Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved

G. Vijay Bhasker Reddy and Michael H. Gold

Under nitrogen-limiting, secondary metabolic conditions, the lignin-degrading basidiomycete *Phanerochaete chrysosporium* rapidly degrades pentachlorophenol. The pathway for the degradation of pentachlorophenol has been elucidated by the characterization of fungal metabolites and oxidation products generated by purified lignin peroxidase (LiP) and manganese peroxidase (MnP). The multi-step pathway is initiated by a LiP- or MnP-catalysed oxidative dechlorination reaction to produce tetrachloro-1,4-benzoquinone. Under primary or secondary metabolic conditions, the quinone is further degraded by two parallel pathways with cross-links. The quinone is reduced to tetrachlorodihydroxybenzene, which can undergo four successive reductive dechlorinations to produce 1,4-hydroquinone, and the latter is o-hydroxylated to form the final aromatic metabolite, 1,2,4-trihydroxybenzene. Alternatively, the tetrachloro-1,4-benzoquinone is converted, either enzymically or nonenzymically, to 2,3,5-trichlorotrihydroxybenzene, which undergoes successive reductive dechlorinations to produce 1,2,4-trihydroxybenzene. Finally, at several points, hydroxylation reactions convert chlorinated dihydroxybenzenes to chlorinated trihydroxybenzenes, linking the two pathways at each of these steps. Presumably, the 1,2,4-trihydroxybenzene produced in each pathway is ring-cleaved with subsequent degradation to CO₂. In contrast to the oxidative dechlorination step, the reductive dechlorinations and hydroxylations occur during both primary and secondary metabolic growth. Apparently, all five chlorine atoms are removed from the substrate prior to ring cleavage.

**Keywords:** pentachlorophenol, reductive dechlorination, hydroxylation, *Phanerochaete chrysosporium*, lignin and manganese peroxidases

INTRODUCTION

Pentachlorophenol (PCP or Pent) was first produced in the 1930s as a wood preservative and it has subsequently been used as a biocide against a wide variety of species (Crosby, 1981). The large-scale use of PCP has led to the contamination of terrestrial and aquatic ecosystems. Owing to its toxicity, PCP is on the list of priority pollutants defined by the US Environmental Protection Agency (Keither & Teilard, 1979; Moos *et al.*, 1983).

The degradation of PCP by soil bacteria (Chu & Kirsch, 1972; Saber & Crawford, 1985; Apajalahti & Salkinoja-Salonen, 1987; Schenk *et al.*, 1989; Radehaus & Schmidt, 1992), particularly by *Mycobacterium chlorophenolicum* (formerly *Rhodococcus chlorophenolicus*) (Apajalahti & Salkinoja-Salonen, 1987; Häggblov *et al.*, 1989, 1994) and *Sphingomonas chlorophenolica* (formerly *Flavobacterium* sp.) (Steiert & Crawford, 1986) has been examined extensively. In both bacterial systems, PCP is first oxidized to tetrachlorodihydroquinone by a PCP monooxygenase (Häggblov *et al.*, 1989; Xun & Orser, 1991; Xun *et al.*, 1992). In *M. chlorophenolicum*, the reaction sequence for the dechlorination of tetrachlorodihydroquinone involves an initial oxidative dechlorination to form trichlorotrihydroxybenzene, which undergoes successive reductive dechlorinations to yield 1,2,4-trihydroxybenzene.

**Abbreviations:** HC/HN, high carbon/high nitrogen; HC/LN, high carbon/low nitrogen; LiP, lignin peroxidase; MnP, manganese peroxidase; PCP, pentachlorophenol.
Phanerochaete chrysosporium, a lignin-degrading white-rot fungus, is known to mineralize several chlorinated phenols, including PCP (Bumpus & Aust, 1987; Hammel & Tardone, 1988; Mileski et al., 1988; Valli & Gold, 1991; Joshi & Gold, 1993; Armenante et al., 1994; Reddy et al., 1998). However, to date, the pathway(s) for PCP degradation has not been adequately elucidated in this organism. Under nutrient nitrogen-limiting conditions, P. chrysosporium produces two extracellular haem peroxidases – lignin peroxidase (LiP) and manganese peroxidase (MnP) – which, along with an H₂O₂-generating system, are involved in the oxidation of lignin and lignin model compounds (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989; Higuchi, 1990). LiP can oxidize PCP to tetrachloro-1,4-dihydroxybenzene (1 mmol) by reacting with N-chlorosuccinimide (1 mmol) as described above. The product (V) was purified by HPLC.

Culture conditions. P. chrysosporium strain OGC101 (Alic et al., 1987) was grown on a conidial inoculum at 37 °C in stationary cultures in 250 ml flasks as described previously (Gold et al., 1982). The medium (25 ml) was as described previously (Kirk et al., 1978; Gold et al., 1982) and contained 2% glucose [high carbon (HC)], 20 mM sodium 2,2-dimethyl succinate [pH 4.5] as the buffer, and either 1:2 mM [low nitrogen (HC/LN)] or 12 mM [high nitrogen (HC/HN)] ammonium tartrate as the nitrogen source. Cultures were incubated under air for 3 days, after which they were purged with 99.9% O₂ at 3-day intervals.

Metabolism of PCP and metabolic intermediates. After 6 days incubation (3 days for HC/HN cultures), the substrates, in 30 μl ethanol, were added to cultures to a final concentration of 250 μM. After the indicated periods, products were extracted, purified and analysed by GC-MS and GC as described previously (Reddy et al., 1998). Quinones were analysed directly by HPLC without prior reduction as described previously (Reddy et al., 1998; Reddy & Gold, 1999).

Enzymes. LiP and MnP were purified from the extracellular medium of acetate-buffered, agitated cultures of P. chrysosporium strain OGC101 as described previously (Gold et al., 1984; Glenn & Gold, 1985; Wariishi & Gold, 1990). The LiP (Gold et al., 1984) and MnP (Glenn & Gold, 1985) concentrations were determined as described previously.

Enzyme reactions. LiP and MnP reactions were performed as described previously (Joshi & Gold, 1993; Reddy et al., 1998). Veratryl alcohol (0.1 mM) was added to stimulate LiP reactions (Kirk & Farrell, 1987; Wariishi & Gold, 1990; Joshi & Gold, 1994). Reaction mixtures were extracted with ethyl acetate, evaporated under N₂, analysed by GC or GC-MS after acetylation as described previously (Joshi & Gold, 1993). For reductive acetylation, sodium dithionite was added to the reaction mixture before extraction. Quinone products were analysed by HPLC without prior reduction. Reaction mixtures were filtered through a Centricon 10 (Amicon) filter prior to direct HPLC analysis. Control reactions were conducted in the absence of either enzyme or H₂O₂ as described previously (Reddy et al., 1998).

Tetrachlorobenzoquinone (II) reduction. Six-day-old P. chrysosporium stationary cultures, grown under HC/LN conditions, were filtered through a Buchner funnel to separate the cells and extracellular medium. The hyphae were washed and resuspended in buffer or in fresh culture medium. Tetrachlorobenzoquinone (II) (0.1 mM) was added to the cell suspension or to the extracellular medium as described previously (Joshi & Gold, 1993; Reddy et al., 1998) and incorporated at 30 °C for 30 min. At the end of the incubation period, the reaction mixtures were acidified, extracted and evaporated as described above and previously (Reddy et al., 1998).

Chromatography and spectrometry. GC-MS was performed at 70 eV on a VG Analytical 7070E mass spectrometer as
described previously (Joshi & Gold, 1993; Reddy et al., 1998). Product quantitation was carried out on an HP 5890 II gas chromatograph. HPLC product analysis was conducted as described previously (Reddy et al., 1998). Products were detected at 285 nm, and product yields on HPLC were quantified using calibration curves obtained with standards.

**RESULTS**

Time courses for the removal of PCP (I) and the key intermediate tetrachlorodihydroxybenzene III (Reddy & Gold, 1999) from cultures of *P. chrysosporium* are shown in Fig. 1. After a 30 h incubation period, only ~10% of the PCP remained in nitrogen-limited (12 mM ammonium tartrate) HC/LN cultures, while ~90% of the substrate remained under nutrient-sufficient (12 mM ammonium tartrate) HC/HN conditions. In contrast, tetrachlorodihydroxybenzene (III), an initial intermediate in PCP degradation, was degraded more rapidly under nitrogen-sufficient conditions than under nitrogen-limited conditions.

**Metabolism of PCP and metabolic intermediates**

Products and yields obtained from the fungal metabolism of PCP (I) and of several intermediates are shown in Fig. 2. Under HC/LN conditions, three products—tetrachlorodihydroxybenzene (III), tetrachlorobenzoquinone (II) and, in trace amounts, pentachloroanisole (IV)—were identified as metabolites of PCP (Fig. 2a).

When tetrachlorodihydroxybenzene (III) was added to cultures, the following metabolites were identified: 2,3,5-trichloro-1,4-dihydroxybenzene (V), trichloro-1,2,4-trihydroxybenzene (VI), dichlorotrihydroxybenzene (VII), tetrachloro-1,4-methoxyphenol (X), tetrachloro-1,4-dimethoxybenzene (IX) and a trace amount of tetrachlorobenzoquinone (II) (Fig. 2b). The metabolite trichlorotrihydroxybenzene (VI) was also formed when tetrachlorobenzoquinone (II) or tetrachlorohydroquinone (III) was incubated in water (data not shown).

When the metabolite trichlorodihydroxybenzene (V) was added to *P. chrysosporium* cultures (Fig. 2c), three major aromatic products were identified: 2,5-dichloro-1,4-dihydroxybenzene (XI), 3,5,6-trichloro-1,2,4-trihydroxybenzene (VI) and dichlorotrihydroxybenzene (VII). The methylated metabolites 2,3,5-trichloro-4-methoxyphenol (XII) and 2,3,5-trichloro-1,4-dimethoxybenzene (XIII) were also detected in trace amounts. When dichlorotrihydroxybenzene (VII) (Fig. 2d) was added to cultures, only 5-chloro-1,2,4-trihydroxybenzene (VIII) could be identified as a metabolite.

Finally, when 2,5-dichloro-1,4-dihydroxybenzene (XI) was added to cultures (Fig. 2e), the following metabolites were identified: 2-chloro-1,4-dihydroxybenzene (XIV), 5-chloro-1,2,4-trihydroxybenzene (VIII), 1,2,4-trihydroxybenzene (XVIII), 2,5-dichloro-1,3,4-trihydroxybenzene (XVII), 2,5-dichloro-1,4-dimethoxybenzene (XV) and 2,5-dichloro-4-methoxyphenol (XVI). The metabolite 2,5-dichloro-1,3,4-trihydroxybenzene (XVII) was also formed in small quantities when 2,5-dichloro-1,4-dihydroxybenzene (XI) was incubated in water.

Under HC/HN conditions, PCP (I) slowly underwent O-methylation to form pentachloroanisole (IV) as the sole product (1% after 3 d incubation). However, under these HC/HN conditions, the transformations of the PCP metabolites tetrachlorodihydroxybenzene (III), trichlorodihydroxybenzene (V), dichlorotrihydroxybenzene (VII) and 2,5-dichlorodihydroxybenzene (XI) were similar to those observed under HC/LN conditions (Fig. 2).

The metabolites were identified by comparing their retention times on GC and their mass spectra with standards (Table 1). The HPLC retention times (in min) for metabolites were as follows: tetrachloro-1,4-dihydroxybenzene (III), 10-3; tetrachloro-1,4-benzoquinone (II), 11-7; trichloro-1,4-dihydroxybenzene (V), 9-5; 2,6-dichlorotrihydroxybenzene (VII), 6-35; 5-chloro-1,2,4-trihydroxybenzene (VIII), 6-2; tetrachloro-1,4-dimethoxybenzene (IX), 5-3; 2,5-dichloro-1,4-dihydroxybenzene (XI), 8-5; 2,5-dichloro-1,4-dimethoxybenzene (XV), 12-4; 1,2,4-trihydroxybenzene (XVIII), 3-4.

**Enzymic oxidation of PCP and tetrachlorodihydroxybenzene**

The MnP- and LiP-catalysed oxidations of PCP and tetrachlorodihydroxybenzene (III) are shown in Fig. 3. Both peroxidases oxidized PCP (I) to yield tetra-
Fig. 2. Metabolites identified from the degradation of PCP and pathway intermediates by *P. chrysosporium*. Cultures were incubated, extracted and products analysed as described in the text. HPLC was used to determine the yields of quinones. GC was used to determine the yields of other compounds. Amounts of substrate remaining (%) or percentage yields of products, following incubations of 6 h, are given in square brackets; t, trace. Roman numerals identifying compounds in the figure correspond to those in the text.
Table 1. Mass spectra of products, or derivatives, from the metabolism of PCP and intermediates by P. chrysosporium cultures and enzyme oxidations

Culture conditions, extractions, product analysis and reaction conditions were as described in Methods. Products from the oxidation of substrates by purified MnP and LiP were identified as described in the text. Reaction conditions and analysis were as described in the text. Retention times and mass spectra of standard compounds were essentially identical to those of the substrates and identified metabolites.

<table>
<thead>
<tr>
<th>Derivatized substrate or metabolite*</th>
<th>GC retention time (min)</th>
<th>Mass spectrum m/z (relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentachloroacetoxybenzene</td>
<td>12.48</td>
<td>312 (3-0), 310 (10-0), 308 (16-5), 306 (10-3), 272 (3-1), 270 (20-3), 268 (64-1), 266 (100), 264 (59-5), 241 (28-8), 239 (8-8), 237 (14-5), 235 (8-3)</td>
</tr>
<tr>
<td>Tetrachloro-1,4-diacetoxybenzene</td>
<td>13.59</td>
<td>334 (3-8), 332 (6-3), 330 (4-8), 292 (6-3), 290 (10-8), 288 (9-3), 252 (11-5), 250 (49-1), 248 (100), 246 (78-1)</td>
</tr>
<tr>
<td>Trichloro-1,2,4-triacetoxybenzene</td>
<td>14.83</td>
<td>316 (5-2), 314 (13-1), 312 (11-1), 274 (10-0), 272 (37-1), 270 (40-7), 232 (33-2), 230 (95-3), 228 (100)</td>
</tr>
<tr>
<td>2,3,5,6-Tetrachloro-1-acetoxy-4-methoxybenzene</td>
<td>12.56</td>
<td>308 (0-5), 306 (3-5), 304 (5-0), 302 (4-1), 266 (11-6), 264 (49-2), 262 (100), 260 (76-5), 251 (58-9), 249 (27-3), 247 (55-7), 245 (43-9)</td>
</tr>
<tr>
<td>2,3,5,6-Tetrachloro-1,4-dimethoxybenzene</td>
<td>11.54</td>
<td>282 (3-1), 280 (9-4), 278 (30-4), 276 (67-3), 274 (41-9), 267 (27-7), 263 (12-3), 263 (54-2), 261 (100), 259 (82-2), 254 (12-5), 213 (16-0), 211 (48-5), 209 (51-1)</td>
</tr>
<tr>
<td>2,3,6-Trichloro-1,4-diacetoxybenzene</td>
<td>12.06</td>
<td>300 (1-1), 298 (28-0), 296 (32-6), 258 (42-2), 256 (12-2), 254 (12-5), 215 (30-9), 214 (96-3), 212 (100)</td>
</tr>
<tr>
<td>2,3,6-Trichloro-1-acetoxy-4-methoxybenzene</td>
<td>10.91</td>
<td>272 (1-9), 270 (4-1), 268 (5-5), 230 (25-1), 228 (94-5), 226 (100), 215 (22-5), 213 (70-7), 211 (72-5), 177 (11-5), 175 (18-6)</td>
</tr>
<tr>
<td>2,3,6-Trichloro-1,4-dimethoxybenzene</td>
<td>10.54</td>
<td>244 (14-4), 242 (40-0), 240 (47-3), 229 (30-8), 227 (97-9), 225 (100), 177 (31-1), 175 (47-3)</td>
</tr>
<tr>
<td>2,6-Dichloro-1,4-di(TMS)benzene</td>
<td>11.08</td>
<td>326 (9-1), 324 (64-4), 322 (90-0), 311 (5-1), 309 (33-2), 307 (48-5), 274 (33-4), 272 (100)</td>
</tr>
<tr>
<td>2,5-Dichloro-1,4-di(TMS)benzene</td>
<td>10.58</td>
<td>326 (11-1), 324 (65-8), 322 (100), 311 (3-9), 309 (26-1), 307 (38-5), 274 (23-2), 272 (68-7)</td>
</tr>
<tr>
<td>Dichlorotriacetoxybenzene</td>
<td>14.01</td>
<td>324 (0-2), 322 (2-1), 320 (3-7), 282 (1-1), 280 (5-1), 278 (10-1), 240 (3-4), 238 (17-3), 236 (22-8), 198 (10-2), 196 (63-8), 194 (100)</td>
</tr>
</tbody>
</table>

* TMS, trimethylsilyloxy.

chlorobenzoquinone (II). Tetrachlorodihydroxybenzene (III) was oxidized by both LiP and MnP to its corresponding benzoquinone. No products indicative of oxidative dechlorination of tetrachlorodihydroxybenzene by LiP or MnP were observed. The benzoquinone (II) was identified by comparing its retention time with that of the standard compound using HPLC. Furthermore, the reduced and acetylated derivative of the benzoquinone (II) was identified by GC-MS (Table 1).

Reduction of tetrachlorobenzoquinone (II)

Six-day-old cultures of P. chrysosporium, as well as 6-d-old washed hyphae, resuspended in buffer, readily converted tetrachlorobenzoquinone (II) to tetrachlorodihydroxybenzene (III) with greater than 80% conversion within 1 h at 37 °C. In contrast, only minimal conversion of the quinone to the hydroquinone (~6% in 1 h) occurred with the filtered extracellular medium from 6-d-old cultures, suggesting that the chloroquinone reduction activity is cell-associated.

DISCUSSION

Tetrachlorodihydroxybenzene (III) was identified as the metabolite of tetrachlorodihydroxybenzene (III) is probably not a significant reaction in the further degradation of this metabolite. Trichlorotrihydroxybenzene (VI) was identified as a metabolite of tetrachlorotrihydroxybenzene (III), and VI was also formed when tetrachlorobenzoquinone (II) was incubated in water. Thus, the formation of VI from III is probably due to the initial oxidative dechlorination of tetrachloroquinone (II) with no oxidative dechlorination products observed, suggesting that the oxidative dechlorination of tetrachlorotrihydroxybenzene (III) probably not a significant reaction in the further degradation of this metabolite. Trichlorotrihydroxybenzene (VI) was also proposed as a metabolite of PCP degradation by the fungus Mycena avenacea (Kremer et al., 1992).

Trichlorotrihydroxybenzene (V) was reductively dechlorinated to yield 2,5-dichlorotrihydroxybenzene (XI). Mass fragmentation patterns of the trimethylsilyloxy derivatives of 2,5-dichlorotrihydroxybenzene and 2,6-dichlorotrihydroxybenzene can be distinguished (Table 1), enabling the observed metabolite to be unequivocally identified as 2,5-dichlorotrihydroxybenzene (XI). In the present study, dichlorotrihydroxybenzene (VII) and 3,5,6-trichlorotrihydroxybenzene (VI) were also metabolites of trichlorotrihydroxybenzene (V), suggesting that V is either enzymically hydroxylated or undergoes oxidation followed by 1,4-addition to produce VI (Joshi & Gold, 1994) and that the latter is reductively dechlorinated to generate VII. Reductive dechlorination of V, followed by hydroxylation of the product XI, would also yield...
VII. The hydroxylation of \( p \)-hydroquinone and chlorohydroquinone has been demonstrated previously (Reddy et al., 1998). Dichlorotrihydroxybenzene (VII) was reductively dechlorinated to produce 5-chloro-1,2,4-trihydroxybenzene as the only product.

Finally, when 2,5-dichlorodihydroxybenzene (XI) was added to cultures, it was further reductively dechlorinated to 2-chloro-1,4-dihydroxybenzene (XIV). Other metabolites included chlorotrihydroxybenzene (VIII), dichlorotrihydroxybenzene (XVIII) and trihydroxybenzene (XVIII). We have reported that 2,6-dichlorodihydroxybenzene also is converted to chlorodihydroxybenzene (Reddy et al., 1998). We also showed that the further reductive dechlorination of chlorodihydroxybenzene (XIV) either precedes or follows hydroxylation of chlorodihydroxybenzene (XIV) to produce the key aromatic metabolite trihydroxybenzene (Reddy et al., 1998). The conversion of 2,5-dichloro-1,4-dihydroxybenzene (XI) to chlorotrihydroxybenzene (VIII) probably occurs via an initial reductive dechlorination to generate XIV, followed by hydroxylation to generate VIII (Fig. 4). Apparently, hydroxylation also is an important reaction in the dechlorination of polychlorophenols.

The methylation of phenols and hydroquinones by \( P. \) chrysosporium cultures has been observed previously during the degradation of various aromatic compounds (Lamar et al., 1990; Valli & Gold, 1991; Joshi & Gold, 1993; Armenante et al., 1994; Reddy et al., 1998). Our results show that PCP (I) was methylated by \( P. \) chrysosporium under HC/HN conditions. However, metabolically stable methylated compounds do not accumulate in HC/LN cultures when PCP is rapidly degraded. Thus, the methylation reaction is probably not a major metabolic step in the degradation of PCP under HC/LN conditions. Under HC/HN conditions, PCP was slowly methylated to form pentachloroanisole as the sole product (~1% product formed after 3 d). The transformation of PCP metabolites, including tetrachlorodihydroxybenzene but not PCP, occurs under HC/HN conditions in a manner similar to that observed under HC/LN conditions (Fig. 2). This suggests that the initial oxidative dechlorination occurs only under HC/LN ligninolytic conditions, but that subsequent steps, including reductive dechlorination and hydroxylation, can occur under either primary or secondary metabolic growth conditions.

Based on our present and previous results, we propose a pathway(s) for the complete dechlorination of PCP (Fig. 4). PCP (I) is first oxidized by LiP or MnP to tetrachlorobenzoquinone (II), which apparently is further degraded by two parallel pathways with several cross-pathway steps. In the first pathway, tetrachlorobenzoquinone (II) is converted to trichlorotrihydroxybenzene (VI) by a 1,4-addition reaction (Joshi & Gold, 1994). The latter can undergo three successive reductive dechlorinations to form trihydroxybenzene (XVIII). In addition, at the trichloro-, dichloro- and monochlorodihydroxybenzene levels, an hydroxylation reaction can redirect the metabolic flow from one pathway to the other (Fig. 4). We showed earlier that trihydroxybenzene is ring-cleaved to form maleyl acetate by a trihydroxybenzene dioxygenase (Rieble et al., 1994). Finally, in previous work (Reddy et al., 1998) we used filtration and plating assays as well as PCR methods to demonstrate that this...
pathway is carried out by the fungus rather than by bacterial contaminants.

PCP degradation has been studied extensively in the prokaryote *S. chlorophenolica*; a PCP monooxygenase and a tetrachlorodihydroxybenzene reductive dehalogenase have been purified from this organism (Xun & Orser, 1991, 1992). However, to our knowledge, this is the first report of a pathway(s) for the complete dechlorination of PCP by a eukaryote. Recently, we published a study on a two-component enzymic system which reductively dechlorinates tetrachlorohydroquinone (III) to produce trichlorohydroquinone (V) in cell-free extracts of *P. chrysosporium* (Reddy & Gold, 1999). This cell-free system also reductively dechlorinates trichlorohydroquinone (V) and dichlorohydroquinone (XI). We are now attempting to purify the two enzyme components of this novel reductive dechlorination system. Finally, this work as well as our previous studies (Reddy *et al.*, 1998; Reddy & Gold, 1999) clearly show that this fungus is able to degrade PCP via a combination of extracellular oxidative and intracellular reductive dechlorination reactions.

ACKNOWLEDGEMENTS

This research was supported by grant DE-FG03-96ER20235 from the US Department of Energy, Division of Energy Biosciences, and grant 2-4722-03 from the US Department of Agriculture.

REFERENCES


Received 13 July 1999; revised 19 October 1999; accepted 20 October 1999.