

Involvement of Cytochrome P450 in Pentachlorophenol Transformation in a White Rot Fungus *Phanerochaete chrysosporium*

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Abstract

The occurrence of cytochrome P450 and P450-mediated pentachlorophenol oxidation in a white rot fungus *Phanerochaete chrysosporium* was demonstrated in this study. The carbon monoxide difference spectra indicated induction of P450 (103 ± 13 pmol P450 per mg protein in the microsomal fraction) by pentachlorophenol. The pentachlorophenol oxidation by the microsomal P450 was NADPH-dependent at a rate of 19.0 ± 1.2 pmol min⁻¹ (mg protein)⁻¹, which led to formation of tetrachlorohydroquinone and was significantly inhibited by piperonyl butoxide (a P450 inhibitor). Tetrachlorohydroquinone was also found in the cultures, while the extracellular ligninases which were reported to be involved in tetrachlorohydroquinone formation were undetectable. The formation of tetrachlorohydroquinone was not detectable in the cultures added with either piperonyl butoxide or cycloheximide (an inhibitor of *de novo* protein synthesis). These results revealed the pentachlorophenol oxidation by induced P450 in the fungus, and it should be the first time that P450-mediated pentachlorophenol oxidation was demonstrated in a microorganism. Furthermore, the addition of the P450 inhibitor to the cultures led to obvious increase of pentachlorophenol, suggesting that the relationship between P450 and pentachlorophenol methylation is worthy of further research.

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Introduction

Pentachlorophenol (PCP) has been widely applied for decades around the world, as a fungicide (mainly as wood preservative), herbicide, defoliant and detergent supplement in soaps [1]. In consequence, PCP and its residues can be found today as a group of major pollutants in many terrestrial and aquatic ecosystems [2,3], and become widespread in humans and other living organisms, including those without direct exposure [4]. In addition to obvious acute effects, PCP can cause birth defects, chromosome abnormalities, blood disorders, nerve damage [4], cancer (confirmed in animal and probable in human) [5] and endocrine disruption [6]. Because of such high toxicity and persistence in the environment, there is now a complete ban on PCP production within the European Union (EU). Moreover, many countries and regions, such as China, USA and EU, classified PCP as a priority pollutant and have recommended restricted use to minimize its impact [7,8].

Biotransformation by microorganisms is an important fate of PCP in the environment and contaminated sites. The white rot fungi, which can decompose lignin, can effectively transform PCP and were widely studied for the treatment of PCP-contaminated soil [9,10] and wastewater [11]. Dechlorination and methylation were the two major pathways of PCP biotransformation and both led to detoxification of PCP. Aerobic dechlorination of PCP is known to be catalyzed by hydroxylase (a flavoprotein in *Flavobacterium sp.*) [12] and various peroxidases, such as laccase

[13], lignin peroxidase (LiP), manganese peroxidase (MnP) [14] in the white rot fungi, and horseradish peroxidase [15]. O-methylation of chlorophenols has been reported to be an environmentally significant alternative to the dechlorination [2]. PCP methylation was found in some bacteria [16], and much more common among fungi including the white rot fungi [1,17].

Cytochrome P450s (P450s) are widely distributed hemoproteins involved in various steps of the biosynthesis of endogenous compounds and in the oxidative detoxification and elimination of many hydrophobic xenobiotics including pollutants, drugs and pesticides [18]. PCP can obviously induce cytochrome P450 (P450) activities in animals and insects [19–21], and was reported to be transformed into tetrachlorohydroquinone (TCHQ) by a human P450 (CYP 3A4) [22]. Involvement of P450-type enzyme in PCP oxidation was suspected according to results of inhibitor assay in *Mucor ramosissimus* [1] and spectrometric evidences in *Mycobacterium chlorophenolicum* (formerly *Rhodococcus chlorophenolicus*) [23]. However, P450-mediated oxidation of PCP was still not elucidated in any microorganism.

The white rot fungus *Phanerochaete chrysosporium* has more than 150 P450 genes, which is one of the largest P450 contingents known to date in fungi [24] and the functions of which are of special interest in recent years [25,26]. In our previous study, we proposed significant induction of *P. chrysosporium* P450 by PCP according to the carbon monoxide (CO) difference spectrum with a peak at 457 nm, and found an increase of PCP degradation with

the presence of P450 inhibitor [27]. In this paper, the P450-mediated oxidation was demonstrated to be involved in PCP transformation by this fungus.

Materials and Methods

Chemicals

PCP, pentachloroanisole (PCA), and piperonyl butoxide (PB) were obtained from Aldrich, and TCHQ was obtained from Supelco. Cycloheximide (CHI) was obtained from Roche.

Microorganisms and growth media

P. chrysosporium strain BKM-F-1767 (ATCC 24725) was maintained on Difco potato dextrose agar (PDA). Difco potato dextrose broth (PDB, 24 g·l⁻¹) was used as nutrient-rich medium.

Inocula were prepared according to the method of Aiken and Logan [28]. Fungi grown for 6 days in a static culture flask were blended and mixed, and then a 0.1-ml aliquot of this suspension was aseptically transferred to a sterile 250-ml flask containing 25 ml of media. The fungi were incubated for 60 h at 37°C.

Then, 0.938 μmol (0.25 mg) of PCP dissolved in 25 μl of acetone was added to each flask to a final concentration of 37.5 μM in the cultures. Two types of controls were prepared, one with sterile culture (autoclaved at 121°C for 20 min) and PCP and the other with intact culture but without PCP. The fungal biomass of the sterile control was approximately equivalent to that in the experimental cultures. All the flasks were incubated at 37°C in darkness (to prevent photooxidation of the PCP). The cultures were routinely examined by microscopy to ensure the absence of bacterial contamination.

Extraction and detection of PCP and the metabolites

Extraction. The flasks were incubated for another 4 days (unless otherwise stated) after addition of PCP.

PCP and the metabolites were extracted according to Reddy et al. [29]. The cultures were added with sodium dithionite to reduce quinone products, acidified with HCl to pH 2, saturated with NaCl, and extracted three times with ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate and concentrated with a rotary vacuum evaporator at 35°C. The residues were acetylated with acetic anhydride-pyridine (2:1) and analyzed by gas chromatography-electron capture detection (GC-ECD) and by GC-mass spectrometry (GC-MS), and then compared with residues from the controls. Quinones were analyzed directly by high-performance liquid chromatography (HPLC) without prior reduction.

HPLC analysis. Reverse phase HPLC analysis of metabolites was conducted as described by Reddy et al. [29], with a Hewlett Packard 1100 series liquid chromatography (Hewlett Packard, Palo Alto, CA, USA) equipped with a Lichrospher 100 RP8 column (Agilent Technologies, Santa Clara, CA, USA), 5 μm (250 mm×4.6 mm). The column was eluted with a linear gradient of 0 to 75% acetonitrile in 0.05% phosphoric acid over 15 min, with a flow rate of 1 ml·min⁻¹. UV absorbance spectra were determined with the diode array spectrophotometer (Hewlett Packard G1315A) and compared to those of standards. PCP and the quinones were detected at 238 nm and 285 nm, respectively.

GC-ECD and GC-MS analysis. GC-ECD analysis was performed on an Agilent 6890N GC with a HP-5 capillary column (30 m×0.25 mm×0.32 μm). GC-MS analysis of derivatized metabolites was performed on a DSQ GC-MS (Thermo Corp., Waltham, MA, USA) with a quadrupole mass filter and a VF-5 MS (Varian, Palo Alto, CA, USA) capillary column (30 m×0.25 mm×0.25 μm), and ran in the electron ionization

mode with electron energy of 70 eV. The injector temperature was 200°C. The column was held at 50°C for 2 min, and programmed to rise to 300°C at 10°C·min⁻¹, after which the temperature was held isothermally at 300°C for 3 min. The derivatized products were identified by comparing their retention times on chromatography and their mass spectra with derivatized standards, and quantitated by using calibration curves obtained with derivatized standards.

Preparation of extracellular and microsomal fractions

To prepare extracellular fractions, the culture supernatants were periodically sampled during the incubation. The samples were centrifuged at 9,000 g for 10 min and filtered through 0.2 μm-pore-size filter to obtain extracellular fractions.

To prepare microsomal fractions for P450 study, some flasks were incubated for 24 hours after addition of PCP dissolved in acetone. Non-induced and acetone-induced cultures were set up as controls for analysis of P450 induced by PCP. The mycelia were harvested by centrifugation (5000 g, 15 min, twice) and the fungal pellets were washed extensively with 0.1 M sodium phosphate buffer (pH 7.5, ice-cold) containing 10 mM EDTA. The microsomal fraction was then prepared according to Masaphy et al. [30]. The pellets were resuspended in 0.1 M sodium phosphate buffer (called buffer B, pH 7.5, ice-cold) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 20% (v/v) glycerol. Then the cells were disrupted by glass beads (0.4~0.6 mm, 1 g mycelium in 1 ml buffer B with 1 g glass beads) using a dismembrator (Sartorius, Mikro-Dismembrator S, Germany) over a period of 2 min with 20-s bursts followed by 20-s cooling on ice. The biomass homogenate was centrifuged at 15,000 g for 15 min twice to remove cell debris, nuclei and mitochondria. Then the supernatant was centrifuged at 105,000 g for 90 min to pellet the microsomal fraction. After washing twice with buffer B, the microsomal fraction was suspended in buffer B and stored at -80°C.

Enzyme activity assay

Cytochrome P450 determination. The P450 contents of microsomal fractions were determined by CO difference spectra as described previously [31–33]. Firstly, the total protein concentrations of the microsomal fractions were determined by the Bradford method [34]. The sample was diluted to about 1 mg·ml⁻¹ protein with buffer B and then added with 1 mM KCN. Subsequently, the sample was dispensed equally into two cuvettes (1 ml per cuvette) and a baseline spectrum was spectrophotometrically recorded in the range of 400~500 nm (Shimadzu, UV-2401PC, Japan). One cuvette was then gently gassed with CO at a rate of 3 ml·min⁻¹ for 40 s, and the other one was gassed with N₂ to the same extent. An equal volume (10 μl) of sodium dithionite solution (400 mg·ml⁻¹) was accurately added into each cuvette, and then a difference spectrum was recorded. The concentrations of P450 were calculated using the extinction coefficient ε_{450–490} value of 91 mM⁻¹·cm⁻¹ [32]. The P450 contents in microsomal fractions were expressed in pmol P450 per mg protein.

Microsomal transformation of PCP. The 1-ml assay solution contained 3 mM MgCl₂, 1 mM NADPH, 5 mg BSA, microsomal fractions from PCP-induced cells (1~1.4 mg of protein) and 37.5 μM PCP in 100 mM potassium phosphate buffer (pH 7.5). Three types of control reactions, no-NADPH control, sterile control and the control using microsomal fractions from non-induced cell, were run parallel. The reaction mixtures were incubated at 37°C overnight, and then extracted with ethyl acetate at pH 2, dried over sodium sulfate, evaporated under N₂ and analyzed by GC-ECD after acetylation. The inhibition of

P450 activity was determined with PB (a P450 inhibitor) added to the concentration of 2 mM in the reaction mixture. The decrease in the amounts of PCP transformed and TCHQ formed was measured by GC-ECD.

Extracellular peroxidases determination. The activities of LiP, MnP and laccase were determined spectrophotometrically by the method of Tien and Kirk [35], Paszczynski et al. [36] and Niku-Paavola et al. [37]. LiP activity in extracellular fractions was assayed with 3,4-dimethoxybenzyl alcohol as the substrate. MnP activity was assayed with Mn^{2+} as substrate. Laccase activity was assayed with 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) as substrate. One unit (U) of enzyme oxidizes 1 μ mol of the substrate per min in the presence of H_2O_2 .

Extracellular enzyme reaction. Two types of extracellular reaction mixtures were set according to the MnP and LiP reaction mixtures reported by Reddy et al. [29]. The first one contained extracellular fractions (1 ml in the 2-ml mixture), PCP (37.5 μ M), $MnSO_4$ (0.2 mM), and H_2O_2 (0.1 mM) in 50 mM sodium malonate (pH 4.5), and the second one had extracellular fractions (1 ml in the 2-ml mixture), PCP (37.5 μ M), veratryl alcohol (0.1 mM), H_2O_2 (0.1 mM) in 20 mM sodium succinate (pH 3.0). Reactions were carried out at 25°C for 4 days. Reaction mixtures were extracted with ethyl acetate, evaporated under N_2 and analyzed by GC-ECD after acetylation as described previously [29].

Inhibitor experiments

The PDB cultures were incubated for 60 h at 37°C in the dark. Then, PB was added to some flasks to a final concentration of 2 mM, while CHI (1.46 mM) and PMSF (0.5 mM) were added to some other flasks to suppress *de novo* synthesis of proteins and to inhibit protease [38]. After another 60-min incubation, the cultures were added with PCP (37.5 μ M), incubated for another 4 days and extracted as described above. Transformation rate of PCP and formation of PCP metabolites were analyzed by GC and compared to those controls without PB or CHI.

The assays were all performed in triplicate and the mean values and standard deviations of the data were presented.

Results

Transformation efficiency and metabolites of PCP in the cultures

In the PDB cultures, the fungus was able to transform $18 \pm 3\%$ (the mean \pm standard deviation of three replicates) of added 37.5 μ M PCP in 1 day ($6.7 \pm 1.1 \mu$ M \cdot day $^{-1}$ on average), and $45 \pm 3\%$ in 4 days ($4.2 \pm 0.3 \mu$ M \cdot day $^{-1}$ on average). The average transformation efficiency was $1.37 \pm 0.23 \mu$ mol \cdot day $^{-1}\cdot$ g $^{-1}$ (μ mol per day per gram dry weight of biomass) in 1 day, and $0.772 \pm 0.047 \mu$ mol \cdot day $^{-1}\cdot$ g $^{-1}$ in 4 days.

After concentration of the acetylated extract, the metabolites were analyzed by comparing the GC profiles (Figure 1) with those of acetylated extracts from the control cultures without PCP. The two metabolites were then identified as PCA and TCHQ, according to comparison of the retention times on GC and the mass spectra with derivatized standards (Figure 2). While $16.9 \pm 1.2 \mu$ M PCP was transformed in the cultures incubated with PCP for 4 days, the concentrations of the products, PCA and TCHQ, were $15.2 \pm 1.2 \mu$ M and $0.31 \pm 0.09 \mu$ M respectively. This result indicated that methylation was the major but not the only way of PCP biotransformation by *P. chrysosporium*.

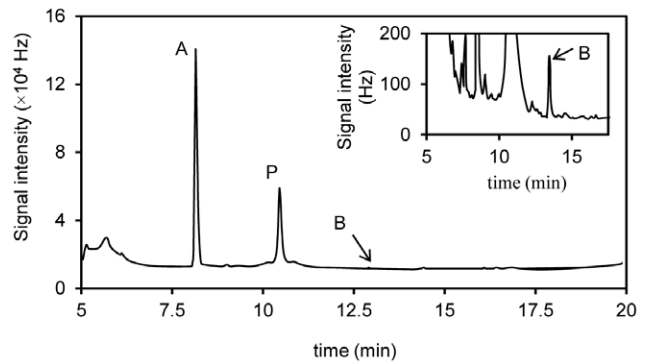


Figure 1. GC-ECD chromatogram of the extracts from the cultures of *Phanerochaete chrysosporium* incubated with pentachlorophenol. After preincubated for 60 h, the 25-ml potato dextrose broth cultures were added with 37.5 μ M pentachlorophenol (dissolved in 25 μ l acetone), and incubated for 4 days at 37°C in darkness. The cultures were then acidified, extracted and acetylated. P, pentachloroacetoxybenzene. A, pentachloroanisole. B, tetrachloro-1,4-diacetoxybenzene (acetylated tetrachlorohydroquinone). The inner figure shows the peak of compound B by scaling up the vertical axis. doi:10.1371/journal.pone.0045887.g001

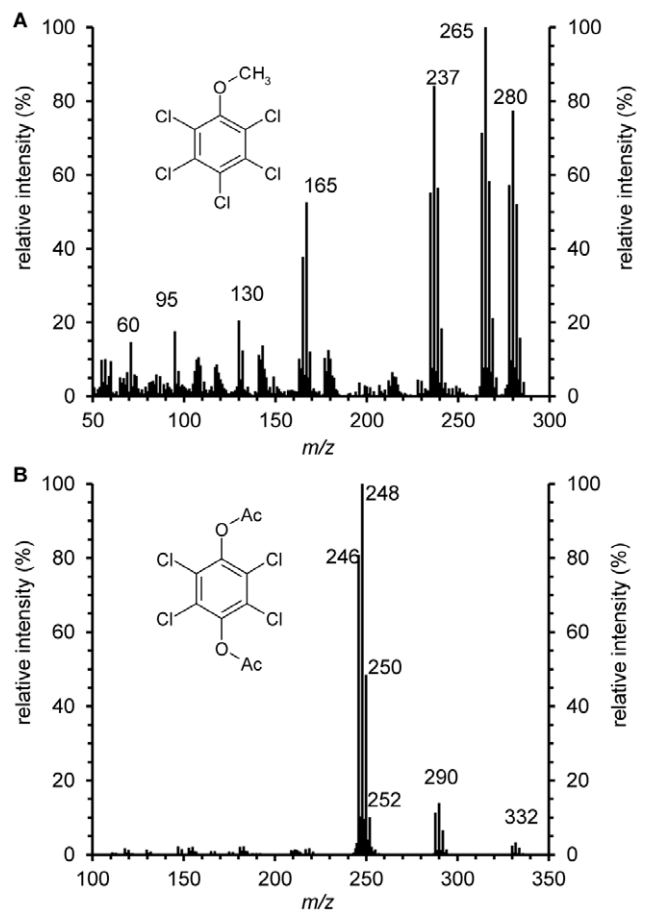


Figure 2. Mass spectra of pentachloroanisole (A) and acetylated tetrachlorohydroquinone (B) produced by *Phanerochaete chrysosporium*. After preincubated for 60 h, the PDB cultures were added with 37.5 μ M pentachlorophenol and incubated for 4 days at 37°C in darkness. The metabolites were extracted, acetylated and analyzed by GC-MS. doi:10.1371/journal.pone.0045887.g002

Role of the extracellular peroxidases

The production of TCHQ indicated PCP oxidation by *P. chrysosporium* which was only reported to be catalyzed by the extracellular ligninases, LiP and MnP [14]. However, the ligninases, LiP, MnP and laccase, were all undetectable in the PDB cultures incubated with PCP for 0, 1, 2, 3 or 4 days. Moreover, neither PCP transformation nor TCHQ were detectable in the reaction mixtures of extracellular fractions from the PDB cultures. These results ruled out detectable involvement of the extracellular ligninases in PCP oxidation in PDB cultures, and suggested the role of some intracellular oxidase which has not been documented to date.

Involvement of induced P450 in PCP transformation

In vitro transformation of PCP by microsomal P450. To demonstrate the existence of P450 during transformation of PCP by *P. chrysosporium*, CO difference spectra of microsomal fractions from PCP-induced cells were recorded. The spectra of microsomal fractions showed peaks at 450 nm (Figure 3A), indicating the occurrence of P450s, the contents of which were 103 ± 13 pmol P450 per mg protein. The spectra of microsomal fractions had major peaks at 420 nm which were attributed to P420, an inactive form of P450. Because P450 was very slight in pure solvent-induced cells (Figure 3B) and not observed in non-induced cells (Figure 3C), the synthesis of P450 was expected to be induced by addition of PCP to the PDB cultures.

The microsomal fraction with active P450 was utilized for *in vitro* transformation of PCP (Table 1). The microsomal fraction of PCP-induced cells from PDB cultures was utilized after 24-h induction and the concentration of P450 in the reaction mixture was up to 102 ± 13 nM. Samples and controls were prepared as described in Materials and Methods. In the reaction mixture containing P450 and NADPH, PCP transformation was significant (18.8 ± 1.2 nM min^{-1}), and 6.5 ± 2.1 μM TCHQ was detected after 10-hour incubation. PB, a P450 inhibitor, was used at 2 mM for inhibition assay. When the mixture was added with PB, PCP transformation rate was decreased by 97% (to 0.6 ± 0.4 nM min^{-1}) and TCHQ was not detectable. Furthermore, PCP transformation was not obvious (at an average rate of 0.5 ± 0.4 nM min^{-1}) in the mixture with P450 but no NADPH. The reaction mixture without detectable P450 (using microsomal fraction from non-induced cell) and the sterile control had no significant difference in PCP

concentration. According to NADPH-dependent PCP transformation, the reaction rate of PCP transformation by the microsomal P450 was up to 19.0 ± 1.2 pmol min^{-1} (mg protein) $^{-1}$.

Effects of the P450 inhibitor and CHI on TCHQ formation in the cultures. The role of P450 was also assessed by examining effect of the P450 inhibitor on PCP transformation in the cultures. We added 2 mM PB to PDB cultures before the addition of 37.5 μM PCP, and TCHQ was not detectable after incubation for 4 days. In contrast, the concentration of TCHQ in the cultures without PB was 0.31 ± 0.09 μM (31 folds higher than the detection limit) after incubation for 4 days (Table 2). This indicated effective inhibition of TCHQ formation by PB.

CHI can suppress *de novo* synthesis of proteins including P450s in the fungi [38]. The PDB cultures was added with 1.46 mM CHI, and then incubated with PCP for 4 days. P450 was not detectable in the microsomal fractions from these cultures. Correspondingly, TCHQ was not detectable either (Table 2). The effects of PB and CHI on TCHQ formation strongly suggesting important role of induced P450 in PCP oxidation.

Increase of PCA by inhibition of P450

When P450-mediated PCP oxidation was inhibited by PB, the transformation rate of PCP and the concentration of PCA in the cultures were increased by $57 \pm 10\%$ and $41 \pm 6\%$ (Table 2), and the biomass was not statistically different from the cultures without PB. When induction of P450 was suppressed by CHI, the transformation rate of PCP and the concentration of PCA remained the same (Table 2), but the biomass was decreased by $17 \pm 5\%$. Accordingly, CHI led to increase of PCP transformation and PCA formation in a unit of biomass, by $22 \pm 7\%$ and $20 \pm 8\%$. These results suggested interesting relationship between P450 and the methylation.

Discussion

In our previous work, the induction of P450 by PCP in *P. chrysosporium* was spectrophotometrically detected, but the peaks were at 457~459 nm in the CO difference spectra [27,39]. In this paper, the method was modified (e.g., KCN was added to mask cytochrome oxidase before CO difference spectra were recorded). As a result, the typical peak at 450 nm was detected in CO difference spectra of the microsomal fractions of PCP-induced

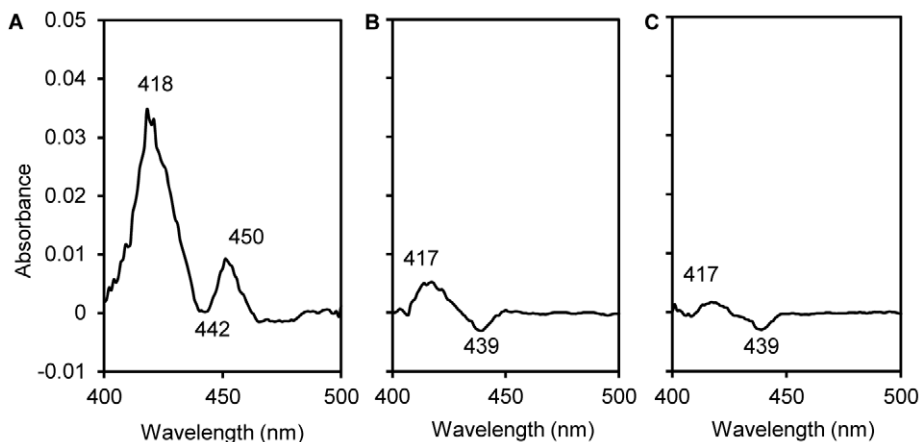


Figure 3. Carbon monoxide difference spectra of microsomal fractions of *Phanerochaete chrysosporium*. The 25-ml potato dextrose broth cultures were added with 37.5 μM pentachlorophenol (dissolved in 25 μl acetone, spectrum A), 25 μl acetone (spectrum B) and without anything (spectrum C), respectively; the protein concentration of every sample tested was 1 mg ml^{-1} . doi:10.1371/journal.pone.0045887.g003

Table 1. Pentachlorophenol (PCP) transformation and concentration of tetrachlorohydroquinone (TCHQ) after incubation of PCP and microsomal fractions of *Phanerochaete chrysosporium* from potato dextrose broth (PDB) cultures.

Initial concentration in reaction mixtures ^a				PCP transformed (μM) ^d	Concentration of TCHQ (μM)	Transformation rate of PCP (nM min^{-1}) ^d
PCP (μM)	P450 (nM)	NADPH (mM)	PB (mM)			
37.5	102 \pm 13 ^b	1	0	11.3 \pm 0.7	6.5 \pm 2.1	18.8 \pm 1.2
37.5	102 \pm 13 ^b	1	2	0.36 \pm 0.24	ND ^e	0.6 \pm 0.4
37.5	102 \pm 13 ^b	0	0	0.30 \pm 0.24	ND	0.5 \pm 0.4
37.5	ND ^c	1	0	0.10 \pm 0.20	ND	0.06 \pm 0.12

Mean \pm standard deviation of the mean, n=3

^aThe reaction mixtures were incubated at 37°C for 10 h

^bThe microsomal fraction of 37.5 mM PCP-induced cells was utilized.

^cThe microsomal fraction of non-induced cells was utilized. ND, P450 was not detectable.

^dThe concentration of PCP transformed and transformation rate of PCP were calculated according to concentrations of residual PCP in the sample and the sterile control after 10-h incubation.

^eND, not detectable ($<0.03\mu\text{M}$).

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cells, while the peak was not present for non-induced cells and very slight for solvent-induced cells. This result convincingly demonstrated induction of active P450 by PCP.

Induction of P450 by PCP has been reported in algae [40], insect [21] and tissues of many animals, such as human, rat [19] and fish [20]. As for bacteria, induction of a P450-type coenzyme by PCP has only been detected in *Mycobacterium chlorophenolicum*, according to the peak at 457 nm in CO difference spectra [23]. However, induction of fungal P450 by PCP has rarely been documented before, except that of *P. chrysosporium* P450 in our previous paper [39]. The white rot fungus *P. chrysosporium* has about 150 P450 genes [24]. Some of these P450s have been shown to be inducible at the transcriptional level by various xenobiotics [26,41–43], while the effects of chlorophenols on these P450s were not reported. In our work, induction of fungal P450 by PCP was first clarified by the appearance of the typical peak at 450 nm in CO difference spectra.

It is known that fungal P450 systems are involved in oxidation of some xenobiotics, e.g., benzoic acid [33], toluene [44], and phenanthrene [45]. The *P. chrysosporium* P450s co-expressed with P450 oxidoreductase in yeasts exhibited broad substrate profiles [25,26]. All these eukaryotic P450s, as well as the PCP oxidizing

P450 from human [22], utilize NAD(P)H as electron donor of the monooxygenation reactions [46,47], whereas most bacterial P450s receive electrons from NADH [18]. In the present study, PCP oxidation was considerable in reaction mixture containing microsomal P450 and NADPH, while PCP transformation was not evident in the mixtures without NADPH or sufficient P450, indicating stringent requirement for P450 and NAD(P)H.

The P450-mediated PCP oxidation was further supported by the results of P450 inhibition assay. PB, a well-known inhibitor of P450s, can significantly inhibit many P450-mediated reactions [45,48,49], and is expected to have no effect on the total activity of *P. chrysosporium* [50]. In our study, PB significantly inhibited PCP transformation and TCHQ formation in the intact cultures, as well as in the microsomal fractions. Furthermore, CHI which suppressed synthesis of P450 also inhibited the TCHQ formation, and the peroxidases which can produce TCHQ from PCP were not detectable in the cultures. Thus, it is strongly suggested that this fungal P450 could catalyze the oxidation of PCP to form TCHQ in the cultures, which is the same as the P450 in human [22].

According to all the evidences described above, we conclude that P450 is involved in PCP transformation by *P. chrysosporium* and

Table 2. Effect of piperonyl butoxide (PB) and cycloheximide (CHI) on the transformation of pentachlorophenol (PCP) and the production of pentachloroanisole (PCA) and tetrachlorohydroquinone (TCHQ) in potato dextrose broth (PDB) cultures of *Phanerochaete chrysosporium*.

Test condition ^a	PCP transformed (μM)	Concentration of PCA		Concentration of TCHQ		Concentration of cells (g l^{-1}) ^c
		(μM)	($\mu\text{mol g}^{-1}$) ^b	(μM)	($\mu\text{mol g}^{-1}$)	
Controls	16.9 \pm 1.2	15.2 \pm 1.2	2.8 \pm 0.3	0.31 \pm 0.09	0.06 \pm 0.02	5.5 \pm 0.3
PB	26.4 \pm 1.7	21.4 \pm 1.0	4.0 \pm 0.2	ND ^d	/	5.4 \pm 0.2
CHI	17.0 \pm 1.0	15.2 \pm 1.0	3.4 \pm 0.2	ND ^d	/	4.5 \pm 0.1

Mean \pm standard deviation of the mean, n=3.

^aThe 25-ml cultures were incubated for 60 h, and then added with 2 mM PB or 1.46 mM CHI. After another 60-min incubation, the cultures were added with PCP (37.5 μM) and incubated for another 4 days before the determination of PCP, PCA and TCHQ by GC-ECD. The cultures without PB or CHI were set as controls. Values represent means \pm standard deviations for three replicates.

^b $\mu\text{mol g}^{-1}$, μmol product per gram dry weight of biomass.

^c g l^{-1} , gram dry weight of biomass per liter culture.

^dND, not detectable ($<0.03\mu\text{M}$).

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it should be the first time that P450-mediated PCP oxidation was demonstrated in a microorganism. The microsomal P450 of PCP-induced cells catalyzed PCP transformation at a reaction rate of $19.0 \pm 1.2 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ (about $1.1 \text{ nmol} \cdot \text{h}^{-1} \cdot (\text{mg protein})^{-1}$). This is similar to the value of $1.75 \sim 2.2 \text{ nmol} \cdot \text{h}^{-1} \cdot (\text{mg protein})^{-1}$ in the case of the P450-type protein in *M. chlorophenolicum* [23], and higher than $8 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{nmol P450})^{-1}$ in microsomal fraction of yeast expressing the human P450 3A43 [22].

Laugero et al. [38] reported that mineralization ratio of PCP was up to 11% while that of PCA was only 2% by *P. chrysosporium* under nutrient-rich condition. Moreover, the TCHQ produced by P450-mediated oxidation was expected to be effectively dechlorinated by *P. chrysosporium* according to Reddy and Gold [14]. Thus, the metabolic pathway initiated by P450 was probably an effective way for PCP mineralization other than methylation.

The present work found PCA was the major product of PCP in *P. chrysosporium*, which is consistent with previous studies [3,38]. PCA was frequently detected in many environments including sediments [51], lake and river waters [52], soils [53] and vegetable materials [54]. Since PCA has a very high bioconcentration factor

(10^4 in fish) and can be rapidly demethylated in both rat and mouse to its more toxic precursor [55], the environmental significance of PCA and other chloroanisoles has been emphasized by several authors [2,56].

Interestingly, we found obvious increase of PCA in the cultures degrading PCP when P450 was inhibited. PCP methylation was the major source of PCA in environments [57]. P450-mediated demethylations of some chloroanisoles have been reported in animals [58], and a O-demethylation of TCA catalyzed by a microsomal P450 to produce 2,4,6-trichlorophenol has been found in a white rot fungus *Phlebia radiata* recently [56]. Thus, it would be of interest to study on the relationship between P450 and PCP methylation, as well as the role of P450s in PCA metabolism in microorganisms.

Author Contributions

Conceived and designed the experiments: DN HW. Performed the experiments: DN. Analyzed the data: DN. Contributed reagents/materials/analysis tools: DN. Wrote the paper: DN HW.

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