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Degradation of 4-nitrophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*

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Abstract The fungal metabolism of 4-nitrophenol (4-NP) was investigated using the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Despite its phenolic feature, 4-NP was not oxidized by extracellular ligninolytic peroxidases. However, 4-NP was converted to 1,2-dimethoxy-4-nitrobenzene via intermediate formation of 4-nitroanisole by the fungus only under ligninolytic conditions. The metabolism proceeded via hydroxylation of the aromatic ring and methylation of phenolic hydroxyl groups. Although the involvement of nitroreductase in the metabolism of 2,4-dinitrotoluene by many aerobic and anaerobic microorganisms including *P. chrysosporium* has been reported, no formation of 4-aminophenol was observed during 4-NP metabolism. The formation of 1,2-dimethoxy-4-nitrobenzene was effectively inhibited by exogenously added piperonyl butoxide, a cytochrome P450 inhibitor, suggesting that cytochrome P450 is involved in the hydroxylation reaction. Thus, *P. chrysosporium* seems to utilize hydroxylation and methylation reactions to produce a more susceptible structure for an oxidative metabolic system.

Introduction

Nitroaromatic compounds, which are widely utilized as dyes, pesticides, insecticides, drugs and explosives (Hallas and Alexander 1983; Schackmann and Muller 1991; Spain et al. 2000), are categorized as environmental pollutants. Nitrophenols, consisting of a huge class of nitroaromatics, are known to accumulate in soil through hydrolysis of organoinsecticides, such as parathion or methylparathion, as well as through the direct use of other nitrophenol derivatives as herbicides. It has been pointed out that nitroaromatics can be widely spread into surface- and ground-water resources, where they cause deleterious effects to biological systems due to their acute toxicity (Spain et al. 2000). Thus, a safe and effective cleanup method for these pollutants should be developed. Bioremediation remains an attractive, albeit slow process, because of its advantages over conventional processes, i.e., minimal impact on the environment and cost effectiveness.

White-rot basidiomycetes are the only organisms known to unilaterally degrade all components of wood, including lignin—one of the most recalcitrant aromatic biomaterials on the earth (Gold et al. 1989; Kirk and Farrel 1987). These fungi are also known to degrade 2,4-dinitrotoluene (2,4-DNT), 2,4,6-trinitrotoluene (TNT), and a variety of polychlorinated phenols (Esteve-Nunez et al. 2001; Fernado et al. 1990; Reddy et al. 1998; Valli and Gold 1991; Valli et al. 1992). Fungal degradation of 2,4-DNT has been extensively studied using the basidiomycete *P. chrysosporium*, with the finding that complete mineralization is initiated by reduction of the nitro group to an amino group, followed by the action of a series of enzymes involved in aromatic degradation, such as peroxidases, quinone reductase, and dioxygenase (Valli et al. 1992). In the past decade, a tremendous amount of information on the fungal metabolism of aromatic compounds has been accumulated, suggesting that white-rot basidiomycetes possess a variety of enzymes to reduce the ionization potential of aromatic moieties (Wariishi 2000). Although nitrophenols contain the ionizable phenolic group, they are known to exhibit a high ionization potential (Li and Hoffman 1999; Ohkura and Hori 1999). Thus, this type of compound is recalcitrant to fungal degradation. In fact, no report on the fungal degradation of nitrophenols has so far been described.

In this study, we examined the ability of one of the best-studied white-rot fungi, *P. chrysosporium*, to degrade 4-nitrophenol (4-NP). 4-NP was not readily oxidized by extracellular peroxidases. Further metabolic studies revealed that *P. chrysosporium* completely degrades 4-NP via a cytochrome P450-mediated aromatic hydroxylation

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reaction, which seems to greatly reduce the ionization potential of 4-NP.

Materials and methods

Chemicals

4-NP, 4-nitroanisole (4-NA), 2,4-DNT, and 2-amino-4-nitrotoluene were obtained from Wako Pure Chemicals (Osaka, Japan). 1,2-Dimethoxy-4-nitrobenzene (DMNB) and 4-nitrocatechol (4-NC) were purchased from Tokyo Kasei (Tokyo, Japan). NADPH was obtained from Sigma (St. Louis, Mo.). All other chemicals were of reagent grade. Deionized water was obtained with a MilliQ Systems (Millipore, Bedford, Mass.).

Culture conditions

P. chrysosporium (ATCC 34541) was grown from conidial inocula at 37°C in a stationary culture under air. The medium (pH 4.5) used in this study was as previously described with 1% glucose (high concentration of carbon source; HC) and either 1.2 (low concentration of nitrogen source; LN) or 12 mM (high concentration of nitrogen source; HN) ammonium tartrate as carbon and nitrogen sources, respectively (Kirk et al. 1978).

Extracellular enzymes

Lignin and manganese peroxidases (LiP and MnP) and laccase were purified from the extracellular medium of *P. chrysosporium* and *Coriolus versicolor*, respectively, as previously described (Wariishi et al. 1987; Wariishi and Gold 1990). Enzymatic reactions were performed as previously reported at 30°C for 15 min (Ichinose et al. 1999; Johjima et al. 1999).

Metabolic reactions

After a 4-day preincubation, 4-NP in water (20 mM) was added to the cultures to a final concentration of 0.25 mM. After additional incubation, the metabolic products were analyzed either by HPLC after homogenization in acetone (the same volume as the medium), centrifugation (25,000 *g*), and filtration (0.45 μm) or by GC-MS after extraction with ethyl acetate (10 ml ×3) at pH 2 or 10, drying over $MgSO_4$, evaporation under N_2. Control experiments were performed using the fungus treated with sodium azide (1 mM) for 30 min prior to the addition of the substrate.

Co-metabolism of 4-NP and 2,4-DNT by *P. chrysosporium*

After a 4-day incubation under HCLN conditions, 4-NP and 2,4-DNT were added simultaneously to the cultures to a final concentration of 0.25 mM each. After additional incubation, the metabolites were analyzed by GCMS as described above.

Preparation of cell free extracts

After a 4-day incubation under HCHN conditions, the mycelial mat was separated from the medium by filtration, washed successively with chilled water, 0.5% (w/v) NaCl, and 100 mM potassium phosphate buffer (pH 7.5), then frozen under liquid nitrogen, and ground to a fine powder with a mortar and pestle (Munir et al. 2001; Rieble et al. 1994). The powder was resuspended in 100 mM potassium phosphate buffer (pH 7.5). Unbroken cells and cell debris were removed by centrifugation at 3,500 *g* for 10 min. The resultant supernatant was filtered through two layers of Miracloth (Calbiochem, San Diego, Calif.) and centrifuged at 17,000 *g* for 30 min.

Nitroreductase assay

Reaction mixture containing 500 μl crude extract, 0.5 mM NADPH, 0.2 mM substrate was incubated for 12 h at 30°C. After filtration (0.45 μm), HPLC analysis was performed. GCMS analysis was also carried out after extraction with ethyl acetate at pH 2 and 10, drying over $MgSO_4$, and evaporation under N_2. Control experiments were performed using reaction mixture without crude extracts or with boiled extracts.

Instrumentation

HPLC analysis was carried out using a Shimadzu STR ODS-II column (4.6×150 mm) with a linear gradient from 20% acetonitrile in water (0.05% phosphoric acid) (isocratic for 5 min) to 100% acetonitrile (21–31 min) at a flow rate of 1.0 ml/min. UV detection was performed at 264 and 316 nm. Products were quantified using calibration curves prepared with authentic standards. A preparative HPLC was carried out with an Inartsil PREP-ODS column (20×250 mm; GL Sciences, Tokyo, Japan) with acetonitrile/water at a flow rate 10 ml/min.

GCMS analysis was performed at 70 eV on a JEOL AMII-15A equipped with GC, and a 30-m fused silica column (DB-5, J & W Scientific, Tokyo, Japan). The oven temperature was programmed from 80 to 320°C at 8°C/min. Products were identified by comparison of their retention times on GC and HPLC and of mass fragmentation patterns with standards.

Results

Effect of extracellular ligninolytic enzymes on 4-NP

The effect of LiP, MnP, and laccase on 4-NP was examined. However, none of these enzymes oxidized 4-NP (data not shown). In the case of the LiP reaction system, the addition of veratryl alcohol or use of a large amount of the enzyme did not cause 4-NP oxidation. The addition of 1-hydroxybenztriazol, a laccase mediator, had no effect on 4-NP oxidation by laccase. Furthermore, 4-NA was not oxidized by LiP, MnP, and laccase.

Metabolism of 4-NP by *P. chrysosporium*

A time-course of the degradation of 4-NP by *P. chrysosporium* is shown in Fig. 1, indicating that 4-NP was degraded only under LN (ligninolytic) conditions, where the dry weight of fungal cells was 3-fold less than that under HN conditions. Subsequent metabolic studies were performed under LN conditions.

Under LN conditions, 4-NP was converted into two products, 4-NA and DMNB, by *P. chrysosporium* (Table 1, Fig. 1). These metabolic intermediates were then added as an exogenous substrate. When 4-NA was utilized as substrate, it was converted to DMNB with trace formation of 4-NP (Fig. 2a). 4-Aminophenol (4-AP) was not detected as a metabolite of either 4-NP or 4-NA. DMNB was metabolized but with no detection of further metabolites (Fig. 2b). A 3-day lag period was also observed for DMNB metabolism.

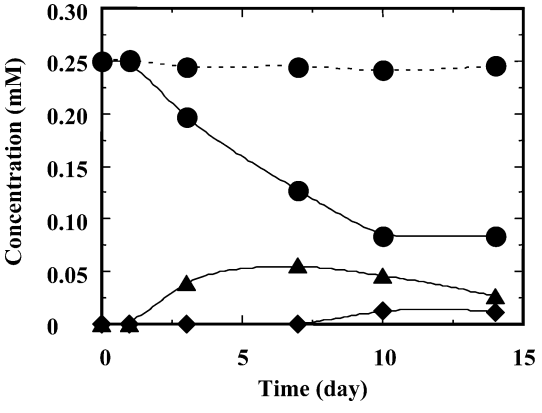

Fig. 1 Metabolism of 4-nitrophenol (4-NP) by *Phanerochaete chrysosporium* under high carbon-high nitrogen (HCHN) (*dashed line*) and high carbon-low nitrogen (HCLN) (*solid line*) conditions. The time-course of substrate disappearance and product formation was monitored using HPLC as described in the text: ● 4-NP, ▲ 4-NA 4-nitroanisole (4-NA), ◆ 1,2-dimethoxy-4-nitrobenzene (DMNB). Metabolic experiments were repeated three times. To better exhibit the quantitative relationship between the substrate and products, a typical result is shown in this figure. SD <11% (n=3)

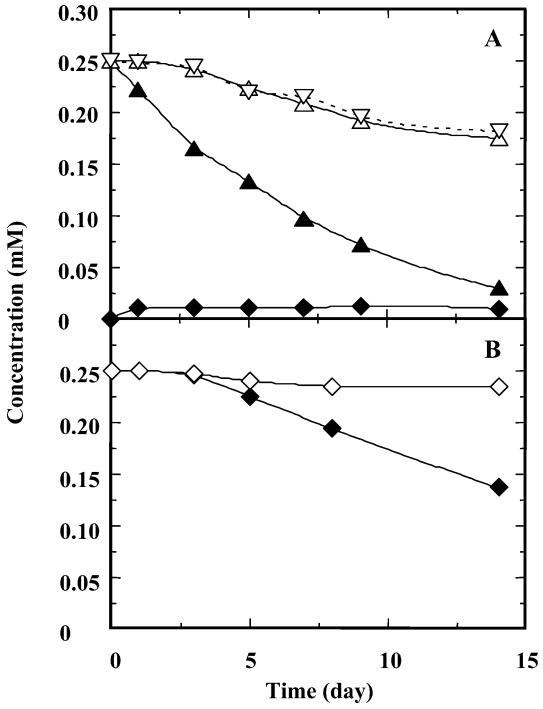

Fig. 2 Metabolism of **A** 4-NA and **B** DMNB by *P. chrysosporium* under HCLN conditions. The time-course of substrate disappearance and product formation were monitored using HPLC as described in the text: ▲ 4-NA, △ 4-NA (control, azide-treated cells), ▽ 4-NA (control without inoculation), ◆ DMNB, ◊ DMNB (control, azide-treated cells). Metabolic experiments were repeated three times. To better exhibit the quantitative relationship between the substrate and products, a typical result is shown in this figure. SD <9% (n=3)

Possible involvement of nitroreductase in 4-NP metabolism

Under conditions where 4-NP is metabolized, 2,4-DNT was fed to the *P. chrysosporium* culture, confirming the formation of 2-amino-4-nitrotoluene (Table 1). The existence of the corresponding enzyme, nitroreductase, has been shown for this fungus (Rieble et al. 1994; Stahl and Aust 1993, 1995). To determine the occurrence of the reduction of 4-NP to 4-AP under conditions where the known nitroreductase is expressed, 4-NP and 2,4-DNT were simultaneously added to the same culture. While 2,4-DNT was reduced to 2-amino-4-nitrotoluene, 4-NP was not converted to 4-AP. On the other hand, 4-NA and DMNB were identified as the metabolic products from 4-NP.

To further confirm the lack of 4-NP reduction by the nitroreductase involved in the 2,4-DNT reduction, cell-free extract was utilized. The cell-free extract, which causes reduction of 2,4-DNT to 2-amino-4-nitrotoluene in the presence of NADPH as previously described (Rieble et al. 1994; Stahl and Aust 1993, 1995), did not catalyze reduction of 4-NP (data not shown).

Table 1 Mass spectra and GC retention times of substrates and the fungal metabolites

Compounds	GC retention time (min)	Mass spectrum m/z (relative intensity)
4-Nitrophenol	15.0	139 M^+ (41.0), 109 (39.2), 93 (21.2), 81 (31.9), 74 (6.0), 65 (100)
4-Nitroanisole	12.4	153 M^+ (78.1), 123 (60.1), 107 (9.9), 92 (89.2), 77 (100), 64 (65.6)
1,2-Dimethoxy-4-nitrobenzene	15.6	183 M^+ (100), 153 (19.5), 107 (44.6), 79 (95.5)
2,4-Dinitrotoluene	14.4	182 M^+ (1.8), 165 (86.0), 119 (27.0), 89 (100), 78 (18.3), 63 (58.0)
2-Amino-4-nitrotoluene	15.5	152 M^+ (78.1), 122 (7.5), 106 (99.0), 94 (20.6), 77 (100)

Effect of cytochrome P450 inhibitor on 4-NP metabolism by *P. chrysosporium*

The effect of a cytochrome P450 inhibitor, piperonyl butoxide (PB), on degradation of 4-NP by *P. chrysosporium* was investigated. Table 2 clearly shows a concentration-dependent inhibition of the disappearance of 4-NP and formation of DMNB. To eliminate the possibility of PB inhibition of the total activity of the fungus, 2,4-DNT was utilized as a substrate, since no involvement of cytochrome P450 has been reported in the metabolism of this compound by *P. chrysosporium* (Valli et al. 1992). The efficiency of 2,4-DNT degradation was the same in either the presence or the absence of 2.5 μM PB (Table 2).

Discussion

No oxidation of 4-NP by either LiP or MnP

Ligninolytic fungi are known to secrete unique, one-electron oxidizing enzymes, such as LiP and MnP, and laccase (Gold et al. 1989; Kirk and Farrel 1987; Wariishi et al. 1987). These extracellular enzymes are also known to play an important role in the degradation of a wide variety of phenolic pollutants (Hirano et al. 2000; Joshi and Gold 1993; Reddy et al. 1998; Valli and Gold 1991). The effect of LiP, MnP, and laccase on 4-NP was examined, and it was found that none of these enzymes were capable of oxidizing 4-NP under physiological conditions. The ionization potential of phenols is known to be affected by substituents. Since a nitro group is a well-known strong electron-withdrawing substituent (Li and Hoffman 1999; Ohkura and Hori 1999), the redox potential of 4-NP seems to be higher than the oxidized intermediates of either LiP or MnP. *P. chrysosporium* was, however, shown to metabolize pentachlorophenol under conditions that are unfavorable for the expression of LiP and MnP (Mileski et al. 1988). Thus, fungal metabolism of 4-NP was examined.

Table 2 Inhibitory effect of piperonyl butoxide (PB) on fungal metabolism of 4-NP and 2,4-DNT[a]

PB[b] (μM)	Substrate remained (μM)		Product formed (μM)
	4-NP	2,4-DNT	DMNB
0	91[c]	22	20
0.25	176	18	16
2.5	235	24	0

[a] *P. chrysosporium* was preincubated in HCLN cultures for 4 days. Substrates (4-NP or 2,4-DNT) were separately added to different cultures with a final concentration of 0.25 mM. After a 10-day incubation, 4-NP remaining and DMNB formed were quantified using HPLC. After a 5-day incubation, the remaining 2,4-DNT was also quantified

[b] PB was dissolved in acetone and added to cultures at the same time as substrate feeding; 100 μl acetone solution was added for the final concentration listed in the table

[c] Values are the mean of data obtained from triplicate experiments. SD <8%

Fungal metabolic pathway of 4-NP

Only under ligninolytic conditions, 4-NP was converted into two products, 4-NA and DMNB, by *P. chrysosporium* (Table 1, Fig. 1). When 4-NA was utilized as an exogenous substrate, it was converted to DMNB with trace formation of 4-NP. Although a possible intermediate, 1-hydroxy-2-methoxy-5-nitrobenzene was not observed during 4-NA metabolism, a metabolic pathway for 4-NP to DMNB with intermediate formation of 4-NA (Fig. 3, Pathway 1) could be proposed.

The conversion of 4-NP to 4-NA was, however, not quantitative after day 5 (Fig. 1). A possible explanation could be attributed to the volatilization of 4-NA shown in Fig. 2a. However, this was observed for exogenously added 4-NA. During 4-NP metabolism, 4-NA was gradually formed (Fig. 1)—most likely produced intracellularly because of the involvement of cytochrome P450 (Table 2). Thus, the effect of 4-NA volatility on non-quantitative conversion of 4-NP was thought to be rather small.

The rate of disappearance of 4-NA was then compared with those of 4-NP and DMNB. The 4-NA disappearance rate was tentatively estimated from Fig. 2a [(▲ − △)/time], indicating that the rate of disappearance of 4-NA was comparable to that of 4-NP. Furthermore, the disappearance rate of DMNB after day 3 was almost the same as that of 4-NA (Fig. 2). Thus, 4-NA formed from 4-NP might be degraded into DMNB and lower metabolites after day 5 without accumulation. The effective mineralization of DMNB by *P. chrysosporium* has been reported (Valli et al. 1992). Furthermore, in the early stage of 4-NP metabolism, the recovery of 4-NA almost equalled the amount of 4-NP consumed (Fig. 1, day 3); thus this pathway was considered as the major pathway. However, 4-NP metabolism seems to stop after day 10 (Fig. 1). A reverse reaction of 4-NA to 4-NP, observed when 4-NA

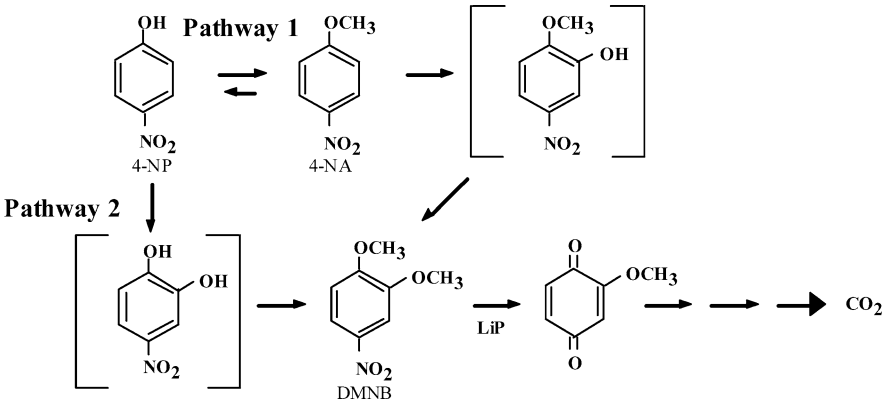

Fig. 3 Proposed metabolic pathway of 4-NP by *P. chrysosporium*

was utilized as exogenous substrate, might at least partially explain the apparent discontinuation of 4-NP disappearance.

4-Nitrocatechol (4-NC) is also a possible intermediate in DMNB formation (Fig. 3, Pathway 2). If 4-NC formation was much slower than that of DMNB, 4-NC would not accumulate during 4-NP metabolism. When 4-NC was added to *P. chrysosporium* culture, it was rapidly converted to DMNB without detection of possible intermediates, 1-hydroxy-2-methoxy-4-nitrobenzene or 1-hydroxy-2-methoxy-5-nitrobenzene (data not shown). Thus, Pathway 2 could not be completely eliminated. The pathway involving nitroreductase was not considered in the present study.

DMNB has been reported to be metabolized to CO_2 and H_2O by *P. chrysosporium* (Valli et al. 1992). Thus, it could be concluded that the white-rot fungus, *P. chrysosporium* is capable of mineralizing 4-NP (Fig. 3). The important steps to initiate 4-NP degradation were the methylation and hydroxylation reactions. The latter was found to be catalyzed by a cytochrome-P450-type enzyme (Table 2). The methylation reaction resulted in the formation of the dimethoxylated derivative (DMNB). A methoxyl group is an electron-donating substituent for an aromatic moiety. LiP oxidation of DMNB was reported to result in the release of a nitro group to form 2-methoxy-1,4-benzoquinone (Valli et al. 1992).

Several bacteria have been reported to degrade 4-NP. *Moraxella* sp. degrades 4-NP through the formation of hydroquinone and β-ketoadipate (Spain et al. 1979, Spain and Gibson 1991). *Arthrobactor* sp. strain JS443 degrades 4-NP through 4-NC formation by the action of monooxygenase; 4-NC is then converted to 1,2,4-trihydroxybenzene and β-ketoadipate (Jain et al. 1994). *P. chrysosporium* was shown to degrade 4-NP via a different pathway, where the key steps seem to be enzymatic modification of the aromatic moiety to form a more susceptible structure for fungal oxidative systems such as extracellular LiP.

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