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Degradation of 2,4-Dinitrotoluene by the Lignin-Degrading Fungus *Phanerochaete chrysosporium*

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Under ligninolytic conditions, the white rot basidiomycete *Phanerochaete chrysosporium* mineralizes 2,4-dinitrotoluene (I). The pathway for the degradation of I was elucidated by the characterization of fungal metabolites and oxidation products generated by lignin peroxidase (LiP), manganese peroxidase (MnP), and crude intracellular cell extracts. The multistep pathway involves the initial reduction of I to yield 2-amino-4-nitrotoluene (II). II is oxidized by MnP to yield 4-nitro-1,2-benzoquinone (XII) and methanol. XII is then reduced to 4-nitro-1,2-hydroquinone (V), and the latter is methylated to 1,2-dimethoxy-4-nitrobenzene (X). 4-Nitro-1,2-hydroquinone (V) is also oxidized by MnP to yield nitrite and 2-hydroxybenzoquinone, which is reduced to form 1,2,4-trihydroxybenzene (VII). 1,2-Dimethoxy-4-nitrobenzene (X) is oxidized by LiP to yield nitrite, methanol, and 2-methoxy-1,4-benzoquinone (VI), which is reduced to form 2-methoxy-1,4-hydroquinone (IX). The latter is oxidized by LiP and MnP to 4-hydroxy-1,2-benzoquinone, which is reduced to 1,2,4-trihydroxybenzene (VII). The key intermediate 1,2,4-trihydroxybenzene is ring cleaved by intracellular cell extracts to produce, after reduction, β-ketoadipic acid. In this pathway, initial reduction of a nitroaromatic group generates the peroxidase substrate II. Oxidation of II releases methanol and generates 4-nitro-1,2-benzoquinone (XII), which is recycled by reduction and methylation reactions to regenerate intermediates which are in turn substrates for peroxidase-catalyzed oxidation leading to removal of the second nitro group. Thus, this unique pathway apparently results in the removal of both aromatic nitro groups before ring cleavage takes place.

Nitroaromatics are very important feedstocks for the synthesis of a wide range of industrial chemicals, including munitions, pesticides, herbicides, and dyes (14, 26). In particular, 2,4- and 2,6-dinitrotoluene are used extensively in the production of polyurethane and explosives. In 1982, the United States alone produced approximately 720 million lb (ca. 327 million kg) of dinitrotoluenes (14). Dinitrotoluenes have been found to be mutagenic in bacterial and mammalian assay systems and carcinogenic in animal studies (39, 46). Because of the large-scale use and toxic nature of dinitrotoluenes, these compounds have been classified as priority pollutants (28).

The white rot basidiomycete fungus *Phanerochaete chrysosporium* is capable of effectively degrading polymeric lignin and lignin model compounds (22, 32). Two extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as an H_2O_2-generating system, are thought to constitute the major components of this organism's lignin-degradative system (22, 32). The degradation of a variety of environmentally persistent pollutants by *P. chrysosporium* has been reported as well (6, 7, 24, 27). In several of these reports, the use of ^{14}C-labeled compounds demonstrated mineralization of the pollutant. Recently, we reported the complete pathway for the degradation of 2,4-dichlorophenol by *P. chrysosporium* and suggested that both LiP and MnP, as well as intracellular enzymes, are involved in this degradation (47). Although the mineralization of ^{14}C-labeled trinitrotoluene by *P. chrysosporium* has been reported (18), the pathway and mechanisms for the degradation of nitroaromatic compounds by this organism have not been elucidated previously. In this report, we examine the reactions involved in the degradation of 2,4-dinitrotoluene and propose a pathway for the fungal metabolism of this important pollutant.

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MATERIALS AND METHODS

Chemicals. 2,4-Dinitrotoluene (I), 2-amino-4-nitrotoluene (II), 4-amino-2-nitrotoluene (III), 2,4-diaminotoluene (IV), 4-nitrocatechol (V), 1,2,4-trihydroxybenzene (VII), 2,5-dihydroxy-1,4-benzoquinone (VIII), 2-methoxy-1,4-hydroquinone (IX), hexafluorophosphoric acid, tetramethyltin, palladium diacetate, 2,4-dinitroaniline, chromotropic acid, 3,5-dinitrobenzoic acid, sulfanilimide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and levulinic acid were obtained from Aldrich or Lancaster (Windham, N.H.). U-^{14}C-ring-labeled 2,4-dinitroaniline (8.2 mCi/mmol) and β-ketoadipic acid (XIII) were obtained from Sigma.

2-Methoxy-1,4-benzoquinone (VI). Compound VI was prepared by the oxidation of compound IX with Ag_2O as described elsewhere (23).

1,2-Dimethoxy-4-nitrobenzene (X). 4-Nitrocatechol (V) (500 mg) was methylated with dimethyl sulfate-K_2CO_3 in refluxing acetone for 5 h as described previously (17). The acetone was evaporated, 50 ml of water was added, the product mixture was extracted with chloroform, and the product was purified by preparative thin-layer chromatography (the solvent system was $CHCl_3$).

4-Nitro-1,2-benzoquinone (XII). XII was prepared from 4-nitro-1,2-dihydroxybenzene (50 mg) by using 1 equivalent of $NaIO_4$ in water (5 ml) (at room temperature for 2 min) (1). The reaction mixture was extracted with ethyl acetate. The organic fraction was dried over Na_2SO_4, and the quinone was isolated via silica gel chromatography (the solvent system was $CHCl_3$).

1,2,4,5-Tetrahydroxybenzene (XI). XI was prepared from 2,5-dihydroxy-1,4-benzoquinone (VIII) as previously described (47).

U-^{14}C-ring-labeled 2,4-dinitrotoluene (I). The specific activity of the U-^{14}C-labeled 2,4-dinitroaniline was adjusted to 4.5 nCi/μmol. The diazonium salt of the ^{14}C-labeled 2,4-dinitroaniline was prepared by adding a slight molar excess of $NaNO_2$ solution to labeled 2,4-dinitroaniline in HCl (1.75 N) at −10°C with vigorous stirring. Subsequently, 2 equivalents of hexafluorophosphoric acid (60%) were added, the mixture was stirred for 5 h, and the hexafluorophosphate diazonium salt of 2,4-dinitroaniline was collected by centrifugation and dried under nitrogen (41). The diazonium hexafluorophosphate (15 mg) thus prepared was added to tetramethyltin (0.5 ml), a catalytic amount of palladium diacetate, and dry CH_3CN (1 ml), and the mixture was stirred at room temperature for 3 h (30). The reaction mixture was added to 10 ml of water and extracted with $CHCl_3$. The organic fraction was dried over Na_2SO_4, and the final product was purified by preparative thin-layer chromatography (the solvent system was $CHCl_3$). The final product, which was essentially pure, had chromatographic properties identical to those of the unlabeled standard on gas chromatography (GC) (retention time = 12.67 min) and high-pressure liquid chromatography (HPLC) (retention time = 7.2 min). The yield of the final product was 15%.

Culture conditions. The organism was grown from a conidial inoculum at 38°C in 25-ml stationary cultures as described elsewhere (16, 21). Unless indicated otherwise, the medium used in this study was as previously described (21, 33) with 2% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen sources, respectively. The medium was buffered with 20 mM sodium 2,2-dimethyl succinate (pH 4.5). Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every 3 days.

Mineralization of 2,4-dinitrotoluene (I). ^{14}C-labeled substrate (4.5×10^4 cpm, 4.5 nCi/μmol) in *N*,*N*-dimethylformamide (25 μl) was added to three replicate cultures on day 6 to avoid inhibition of growth by I. Flasks were fitted with ports which allowed periodic purging with O_2 and trapping of $^{14}CO_2$ (21, 33) in a basic scintillation fluid as previously described (33). The efficiency of $^{14}CO_2$ trapping after purging for 10 min was greater than 98%. Counting efficiency (>70%) was monitored with an external standard.

Metabolism of 2,4-dinitrotoluene and metabolic intermediates. After 6 days of incubation, the substrates in acetone (20 μl) were added to duplicate cultures to a final concentration of 250 μM. After the indicated additional intervals (2, 24, or 48 h), cultures were filtered through a Büchner funnel. The mycelial mat and the extracellular medium were then extracted separately with ethyl acetate (21). The total organic fraction was washed with 10 ml of water, dried over sodium sulfate, and evaporated under reduced pressure. The products were dissolved in methanol (250 μl), and the quinone products were reduced with sodium dithionite. The products were analyzed either directly or after derivatization. Trimethylsilylation of the reduced products was carried out in bis(*N*,*O*-trimethylsilyl)trifluoroacetimide-pyridine (2:1). Acetylation of reduced products was carried out in acetic anhydride-pyridine (1:1).

Peroxidases. LiP and MnP were purified from the extracellular medium of an acetate-buffered agitated culture of *P. chrysosporium* OGC101 (2) as described elsewhere (19, 20, 48, 49). The LiP concentration was determined at 408 nm by using an extinction coefficient of 133 mM^{-1} cm^{-1} (20). The specific activity of the purified LiP was 18 nmol μg^{-1} min^{-1} by the veratryl alcohol oxidation assay (20, 32). The MnP concentration was determined at 406 nm by using an extinction coefficient of 129 mM^{-1} cm^{-1} (19). The specific activity of the purified MnP was 85 nmol μg^{-1} min^{-1} by the Mn(II) lactate assay (19, 48).

Intracellular enzyme preparation. Six-day-old cells grown under nitrogen-limiting conditions were filtered and washed with ice-cold 0.5% NaCl. The cells (10 g [wet weight]) were ground with 15 g of acid-washed sand with a mortar and pestle. Subsequently, 50 mM sodium phosphate buffer (pH 7.0) (20 ml) was added and the mixture was stirred at 4°C for 15 min. The crude extract was centrifuged at 15,000 × *g*, and the supernatant was concentrated by ultrafiltration with an Amicon PM-10 membrane. The final protein concentration was determined to be 1 mg/ml (43).

Enzyme reactions. LiP reaction mixtures (2 ml) consisted of enzyme (5 μg), substrate (100 μM), and H_2O_2 (100 μM) in 20 mM sodium succinate (pH 3.0). MnP reaction mixtures consisted of enzyme (5 μg), substrate (100 μM), $MnSO_4$ (100 μM), and H_2O_2 (200 μM) in 50 mM sodium malonate (pH 4.5). Reactions were carried out at 30°C for 15 min. Enzyme reactions were conducted in duplicate.

Ring cleavage of 1,2,4-trihydroxybenzene. The reaction mixture (10 ml) contained 1 ml of concentrated cell extract, 1,2,4-trihydroxybenzene (25 μmol), NADPH (10 μmol), glucose 6-phosphate (25 μmol), and glucose 6-phosphate dehydrogenase (15 U) in 50 mM sodium phosphate (pH 7.0). The reaction mixture was stirred slowly at 28°C for 30 min as described elsewhere (9). At the end of the reaction, the pH was adjusted to 2.0 and the mixture was immediately extracted with ethyl acetate. The organic fraction was washed with water, dried over sodium sulfate, and evaporated. After silylation, the product was analyzed by GC-mass spectrometry. Control reactions with boiled extracts were also conducted.

Detection of methanol released in enzyme reactions. Two different procedures were used to detect methanol as a product of the enzyme reactions. (i) Reaction mixtures contained substrate (500 μM), $MnSO_4$ (500 μM), MnP (25 μg), and H_2O_2 (1 mM) in 5 ml of 50 mM sodium malonate (pH 4.5). Reactions were carried out at 30°C for 60 min. Control reactions in which either MnP or H_2O_2 was omitted were also conducted. Subsequently, 500 μl of H_2SO_4 (18 M) followed by 3,5-dinitrobenzoic acid (500 μM final concentration) was added to form the corresponding methyl benzoate. The reaction mixture was incubated at 90°C for 3 h. The mixture was extracted with ethyl acetate, washed with water, dried over Na_2SO_4, evaporated under reduced pressure, and analyzed for methyl-3,5-dinitrobenzoate (29). (ii) A 0.5-ml volume of 5% phosphoric acid and 1.0 ml of 5% $KMnO_4$ were added to 1 ml of the reaction mixture described above. The solution was stirred slowly at room temperature for 10 min to ensure the oxidation of methanol to formaldehyde. Sodium bisulfite (saturated solution) was added dropwise to remove the excess $KMnO_4$. The solution was cooled in an ice bath, after which 4 ml of concentrated H_2SO_4 followed by 0.5 ml of 2% chromotropic acid was added. The mixture was incubated at 60°C for 15 min, the A_{570} was measured, and the amount of methanol generated was determined as described elsewhere (3).

Detection of nitrite released in enzyme reactions. The reaction mixture used for the detection of nitrite was identical to that used for the detection of CH_3OH. Identical control reactions in which either MnP or H_2O_2 was omitted were also carried out. A 100-μl volume of sulfanilamide (1.7% in 2 N HCl) was added at the end of the enzymatic reaction, and

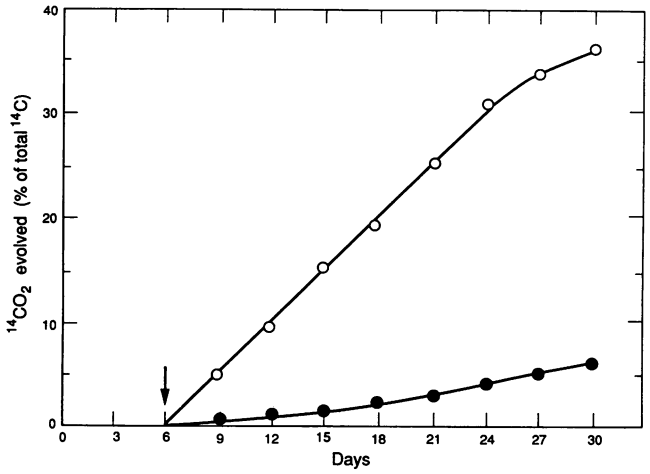

FIG. 1. Effect of nitrogen concentration on the mineralization of U-[^{14}C]-ring-labeled-2,4-dinitrotoluene. Triplicate stationary cultures containing 1.2 mM (○) and 12 mM (●) ammonium tartrate were inoculated with conidia and incubated for 6 days at 37°C, after which radiolabeled substrate was added (↓). Flasks were purged with O_2, and evolved $^{14}CO_2$ was trapped and counted as described in the text. Results are averages obtained from triplicate cultures. Variance in individual flasks from the average was less than 10% of the radioactivity counted.

the resultant mixture was incubated at room temperature for 5 min. Subsequently, *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added, and the A_{540} was measured after 10 min. The concentration of NO_2^- was determined by using a standard curve (25).

Chromatography and spectrometry. GC-mass spectrometry was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 25-m-long fused silica column (DB-5; J & W Science). The oven temperature was programmed from 80 to 320°C at 10°C/min. Quantitation of aromatic products was carried out on an HP gas chromatograph with a flame ionization detector and calculated by using standard curves. Substrates and products were also analyzed by HPLC with a Beckman C_8 ultrasphere column and a linear gradient from 30% methanol in water to 100% methanol. The 4-nitro-1,2-benzoquinone was analyzed by HPLC (retention time = 2.8 min) and quantitated by using standard curves.

RESULTS

Mineralization of 2,4-[^{14}C]dinitrotoluene (I) by cultures of *P. chrysosporium* is demonstrated in Fig. 1. After a 24-day incubation period, approximately 34% of the substrate was degraded to $^{14}CO_2$ in nitrogen-limited (1.2 mM ammonium tartrate) cultures, whereas only about 7% of the substrate was mineralized in nitrogen-sufficient (12 mM ammonium tartrate) cultures.

Metabolism of substrates. Products and yields obtained from the *P. chrysosporium* metabolism of various substrates and intermediates are diagrammed in Fig. 2. Three aromatic products were identified as *P. chrysosporium* metabolites of I: 2-amino-4-nitrotoluene (II), 4-amino-2-nitrotoluene (III), and 2,4-diaminotoluene (IV). Since 2-amino-4-nitrotoluene was found in the greatest yield, the metabolism of this intermediate was examined further. As shown in Fig. 2, 4-nitrocatechol (V), 1,2-dimethoxy-4-nitrobenzene (X), and

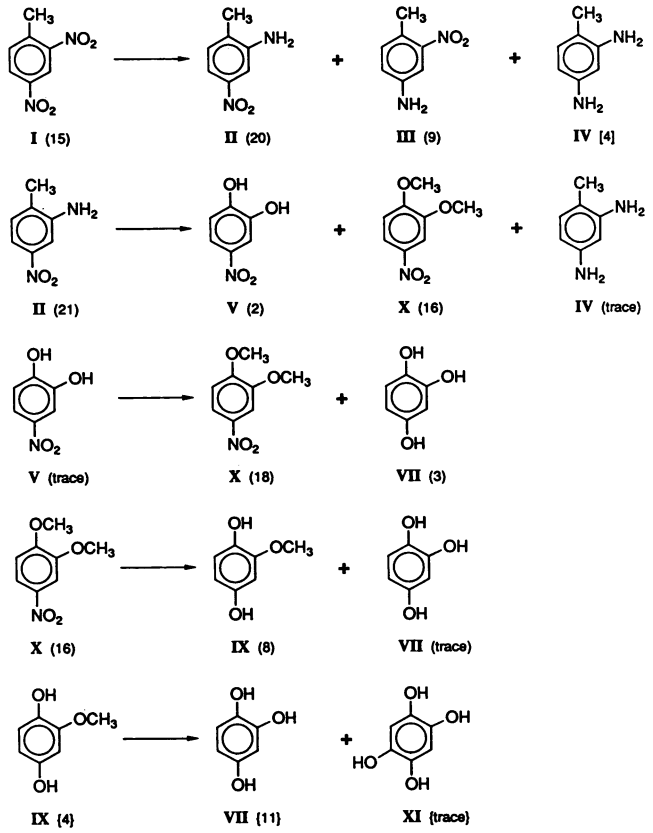

FIG. 2. Metabolites identified from the degradation of 2,4-dinitrotoluene and pathway intermediates. Duplicate cultures were incubated and extracted, and products were analyzed as described in the text. HPLC and GC were used to determine yields. Average mole percent yields from duplicate cultures of products or remaining substrate for incubations of 2 h (in braces), 24 h (in parentheses), and 48 h (in brackets) are indicated. Variance from the average was less than 10%.

2,4-diaminotoluene (IV) were identified as fungal metabolites of 2-amino-4-nitrotoluene (II). Several of the metabolites identified above were also added to fungal cultures. 4-Nitrocatechol (V) was metabolized to 1,2-dimethoxy-4-nitrobenzene (X) and 1,2,4-trihydroxybenzene (VII). 1,2-Dimethoxy-4-nitrobenzene (X) was metabolized to 2-methoxy-1,4-hydroquinone (IX) and 1,2,4-trihydroxybenzene (VII). Finally, 2-methoxy-1,4-hydroquinone (IX) was metabolized to 1,2,4-trihydroxybenzene (VII) and 1,2,4,5-tetrahydroxybenzene (XI). Metabolites were identified by comparing their retention times on GC and by comparing their mass spectra with standards. The mass spectra and GC retention times of these metabolites and enzyme reaction products or their derivatives are listed in Table 1. In all cases, the mass spectra and retention times of metabolites and enzyme reaction products were essentially identical to those of chemically synthesized standards.

Enzymatic oxidation of substrates and metabolic intermediates. The peroxidase oxidation products of 2-amino-4-nitrotoluene (II) and several identified metabolites are diagrammed in Fig. 3. MnP, but not LiP, oxidized 2-amino-4-nitrotoluene (II) to 4-nitro-1,2-benzoquinone. Methanol was also detected as a product of this reaction. The release of methanol was detected via the formation of methyl-3,5-

TABLE 1. Mass spectra of fungal metabolites and enzyme reaction products and their derivatives[a]

Substrate or metabolite	GC retention time (min)	Mass spectrum *m/z* (relative intensity)
2,4-Dinitrotoluene (I)	12.67	182 (16), 165 (100), 119 (10.6), 89 (36.2), 77 (13.8), 63 (19.1)
2-Amino-4-nitrotoluene (II)	13.50	152 (100), 106 (70.2), 94 (7.5), 79 (38.3), 77 (44.6)
4-Amino-2-nitrotoluene (III)	12.78	152 (100), 135 (64.9), 122 (13.8), 107 (72.3), 94 (7.5), 79 (36.2), 77 (79.7)
2,4-Diaminotoluene (IV)	10.70	122 (100), 121 (89.4), 105 (13.8), 94 (13.8), 77 (10.6), 67 (5.3), 61 (8.5), 57 (8.5)
4-Nitro-1,2-di(TMS)benzene[b]	15.40	299 (10.6), 284 (8.5), 269 (6.4), 104 (6.4), 74 (41.5), 73 (100)
1,2-Diacetoxy-4-nitrobenzene	15.78	239 (14.9), 197 (100), 155 (71.3), 139 (21.3), 125 (33), 109 (14.9), 79 (45.7)
1,2-Dimethoxy-4-nitrobenzene (X)	13.54	183 (100), 168 (5.3), 152 (16), 137 (22.3), 125 (10.6), 107 (25.5), 92 (36.2), 79 (100), 77 (87.2), 63 (35)
2-Methoxy-1,4-di(TMS)benzene	12.84	284 (72), 269 (122), 254 (80), 239 (10.2), 112 (10.2), 89 (9.1), 73 (100)
1,4-Diacetoxy-2-methoxybenzene	14.11	224 (5.2), 182 (15.2), 140 (100), 135 (26.2), 97 (10.2), 69 (15.2)
1,2,4-Tri(TMS)benzene	13.43	342 (28.6), 327 (26), 312 (1.2), 239 (12.1), 73 (100)
1,2,4-Triacetoxybenzene	15.20	252 (3.2), 210 (10.6), 197 (10.6), 168 (25.5), 155 (7.4), 126 (100), 97 (53)
1,2,4,5-Tetra(TMS)benzene	15.45	430 (26.9), 415 (2.0), 355 (1.5), 342 (2.6), 215 (4.6), 179 (4.6), 147 (21.9), 73 (100)
1,2,4,5-Tetraacetoxybenzene	18.23	310 (10.6), 268 (20.2), 226 (69.1), 184 (84.0), 142 (100), 113 (10.6), 69 (20)
β-Ketoadipic acid [tri(TMS) derivative] (XIII)[c]	14.84	376 (15.4), 361 (73), 317 (15.4), 286 (38.5), 259 (15.4), 231 (38.5), 169 (88.5), 147 (65.4), 125 (15.4), 73 (100)

[a] Products identified from the *P. chrysosporium* metabolism of 2,4-dinitrotoluene and intermediates. Cultures were incubated and extracted and products were analyzed as described in the text. Also, products from the oxidation of various intermediates by LiP, MnP, and crude cell extracts were identified. Reaction conditions and analysis were as described in the text. In all cases, the retention times and mass spectra of standard compounds were essentially identical to those of the substrates and metabolites.

[b] TMS, trimethylsilyloxy.

[c] Under the basic conditions used in derivatization, β-ketoadipic acid exists in its enolic form, 3-hydroxy-hex-2-ene-1,6-dioic acid; hence, we obtain the tri(TMS) derivative of β-ketoadipic acid for both the experimentally produced and the standard compounds.

dinitrobenzoate. Mass spectrum *m/z* (relative intensity) values are 226 (10.7), 197 (32), 195 (100), 183 (1.9), 149 (15.2), 75 (38), and 74 (10). No methanol was detected when either enzyme or H_2O_2 was omitted from the reaction mixture. A quantitative spectrophotometric assay demonstrated that 0.8 equivalent of methanol was released for each equivalent of substrate oxidized.

MnP, but not LiP, also oxidized 4-nitrocatechol (V) to 4-nitro-1,2-benzoquinone (XII) and 2-hydroxy-1,4-benzoquinone. The latter was detected after reduction as 1,2,4-

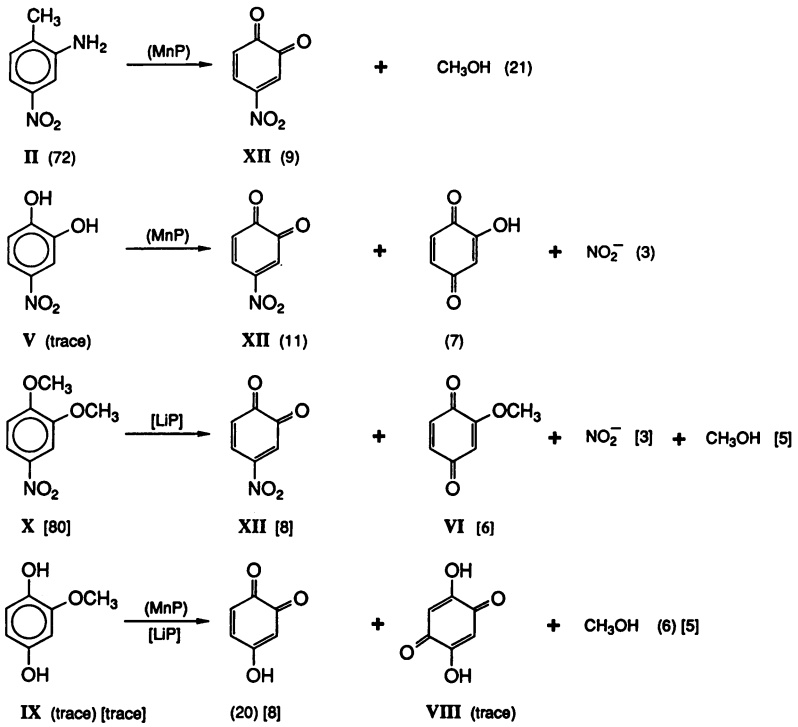

FIG. 3. Products identified from the oxidation of 2-amino-4-nitrotoluene and several other intermediates by purified LiP and MnP. Reaction conditions and identification of products were as described in the text. Mole percent product yields from the reaction with MnP (in parentheses) and LiP (in brackets) are indicated. No oxidized aromatic products, NO_2^-, or methanol was detected when either H_2O_2 or enzyme was omitted from the reactions.

trihydroxybenzene (VII). NO_2^- was also detected as a product of this reaction. Under the conditions used, LiP was not able to oxidize either of the metabolic intermediates (II or V). Because of their instability, no attempt was made to detect 2-hydroxy-1,4-benzoquinone or 4-hydroxy-1,2-benzoquinone prior to reduction and derivatization.

LiP, but not MnP, slowly oxidized 1,2-dimethoxy-4-nitrobenzene (X) to 4-nitro-1,2-benzoquinone (XII) and 2-methoxy-1,4-benzoquinone (VI). Both methanol and NO_2^- were detected as products of the reaction. Finally, both LiP and MnP oxidized 2-methoxy-1,4-hydroquinone (IX) to 4-hydroxy-1,2-benzoquinone and 2,5-dihydroxy-1,4-benzoquinone (VIII). Methanol was also detected as a product of this reaction. For each of the reactions described above, no oxidized aromatic product, NO_2^-, or methanol was detected when H_2O_2 or enzyme was omitted from the reaction mixture or when the enzyme was boiled for 2 min prior to the reaction.

When the oxidation of 1,2,4-trihydroxybenzene was carried out with the crude cell extract in the presence of NADPH and an NADPH-regenerating system, the formation of β-ketoadipic acid was demonstrated by GC-mass spectrometry (Table 1). The yield of β-ketoadipic acid was ~24 mol% of the starting substrate. A small amount (1.9 mol%) of levulinic acid was also obtained. Mass spectrum *m/z* (relative intensity) values for di(trimethylsilyloxy) derivative are 260 (9.5), 245 (4.3), 143 (83), and 73 (100). The same ratio of β-ketoadipic acid to levulinic acid was observed when standard β-ketoadipic acid was incubated in the reaction buffer and then extracted under identical conditions. The nonenzymatic decarboxylation of β-ketoadipic acid to levulinic acid has been reported previously (12).

DISCUSSION

White rot basidiomycetous fungi are primarily responsible for the initiation of the depolymerization of lignin in wood (11, 22, 32). The best-studied white rot fungus, *P. chrysosporium*, degrades lignin during secondary metabolic (idiophasic) growth (11, 22, 32). Under ligninolytic conditions, *P. chrysosporium* secretes two heme peroxidases (LiP and MnP) in addition to an H_2O_2-generating system (22, 32). These two peroxidases appear to be primarily responsible for the oxidative depolymerization of this heterogeneous, random phenylpropanoid polymer. Recent work has also demonstrated that *P. chrysosporium* is capable of mineralizing many persistent environmental pollutants (6, 7, 15, 24), including trinitrotoluene (18). However, to date, only the *P. chrysosporium* degradation pathway for the pollutant 2,4-dichlorophenol has been elucidated in detail (47). In our report, we showed that oxidative, reductive, and methyl transfer reactions were involved in the degradation of 2,4-dichlorophenol (47).

The metabolism of 2,4-dinitrotoluene in both mammalian and microbial systems has been examined previously (39). Reduction of the *ortho* and the *para* nitro groups by rat hepatic postmitochondrial supernatant and microsomal fractions has been reported (13, 34). However, the major 2,4-dinitrotoluene metabolite in rats appears to be 2,4-dinitrobenzyl alcohol, which is excreted as its glucuronide conjugate (13, 40). Both the yeast *Rhodotorula glutinis* (34) and the fungus *Microsporium* sp. (36) reduced 2,4-dinitrotoluene to 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. Thus, in these studies an important first step in the metabolism of 2,4-dinitrotoluene is reduction of the 2- or 4-nitro group to the amino function. Beyond this initial step, little has been reported previously about the degradation of this compound.

Our results demonstrate that *P. chrysosporium* extensively mineralizes 2,4-dinitrotoluene only under nutrient nitrogen-limiting conditions (Fig. 1), suggesting that the lignin-degradative system is (at least in part) responsible for the degradation of this pollutant. Sequential identification of the primary metabolites produced during 2,4-dinitrotoluene degradation and subsequent identification of the secondary metabolites after addition to cultures of synthesized primary metabolites have enabled us to propose a pathway for the degradation of 2,4-dinitrotoluene. The first step in the pathway is the reduction of I to either 2-amino-4-nitrotoluene (II) or 4-amino-2-nitrotoluene (III). The subsequent reduction of the monoamino-mononitrotoluenes apparently also takes place, since we have identified 2,4-diaminotoluene (IV) as a *P. chrysosporium* metabolite of I. Presumably, the organism takes up the substrate and the reduction takes place intracellularly. Enzymes involved in the reduction of aromatic nitro groups have been characterized from microorganisms (4, 5, 31). In addition, oxygen-insensitive and oxygen-sensitive aromatic nitroreductases from *Escherichia coli* (38) and mammalian microsomes (37) have been studied. The oxygen-sensitive enzymes catalyze the one-electron reduction of the substrate to form the nitroaromatic-anion free radical which reacts with molecular oxygen to form superoxide and the parent nitroaromatic in a futile cycle (38).

P. chrysosporium cultures convert 2-amino-4-nitrotoluene to 4-nitro-1,2-benzoquinone (XII) and 4-nitro-1,2-dimethoxybenzene (X), suggesting that 2-amino-4-nitrotoluene (II) is oxidized by either LiP or MnP. Results of enzyme reactions (Fig. 3) confirmed that MnP oxidizes 2-amino-4-nitrotoluene (II) to 4-nitro-1,2-benzoquinone (XII). Nitroquinones are not substrates for either LiP or MnP, and, like chloroquinones, they are strong oxidizing agents. Thus, the nitroquinone intermediates in fungal cultures could be converted to the corresponding hydroquinone either nonenzymatically or enzymatically. Methylation of hydroquinone intermediates was previously observed in our study of 2,4-dichlorophenol degradation (47).

Both 4-nitro-1,2-hydroquinone (V) and 1,2-dimethoxy-4-nitrobenzene (X) are metabolized by *P. chrysosporium* (Fig. 2), suggesting that they are substrates for either LiP or MnP. MnP oxidizes 4-nitro-1,2-hydroquinone to 4-nitro-1,2-benzoquinone (XII) and to 2-hydroxy-1,4-benzoquinone, releasing NO_2^- in the process. LiP oxidizes, 1,2-dimethoxy-4-nitrobenzene (X) to 4-nitro-1,2-benzoquinone (XII) and 2-methoxy-1,4-benzoquinone (VI), also releasing NO_2^- and methanol in the process. This demonstrates that LiP is capable of oxidizing nitrodimethoxybenzenes and chlorodimethoxybenzenes (47), although at a rate lower than that for dimethoxybenzene (29).

P. chrysosporium also rapidly metabolizes 4-nitro-1,2-hydroquinone (V) to 1,2-dimethoxy-4-nitrobenzene (X) and 1,2,4-trihydroxybenzene (VII), confirming that 2-amino-4-nitrotoluene is probably first oxidized to 4-nitro-1,2-benzoquinone (XII) by MnP and then the latter is reduced to the hydroquinone (V). Subsequently, 4-nitro-1,2-hydroquinone (V) is oxidized by MnP to yield 2-hydroxy-1,4-benzoquinone, which is reduced to form 1,2,4-trihydroxybenzene (VII) (Fig. 4). V is also methylated to yield 1,2-dimethoxy-4-nitrobenzene (X), which is in turn a substrate for LiP. Oxidation of 1,2-dimethoxy-4-nitrobenzene (X) by LiP yields 2-methoxy-1,4-benzoquinone (VI) (Fig. 3), which is subsequently reduced to the hydroquinone (IX) (Fig. 2). This intermediate is oxidized by both LiP and MnP to 2-hydroxy-

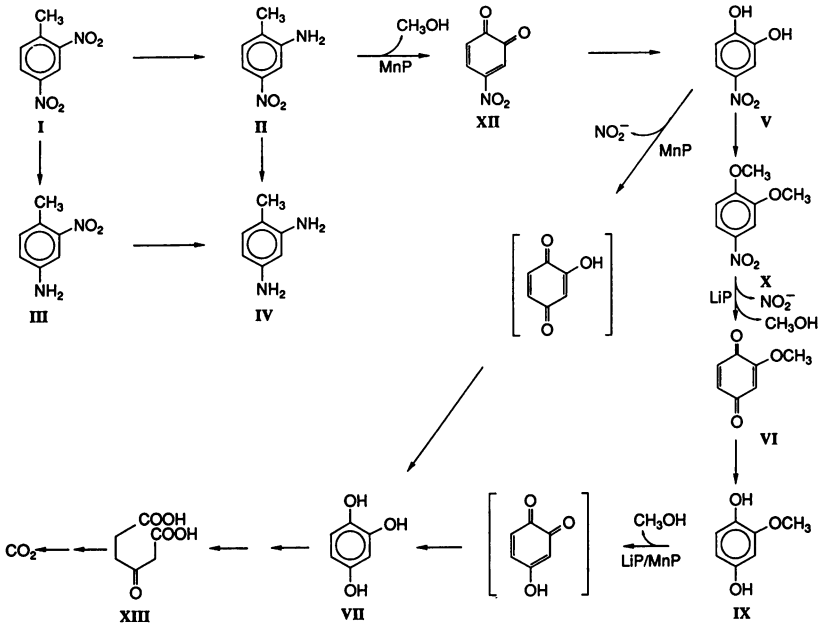

FIG. 4. Proposed pathway for the degradation of 2,4-dinitrotoluene by *P. chrysosporium*.

1,4-benzoquinone (Fig. 3), which is subsequently reduced to 1,2,4-trihydroxybenzene (VII). Using a crude intracellular enzyme preparation, we confirmed (9) that trihydroxybenzene is ring cleaved by a 1,2-dioxygenase to produce, after subsequent reduction, β-ketoadipic acid. The likely intermediate maleylacetic acid, not identified in this crude system because of its presumed instability, was probably reduced to yield β-ketoadipic acid (9, 44, 45). β-Ketoadipic acid would be readily metabolized to CO_2. The biodegradation of 4-amino-2-nitrotoluene (III) and 2,4-diaminotoluene (IV) by *P. chrysosporium* cultures was not pursued, but presumably similar reactions are involved.

Our results suggest that *P. chrysosporium* elaborates a general pathway for the degradation of nitroaromatic compounds which involves the initial reduction of an aromatic nitro group to an aromatic amine. Subsequent oxidation of the amine by MnP generates 4-nitro-1,2-benzoquinone (XII). This intermediate undergoes a cycle of reduction and methylation generating 4-nitro-1,2-hydroquinone (V) and 1,2-dimethoxy-4-nitrobenzene (X). The former is a substrate for MnP, and the latter is a substrate for LiP. Oxidation of compound V by MnP yields 2-hydroxy-1,4-benzoquinone, which is reduced to form 1,2,4-trihydroxybenzene (VII). Oxidation of X by LiP yields 2-methoxy-1,4-benzoquinone (VI), which is subsequently metabolized to VII.

Presumably, parts of the proposed pathway evolved for the degradation of lignin metabolites. For example, the oxidative decarboxylation of the lignin metabolite vanillic acid yields 2-methoxy-1,4-hydroquinone (8, 50) and 2-methoxy-1,4-benzoquinone (35). 2-Methoxy-1,4-benzoquinone (VI) is also a product of the LiP-catalyzed oxidation of the *P. chrysosporium* metabolite veratryl alcohol (42). Reduction of 2-methoxy-1,4-benzoquinone to its corresponding hydroquinone (IX) in cell extracts has been measured (10). It is likely that the peroxidase oxidations described above would also result in the formation of oligomeric products. However, *P. chrysosporium* is well adapted to degrade the aromatic polymer lignin. Thus, it is likely that any oligomers which are formed would be degraded subsequently.

In the pathway described above, both nitro groups are removed before ring cleavage by a 1,2-dioxygenase occurs. An initial reduction of one nitro group to an aromatic amine activates the pollutant for attack by a peroxidase. The oxidized product, an orthoquinone, undergoes a cycle of reduction and methylation which again generates several peroxidase substrates. A second oxidation of 4-nitro-1,2-hydroquinone (V) by MnP removes the second nitro group from the ring. Alternatively, a second oxidation of the intermediate 1,2-dimethoxy-4-nitrobenzene (X) by LiP removes the second nitro group from the ring. We recently demonstrated *P. chrysosporium* activation of compounds by cycles of reduction and methylation in the degradation of 2,4-dichlorophenol (47). Preliminary results (47a) suggest that 2,4,6-trinitrotoluene is degraded via similar pathways. We are attempting to isolate and characterize the nitroreductase(s), quinone reductase(s), methyltransferase(s), and 1,2-dioxygenase(s) implicated by the results of this study.

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REFERENCES

1. **Adler, E., and R. Magnusson.** 1959. Periodate oxidation of phenols. Acta Chem. Scand. **12**:505–519.
2. **Alic, M., C. Letzring, and M. H. Gold.** 1987. Mating system and basidiospore formation in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **53**: 1464–1469.
3. **Boos, R. N.** 1948. Quantitative colorimetric microdetermination of methanol with chromotropic acid reagent. Anal. Chem. **20**:964–965.
4. **Bryant, C., and M. Deluca.** 1991. Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. J. Biol. Chem. **266**:4119–4125.
5. **Bryant, D. W., D. R. McCalla, M. Leckshma, and P. Canenville.** 1981. Type I nitroreductases of *Escherichia coli*. Can. J. Micro-

biol. **27**:81–86.
6. **Bumpus, J. A., and S. D. Aust.** 1987. Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin-degrading system. Bioessays **6**:166–170.
7. **Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science **228**:1434–1436.
8. **Buswell, J. A., P. Ander, B. Pettersson, and K.-E. Eriksson.** 1979. Oxidative decarboxylation of vanillic acid by *Sporotrichum pulverulentum*. FEBS Lett. **103**:98–101.
9. **Buswell, J. A., and K.-E. Eriksson.** 1979. Aromatic ring cleavage by the white rot fungus *Sporotrichum pulverulentum*. FEBS Lett. **104**:258–260.
10. **Buswell, J. A., and K.-E. Eriksson.** 1988. NAD(P)H dehydrogenase (quinone) from *Sporotrichum pulverulentum*. Methods Enzymol. **161**:271–274.
11. **Buswell, J. A., and E. Odier.** 1987. Lignin degradation. Crit. Rev. Biotechnol. **6**:1–60.
12. **Chapman, P. J., and D. W. Ribbons.** 1976. Metabolism of resorcinylic compounds by bacteria: alternative pathways for resorcinol catabolism in *Pseudomonas putida*. J. Bacteriol. **125**:985–998.
13. **Decad, G. M., M. E. Graichen, and J. D. Dent.** 1982. Hepatic microsomal metabolism and covalent binding of 2,4-dinitrotoluene. Toxicol. Appl. Pharmacol. **62**:325–334.
14. **Dunlap, K. L.** 1979. Nitrobenzene and nitrotoluenes, p. 916–932. *In* H. F. Mark, D. F. Othmer, C. G. Overberg, and G. T. Seaborg (ed.), Encyclopedia of chemical technology, 3rd ed., vol. 15. John Wiley & Sons, Inc., New York.
15. **Eaton, D. C.** 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **7**:194–196.
16. **Enoki, A., G. P. Goldsby, and M. H. Gold.** 1981. β-Ether cleavage of the lignin model compound 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether and derivatives by *Phanerochaete chrysosporium*. Arch. Microbiol. **129**:141–145.
17. **Feiser, L. F., and M. Feiser.** 1967. Reagents for organic synthesis, vol. 1. John Wiley & Sons, Inc., New York.
18. **Fernando, T., J. A. Bumpus, and S. D. Aust.** 1990. Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **56**:1666–1671.
19. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242**:329–341.
20. **Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn.** 1984. Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234**:353–362.
21. **Gold, M. H., M. B. Mayfield, T. M. Cheng, K. Krisnangkura, A. Enoki, M. Shimada, and J. K. Glenn.** 1982. A *Phanerochaete chrysosporium* mutant defective in lignin degradation as well as several other secondary metabolic functions. Arch. Microbiol. **132**:115–122.
22. **Gold, M. H., H. Wariishi, and K. Valli.** 1989. Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*. ACS Symp. Ser. **389**:127–140.
23. **Haemmerli, S. D., H. E. Schoemaker, W. H. Schmidt, and M. S. A. Leisola.** 1987. Oxidation of veratryl alcohol by the lignin peroxidase of *Phanerochaete chrysosporium*. FEBS Lett. **220**:149–154.
24. **Hammel, K. E.** 1989. Organopollutant degradation by ligninolytic fungi. Enzyme Microb. Technol. **11**:776–777.
25. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
26. **Hartter, D. R.** 1985. The use and importance of nitroaromatic chemicals in the chemical industry, p. 1–13. *In* D. E. Rickert (ed.), Toxicity of nitroaromatic compounds. Chemical Industry Institute of Toxicology series. Hemisphere Publishing Corp., Washington, D.C.
27. **Huynh, V.-B., H.-M. Chang, T. W. Joyce, and T. K. Kirk.** 1985. Dechlorination of chloro-organics by a white-rot fungus. Tappi J. **68**:98–102.
28. **Keither, L. H., and W. A. Telliard.** 1979. Priority pollutants. I. A perspective view. Environ. Sci. Technol. **13**:416–423.
29. **Kersten, P. J., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxy benzenes. J. Biol. Chem. **260**:2609–2612.
30. **Kikukawa, K., K. Kono, F. Wada, and T. Matsuda.** 1983. Reaction of diazonium salts with transition metals. 8. Palladium-catalyzed carbon-carbon coupling of arenediazonium salts with organotin compounds. J. Org. Chem. **48**:1333–1336.
31. **Kinouchi, T., and Y. Ohnishi.** 1983. Purification and characterization of 1-nitropyrene nitroreductases from *Bacteroides fragilis*. Appl. Environ. Microbiol. **46**:596–604.
32. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41**:465–505.
33. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**: 227–285.
34. **Kozuka, H., M. Mori, K. Katayama, T. Matsuhashi, T. Miyahara, Y. Mori, and S. Nagahara.** 1978. Studies on the metabolism and toxicity of dinitrotoluenes—metabolism of dinitrotoluenes by *Rhodotorula glutinis* and rat liver homogenate. Eisei Kagaku **24**:252–259.
35. **Krisnangkura, K., and M. H. Gold.** 1979. Peroxidase catalyzed oxidative decarboxylation of vanillic acid to methoxy-*p*-hydroquinone. Phytochemistry **18**:2019–2021.
36. **McCormick, N. G., J. H. Cornell, and A. M. Kaplan.** 1978. Identification of biotransformation products from 2,4-dinitrotoluene. Appl. Environ. Microbiol. **35**:945–948.
37. **Orna, M. V., and R. P. Mason.** 1989. Correlation of kinetic parameters of nitroreductase enzymes with redox properties of nitroaromatic compounds. J. Biol. Chem. **264**:12379–12384.
38. **Peterson, F. P., R. P. Mason, J. Housepian, and J. L. Hotzman.** 1979. Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. J. Biol. Chem. **254**: 4009–4014.
39. **Rickert, D. E., B. E. Butterworth, and J. A. Popp.** 1983. Dinitrotoluene acute toxicity, oncogenicity, genotoxicity and metabolism. Crit. Rev. Toxicol. **13**:217–234.
40. **Rickert, D. E., and R. M. Long.** 1981. Metabolism and excretion of 2,4-dinitrotoluene in male and female Fischer 344 rats after different doses. Drug Metab. Dispos. **9**:226–232.
41. **Rutherford, K. G., and W. Redmond.** 1973. 1-Bromo-3-fluorobenzene, p. 133–134. *In* H. E. Baumgarten (ed.), Organic synthesis, vol. 5. John Wiley & Sons, Inc., New York.
42. **Schoemaker, H. E., E. M. Meijer, M. S. A. Leisola, S. D. Haemmerli, R. Waldner, D. Sanglard, and H. W. H. Schmidt.** 1989. Oxidation and reduction in lignin biodegradation, p. 454–471. *In* N. G. Lewis and M. G. Paice (ed.), Plant cell wall polymers: biogenesis and biodegradation. American Chemical Society, Washington, D.C.
43. **Smith, P. K., R. J. Krohm, G. T. Hermanson, A. K. Malhia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
44. **Spain, J. C., and D. T. Gibson.** 1991. Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. **57**:812–819.
45. **Spain, J. C., O. Wyss, and D. T. Gibson.** 1979. Enzymatic oxidation of *p*-nitrophenol. Biochem. Biophys. Res. Commun. **88**:634–641.
46. **U.S. Public Health Service.** 1989. Toxicological profile for 2,4-DNT and 2,6-DNT. Agency for Toxic Substances and Disease Registry, Atlanta.
47. **Valli, K., and M. H. Gold.** 1991. Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrys-*

osporium. J. Bacteriol. **173**:345–352.
47a. **Valli, K., D. K. Joshi, and M. H. Gold.** Unpublished data.
48. **Wariishi, H., H. B. Dunford, I. D. MacDonald, and M. H. Gold.** 1989. Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*: transient-state kinetics and reaction mechanism. J. Biol. Chem. **264**:3335–3340.
49. **Wariishi, H., and M. H. Gold.** 1990. Lignin peroxidase compound III. Mechanism of formation and decomposition. J. Biol. Chem. **265**:2070–2077.
50. **Yajima, Y., A. Enoki, M. B. Mayfield, and M. H. Gold.** 1979. Vanillate hydroxylase from the white rot basidiomycete *Phanerochaete chrysosporium*. Arch. Microbiol. **123**:319–321.