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Metabolism of 2,4,6-trinitrotoluene by the white-rot fungus *Bjerkandera adusta* DSM 3375 depends on cytochrome *P*-450

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Abstract Degradation of 2,4,6-trinitrotoluene (TNT) by the white-rot fungus *Bjerkandera adusta* DSM 3375 was studied in relation to extracellular ligninolytic activities. The Mn(II)-dependent peroxidase, the only ligninolytic enzyme detectable, reached a maximum activity of 600 ± 159 U/l after incubation in mineral medium with a sufficient nitrogen source. In contrast, the highest extent of [^{14}C]TNT mineralization was detected in malt extract broth, so that the ability of *B. adusta* to mineralize TNT did not parallel ligninolytic activity. The microsomal fraction of cells grown in the presence of TNT was found to contain 11 pmol cytochrome *P*-450/mg protein. In cells grown without TNT, no microsomal cytochrome *P*-450 could be found. Instead, 14 pmol *P*-450/mg protein was present in the cytosolic fraction of these cells. Cytochrome *P*-450 apparently affected the TNT metabolism, as shown by inhibitory studies. Addition of the cytochrome *P*-450 inhibitor piperonyl butoxide diminished the $^{14}CO_2$ release from 21% to 0.9%, as determined after 23 days of incubation, while 1-aminobenzotriazole and metyrapone decreased the mineralization to 8.6% and 6.3% respectively. Mass-balance analysis of TNT degradation in liquid cultures revealed that, by inhibition of cytochrome *P*-450, the TNT-derived radioactivity associated with biomass and with polar, water-soluble metabolites decreased from 93.9% to 15.0% and the fraction of radiolabelled metabolites extractable with organic solvents fell to 92.6%. The TNT metabolites of this fraction were identified as aminodinitrotoluenes, indicating that this initial transformation product of TNT may function as a substrate for cytochrome-*P*-450-dependent reactions in *B. adusta*.

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Introduction

The area of former production plants for 2,4,6-trinitrotoluene (TNT) is often contaminated with this explosive and its precursors, which are known to be toxic (Koss et al. 1989) and mutagenic (Won et al. 1976). Although the three nitro groups of TNT cause this aromatic compound to be highly persistent in natural environments, transformations of TNT have been observed; they are catalysed by bacteria under aerobic (McCormick et al. 1976; Boopathy et al. 1994) as well as under anaerobic (Boopathy and Kulpa 1992; Preuss et al. 1993) conditions. Moreover, the white-rot fungus *Phanerochaete chrysosporium* was shown to mineralize TNT to CO_2 (Fernando et al. 1990). Further studies revealed that degradation by *Ph. chrysosporium* occurs to a higher extent when cultivation allows the expression of the extracellular, ligninolytic enzyme system, indicating the involvement of lignin peroxidase in the breakdown of TNT (Michels and Gottschalk 1994). The expression in *Ph. chrysosporium* of this enzyme system with lignin peroxidase and Mn(II)-dependent peroxidase as major constituents depends on culture conditions (Jeffries et al. 1981; Kirk et al. 1978) and is highly species-specific (Waldner et al. 1988; Kaal et al. 1993). Also the Mn(II)-dependent peroxidase of the white-rot basidiomycete *Phlebia radiata* has been shown to be involved in the oxidation of 4-hydroxylamino-2,6-dinitrotoluene, an intermediate of TNT breakdown (Van-Aken et al. 1999).

Although the high degradative capability of white-rot fungi has often been related to the activity of their ligninolytic enzyme system, breakdown of pentachlorophenol (Mileski et al. 1988), 2,4,5-trichlorophenoxyacetic acid

(Yadav and Reddy 1992), benzene and other aromatic compounds (Yadav and Reddy 1993) by *Ph. chrysosporium* could not be attributed to this extracellular activity, suggesting the involvement of an intracellular enzyme. Cytochrome *P*-450 monooxygenase is a well known and extensively studied enzyme in mammalian liver and in other eukaryotes and prokaryotes with the ability to oxidize several xenobiotics (Bernhardt 1995). The first fungal cytochrome *P*-450 was detected in *Cunninghamella bainieri* (Ferris et al. 1976), and the hydroxylation of aryl hydrocarbons was attributed to the activity of this cytochrome. In white-rot fungi, *P*-450 enzymes have been shown to be involved in the degradation of phenanthrene by *Pleurotus ostreatus* (Bezalel et al. 1997), atrazine by *Pl. pulmonarius* (Masaphy et al. 1996a) and benzo[*a*]pyrene by *Ph. chrysosporium* (Masaphy et al. 1996b).

In this study we show that the white-rot fungus *Bjerkandera adusta* is able to mineralize TNT to a considerable extent and that mineralization does not involve extracellular enzymatic activities but most likely intracellular cytochrome *P*-450.

Materials and methods

Chemicals

Unlabelled 2,4,6-trinitrotoluene was obtained from Dynamit Nobel AG (Troisdorf, Germany). 2-Amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were obtained from the Institute for Organic Chemistry of the Georg-August University (Göttingen, Germany). Malt extract was purchased from Merck (Darmstadt, Germany). Vanillylacetone, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate), piperonyl butoxide, 1-aminobenzotriazole and metyrapone were from Aldrich (Steinheim, Germany). Uniformly ^{14}C-labelled glucose was from Amersham Buchler GmbH (Braunschweig, Germany), ring-labelled [^{14}C]TNT and ^{14}C-labelled aminodinitrotoluene were synthesized as described earlier (Michels and Gottschalk 1993).

Microorganism and culture conditions

B. adusta DSM 3375 was maintained at 30 °C on 2% (w/v) malt-extract/agar plates. Mineral media were prepared according to Tien and Kirk (1988) but were modified by raising the concentration of ammonium tartrate under nitrogen-limited conditions to 0.44 g/l and by leaving out veratryl alcohol. For incubation in nitrogen-sufficient medium without manganese, the stock solution of trace elements was prepared without $MnSO_4$. The complex medium consisted of 2% (w/v) malt extract. Experiments were performed in 100-ml or 2-l conical flasks containing 10 ml or 800 ml medium under an atmosphere of air. For radioactive experiments the flasks were sealed with a screw cap and were flushed with air every 3–4 days. Inoculation of liquid cultures was done with an agar plug obtained from the outer layer of a 6- to 8-day-old culture grown at 30 °C on malt-extract/agar plates. Incubation of all cultures was done on a rotary shaker (120 rpm, 2.5 cm in diameter) at 30 °C.

Enzyme assays

All enzymes were determined spectrophotometrically (UVikon 710, Kontron Instruments, Neufahrn, Germany) at 30 °C. Lignin peroxidase activity was measured according to Tien and Kirk (1988) by monitoring the oxidation of veratryl alcohol to veratryl aldehyde. Mn(II)-dependent peroxidase was assayed as described by Paszczynski et al. (1986) with vanillylacetone [4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one] as substrate and Mn(II)-independent peroxidase as described by de Jong et al. (1992) following the oxidation of phenol red. Aryl alcohol oxidase activity was measured according to Kimura et al. (1990) by monitoring the oxidation of veratryl alcohol in the absence of H_2O_2. The oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) was recorded as a measure of laccase activity (Bourbonnais and Paice 1990). Measurements of the five different enzymes in the culture supernatant were done over the course of 18 days. The sample volume withdrawn daily from the supernatant was 50 µl in each case.

Mineralization of [^{14}C]TNT and [^{14}C]glucose

On day 6 of the incubation, 30.5 nCi ring-labelled [^{14}C]TNT (87 µM) and 18.2 nCi uniformly labelled [^{14}C]glucose (111 µM) were added to 10-ml cultures in 100-ml conical flasks. The $^{14}CO_2$ released was trapped in an alkaline scintillation cocktail by flushing the cultures with air every 3–4 days as described previously (Michels and Gottschalk 1994). For cytochrome *P*-450 inhibition studies, 1-aminobenzotriazole (700 µM), metyrapone (566 µM) or piperonyl butoxide (87 µM) was added 30 min before the radioactive substrate.

Determination of cytochrome *P*-450

The biomass was filtered after growth and washed with 0.9% (w/v) NaCl. The mycelium was then resuspended at a 1:5 (w/v) dilution in buffer A (20 mM TRIS/HCl, pH 8.2, 10% glycerol w/v, 1 mM EDTA, 0.5 mM dithioerythritol, 0.5 mM phenylmethylsulfonyl fluoride) and mechanically disrupted with an Ultra Turrax homogeniser (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at half maximal velocity (8000–9500 U/min). After cell breakage by French pressing (5.52×10^6 Pa; SLM, Aminco Instruments Inc., Rochester, USA) cell debris and mitochondria were removed by centrifugation at 6000 *g* for 10 min and at 25 000 *g* for 30 min. Separation of the cytosolic and microsomal fractions was performed by ultracentrifugation at 250 000 *g* for 1 h. After washing, the microsomal pellet was resuspended in buffer A. The determinations of cytochrome *P*-450 and of the protein concentration were performed as described earlier (Omura and Sato 1964; Bradford 1976). Spectrophotometric studies were carried out on a Uvicon 941 Plus double-beam spectrophotometer (Kontron AG, Zürich, Schweiz).

Mass-balance experiments

Mass-balance analysis of liquid cultures was performed 12 days after 30.5 nCi ring-labelled [^{14}C]TNT and piperonyl butoxide (each 87 µM) had been added to 6-day-old mycelia. For extraction, mycelia of a 10-ml malt extract culture were blended with a dispenser (IKA Ultra Turrax T25, Janke & Kunkel, Staufen, Germany) at half-maximal speed and the resulting homogenate was extracted twice with 2 ml hexane. The remaining aqueous phase was acidified to pH 1.5 with 6 M HCl and then extracted with 2 ml methylene chloride three times. The biomass was separated from the aqueous layer by filtration through a glass-fiber filter and was dried for quantification at 100 °C for 1 h. Radioactivity incorporated into the biomass was determined by combustion as described (Amato 1983). The hexane and methylene chloride extracts were separately pooled and dried under a gentle stream of nitrogen and the material remaining was dissolved in 0.5 ml methanol. Aliquots of 50 µl were taken from each extract and from the aqueous phase for liquid scintillation counting in 6 ml Opti-Fluor (Packard, Frankfurt, Germany).

Analysis of TNT metabolites

The hexane and methylene chloride extracts of cultures grown in the presence of the inhibitor piperonyl butoxide were analyzed by

HPLC. The HPLC system was equipped with a reversed-phase column (C_{18}, 5 mm, 250 × 4 mm; Macherey & Nagel, Düren, Germany) and was adjusted to a flow rate of 0.6 ml/min. Elution was done with a linear gradient of methanol from 32% to 100% (0–20 min) followed by isocratic elution with 100% methanol (20–25 min). Then linear elution was done from 100% to 32% (v/v) methanol (25–40 min), followed by isocratic elution at 32% (v/v) methanol (40–60 min). Absorption was recorded at 254 nm. Samples comprising 10 µl hexane extract and 25 µl methylene chloride extract, each diluted 1:4 with methanol, were injected for analysis. Fractions (0.6 ml) were collected at 60-s intervals and were measured by liquid scintillation counting. Identification of 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene was done by comparison of retention times to those of standard substances. The control experiment was performed in a 10-ml malt extract culture, incubated for 6 days and autoclaved before piperonyl butoxide (87 µM) and ^{14}C-labelled aminodinitrotoluene (100 µM, as a mixture of both isomers) were added. After 3 days of incubation, extraction with hexane and methylene chloride followed, as described above for mass-balance analysis.

Results

Enzymatic activity in agitated cultures of *B. adusta*

The activity of five extracellular enzymes, of lignin peroxidase: Mn(II)-dependent and Mn(II)-independent peroxidase, aryl-alcohol oxidase and laccase, was tested in liquid cultures of *B. adusta* DSM 3375. The strain was cultivated in nitrogen-sufficient or -limited medium as well as in malt extract broth. During the entire incubation period of 18 days the only detectable enzyme was the Mn(II)-dependent peroxidase (Fig. 1). This enzyme reached the highest activity of 600 ± 159 U/l at day 8 in mineral medium with sufficient nitrogen. In none of the other culture media did Mn(II)-dependent peroxidase attain comparable activities. Cultivation of the fungus in a manganese-deficient, nitrogen-rich medium resulted in the complete absence of detectable peroxidase activity.

Mineralization experiments

Since the extent of TNT mineralization by *Ph. chrysosporium* is strongly enhanced by the extracellular enzyme system (Michels and Gottschalk 1994), the effect of the Mn(II)-dependent peroxidase on the [^{14}C]TNT mineralization efficiency of *B. adusta* was tested. The cumulative $^{14}CO_2$ release from [^{14}C]TNT (87 µM) under various culture conditions is depicted in Fig. 2. In malt extract broth 11.7% of the $^{14}CO_2$ was released 6 days after addition of [^{14}C]TNT, while in mineral media the values were 5.1%–7.4% $^{14}CO_2$. A significant difference in TNT mineralization could not be detected in media with high or with completely repressed activity of Mn(II)-dependent peroxidase.

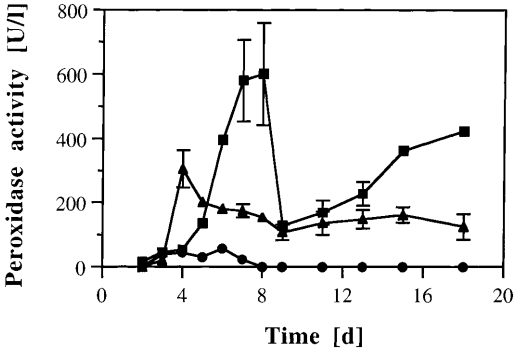

Fig. 1 Time course of Mn(II)-dependent peroxidase activity in the culture supernatant. Incubation in nitrogen-sufficient (■), nitrogen-limited (●) or malt extract medium (▲)

Intracellular localization of cytochrome *P*-450

Known extracellular enzyme activities could not be correlated with the rate and extent of $^{14}CO_2$ release from TNT and intracellular TNT oxidation had to be taken into account. Therefore, the cytochrome *P*-450 content in the microsomal and cytosolic fractions of cells grown with or without TNT was determined by carbon monoxide difference spectra. The microsomal fraction of cells grown with TNT present was fount to contain 11 pmol cytochrome *P*-450 mg^{-1} protein, while *P*-450 was not detectable in the cytosol (Fig. 3). In cells grown without TNT, however, the *P*-450 content in the microsomal fraction was below the limit of detection. Instead, 14 pmol cytochrome *P*-450 mg protein^{-1} was found in the cytosolic fraction (data not shown). We also observed, for both culture conditions, a major peak at 420 nm in the microsomal and cytosolic fractions, which may represent other haem proteins or degradation products of cytochrome *P*-450.

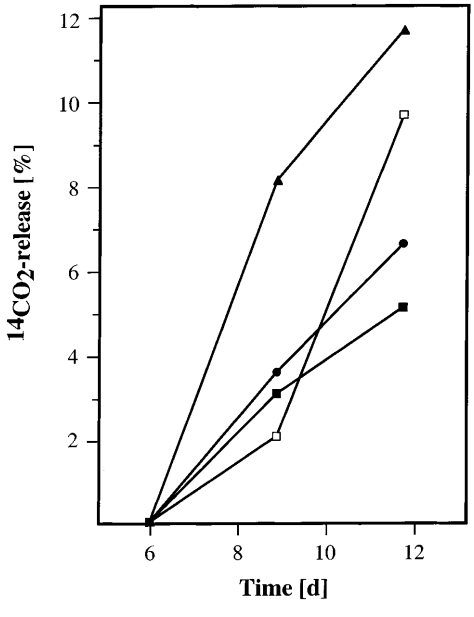

Fig. 2 Mineralization of 30.5 nCi [^{14}C]trinitrotoluene (87 µM) under various culture conditions: in nitrogen-sufficient (■) or nitrogen-limited (●) mineral medium with Mn(II), in nitrogen-sufficient, Mn(II)-free mineral medium (□) and in malt extract broth (▲). The radiolabelled substrate was added on day 6 of incubation

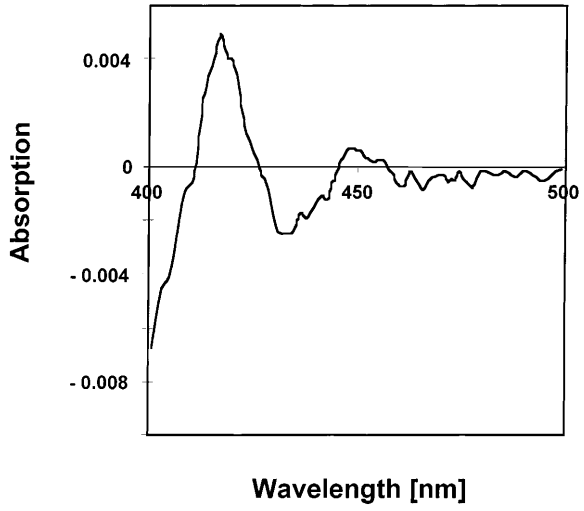

Fig. 3 The reduced carbon monoxide difference spectrum of the microsomal fraction of *Bjerkandera adusta* grown in the presence of trinitrotoluene indicating the presence of cytochrome *P*-450

Mineralization of TNT in the presence of inhibitors of cytochrome *P*–450

Because of the presence of cytochrome *P*-450 in *B. adusta*, we tested whether inhibitors of the intracellular haemoprotein cytochrome *P*-450 affected the mineralization of TNT. As shown in Fig. 4A, the cumulative mineralization decreased from 21% $^{14}CO_2$ on day 23 of incubation to 8.6%, 6.3% and 0.9% in the presence of the cytochrome-*P*-450-specific inhibitors 1-aminobenzotriazole, metyrapone and piperonyl butoxide respectively. In contrast, these inhibitory effects could not be detected during mineralization of [^{14}C]glucose, as is apparent from Fig. 4B. The $^{14}CO_2$ release of 39.7% within 23 days remained unaffected by the addition of 1-aminobenzotriazole or metyrapone; piperonyl butoxide, however, caused an enhanced $^{14}CO_2$ release of 46.1% from [^{14}C]glucose within the same period of time.

Mass-balance analysis of TNT metabolism

[^{14}C]TNT was added to cultures of *B. adusta*, which were then subjected to an extraction and separation procedure immediately after the addition of [^{14}C]TNT and after 12 days. The results are summarized in Table 1. The control experiment showed that TNT was completely removed from the aqueous system by organic solvent extraction. After 12 days, TNT or the corresponding apolar degradation products were hardly present, and the radioactivity resided in water-soluble products or in the cellular components, 13.3% being present in the form of $^{14}CO_2$. Piperonyl butoxide not only inhibited the total oxidation of TNT to CO_2 but also the formation of polar compounds. HPLC analysis revealed that 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene accumulated under these conditions.

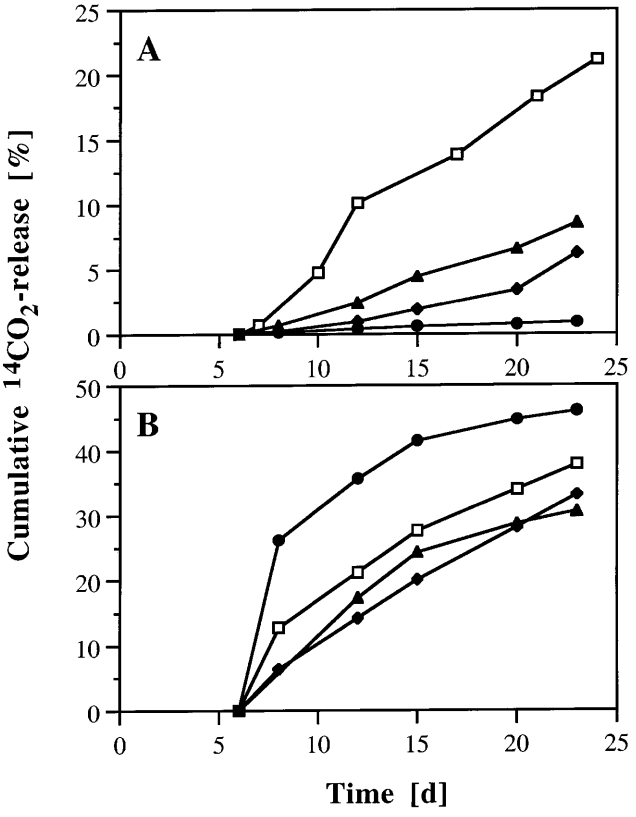

Fig. 4A, B Effect of cytochrome *P*-450 inhibitors on the mineralization of (**A**) 30.5 nCi [^{14}C]trinitrotoluene (87 μM) and (**B**) 18.2 nCi [^{14}C]glucose (111 μM), in malt extract broth without inhibitors (□), and with the addition of 700 μM 1-aminobenzotiazole (◆), 87 μM piperonyl butoxide (●) or 566 μM metyrapone (▲). The labelled substrates as well as the inhibitors were added on day 6 of the incubation

Discussion

Early investigations on the ligninolytic system of white-rot fungi such as *Ph. chrysosporium* indicated that this enzyme system is expressed under nutrient starvation, e.g. nitrogen limitation (Jeffries et al. 1981). Later it was shown for several *Bjerkandera* strains that extracellular

Table 1 Percentage of radioactivity recovered in various fractions obtained after degradation of [^{14}C]trinitrotoluene (*TNT*) by *Bjerkandera adusta* DSM 3375. The control was obtained by extracting a 10-ml culture immediately after addition of [^{14}C]TNT. Test cultures were extracted 12 days after addition of [^{14}C]TNT

Fraction	Radioactivity recovered (%)		
	Control	TNT	TNT + piperonyl butoxide
Hexane	62.2	< 0.1	47.9
Methylene chloride	25.5	3.9	44.7
Aqueous	< 0.1	38	1.1
Biomass	1.0	55.9	13.9
CO_2	< 0.1	13.3	0.6
Total recovery	88.7	111.1	108.2

enzymes are not expressed under nitrogen starvation but under nitrogen-rich conditions (Kimura et al. 1990; Kaal et al. 1993). Under these conditions, Mn(II) was found to be the stimulatory element for the expression of Mn(II)-dependent peroxidase and lignin peroxidase (Mester et al. 1995). A stimulatory effect of Mn(II) on the activity of Mn(II)-dependent peroxidase in nitrogen-rich medium was also detected in our investigations with *B. adusta* DSM 3375, but neither lignin peroxidase nor aryl-alcohol oxidase nor Mn(II)-independent peroxidase activities could be found with our strain, in contrast to reports on other *Bjerkandera* strains (Muheim et al. 1990; de Jong et al. 1992), confirming the great diversity of extracellular enzymatic activities in white-rot fungi.

The mineralization of xenobiotics by white-rot fungi is often directly correlated with their ligninolytic activity (Bumpus and Aust 1987). But several results have indicated that even the model organism *Pl. chrysosporium* is able to metabolize xenobiotics like pentachlorophenol or 2,4,5-trichlorophenolic acid under conditions that are unfavourable for expression of extracellular ligninolytic enzymes (Mileski et al. 1988; Yadav and Reddy 1992, 1993). The findings of our study suggest that mineralization of TNT by *B. adusta* DSM 3375 is also not dependent on the expression of extracellular enzymes.

It has been shown, for several white-rot fungi, that cytochrome *P*-450 enzymes are involved in the degradation of xenobiotics like atrazine, phenanthrene or benzo[*a*]pyrene (Masaphy et al. 1996a, b; Bezalel et al. 1997). The atrazine degradation in *Pl. pulmonarius* depends on the Mn(II) concentration. Mn(II) enhances the interaction between atrazine and cytochrome *P*-450 and, therefore, increases the biotransformation of this xenobiotic. Furthermore, the cytochrome *P*-450 content in the microsomal and cytosolic fractions increases at elevated Mn(II) concentrations (Masaphy et al. 1996a). In *Trichosporon cutaneum*, the soluble cytochrome *P*-450 catalyses the conversion of salicylic acid to catechol. The cytochrome *P*-450 content in salicylate-grown cells is fivefold higher than that in cells grown without salicylate as the sole source of carbon and energy (Yang et al. 1997). Our spectroscopic data indicate that *P*-450 synthesis is also induced in *B. adusta*. While we were not able to detect microsomal *P*-450 in cells grown without TNT, we could clearly demonstrate the presence of this enzyme in the microsomal fraction of TNT-grown cells. On the other hand, the cytosolic cytochrome *P*-450 detectable in unstressed cells could not be identified in TNT-grown cells. This may be due to the toxic effects of TNT metabolites reported earlier (Spain 1995). Further experiments will have to elucidate the role of TNT in the induction of cytochrome *P*-450 synthesis and its localization in this organism.

Cytochrome *P*-450 participates in the initial steps of TNT reduction by microsomal fractions (Leung et al. 1995), but an involvement of cytochrome *P*-450 in the biological breakdown of TNT by white-rot fungi has not been reported before. The degradation of several xenobiotics by white-rot fungi, however, proceeds with the participation of this haemoprotein. A cytochrome-*P*-450-dependent enzyme is apparently involved in the biotransformation of the cyclodiene pesticide endosulfan (Kullman and Matsumura 1996), of phenanthrene (Sutherland et al. 1991) or benzo[*a*]pyrene (Masaphy et al. 1996b) by *Ph. chrysosporium* and in the mineralization of aromatic hydrocarbons by *Pl. ostreatus* (Bezalel et al. 1996). Spectroscopic evidence for various cytochrome *P*-450 isoenzymes indicates a substrate-specific involvement in degradation processes; over 300 isoenzymes have been characterized so far (Bernhardt 1995).

Addition of piperonyl butoxide to liquid cultures of *B. adusta* caused a complete inhibition of aminodinitrotoluene transformation, making it likely that 2-amino-, 4-aminodinitrotoluene or both are the substrates for cytochrome *P*-450. Although the electrophilic character of these compounds would prevent a direct hydroxylation on the aromatic ring system, as is known for polyaromatic hydrocarbons (Ferris et al. 1976), aminodinitrotoluene possesses several active sites that might react with a monooxygenase. Hydroxylation of amino groups (Thorgeirsson et al. 1973), oxidative demethylation (Czygan et al. 1973) and alkyl side-chain oxidation (Cox et al. 1996) are known reactions of cytochrome *P*-450. TNT oxidation in *B. adusta* with the likely participation of cytochrome *P*-450 leads to the formation of polar intermediates and further to CO_2. The structure of the radiolabelled compounds that accumulated in the aqueous fraction still remains to be elucidated.

The involvement of cytochrome *P*-450 in the degradation of TNT by *B. adusta* caused an almost complete metabolism of TNT, with only 3.9% remaining in the organic phase after 12 days of incubation. Such a high percentage has not been reported for the degradation of TNT by any other organism, demonstrating that cytochrome *P*-450 exhibits effective reactions leading to ring cleavage. Therefore, further investigations of TNT metabolism by *B. adusta* should focus on the cytochrome *P*-450 reaction products and ring-cleavage substrates. One advantage of the *B. adusta* system could be that not only the primary reduction steps but also the subsequent oxidation reactions take place intracellularly. This makes diffusion processes, in addition to the intitial TNT uptake, unnecessary.

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