ORIGINAL

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Identification of cytochrome P450 monooxygenase genes from the white-rot fungus *Phlebia brevispora*

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Abstract

Three cytochrome P450 monooxygenase (CYP) genes, designated *pb*-1, *pb*-2 and *pb*-3, were isolated from the white-rot fungus, *Phlebia brevispora*, using reverse transcription PCR with degenerate primers constructed based on the consensus amino acid sequence of eukaryotic CYPs in the O_2-binding, meander and heme-binding regions. Individual full-length CYP cDNAs were cloned and sequenced, and the relative nucleotide sequence similarity of *pb*-1 (1788 bp), *pb*-2 (1881 bp) and *pb*-3 (1791 bp) was more than 58%. Alignment of the deduced amino acid (aa) sequences of *pb*-1-*pb*-3 showed that these three CYPs belong to the same family with > 40% aa sequence similarity, and *pb*-1 and *pb*-3 are in the same subfamily, with > 55% aa sequence similarity. Furthermore, *pb*-1-*pb*-3 appeared to be a subfamily of CYP63A (CYP63A1-CYP63A4), found in *Phanerochaete chrysosporium*. The phylogenetic tree constructed by 500 bootstrap replications using the neighbor-joining method showed that the evolutionary distance between *pb*-1 and *pb*-3 was shorter than that between *pb*-2 and *pb*-1 (or *pb*-3). Exon-intron analysis of *pb*-1 and *pb*-3 showed that both genes have nearly the same number, size and order of exons and the types of introns, also indicating both genes appear to be evolutionarily close. It is interesting that the transcription level of *pb*-3 was evidently increased above the *pb*-1 transcription level by exposure to 12 coplanar PCB congeners and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, though the two genes were evolutionarily close.

Keywords: cytochrome P450 monooxygenase, *Phlebia brevispora*, gene cloning, real-time RT-PCR, dioxins, CYP63A

Introduction

Cytochrome P450 enzymes (CYPs) constitute a large superfamily of heme-containing monooxygenases that are widely distributed in all kingdoms of life (Nelson 2009). CYPs are involved in the metabolism of a wide variety of endogenous and xenobiotic compounds by catalyzing regio- and stereospecific monooxygenation with an oxygen atom generated from molecular oxygen. Mammalian CYPs have been studied extensively because of their leading role in drug and xenobiotic metabolism and detoxification (Allis et al. 2002; Inouye et al. 2002; McGraw JE and Waller 2006; Shimada 2006; Vrba et al. 2004; Warner et al. 2009; Yamazaki 2000; Zhang et al. 2006). CYPs from bacteria, yeast and fungi have also been well studied in the biosynthesis of essential compounds like ergosterol, which is a constituent of fungal cell membranes, and in the detoxification and biodegradation of a broad spectrum of environmental chemical pollutants (Kelly et al. 1997; Kelly et al. 2003; Lamb et al. 2000; Seth-Smith et al. 2008; van den Brink et al. 1998).

The wood-rotting *Basidiomycetes*, white-rot fungi, have been extensively used for biodegradation of various chemical pollutants. The ability to degrade such structurally diverse chemical pollutants has generally been attributed to a lignin-degrading enzyme system, including mainly lignin peroxidase, manganese-dependent peroxidase and laccase produced by these fungi (Cameron et al. 2000; Fujihiro et al. 2009; Han et al. 2004; Mayer and Staples 2002; Takagi et al. 2007; Van Aken et al. 1999). However, several studies pointed out that white-rot fungi are capable of degrading certain xenobiotics under culturing conditions that did not induce the production of lignin peroxidase, manganese-dependent peroxidase or laccase

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(Bumpus and Brock 1988; Mileski et al. 1988; Yadav and Reddy 1993; Yadav et al. 1995). Therefore, besides such lignin-degrading enzymes, alternative oxygenases, CYPs, are apparently involved in catalyzing degradation of several xenobiotics. In particular, several specific CYPs from *Phanerochaete chrysosporium*, the model white-rot fungus, have been studied in the metabolism of xenobiotics (Chigu et al. 2010; Kasai et al. 2010; Matsuzaki and Wariishi 2005; Ning et al. 2010; Subramanian and Yadav 2009; Syed et al. 2010). Since whole genome sequencing of *P. chrysosporium* has been completed, the molecular diversity of CYPs and the presence of at least 150 CYP genes have been elucidated (Nelson 2009).

A previous report described the fungal metabolism of coplanar PCBs (Co-PCBs) by the white-rot fungus *Phlebia brevispora* (Kamei et al. 2006). In addition, the monomethoxylated metabolite was detected in cultures containing each congener by gas chromatography and mass spectrometry, suggesting the involvement of CYP in the transformation of Co-PCBs to methoxylated compounds via hydroxylation. This result led us to search for the CYP system in *P. brevispora* involved in the metabolism of xenobiotics. Here, we describe the identification, cloning, and sequence analysis of three CYP genes from *P. brevispora*.

Materials and methods
Chemicals
Twelve Co-PCB congeners and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were purchased from Wellington Labs (Ontario, Canada). Each congener was mixed in dimethylsulfoxide (DMSO) at a concentration of 2 μg/ml for experimental use.

Strain and culture conditions
P. brevispora TMIC33929 was obtained from the Tottori Mycological Institute (Tottori, Japan). The fungus was maintained as a culture on potato dextrose agar medium (Difco Laboratories, MI, USA). The fungus was grown on a potato dextrose agar plate at 26°C for 2 weeks. Then, the fungus mycelium was inoculated into Kirk liquid medium and incubated statically at 26°C for 2 to 3 weeks; an additional incubation was carried out for 2 days in Kirk liquid medium (Tien and Kirk 1988) containing all

of 12 Co-PCB congeners and TCDD at a concentration of 0.25 ng/ml each. Fungal mycelium was harvested from cultures by vacuum filtration and ground in a mortar and pestle with the aid of liquid nitrogen. The ground mycelium was immediately used for RNA preparation.

Construction of degenerate primers for cDNA isolation of CYP genes
In a previous study to search for unknown CYP genes in cultures of *P. chrysosporium*, a degenerate primer set was constructed based on the relatively conserved consensus aa sequences across eukaryotic CYPs in the O_2-binding and heme-binding regions (Kullman and Matsumura 1997). Hence, for the first round of PCR of CYP genes, we used the same degenerate forward and a slightly modified reverse primer (see Table 1) from that used in the study of *P. chrysosporium*. For the second nested PCR of CYP genes, a degenerate forward primer was constructed based on the relatively conserved consensus aa sequence between the CYP O_2-binding region and the CYP heme-binding region, which is called a meander region (Hasemann et al. 1995), as shown in Table 1. The degenerate reverse primer used in the second PCR was constructed for a region slightly upstream of the heme-binding region.

Isolation, cloning and sequencing of partial cDNA fragments of CYP genes
Total RNA as a template for reverse transcription (RT)-PCR was prepared from the ground mycelium using an RNeasy Plant Mini kit (QIAGEN Sciences, MD, USA). The RT mixture (13 μl), containing 1 μl total RNA (> 50 ng), 1 μl oligo(dT)$_{12-18}$ (0.25 μg), 4 μl dNTP mixture (2.5 mM) and 7 μl sterile water, was heated at 65°C for 5 min and incubated on ice for 1 min. After addition of 4 μl 5 × first-strand buffer, 1 μl dithiothreitol (0.1 M), 1 μl RNase inhibitor and 1 μl SuperScript III reverse transcriptase (200 units) (Invitrogen Corp., CA, USA) to a total volume of 20 μl, the reaction mixture was incubated at 50°C for 60 min, then at 70°C for 15 min. Finally, 20 μl sterile water was added to the reaction mixture, which was stored at -20°C. The first PCR for CYP cDNA amplification was performed in a reaction mixture (20 μl) containing 2 μl cDNA, 1 μl each of the

Table 1 Degenerate primers used for the searching for partial CYP cDNAs from *P. brevispora*

Target gene	Forward primer	Reverse primer
pb-1 (first PCR)	5'(A/C/T)TIIIIG(C/G)IGGI(A/C/G)(A/G)I(C/G)AIACIACIGC-3'	5'-CCI(A/G)(A/G/T)(A/G)CAIIII(C/T)(G/T)II(C/G/T)ICCII(A/C/G)I (C/T)(C/T)(A/G)AAIGG-3'
pb-1 (second PCR)	5'-T(G/T)(C/G/T)(A/C/G)(A/G)IC CI GAI(A/C/G)IT(G/T)(C/G/T)(C/G/T)T-3'	5'-(C/T)(G/T)IIIICCII(A/C/G)I(C/T)(C/T)(A/G)AAIGGIA(A/G/T)I T(A/G)-3'
pb-2, *pb*-3	5'-(C/T)TIAA(C/T)ATI(A/C/T)TI(A/C/T)TIGCIGGI(A/C)GIGA(C/T)ACIAC-3'	5'-(C/T)TGICCIA(A/G)(A/G)CA IA(A/G/T)IC(G/T)IGGICCIGC-3'

degenerate forward and reverse primers (10 μM), 2 μl 10 × Ex Taq buffer, 2 μl dNTP mixture (2.5 mM), 0.2 μl Ex Taq HS (TaKaRa Bio Inc., Shiga, Japan) and 11.8 μl sterile water. The cycling conditions used for the first PCR were as follows: 98°C for 3 min, followed by 30 cycles of 98°C for 30 s, 53°C for 30 s and 72°C for 120 s, with a final step at 72°C for 7 min. The second nested PCR was performed with the first PCR mixture as a template and degenerate primers for the second PCR according to the following procedure: 98°C for 3 min, followed by 30 cycles of 98°C for 30 s, 50°C for 30 s and 72°C for 120 s, with a final step at 72°C for 7 min. This two-round PCR led to the isolation of a single PCR fragment, which had high sequence homology to CYP genes from *P. chrysosporium* in BLAST homology searches. Cloning of the partial cDNA fragment for the CYP gene was performed using a Mighty TA-cloning system (TaKaRa Bio Inc.). The reaction mixture, containing 2 μl of the partial cDNA fragment, 0.5 μl pMD20-T vector and 2.5 μl ligation Mighty-Mix was incubated at 16°C for 30 min, then added to competent DH10B *E. coli* (Invitrogen Corp., CA, USA) for transformation. The transformed cells were screened in LB medium containing X-gal, IPTG and ampicillin according to the *LacZ* blue/white screening method. The cloned partial cDNA fragment was prepared from a white transformed colony grown in LB medium containing ampicillin (100 μg/ml) at 37°C overnight using a QIAprep spin miniprep kit (QIAGEN Sciences). The cloned partial cDNA fragment was sequenced according to the dye-terminator method (Sanger and Coulson 1975).

Unknown 5′- and 3′-end sequence determination of cDNAs

The 5′- and 3′-end sequences were determined using a SMARTer RACE cDNA amplification system (Clontech Laboratories Inc., CA, USA). According to the manufacturer's instructions, 5′-RACE-ready cDNA and 3′-RACE-ready cDNA were separately prepared from total RNA (10 ng to 1 μg). The CYP cDNA-specific primers for 5′-RACE and 3′-RACE PCR were respectively designed according to the base sequence of partial cDNA as follows: 5′-RACE, 5′-TCGAGCGCGATAGTGTC-GAAGTGCTGCAGC-3′ (first PCR) and 5′-TGTACGCGAACTGCTGGCCGAGGCAGATG-3′ (nested PCR); 3′-RACE, 5′-TCGACGAACGTCTGCA-CAAGCACCTGACAC-3′ (first PCR) and 5′-AGCACCTGACACCGAACCCATTCATC-3′ (nested PCR). The cycling conditions used for the both rounds of PCR were: 98°C for 3 min, followed by 30 cycles of 98°C for 30 s, 68°C for 30 s and 72°C for 120 s, with a final step at 72°C for 7 min. The cloning and sequencing methods were the same as described in the Materials and methods subsection: *Isolation, cloning and sequencing of partial cDNA fragments of CYP genes.*

Cloning and sequencing of full-length cDNAs

Full-length CYP cDNAs were cloned using a universal cloning method based on the site-specific recombination system of bacteriophage lambda (Invitrogen Corp.). Based on the 5′- and 3′-end sequences, one primer set for cloning of full-length CYP cDNA was designed to the 5′-UTR region for the forward primer and to the 3′-UTR region for the reverse primer. According to the manufacturer's instructions, CYP gene specific forward and reverse primers, attached by special sequences called attB1 (5′-GGGGACAAGTTTGTACAAAAAAG-CAGGCTTC-3′) and attB2 (5′-GGGGACCACTTTGTA-CAAGAAAGCTGGGT-3′) were constructed as follows: forward, 5′-GGGGACAAGTTTGTACAAAAAAG-CAGGCTTCTCTCGACGGAGCCAAGTT GCCTG TATC-3′; reverse, 5′-GGGGACCACTTTGTACAA-GAAAGCTGGGTTCGTCCAAATACAAGATGAAT CGCGCTAC-3′. PCR for full-length CYP cDNA was performed in a reaction mixture (50 μl) containing 1 μl cDNA, 1 μl each of the attB1-forward and attB2-reverse primers (10 μM), 25 μl PrimeSTAR Max DNA polymerase (TaKaRa Bio Inc.) and 22 μl sterile water. The cycling conditions used for PCR were: 98°C for 3 min, followed by 35 cycles of 98°C for 20 s, 61°C for 10 s and 72°C for 120 s, with a final step at 72°C for 7 min. The cloning of full-length CYP cDNA was performed using a reaction mixture containing 1-2 μl amplified PCR product (15-150 ng), 1.5 μl cloning vector (P-DONR221, 100 ng/μl), 4.5-5.5 μl TE buffer (pH 8.0) and 2 μl BP Clonase II enzyme mix (Invitrogen Corp.). The reaction mixture was incubated at 25°C for 60 min, and 1 μl proteinase K was added to stop the reaction. For transformation of *E. coli*, 1 μl of the reaction mixture was added to competent DH10B cells. The transformed cells were screened in LB medium containing kanamycin (100 μg/ml) at 37°C overnight. Full-length CYP cDNA was sequenced according to the dye-terminator sequencing method. The aa sequence was deduced by GENETYX ver.8 software (GENETYX Corp., Tokyo, Japan)

Isolation, cloning and sequencing of full-length CYP genes from genomic DNA

The cloning and sequencing of full-length CYP genes from genomic DNA was performed using the same procedure as that described in the Materials and methods subsection: *Cloning and sequencing of full-length cDNAs* except that the cDNA was replaced with genomic DNA as the template in the reaction mixture. The genomic DNA was prepared from the ground mycelium of *P. brevispora* using a DNeasy Plant Mini kit (QIAGEN Sciences).

Quantitative analysis of gene transcripts by real-time RT-PCR

Total RNA as a template for real-time quantitative RT-PCR was prepared from *P. brevispora* exposed to all 12 Co-PCB congeners and TCDD for 2 days at a final concentration of 0.5 ng/ml in Kirk liquid medium using an RNeasy Plant Mini kit. As a control experiment, DMSO was added into Kirk liquid medium instead of the 12 Co-PCB congeners and TCDD. Target gene-specific primers for quantification of transcripts were constructed based on < 300 bp amplicons using online technical support for design of real-time PCR assays (Roche Applied Science, Bavaria, Germany). The 18S rRNA gene was used as an internal control gene in RT-PCR. The constructed primers and amplicon lengths were: *pb-1*, 5'-CGCGTACAACGAGATGTCA-3' (forward), 5'-GAGCGCGATAGTGTCGAAGT-3' (reverse) and 64 bp (amplicon); *pb-2*, 5'-TCATCTTCGTGCCCTTCAAT-3' (forward), 5'-ACGACGCTTCGTTGTATGC-3' (reverse) and 72 bp (amplicon); *pb-3*, 5'-TTCTAT-GACGCGCCCTTT-3' (forward), 5'-CATGCCTATC-GAACACCTCA-3' (reverse) and 65 bp (amplicon); 18S rRNA, 5'-AACTTAAAGGAATTGACGGAAGG-3' (forward), 5'-TGAGTTTCCCCGTGTTGAG-3' (reverse) and 77 bp (amplicon). The RT reaction was performed as described in the Materials and methods subsection: *Isolation, cloning and sequencing of partial cDNA fragments of CYP genes* except that oligo(dT)1_{2-18} primers were replaced with random primers in the reaction mixture. Real-time quantitative RT-PCR was performed by the detection of the nonspecific dye SYBR Green, which binds to any double-stranded DNA, using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture (25 μl), containing 2 μl cDNA, 2.5 μl each of the target gene-specific forward and reverse primers (1 μM), 12.5 μl 2 × SYBR Premix Ex Taq II (TaKaRa Bio Inc.), 0.5 μl ROX Reference Dye II and 5 μl sterile water, was put into a 96-well reaction plate, which was set in the 7500 Fast Real-Time PCR System. The cycling conditions used were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60 C for 40 s. The number of gene transcripts was estimated using a λ, polyA$^+$RNA (Takara Bio Inc.) as a standard reference RNA. The amplicon from a λ, polyA$^+$RNA was quantified based on the SYBR green fluorescence signal. The standard curve was constructed by plotting threshold cycle values (Y-axis), which correspond to the number of PCR cycles needed to reach the threshold fluorescence, against log number of RNA molecules (X-axis). The number of gene transcripts in each of the DMSO-treated control and the12 Co-PCB, TCDD-exposed culture was individually estimated using an equation of the constructed standard curve, Y = -3.1815X + 34.935, R^2 = 0.99765.

Results

Isolation and sequence analysis of cDNAs for CYP genes *pb*-1, *pb*-2 and *pb*-3

A single cDNA fragment that had an approximate length of 100 bp was obtained by nested PCR, as shown in Figure 1. This cDNA fragment showed high nucleotide sequence homology with the CYP63 family from *P. chrysosporium* (Yadav et al. 2003). Hence, we designated this CYP gene from *P. brevispora pb*-1. Because of high nucleotide sequence homology between *pb*-1 and CYP63, degenerate primers were constructed to search for CYP genes in addition to *pb-1* based on the highly conserved consensus sequences in the O_2-binding region and heme-binding region of CYP63 (Yadav et al. 2003), as shown in Table 1. As a result of RT-PCR with these degenerate primers, two more CYP genes (*pb*-2, *pb*-3) were obtained. The nucleotide sequences of the 5'- and 3'-ends of the cDNA for *pb*-1, *pb*-2 and *pb*-3 were determined by a SMARTer RACE cDNA amplification system, and finally, full-length cDNAs of *pb*-1 (1788 bp), *pb*-2 (1881 bp) and *pb*-3 (1791 bp) were obtained.

Figure 1 Identification of a partial CYP cDNA from *P. brevispora* by the agarose gel electrophoresis. PCR products were separated with 2% agarose gel for 60 min at 100 V. Each electrophoretogram shows the result of PCR with forward/reverse primers (Lane 1), a forward primer (2), a reverse primer (3), and 100 bp ladder markers (M), respectively. The partial cDNA for CYP gene, *pb*-1, having an approximate length of 100 bp is marked by a white dot.

Table 2 Amino acid sequence similarities among CYPs from *P. brevispora* and *P. chrysosporium*

	pb-1	*pb*-2	*pb*-3	CYP63A1	CYP63A2	CYP63A3
pb-2	47.4	*pb*-2				
pb-3	61.4	46.5	*pb*-3			
CYP63A1	60.3	46.9	64.4	CYP63A1		
CYP63A2	60.6	48	60.4	58.6	CYP63A2	
CYP63A3	63.7	49.1	61.7	59.3	84.9	CYP63A3
CYP63A4	48	62.4	46.9	49.6	47.5	49.7

Amino acid sequences of CYP63A1-CYP63A4 from *P. chrysosporium* used for the similarity analysis were derived from DNA data base as described in Figure 3. Each number represents the percent similarity.

The nucleotide sequence similarities of these three genes were 60.9% between *pb*-1 and *pb*-2, 64.6% between *pb*-1 and *pb*-3, and 57.9% between *pb*-2 and *pb*-3. The nucleotide sequences of the three CYP cDNAs have been registered in the DNA Data Bank of Japan (DDBJ) and are available under the accession numbers AB634456, AB634457 and AB634458 for *pb*-1, *pb*-2 and *pb*-3, respectively.

Deduced aa sequence and protein analysis

The aa sequence similarities of *pb*-1, *pb*-2 and *pb*-3 are shown in Table 2. The percentage of aa sequence similarity was 47.4% between *pb*-1 and *pb*-2, 61.4% between *pb*-1 and *pb*-3, and 46.5% between *pb*-2 and *pb*-3. The overall aa sequence alignments showed a lower similarity in the N-terminal region (ca. < 140 aa) than in the C-terminal region. Although the aa sequence similarity was lower between *pb*-1 and *pb*-2 and between *pb*-2

and *pb*-3, the aa sequences around the meander and heme-binding regions were highly conserved in the three CYP genes (Figure 2). Furthermore, *pb*-1 and *pb*-3 also showed high aa sequence similarity to the CYP63 subfamily, CYP63A1-CYP63A3 (Doddapaneni et al. 2005; Doddapaneni and Yadav 2004), on the other hand, *pb*-2 showed high aa sequence similarity to CYP63A4 (Nelson 2009), as shown in Table 2. Phylogenetic analysis was performed for *pb*-1 through *pb*-3 and CYP63A1 through CYP63A4 using the neighbor-joining method in *MEGA* version 5 software (Tamura et al. 2011). A phylogenetic tree was constructed by 500 bootstrap replications, as shown in Figure 3. As a result, three clades appeared with high bootstrap values. CYP *pb*-1 and CYP63A1 were siblings in 98% of the bootstrap replications, and CYP *pb*-2 and CYP63A4 were siblings in 98% of the bootstrap replications. CYP *pb*-3 was grouped in a clade that included CYP63A2 and CYP63A3 in 67% of the bootstrap replications. The deduced CYP proteins for *pb*-1, *pb*-2 and *pb*-3 had estimated molecular weights of approximately 68,400, 71,300 and 68,100 and isoelectric points of 8.46, 6.56 and 6.93, respectively. The short sequences of hydrophobic aa (ca. 30 bp) at the *N*-terminal site found in all three CYP proteins are probably signal peptides for membrane binding.

Cloning and sequence analysis of genomic CYP genes *pb*-1, *pb*-2 and *pb*-3

The full-length CYP gene, *pb*-1, had 16 exons and 15 introns, leading to a predicted length of 2668 bp, as shown in Figure 4. Each exon varied in size from 13 bp


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pb-1  MFA-------LPQAYGLFLQQIFFLISPAVLIAGIVSLVTRRIGIDLPLWAIAIGSVFSLPFVHRIRAWSYYRSMQHKAARLGAVLPPQWRTSKIGNTDVLRMLMEAWREGFLSDHLWEK 113
pb-2  MALTTSLPPGILFLLS-NLPQVVLPPLAASLLLYALRVL---YGLAPPAWLAALAYLLSWPLAWTFDVQYRDWRIQREARARGAECPPRIPSKAIGGVDLIKQFGKDMEKRIIAYRHWDF 116
pb-3  MAL-------IPFTLSLVLEQFAKLIYPGVLLSVALIVLDRASVVHVPGWAIAVIVLTGIIPYHIAAARYRLWRDARKAASLGAILPPRWEGKAIGSRDLLKMLDHAWQHGYLSDQLWEK 113
             *       * *         *                   * *    *                * **   **  **  *                                      *
pb-1  MHELGQTYMVYVLWDPNYVTSDANVVKTILATDFGNYVKGPKFRTPMKSVLGSGVFNSDGDMWKFHRSMTRPFFSRERISHFELFDKYAERAMKRMKERMNSGYA------VDFQDLISR 227
pb-2  IKEFGPTYNLRVLFENRIFTAEPEYIKRILATEFPNYEKGPDFTFQMRTLLGTGVFNSDGDMWKFHRGMTRPFFSRDRISHFDIFDRHADAALGLMRARMAEGCAIDWQ------DLVSR 230
pb-3  FSELGSTYDLYIFWAHDYCTTDVNIVKSVLATEFDNWVKGKGFDSFMRSVLGTGVFNADGDLWKFHRSMTRPFFSRERIHHYEVFDRHAEEAISKMKDRLREGYA------VNFADAISR 227
         * *  **       *   *        **** ***  *  *   *  ** *****  ** ***** ***** ****** *    **  **  *   *  **   *           *   * **
pb-1  FTLDSASEFLFGASVNSL-ESSLPY-PFHAPASLREQPPT--------------QAEEFAKAFAAAQTVISARARLGHIWPWFELAGSKTAPFMRIVNAFLQPILEEA--LRKAKEEKA 328
pb-2  FTLDSASEFLFGQDVRSL-SAPLPYPPFPSASALSPLPFSSQEAYDAYTAQASTHPANRFAQSFLDAQITSARRSRFTRAWPLMEFWGDKVKRGLDVVDGFIDPLCEAAVRRERERRLRE 349
pb-3  FTLDTATEFLFNKCVHSLRDSTLPY-PHNAPPMLHDLPLS--------------AADQFARAFMDAQGAISARARTGWVWPWFEIFRSKTAAPMRVVDAFVAPILKQA--LERAKIAKE 329
         **** * ****     * **      ***  *        *          *          * **  * *    * *    **  *      *       *  *   *  *   *
pb-1  SGKGVEKKD-----GVLEDDDTLLDYLVRYTSDPVVLHDEVLNIMI AGRDTTA ATLTIVVYFLSQYPDVLRRLREEILIQVGPSRRPTYDDIREMKFLRAVLNETLRLYPSVPWNVRYAV 443
pb-2  GGGGDGASAKEKEKEEVGEEETLLEHLVKLTDDPQIVHDETLNILL AGRDTTA ITLTMAAYMLALHPHVLKRLRDEVQTRVG-ARRPTYDDIREMKYLRAVLRAFINEVLRLYPPVFNVRYAI 468
pb-3  SGLVGGSKA-----DEIKDDECLLDHLVRHTTDPVILHDEVLNIMI AGRDTTA GMMTVTVYFLSQYPNVLKRLREEILEKVGPINRPTYDDIREMKYLRAVLNESMRLYPAVPWNMRYAV 444
         *              ** ** ***  ** ***  ***** ***** *******    *    * *   ** **** *   **  *********** ***  *** ** *  ***
                                                  O2-binding
pb-1  NDGTIPSSDP-EKPFFVAAGTPVSYSVHCMHRRKDYWGPDAEE FDPDRF IDERLHKYLTPNPFIFL PFNAGPRICLG QQFAYNEMSFFLIRLLQHFDTIALDVDSQPSSSRRPEWKGLP 562
pb-2  NATTWPSLVPGGKPFYVPAGVRCLYSVFIMHRRKDLWGPDADK FDPDRF LDERLHRYLTPNPFIFV PFNAGPRICLG QQFAYNEASFMLIRLLQRVSHIEL-VQEVNPAAVPPPEWGVSG 587
pb-3  QDALLPNSEPNGKPFYIPAGASVSYSVHCMHRRKDYWGPDAEE FDPDRF LDHRLHKYLTPNPFIFL PFNAGPRICLG QQFAYNEMSFFIVRLLQNFETISLDIDAQPPDTRPPAEWAGAP 564
         *   *   ***  **     ***  *  *** ** * *********   ****** ***        *** *********** **     ****   **          *   **
                                                     Meander              Heme-binding
pb-1  GRKGVEKFWPKTHLTLYSEGGMWVK------MGEVQNDAE 596
pb-2  GSDGSDKVVFRGHLTMYVQGGLWVKMTEEPLAGEDAEADV 627
pb-3  GRKGVEKFWPKAHLTVYADGGLWVK------FKEVSSDI- 597
         * *  *       *** *  ** ***           *
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Figure 2 Amino acid sequence alignments of CYP *pb*-1, *pb*-2 and *pb*-3 from *P. brevispora*. The degenerate forward and reverse primers used for the searching for partial CYP cDNAs were constructed based on the consensus motifs squared in the alignments.
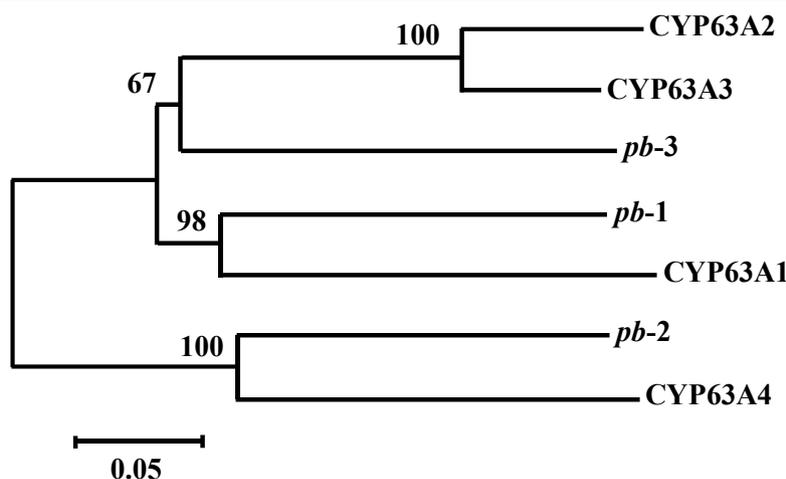
Figure 3 Phylogenetic tree constructed using amino acid sequence alignments of *pb*-1-*pb*-3 and CYP63A1-CYP63A4. The phylogenetic tree was constructed by 500 bootstrap replications and the neighbor-joining method. Amino acid sequences for CYP63A1-CYP63A4 from *P. chrysosporium* were derived from the CYP DNA data base (Nelson DR (2009) The cytochrome p450 homepage. Hum Genomics 4: 59-65).

to 400 bp; however, the size of the 15 introns was generally around 60 bp (Table 3). The full-length CYP genes, *pb*-2 and *pb*-3, were respectively obtained using attB-sequence attached primer set prepared as follows: *pb*-2, 5'-GGGGACAAGTTTGTACAAAAAAG-CAGGCTTCACATGGGGACGTCGTCAGG-3' (forward), 5'-GGGGACCACTTTGTACAAGAAAGCTGGG TTCCCACATAGATACGGCCATC-3' (reverse); *pb*-3, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTT CTCGAAAGGCGAGCGTCTCAATTAC-3' (forward), 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCG-GATTCTCCTTTGAATTTGT TCAC-3' (reverse). CYP *pb*-2 had 11 exons and 10 introns, with a length of 2871 bp, and *pb*-3 had 16 exons and 15 introns, with a length of 2595 bp. As shown in Table 3, the number, size and order of exons was the same in *pb*-1 and *pb*-3, except for three exons of 400, 72 and 45 bp in *pb*-1. Although each intron that was similar in size in *pb*-1 was slightly larger than the corresponding intron in *pb*-3, each type of intron was in the same order in *pb*-1 and *pb*-3. On the other hand, *pb*-2 was quite different from the other two CYP genes in all properties of exons and introns. The intron type was defined as follows: type 0, lies between two codons; type I, lies after the first base in the codon; type II, lies after the second base in the codon. The relative occurrence of the three intron types was 26.7% (type 0), 46.7% (type I) and 26.7% (type II) for *pb*-1 and *pb*-3, and 40% (type 0), 50% (type I) and 10% (type II) for *pb*-2.

Effect of exposure to dioxins on transcription levels of *pb*-1, *pb*-2 and *pb*-3

The effect of exposure to 12 Co-PCB congeners and TCDD on transcription levels of *pb*-1, *pb*-2 and *pb*-3 was investigated using real-time quantitative RT-PCR to monitor the fluorescent intensity of SYBR Green. The ratio of transcription levels following exposure to 12 Co-PCB congeners and TCDD to that following a control exposure to DMSO, the solvent for the dioxins, is represented in Figure 5. Among the three CYP genes, the transcription of *pb*-3 was evidently upregulated 2- to 3-fold by exposure to the 12 Co-PCB congeners and TCDD. The transcription rate of *pb*-2 was slightly increased; however, *pb*-1 transcription was unchanged.

Discussion

Kamei et al. (2006) reported the congener-specific metabolism of 3,3',4,4'-tetrachlorobiphenyl, 2,3,3',4,4'-penta-chlorobiphenyl, 2,3',4,4',5-pentachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl and 2,3',4,4',5,5'-hexa-chlorobiphenyl in 11 Co-PCBs by *P. brevispora* and the detection of methoxylated metabolites in the culture containing each congener, suggesting that these metabolites are probably produced via hydroxylation of Co-PCBs catalyzed by CYPs. To investigate the involvement of CYPs with the metabolism of dioxins, we first searched for CYP cDNA in *P. brevispora*. There is little information concerning CYP genes from *P. brevispora*; however, some useful information about nucleotide sequences of CYP cDNAs from *P. chrysosporium* is available. Hence, two sets of degenerate primers were constructed to search for CYP cDNAs from *P. brevispora*, as shown in Table 1, based on the nucleotide sequence of CYP cDNAs from *P. chrysosporium* presented by Kullman and Matsumura (1997), and the nucleotide sequences registered on the cytochrome P450 homepage organized by Nelson (2009). We describe three unique full-length cDNAs encoding CYP genes

```
                                              Exon 1
ACGGAGCCAAGTTGCCTGTATCCACCATGTTCGCCCTCCCGCAGGCTTATGGACTCTTCCTGCAGCAGATATTCTTCTTGATCTCGCCCGCTGT  68

GCTTATAGCAGGCATTGTAAGCCTTGTAACAAGGCGCATTGGGATTGATCTCCCCCTTTGGGCCATCGCCATCGGATCCGTGTTCAGCCTACCG  162

TTCGTACATCGCATAAGAGCATGGTCTTACTACCGGTCCATGCAGCACAAGGCCGCTCGCCTTGGTGCTGTCCTCCCGCCCCAGTGGAGAACGA  256
                                                                          Intron 1 (I)
GCAAGATAGGCAACACTGATGTTCTCAGAATGCTAATGGAAGCGTGGCGTGAAGGTTTCTTGAgtacgatcctgcaaaggttggccagagtccc  350
                       Exon 2
gttcctgtgctgacgctggtatgtagGTGACCATCTATGGGAGAAGATGCACGAACTCGGGCAGACATACATGGTCTATGTCCTGTGGGACCCT  444
                   Intron 2 (0)                                                        Exon 3
AATTATGTTACCTCGGATGCGAACGTGGTCAAGgttgttataccgacgactcacgtccgagatgatgctgactatagactatacagACGATCCT  538
               Intron 3 (I)                                        Exon 4
GGCTACTGATTTTGGCAACTATGTGAAAGgtatgatccacatatgctatgctctcacctagtctaacgcatcatgaagGCCCAAAGTTCCGCAC  632
                         Intron 4 (I)
GCCTATGAAGTCGGTGTTAGGCTCCGGCGTATTCAACTCAGATGgttcgtgtaagcgtcgtcctgggttattcacgtcacttatgttgtgcgac  726
         Exon 5         Intron 5 (II)                                           Exon 6
tatcgcagGCGATATGTGGAAgtgggtcaccaggcaaaatcagttttcaagtcttaatgattgggaattgtgtatagGTTTCACCGCTCCATG  820
                                                         Intron 6 (I)
ACCAGACCATTCTTCAGCCGTGAAAGAATAAGCCATTTCGAGCTATTTGATAAGTATGCTGgtgagtagcatgcgctttcagaaccaggaacaa  914
                     Exon 7
tcatatgcattcttgtgtgattgtctttccagAACGTGCTATGAAGAGAATGAAAGAGCGCATGAATTCAGGCTATGCCGTTGACTTCCAGgtt  1008
   Intron 7 (0)                                       Exon 8
cgttgatacccatcaatgaagcgtgagcctataatattgacatcgaaatctcacttcagGACCTCATTTCGCGCTTCACACTTGACTCCGCCTC  1102

TGAGTTCCTCTTCGGCGCATCCGTGAATAGCCTCGAGAGCTCGCTGCCCTACCCATTCCACGCTCCCGCGAGCCTACGCGAGCAGCCGCCGACA  1196

CAGGCGGAGGAGTTTGCCAAGGCGTTCGCTGCGGCGCAGACGGTTATCTCCGCGCGTGCGAGGCTCGGACACATATGGCCCTGGTTCGAGCTCG  1290

CGGGCTCCAAGACCGCTCCGTTCATGCGCATTGTCAATGCGTTCCTGCAGCCCATCCTCGAGGAGGCGCTGCGGAAAGCAAAGGAAGAGAAGGC  1384
                                                                          Intron 8 (I)
TTCGGGCAAGGGGGTCGAGAAGAAGGACGGCGTGCTCGAGGACGACGACACCCTCCTGGACTACCTTGTGCGCTATACCAGCGgtgggttttga  1478
                                                     Exon 9
gtttctgggcaccgcgttcctggagcttcatcggcaggttactaatcatgcgcatagACCCGGTTGTCTTGCATGATGAGGTGCTGAACATCAT  1572
         Intron 9 (0)                                            Exon 10
GATCGCAGGCCGCGATACGgtacgcacggctatttcggtgtttgatcttcaattttctgacgcgcaatgcatatcacagACCGCAGCTACATTG  1666

ACCATCGTCGTCTACTTCCTGTCTCAGTATCCTGATGTCCTGCGGCGGCTGCGCGAGGAGATCCTGATTCAGGTCGGGCCCTCGCGGCGTCCTA  1760
                                                       Intron 10 (I)
CGTATGATGATATCCGGGAGATGAAGTTCCTGAGGGCGGTTTTGAACGgtcagctggcttatctatttctctgggaatccaagctcatatattg  1854
       Exon 11                   Intron 11 (II)
acagAAACGCTGAGATTGTACCCTTCGGTgtaagtggactagttccaagttgtagaaatagtactgacgtagagataacttgtgggtgcgcagA  1948
   Exon 12         Intron 12 (II)                                     Exon 13
CCCTGGAATGTGCGgtatgtcataagctcccctagggtgtcgatactgtgtcttacgcccttttgaagATATGCTGTGAACGACGGCACCATCCC  2042
                     Intron 13 (II)
AAGCTCGGACCCCGAGAAGCCTTTCTTCGTTGCTGCAGGGACACCgtcagtgcatttgattccttgagttgtctttgacattgaccattcactt  2136
       Exon 14                                          Intron 14 (I)
caatacccagAGTCTCGTATTCTGTCCATTGTATGCATCGTCGAAAAGACTACTGGGGTCCGGATGgtacgtcaaacgctgaactcgtacatac  2230
                             Exon 15
tcactcgctcacttccccgcctccacccacagCCGAGGAATTCGATCCGGACCGCTTCATCGACGAACGTCTGCACAAGTACCTGACACCGAAC  2324

CCATTCATCTTCCTCCCCTTCAACGCCGGCCCGCGCATCTGCCTCGGCCAGCAGTTCGCGTACAACGAGATGTCATTCTTCCTCATCCGGCTGC  2418

TGCAGCACTTCGACACTATCGCGCTCGACGTGGACTCCCAGCCGTCCTCGTCGCGCCCACGCCCTGAGTGGAAGGGCTTGCCGGGACGGAAGGG  2512
                                                         Intron 15 (0)
TGTCGAGAAGTTCTGGCCGAAGACGCATTTGACGTTATATTCGGAGgtaagaggatttattcgttgtgaagtcagtgattagggacgtgaatac  2606
       Exon 16
tgattttgttctggtagGGTGGAATGTGGGTCAAAATGGGCGAAGTTCAGAATGACGCAGAGTAAATGTATTGGATTGGGATTGTAAGACTCGT  2700

GAATACCCCCTGGATCATAGTCTGTATTCTTGCATG                                                           2736
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Figure 4 Nucleotide sequence of CYP gene *pb*-1 from *P. brevispora*. Base numbering begins with the putative initiation codon ATG encoding the amino acid methionine. Exon and intron bases are indicated in upper and lower case letters, respectively. Exon nucleotide sequences are shadowed and numbered from 5′ end. Intron nucleotide sequences are also numbered from the 5′ end in *italics* followed by the intron type *(0, I or II)*. The predicted sequence for the signal peptide is underlined.

Table 3 Exon-intron organization in CYP genes *pb*-I, *pb*-2 and *pb*-3

pb-1		*pb-2*		*pb-3*	
Exon	Intron (Type*)	Exon	Intron (Type)	Exon	Intron (Type)
319		328		319	
	57(I)		67(I)		57(I)
101		71		101	
	53(0)		158(0)		53(0)
37		67		37	
	49(I)		54(I)		49(I)
60		60		60	
	58(I)		62(I)		49(I)
13		149		13	
	57(II)		283(0)		55(II)
77		469		77	
	65(I)		79(I)		47(I)
59		56		59	
	62(0)		68(0)		58(0)
400		179		403	
	68(I)		85(II)		64(I)
56		146		56	
	60(0)		68(I)		54(0)
157		116		157	
	50(I)		66(0)		52(I)
25		240		25	
	64(II)				53(II)
15				15	
	53(II)				50(II)
72				75	
	59(II)				48(II)
56				56	
	60(I)				57(I)
296				296	
	65(0)				58(0)
45				42	
1788	880	1881	990	1791	804
	2668		2871		2595
Type 0	4 (26.7%)	Type 0	4(40%)	Type 0	4 (26.7%)
Type I	7 (46.7%)	Type I	5 (50%)	Type I	7 (46.7%)
Type II	4 (26.7%)	Type II	1 (10%)	Type II	4 (26.7%)

The number in the table represents the bp length each of exon and intron.
*Type: representing presence of the intron between codons(Type 0), after the first base in the codon (Type I), and after the second base in the codon (Type II)

pb-1, *pb*-2 and *pb*-3 in *P. brevispora*. As a result of BLAST nucleotide sequence homology searching of these three CYP cDNAs, we found they were closely related to the members of the representative multigene family CYP63, CYP63A1-CYP63A4, found in *P. chrysosporium* (Doddapaneni et al. 2005; Doddapaneni and Yadav 2004; Nelson 2009). CYPs are classified and named based primarily on the level of aa sequence similarity. A family is generally defined as those CYPs having > 40% aa sequence similarity, and a subfamily is defined as those CYPs having > 55% aa sequence similarity. The deduced aa sequence alignments of *pb*-1-*pb*-3 showed that these three CYPs are members of the same family, and *pb*-1 and *pb*-3 are in the same subfamily. Furthermore, *pb*-1 and *pb*-3 appeared to belong to the subfamily of CYP63A1-CYP63A3, and *pb*-2 to CYP63A4.

Phylogenetic analysis of *pb*-1-*pb*-3 and CYP63A1-CYP63A4 with deduced aa sequence alignment using a neighbor-joining method also indicates that the phylogenetic tree is constituted of three clades and each *pb*-1-*pb*-3 belongs to a different one of the three clades. Another phylogenetic analysis using a maximum likelihood method showed a different phylogenetic tree from that of the neighbor-joining method, indicating that *pb*-3 is grouped in the clade with *pb*-1 and CYP63A1 in 81% of the bootstrap replications (data not shown). In both phylogenetic trees, the evolutionary distance between *pb*-1 and *pb*-3 was shorter than that between *pb*-2 and *pb*-1 (or *pb*-3). In addition to having a short evolutionary distance between *pb*-1 and *pb*-3, these two genes were closely located on the genomic DNA. A PCR fragment that had an approximate length of 700 bp was detected with the *pb*-3 forward primer (5'-AGGAT-TATGGGTCAAGTTCAAGGAAG-3') and *pb*-1 reverse primer (5'-CTTATGGACTCTTCCTGCAGCAGAT-3'), indicating that *pb*-3 is located upstream of *pb*-1 with a 613 bp interspace region in the same orientation (data not shown). Exon-intron analysis of *pb*-1 and *pb*-3 indicated that 13 of the 16 exons of the genes were similar in size and order; the exceptions were three exons: 8 (400 vs. 403 bp), 13 (72 vs. 75 bp) and 16 (45 vs. 42 bp), numbered according to the nucleotide sequence of *pb*-1 (Figure 4, Table 3). Relatively small and similarly sized introns were found in both *pb*-1 (49-68 bp) and *pb*-3 (47-64 bp), and the order of the types of introns in *pb*-1 was the same as in *pb*-3 (Table 3). From these results, the presence of some interesting variants, which were found in CYP63A1 by Yadav et al. (2003), would also be expected in the CYP genes of *P. brevispora*. In a study of intron-exon organization using the numerous *Arabidopsis* CYP genes, the intron position and type were well conserved among both subfamily and family, suggesting that intron position and type can be correlated with phylogenic relations and CYP functions among the subfamily and family (Paquette et al. 2000).

Searching for CYP genes involved in the metabolism of dioxins in *P. brevispora* is an objective of our studies; one CYP gene (*pb*-3) found in *P. brevispora* was especially upregulated at the level of transcription following exposure to 12 Co-PCB congeners and TCDD. To detect precisely the change in transcription rates of CYP genes by exposure to 12 Co-PCB congeners and TCDD, a control
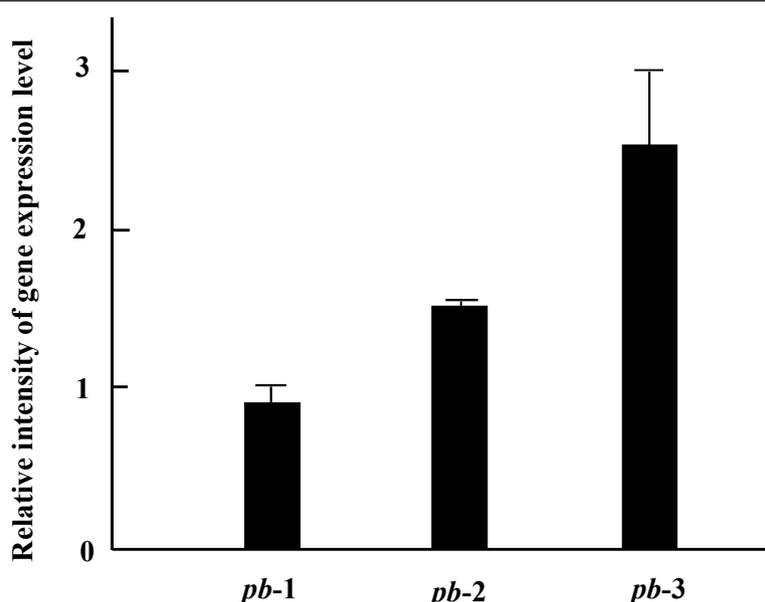
Figure 5 Effect of exposure to Co-PCBs and TCDD on transcription levels of CYPs from *P. brevispora*. Each bar represents the ratio of the transcription level of CYP gene following exposure to 12 Co-PCB congeners and TCDD to that following a control exposure to DMSO. Each bar represents the mean and SD of five determinations.

gene that is not influenced by these chemicals at transcription is essential for correcting the initial level of cDNA in real-time quantitative RT-PCR. The 18S rRNA gene was not influenced in the transcription step by 12 Co-PCB congeners and TCDD in preliminary experiments; hence, the 18S rRNA gene was used as an internal control gene. It is interesting that only the transcription level of *pb*-3 was evidently increased by exposure to these 12 Co-PCB congeners and TCDD, though *pb*-3 and *pb*-1 were evolutionarily close. In a previous study of xenobiotic induction of CYP63A1 and CYP63A2, some xenobiotics including PCB (Aroclor 1254), appeared to be responsible for the induction of only one gene (Doddapaneni and Yadav 2004). It seems that xenobiotic induction is not due to the phylogenetic correlation between the CYP genes, but rather due to the presence of the transcription regulatory site, e.g., xenobiotic response elements, located upstream of the CYP genes.

In this study, we have described the presence of three CYP genes in a white-rot fungus, *P. brevispora*; one of these genes was upregulated on exposure to dioxins. However, it is not obvious whether this upregulated CYP gene is involved in the metabolism of dioxins; so further experiments must be carried out to elucidate the correlation of CYP gene expression with the metabolism of dioxins.

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