI	236 (62), 218 (100), 205 (23), 189 (68), 176 (17), 95 (23), 46 (26)

[a] TSP, thermospray ionization; EI, electron impact ionization.

TABLE 2. 500-MHz ^{1}H-NMR spectral parameters of metabolites formed from pyrene by *C. stipitaria*

Compound	Proton assignments (ppm)[a] and coupling constants (Hz)
I	8.16 (2-H), 8.33 (3-H), 8.08 (4-H),[b] 8.01 (5-H),[b] 8.18 (6-H),[c] 7.98 (7-H), 8.17 (8-H),[c] 8.07 (9-H), 8.56 (10-H); $J_{2,3}$ = 8.5 Hz, $J_{4,5}$ = 8.9 Hz, $J_{7,8}$ = 7.5 Hz, $J_{9,10}$ = 9.2 Hz
II	5.07 (4-H/5-H), 7.65 (2-H/7-H), 7.83 (9-H/10-H), 7.87 (1-H/8-H), 7.9 (3-H/6-H); $J_{1,2}$ = 7.3 Hz, $J_{2,3}$ = 7.9 Hz

[a] In acetone-d_6 downfield from TMS.
[b,c] Interchangeable.

^{1}H-NMR analysis of metabolite I (Table 2) confirmed that the sulfate group is linked to C-1. The 500-MHz ^{1}H spectral assignments of metabolite I were as follows (chemical shift in parts per million, multiplicity, number of protons, coupling constant in hertz if observed, assignment): 8.16 (d, 1, $J_{2,3}$ = 8.5 Hz, 2-H), 8.33 (d, 1, 3-H), 8.08 (d, 1, $J_{4,5}$ = 8.9 Hz, 4-H), 8.01 (d, 1, 5-H), 8.18 (d, 1, $J_{6,7}$ = 7.5 Hz, 6-H), 7.98 (t, 1, $J_{6,7}$ = $J_{7,8}$ = 7.5 Hz, 7-H), 8.17 (d, 1, 8-H), 8.07 (d, 1, $J_{9,10}$ = 9.2 Hz, 9-H), and 8.56 (d, 1, 10-H).

Upon treatment of metabolite I with arylsulfatase and saccharolactone (to inhibit β-glucuronidase activity), 1-hydroxypyrene was obtained as the product of deconjugation. 1-Hydroxypyrene is a well-known transformation product of the microbial metabolism of pyrene and was identified by its HPLC elution time and its UV absorption spectrum. Thus, metabolite I was identified as 1-pyrenylsulfate.

The UV absorption spectrum of metabolite II (Fig. 3B) shows absorption maxima at 221 and 259 nm. The similarity of the UV spectrum to that of phenanthrene indicates that the metabolite contains the basic aromatic structure of a phenanthrene. The mass spectrum shows an M$^+$ molecular ion at *m/z* 236, a base peak at *m/z* 218 (M$^+$ − 18, H_2O loss), and fragment ions at *m/z* 205, 189, 176, 95, and 46. The HPLC retention time, UV-visible absorption spectra, and mass spectrum suggested that metabolite II was a pyrene-dihydrodiol. The definite structure, however, was confirmed by ^{1}H-NMR spectroscopy. The 500-MHz ^{1}H spectral assignments of metabolite II were as follows (chemical shift in ppm, multiplicity, number of protons, coupling constant in Hz if observed, assignment): 5.07 (s, 2, 4-H/5-H), 7.65 (dd, 2, $J_{1,2}$ = 7.3 Hz, $J_{2,3}$ = 7.9 Hz, 2-H/7-H), 7.83 (s, 2, 9-H/10-H), 7.87 (d, 2, 1-H/8-H), 7.9 (d, 2, 3-H/6-H). These chemical shifts and coupling patterns were identical to those shown in previous studies of pyrene *trans*-4,5-dihydrodiol formed by a *Mycobacterium* species (16).

In addition to 1-pyrenylsulfate, small amounts of 1-hydroxypyrene, the probable precursor of the sulfate conjugate, were detected in mycelial extracts. 1-Hydroxypyrene (metabolite III) was identified by comparison of its HPLC elution time and its UV spectrum with the data for 1-hydroxypyrene described by Cerniglia et al. (11).

When *C. stipitaria* JK 364 was incubated with [^{14}C]pyrene for 7 days, 40% of the labeled substrate was converted into

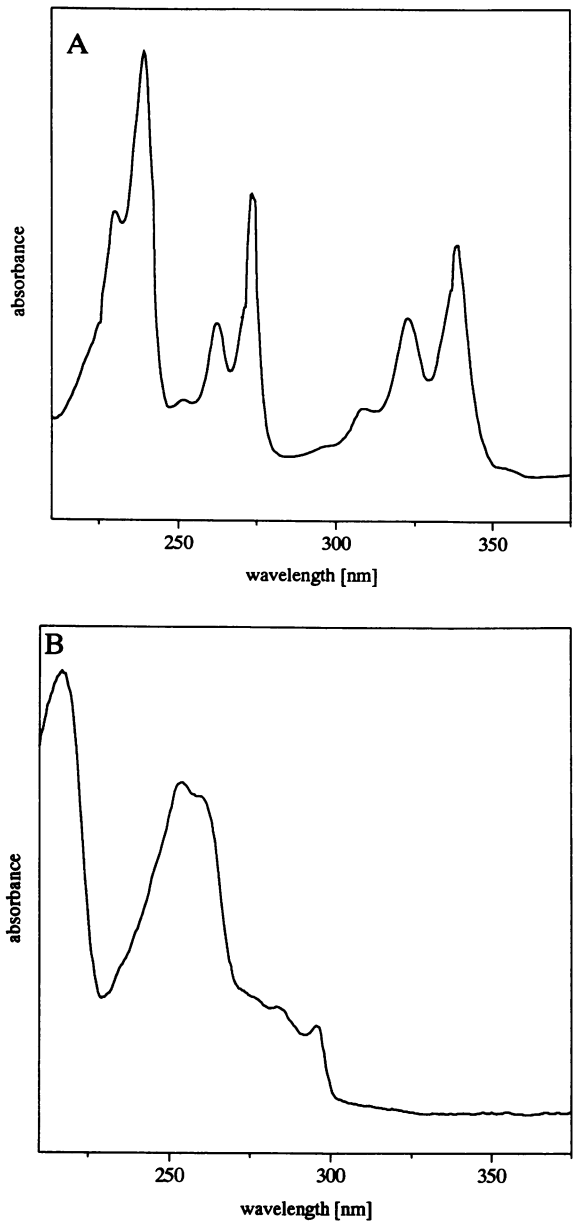

FIG. 3. UV absorption spectra of the isolated metabolites produced from pyrene by *C. stipitaria*. (A) Metabolite I, 1-pyrenylsulfate; (B) metabolite II, pyrene *trans*-4,5-dihydrodiol.

metabolites extractable with organic solvent. 1-Pyrenylsulfate was the predominant metabolite, representing 33% of all extractable metabolites. Pyrene *trans*-4,5-dihydrodiol accounted for 17%, and 1-hydroxypyrene accounted for 5.6%. Of the organic solvent-extractable metabolites, 44% were highly hydrophilic, with HPLC retention times between 2 and 12 min, and have not yet been identified.

DISCUSSION

C. stipitaria JK364 was found to metabolize pyrene to 1-pyrenylsulfate as the major metabolite, as well as to *trans*-4,5-dihydro-4,5-dihydroxypyrene. To our knowledge, both are novel metabolites in the fungal metabolism of pyrene. In addition, small amounts of 1-hydroxypyrene, the probable precursor of the sulfate conjugation product, were detected.

Previous studies on the enzymatic reactions involved in the transformation of PAHs showed that fungi under nonligninolytic conditions metabolize PAHs via pathways that are generally similar to those used by mammalian systems (8, 13). The initial oxidation of an aromatic ring is catalyzed by a cytochrome P450-containing monooxygenase to form an arene oxide. Next, either an epoxide hydrolase catalyzes the formation of a *trans*-dihydrodiol or phenols are formed by a nonenzymatic rearrangement (2, 3, 19). These "primary" metabolites then undergo further transformations and form glucoside, sulfate, or glucuronide conjugates (6, 25). The metabolites isolated from cultures of *C. stipitaria* JK364 can be arranged in the scheme given in Fig. 4. Pyrene oxides as transient metabolites were not found. However, PAH arene oxides are generally unstable in aqueous solution, and isomerization to phenols or hydration to *trans*-dihydrodiols occurs very fast (9, 19, 26).

Oxidation at the C-1 position of pyrene results in the formation of 1-hydroxypyrene, which is conjugated with sulfate to 1-pyrenylsulfate. By oxidation at the 4,5-positions (K region) of pyrene, a *trans*-4,5-dihydrodiol is formed. The reaction sequences leading to the metabolites formed by strain JK375 are also included in Fig. 4.

Conjugation reactions of phenolic PAHs are generally considered to be important in the detoxification of xenobiotic compounds, since their water solubility facilitates excretion of the compounds (6, 13). 1-Pyrenylsulfate, however, the predominant metabolite formed by *C. stipitaria* JK 364, was found only in the mycelia and was not excreted to the culture filtrate. This could be due to a localization of the sulfate in vacuoles in the cells, as has been reported for different intermediates and secondary metabolites (22).

Oxidation of the K region (4,5 positions) on pyrene is a major site of metabolism in mammals. In previous studies on the fungal metabolism of PAH, oxidation at the K region of pyrene by the fungus *Cunninghamella elegans* could not be observed (3, 11). In contrast to these results, *C. stipitaria* JK364 oxidizes pyrene at the 4,5 positions to form a pyrene *trans*-4,5-dihydrodiol, which is also known as a bacterial metabolite (16). From mammalian metabolism of pyrene, it is known that pyrene *trans*-4,5-dihydrodiol is a nontoxic metabolite which can be eliminated directly or can be transformed to glucoside, sulfate, or glucuronide conjugates (18, 24).

The role of litter-decomposing and plant-inhabiting basidiomycetes in the transformation of PAHs has largely been overlooked. The present study, however, shows that grass-inhabiting basidiomycetes are able to perform the initial oxidation of a recalcitrant four-ring PAH under nonligninolytic conditions by transforming the compound into nontoxic metabolites.

In contrast to strain JK364, *C. stipitaria* JK375 was found to metabolize pyrene to 1-hydroxypyrene and 1,6- and 1,8-dihydroxypyrene, as well as 1,6- and 1,8-pyrenequinone (21), as shown in Fig. 4. In this case, however, no detoxification was achieved. It clearly illustrates that biotransformation and metabolization of PAHs by basidiomycetes is not only species specific but also strain specific.

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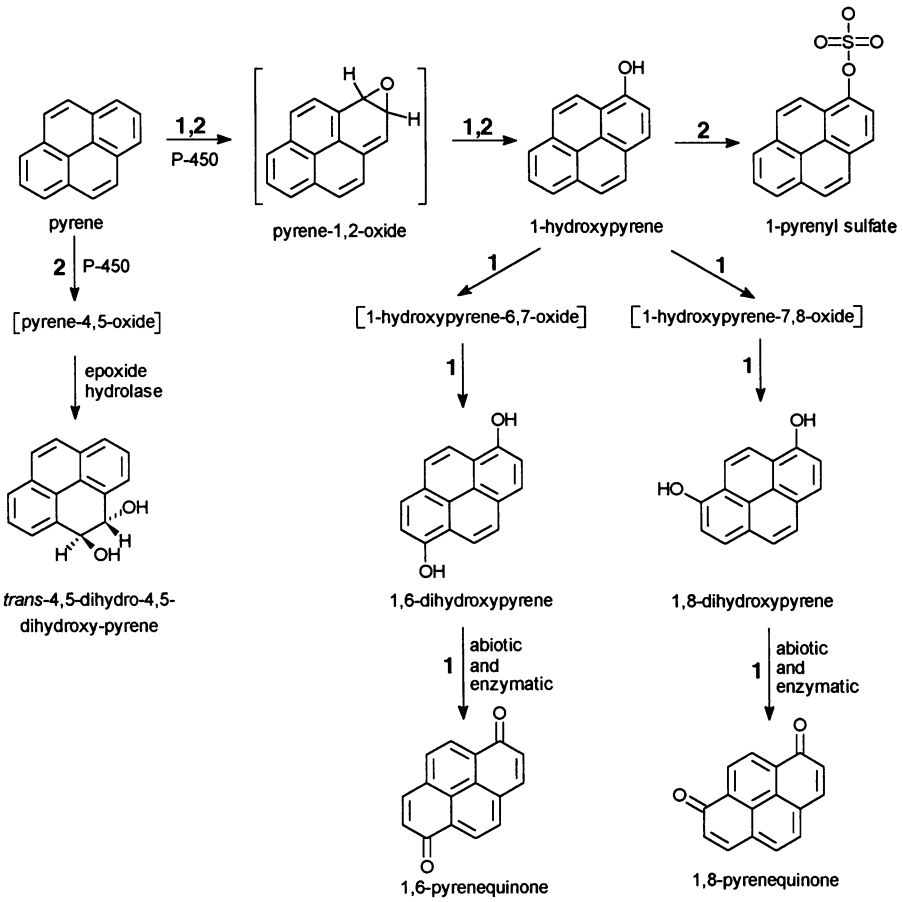

FIG. 4. Proposed pathways for the initial reactions in the metabolism of pyrene by two strains of *C. stipitaria*: **1**, JK375; **2**, JK364.

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