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DEGRADATION OF THE POLYCYCLIC AROMATIC HYDROCARBON BENZO[A]PYRENE BY A NON-WHITE ROT FUNGUS *FUSARIUM SOLANI*: A NEW METABOLIC PATHWAY?

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Abstract

In previous studies, we reported that telluric fungal strains isolated from fuel-contaminated compost showed interesting potential for soil bioremediation. In particular, we isolated a Deuteromycete fungus *Fusarium solani* that was able to grow in liquid medium with BaP as sole carbon source and to mineralize this PAH. In a batch fermentor, [7,10-¹⁴C]benzo[a]pyrene mineralization occurred rapidly at early stages of fermentation (15 hr) during the germination of fungal spores and was not a continuous process as mineralization occurred in a biphasic pattern. Moreover, *F. solani* showed an average rate of mineralization about 65 µg/g dry weight/day within 11 days of incubation, comparable as mineralization rate obtained with a white rot fungus *Phanerochaete chrysosporium*.

In order to study the enzymatic mechanisms involved in the successive steps of BaP degradation, we developed an indirect approach by using inhibitors of enzymes. As is well known, two main enzyme groups are involved in the initial attack on PAHs by fungi: the intracellular cytochrome P-450 monooxygenases and the extracellular lignin peroxidases. We used either specific inhibitors of peroxidases (i.e. salicylhydroxamic acid) and of cytochrome P-450 (i.e. cyclopropylamine) or inhibitors of both enzymes (i.e. potassium cyanide). Surprisingly, no expected decrease of BaP degradation was observed with most inhibitors tested but on the contrary, more BaP was degraded with the cytochrome P-450 inhibitor, cyclopropylamine (4 % enhancement), with the peroxidases inhibitor, salicylhydroxamic acid (7 % enhancement), or with potassium cyanide (9 % enhancement). Only the butylated hydroxytoluene, which acts as a free radical scavenger, inhibited BaP degradation. For one, these experiments suggest that, according to the mechanism of action of the different compounds tested, classical hemoproteins (peroxidases and cytochrome P-450) might have not been only involved in the

biotransformation of benzo[a]pyrene by *Fusarium solani*. For other, the inhibition of these enzymes, which use H₂O₂ as cosubstrat, might have resulted in an increase of hydrogen peroxide availability in the fungal culture and therefore might have stimulated benzo[a]pyrene biotransformation by *F. solani*. Further research is in progress to determinate if this original metabolic pathway used by this fungus is due not only to non-hemoproteins enzymes but also to an increase of Fenton-like reactions correlated with H₂O₂ concentration in fungal cultures.

Keywords: benzo[a]pyrene, *Fusarium solani*, degradation, hydrogen peroxide.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed and relocated in the environment as a result of the incomplete combustion of organic matter, emission sources, automobile exhausts, stationary matter (e.g. coal-fired, electricity-generating power plants), domestic matter (e.g. tobacco smoke and residential wood or coal combustion), area source matter (e.g. forest fires and agricultural burning) and also in food. These compounds represent an important class of environmental pollutants, of which some are known to be mutagenic and carcinogenic [1]. Due to their hydrophobicity, most PAHs especially high-molecular-weight ones such as pyrene, benzo[a]anthracene and benzo[a]pyrene (BaP) persist in soils and sediments and therefore are less available for biological uptake. Among the processes whereby these compounds are removed from the environment, microbial degradation plays a major role in the remediation of contaminated sites [2, 3]. Numerous previous studies focused on the ability of bacteria and fungi to partially degrade, cometabolically oxidize, or mineralise high-molecular-weight PAHs. In soils, due to their mycelial growth habit, filamentous fungi might have a competitive advantage over bacteria especially with respect to the colonization of insoluble substrates. Moreover, the high surface-to-cell ratio of filaments maximizes both mechanical and enzymatic contact with the environment [4]. We focused therefore on isolating telluric fungi and evaluating their degradative ability on benzo[a]pyrene, a high molecular weight PAH, for clean up of coal tar contaminated soils.

In previous studies, we reported that telluric fungal strains isolated from fuel-contaminated compost showed interesting potential for soil bioremediation. In particular, we isolated a Deuteromycete fungus *Fusarium solani* that was able to grow in liquid medium with BaP as sole carbon source and to mineralize this PAH [5]. In a batch fermentor, [7,10-¹⁴C]benzo[a]pyrene mineralization occurred rapidly at early stages of fermentation (15 hr) during the germination of fungal spores and was not a continuous process as mineralization occurred in a biphasic pattern [6]. Moreover, *F. solani* showed an average rate of mineralization about 65 µg/g dry weight/day within 11 days of incubation, comparable as mineralization rate obtained with a white rot fungus *Phanerochaete chrysosporium* [7].

Our objective in the research described herein was to elucidate the enzymatic mechanisms involved in the successive steps of BaP degradation by *Fusarium solani*. Therefore we developed an indirect approach by using inhibitors of degradative enzymes. As is well known, two main enzyme groups are involved in the initial attack on PAHs by fungi: the intracellular cytochrome P-450 monooxygenases and the extracellular lignin peroxidases [8]. We used either specific inhibitors of peroxidases or of

cytochrome P-450 or inhibitors of both enzymes. Our preliminary results of the BaP degradation pathway of *F. solani* were discussed herein.

MATERIALS AND METHODS

Chemicals

Benzo[a]pyrene (BaP) was obtained from Acros Organics (Noisy-Le-Grand, France). A 16 EPA-PAH kit was purchased from Restek (Evry, France). Dichloromethane (DCM), N-, N'-dimethylformamide (DMF), ethyl acetate, other solvents and chemicals, except when specified, were obtained in the highest purity available from Merck (Darmstadt, Germany). All the solvents we used were high-performance chromatography (HPLC). All other chemicals were of the highest purity available. The inhibitors used (cyclopropylamine, salicylhydroxamic acid, potassium cyanide, piperonyl butoxyde, butylated hydroxytoluene) were purchased from Sigma Aldrich (St Quentin Fallavier, France).

TABLE 1. Compounds used for BaP degradation studies in presence of *Fusarium solani*

Abbreviation	Compound	[C] mM	Activity
CYC	Cyclopropylamine	1	Inhibition of Cytochrome P-450 [9]
SAL	Salicylhydroxamic acid	5	Specific peroxidases inhibitor [10]
KCN	Potassium cyanide	5	Both enzymes (P-450 and peroxidases) inhibitor [9]
PIP	Piperonyl butoxyde	1	Both enzymes (P-450 and peroxidases) inhibitor [9, 11]
BHT	Butylated hydroxytoluene	5	P-450 inhibitor and antioxidant [9, 12]

Media

Malt Yeast Extract Agar (MYEA) medium contained 1.8% Malt extract, 0.2% Yeast Extract and 1.5% Agar. The standard basal medium used for the benzo[a]pyrene degradation studies was the Mineral salts Medium (MM) [6] consisting of (g.l⁻¹): glucose, 10.0; KCl, 0.25; NaH₂PO₄·2H₂O, 6.47; Na₂HPO₄·7H₂O 15.68; MgSO₄·7H₂O, 0.5; NH₄NO₃, 1; ZnSO₄·7H₂O, 0.1; MnCl₂·4H₂O, 0.01; FeSO₄·7H₂O, 0.1; CuSO₄·5H₂O, 0.05; CoCl₂·6H₂O, 0.01; MoO₃, 0.02, chloramphenicol, 0.5.

Organism

This study was carried out with *Fusarium solani* F33 previously isolated from petroleum-contaminated soil [5]. Stock cultures were maintained on malt yeast extract agar slants at 18°C and subcultured every 3 months.

BAP degradation in liquid cultures

Growth medium was prepared by adding BaP (5 10⁻⁴ mol.l⁻¹) dissolved in 1 ml DMF in 250-ml empty Erlenmeyer flasks. After total evaporation of the organic solvent, the MM- medium (50 ml per flask) was added. The flasks were sterilized at 121°C for 20 min. For inhibitor experiments, each compound

described in Table 1 was added to MM-medium. In control biodegradation treatment, no inhibitor was used. Inoculation was performed by adding a spore suspension of *Fusarium solani* (aged 10 days), prepared as described previously [5], so as to obtain a final concentration of 10^4 spores per ml of culture medium. To detect abiotic degradation of benzo[a]pyrene, flasks without fungi were prepared and processed analogously. In order to detect adsorbed BaP on fungal hyphae (extraction controls), blank flasks (containing 50 ml MM without BaP) were inoculated with *F. solani*. At the end of incubation (15 days), the obtained mycelia were suspended in 50 ml MM with BaP ($5 \cdot 10^{-4}$ mol.l⁻¹), stirred for 4 hr with reciprocation shaking (90 min^{-1}) at 4°C until equilibrium was reached. These treatments allowed us to determine the adsorption processes on hyphae, used in further calculations of BaP degradation. All treatments were incubated at 25°C with a 12 h photoperiod for 14 days with reciprocation shaking (Laboshake, Gerhardt, 90 min^{-1}). Triplicates were run for each treatment.

BaP extraction

At the end of incubation, the cultures were lyophilized during three days. Total lyophilized cultures were scraped and extracted for 16 h in a Soxhlet apparatus with DCM in order to remove adsorbed and non-metabolized BaP on hyphae. Organic fractions were concentrated in 100 ml DCM/ethyl acetate (50:50, v/v). BaP concentrations were determined by HPLC.

HPLC analysis

BaP concentrations were measured on a Waters 2690 system fitted with a Waters Symmetry^R, C18, 5 μm , 100 Å column and a Waters 996 Photo Diode Array Detector. The separation was achieved with a 5 min linear gradient of acetonitrile/water (60:40 to 100:0, v/v) at a solvent flow rate of 0.3 ml.min⁻¹. Concentrations were determined by UV absorbance at 254 nm.

The percentage of benzo[a]pyrene biodegradation was given by the formula:

$$\left[\frac{(m_{\text{EC}} - m_{\text{T}})}{m_{\text{EC}}} \right] \cdot 100$$
, in which m_{EC} was the quantity of BaP obtained in extraction controls (taking account adsorption processes on hyphae) and m_{T} was the quantity of BaP obtained in each treatment. The mean (m) and the standard deviation (SD) were calculated and fitted in Figure 1 according to the formula $m \pm \text{SD}$.

RESULTS AND DISCUSSION

The purpose of this study was to investigate the metabolization of BaP by a Deuteromycete fungus *F. solani* in liquid medium. No BaP degradation was recovered from abiotic control flasks that were incubated with BaP but without mycelium. Moreover, in order to estimate BaP biodegradation by this fungus, percentage degradations were calculated taking account of PAH sorption phenomena obtained in cultures conducted with the intact fungus (extraction controls). In fact, these sorption phenomena to fungal mycelium are known to be significant factors in PAH disappearance in cultures [13]. Therefore,

PAH disappearance in inoculated treatment was interpreted as biodegradation, since the decrease in PAH concentration exceeded the amount that disappear from abiotic and extraction controls.

In treatment without inhibitors, weak degradation percentages of BaP were obtained with *F. solani* (7 %) after 15 days of incubation. Similar experiments were performed to assess the effects upon BaP degradation with different inhibitors (Fig. 1). Surprisingly, no decrease of BaP degradation was observed with most compounds used. The specific cytochrome P-450 inhibitor, cyclopropylamine and the peroxidases inhibitor, salicylhydroxamic acid, did not inhibit BaP degradation by *F. solani*, but on the contrary, more BaP was degraded (about 11.2% and 13.8% respectively). According to literature, these results were unexpected. Indeed, extensive work conducted on PAH degradation by fungi clearly underlined the involvement, in the initial attack on PAHs by fungi, of these both enzyme systems, either cytochrome P-450 monooxygenase, as described in mammalian PAH metabolism, or peroxidases [8, 14]. The extracellular lignin peroxidase enzymes, produced by white rot fungi (e.g. *Phanerochaete*, *Trametes*, *Bjerkandera*) have been shown to oxidize various organopollutants with structural similarities to lignin (e.g. PAH such as phenanthrene, pyrene, benzo[a]pyrene ...) [15]. In our study, the inhibition of either enzyme system stimulated BaP degradation. In addition, the common feature of both of these enzyme systems is the requirement of hydrogen peroxide. Indeed, cytochrome P-450 could also act as a peroxidase by catalysing hydroperoxide-dependent substrate oxidation [16]. The use of cyclopropylamine and salicylhydroxamic acid could therefore cause an increase of hydrogen peroxide availability in the fungal culture.

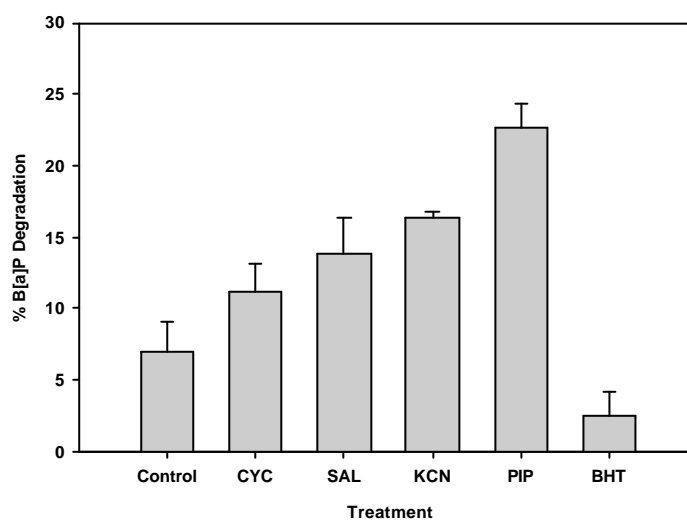


FIGURE 1. Benzo[a]pyrene degradation by *Fusarium solani* in Mineral salts Medium supplemented with $5 \cdot 10^{-4}$ mol.l⁻¹ BaP during 14 days of incubation, either in absence (control) or in presence of different heme-protein inhibitors (abbreviations are detailed in Table 1). Results are expressed in mean \pm standard deviation of BaP degradation determined in triplicates.

This hypothesis was further investigated by experiments conducted with two other inhibitors: potassium cyanide, which was known as an inhibitor of all heme-containing proteins (including therefore peroxidases and cytochrome P-450) and piperonyl butoxyde. This last compound was particularly interesting due to its capacity to inhibit both mechanisms of cytochrome P-450, acting either as a

NADPH-dependent peroxidase or as a peroxygenase [16]. Moreover, this compound was also recently described as an inhibitor of peroxidases [11]. As expected, potassium cyanide and piperonyl butoxyde stimulated significantly BaP degradation with 16% and 22% degradation rates obtained respectively. Consequently, it might seem again that an inactivation of H_2O_2 -dependent enzymes was correlated with an enhancement of BaP degradation. This result, along with the previous described above (obtained with cyclopropylamine and salicylhydroxamic acid), suggested that the enhancement of hydrogen peroxide availability in the fungal cultures could play an important role in the degradation of benzo[a]pyrene. This increase could be a prerequisite for stimulation of an alternative metabolic pathway involved in BaP degradation by *Fusarium solani*. These results also suggested that this metabolic pathway was clearly independent of heme-containing enzymes.

At this time, we might hypothesize that the enhancement of H_2O_2 concentration could induce formation of reactive oxygen species (ROS), such as hydroxyl radicals ($^{\bullet}OH$), which might be the agents that initiate benzo[a]pyrene oxidation. The hypothesis that extracellular ROS, especially hydroxyl radicals ($^{\bullet}OH$), acting as small non-enzymatic agents are involved in wood decay by fungi, has been redeveloped in recent works, after having been somewhat neglected in the last twenty years, probably because of increasing interest in fungal peroxidases with ligninolytic properties [17]. It has been proposed that these fungi use a Fenton system ($Fe^{2+} + H_2O_2$) to generate hydroxyl radical ($^{\bullet}OH$) known as powerful oxidant. Our results suggested that a similar free radical generating system, correlated to the enhancement of H_2O_2 ratio in the fungal cultures, could be a key component in the BaP biodegradation by *F. solani* in experiments conducted with inhibitors. We illustrate this proposed metabolic pathway diagrammatically in Figure 2.

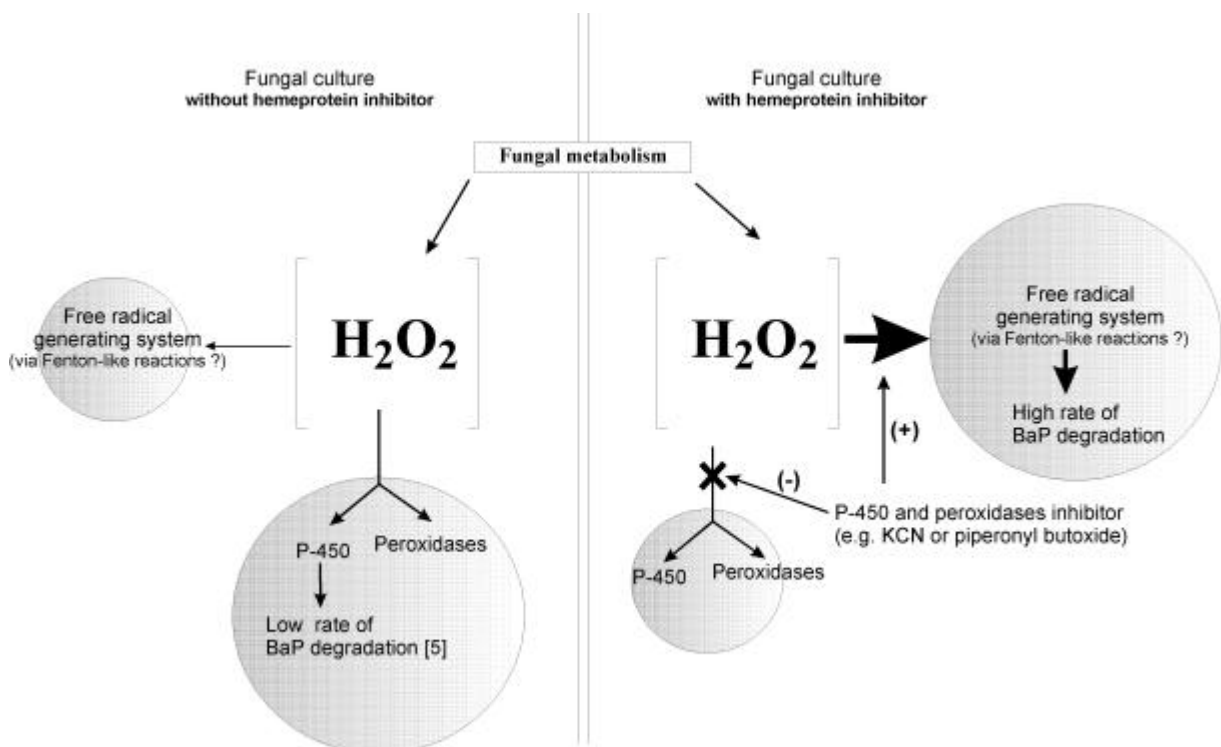


FIGURE 2. Hypothetical pathway for the benzo[a]pyrene degradation by *Fusarium solani*: (1) by enzymes (cytochrome P450 and peroxidases) and/or (2) by free radical generating system, either in absence or in presence of different hemeprotein inhibitors (explanations are detailed in the text). Surfaces of gray circles are proportional to the hydrogen peroxide consumption by the different systems (e.g. enzymes or free radical generating system).

This hypothesis was reinforced with degradation assays conducted with butylated hydroxytoluene, which is not only a specific cytochrome P-450 inhibitor but especially a free radical scavenger, commonly used as food preservative [12, 18]. In this case, the BaP degradation in cultures of *F. solani* incubated with butylated hydroxytoluene was inhibited and reduced to 2.5% degradation rate. This result would appear to suggest that the use of an antioxidant compound might eliminate ROS and subsequently their powerful oxidation potential of benzo[a]pyrene.

In conclusion, our study permitted to show a hypothetical alternative metabolic pathway, which is independent of classical hemeproteins enzyme systems (cytochrome P-450 and peroxidases) involved in PAH metabolism by fungi. This alternative metabolic pathway, based on free radicals production, might present two important advantages with regard to PAH degradation: firstly, the free-radical mechanism is highly non specific and nonstereoselective. This is precisely the type of mechanism that one would select for site decontamination, because contamination by only one compound is likely to be the exception rather than the rule [15]. Secondly, in our experiments, the percentage of benzo[a]pyrene degradation by *Fusarium solani* was significantly enhanced when this alternative metabolic pathway was stimulated. Our picture of fungal free radical degradation mechanism remains incomplete and requires further investigations to confirm this hypothetical mechanism. This free radical generating system might have potential use in a variety of pollution control applications.

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