Rat Cytochrome P_{450}-Mediated Transformation of Dichlorodibenzo-P-Dioxins by Recombinant White-Rot Basidiomycete *Coriolus hirsutus*

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Rat cytochrome P450, CYP1A1, has been reported to play an important role in the metabolism of mono-trichlorodibenzo-*p*-dioxins (M-TriCDDs). To breed lignin (and M-TetraCDDs)-degrading basidiomycete *Coriolus hirsutus* strains producing rat CYP1A1, an expression cassette [*C. hirsutus gpd* promoter-*C. hirsutus gpd* 5'-portion (224-bp of 1st exon-8th base of 4th exon)-rat *cyp1a1* cDNA-*Lentinula edodes priA* terminator] was constructed and inserted into pUCR1 carrying the *C. hirsutus arg1* gene. The resulting recombinant plasmid, MIp5-(cyp1a1+arg1) was introduced into protoplasts of *C. hirsutus* monokaryotic strain OJ1078 (Arg^-, Leu^-), obtaining three good Arg^+ transformants. These transformants [ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1)] were estimated to carry nine, six, and seven copies of the expression cassette on their chromosomes, respectively. Immunoblot analysis revealed that the three transformants produce similar amounts of rat CYP1A1 enzyme. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and recipient OJ1078 were cultivated in a liquid medium containing 2,7/2,8 (at a ratio of 1:1)-dichlorodibenzo-*p*-dioxins (2,7/2,8-DCDDs) and the amount of intra- and extracellular 2,7/2,8-DCDDs remaining was measured. The results showed that all three transformants efficiently transform 2,7/2,8-DCDDs through the action of the recombinant rat CYP1A1 enzyme.

1. Introduction

Chlorinated dibenzo-*p*-dioxins (CDDs) have been of public concern for two decades because of their toxicity in animal tests (Safe 1990; Schecter et al.

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1987). Extracellular lignin-degrading enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP) produced by white-rot basidiomycete fungi have been reported to be involved in transformation of various CDDs (and various chlorophenols etc.) (Armenante et al. 1994; Bumpus et al. 1985; Joshi and Gold 1993; Joshi and Gold 1994; Reddy and Gold 2000; Takada et al. 1996; Valli et al. 1992). Through various metabolic pathways, the white-rot basidiomycete fungi convert the chlorinated aromatic compounds to CO_2 and H_2O. We have recently produced monokaryotic strains of the white-rot basidiomycete *Coriolus hirsutus* with high MnP or LiP activity (Yamazaki et al. 2004a; Yamazaki et al. 2004b). The culture supernatants of these strains showed higher transformation activities of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) and pentachlorophenol.

To date, the metabolism of various CDDs has been studied *in vivo* using experimental animals (Hu and Bunce 1999a; Poiger et al. 1982; Rose et al. 1976; Tulp and Hutzinger 1978; Van den Berg et al. 1994; Wroblewski and Olson 1985). The insertion of a single oxygen atom into the dioxin molecule to form an epoxide by cytochrome P450 (CYP) is considered to be the initial reaction in the metabolism of various CDDs. Hu and Bunce (1999b) suggested that mammalian CYP1A1 and CYP1A2 play an important role in the metabolism of mono-tri (M-Tri)CDDs. In vivo studies suggested that the CYP-dependent metabolism includes multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, hydroxylation with elimination of a chloride substituent, and opening of the dioxin ring (Sakaki et al. 2002). All of these reactions appear to be reactions aimed at detoxifying M-TriCDDs. Thus, CYPs seem very likely to be key enzymes for metabolism of M-TriCDDs in mammals. So far this CYP-catalyzed metabolism of M-TriCDDs has been not reported in basidiomycete (and ascomycete) fungi, even though the CYPs of white-rot basidiomycete *Phlebia lindtneri* have been implicated in the catalysis of a simple mono-hydroxylation of non-chlorinated DD; the *in vivo* mono-hydroxylation has been shown to be inhibited by CYP inhibitors (Mori and Kondo 2002), and the CYPs of the white-rot basidiomycete *Lentinula edodes* (Akiyama et al. 2002, 2004), *Phanerochaete chrysosporium* (Van den Brink et al. 1998) and *Pleurotus pulmonarius* (Maspahy et al. 1999) have been shown to catalyze the conversions of benzo(a)pyrene and 7-ethoxycoumarin. In this paper, we attempted to breed *C. hirsutus* strains that produce mammalian

CYP and metabolize chlorinated dioxin molecules efficiently within mycelial cells. If such strains could be successfully bred, and used together with strains producing large amounts of extracellular LiP and MnP, various CDDs may be metabolized more efficiently. The rat *cyp1a1* cDNA encoding the CYP1A1 enzyme that transforms 2,7-DCDD, 2,8-DCDD, and 2,3,7-TriCDD (Murakami et al. 1990) was used for breeding of *C. hirsutus*. We constructed a chromosome-integrating recombinant plasmid carrying an expression cassette for rat CYP1Al and introduced it into protoplasts of monokaryotic strain of *C. hirsutus*. Here, we report successful production of strains that produce the rat CYP1A1 enzyme and transform 2,7/2,8(at a ratio of 1:1)-DCDDs (hereafter referred to simply as 2,7/2,8-DCDDs).

2. Results

2.1. *Transformation of C. hirsutus Arg⁻ Leu⁻ auxotrophic monokaryotic strain OJ1078 with MIp5-(cyp1a1+arg1)*

MIp5-(cyp1a1+arg1) contains the rat CYP1A1-expression cassette (*C. hirsutus gpd* promoter-*C. hirsutus gpd* 5'-portion-rat *cyp1a1* cDNA-*L. edodes priA* terminator) and the selection marker *C. hirsutus arg1* gene (Tsukamoto et al. 2003), as shown in Fig. 1.

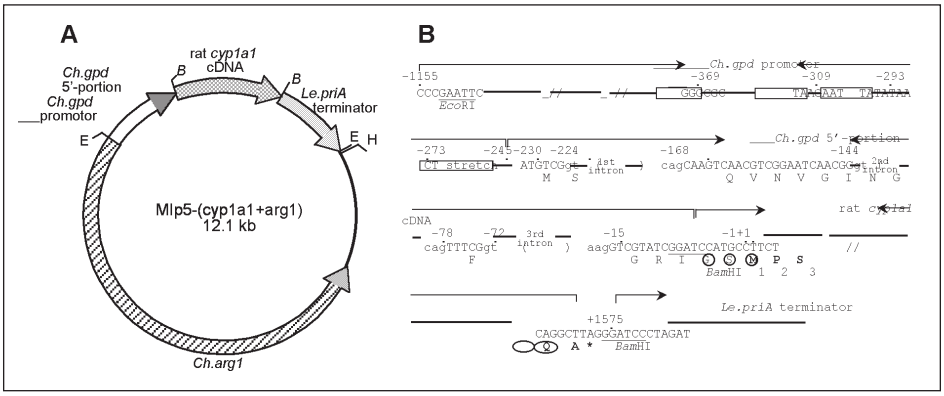

Figure 1. Structure of the rat CYP1A1 (524 amino acid residues)-expressing recombinant plasmid MIp5-(cyp1a1+arg1) (A), the structural features of the *C. hirsutus gpd (Ch.gpd)* promoter, the *Ch.gpd 5'*-portion (224-bp sequences of 1st exon-8th baseof 4th exon), and the nucleotide sequences at the fusion junction between the *Ch.gpd 5'*-portion, rat *cyp1a1* cDNA and the *L. edodes priA (Le.priA)* terminator (B)

This recombinant plasmid was introduced into protoplasts of *C. hirsutus* monokaryotic strain OJ1078 (Arg^-, Leu^-), obtaining 20 Arg^+ transformants. Of these, three Arg^+ transformants showed almost the same growth rates as that of recipient strain OJ1078. Total DNA from the three transformants was digested with *Eco*RI and subjected to Southern-blot analysis using a mixed probe [^{32}P-labelled 1,575-bp rat *cyp1a1* cDNA and 1,059-bp *C. hirsutus ras* (*Ch.ras*) gene; Yamazaki et al. 2004]. Our previous study has shown that *C. hirsutus* genome contains a single copy of the *ras* gene, and an *Eco*RI-digest of chromosomal DNA gives a *ras* signal at a position corresponding to 2.8 kb (Yamazaki et al. 2004b). As shown in Fig. 2, *Eco*RI-digests of three Arg^+ transformants, namely ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1)(lane 3), and ChTF5-6(CYP1A1)(lane 4), gave an intense signal at 3.9 kb, corresponding to the size of the rat CYP1A1-expression cassette (in addition to the 2.8-kb *ras* signal). An *Eco*RI-digest of the control OJ1078 (lane 1) showed only the *ras* signal. To estimate the copy number of the rat CYP1A1-expression cassette in the chromosomes of ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1), the radioactivity of the 3.9-kb rat *cyp1a1* band was compared with that of the 2.8-kb *ras* band. The specific radioactivities of the two probes of rat *cyp1a1* cDNA and *Ch.ras* gene were similar. The data suggested that ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) carry nine, six, and seven copies of the rat CYP1A1-expression cassette on their chromosomes, respectively.

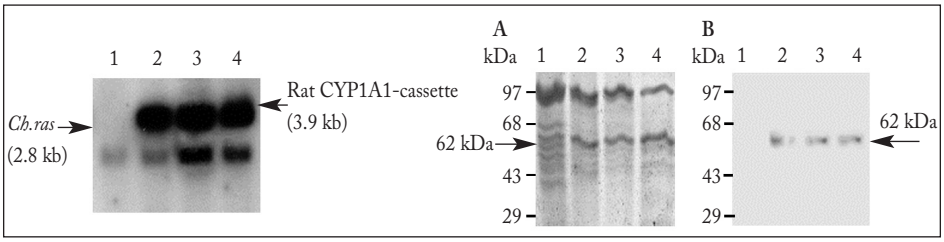

Figure 2 (Left). Southern-blot analysis of the *Eco*RI-digests of total DNAs prepared from the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. Lanes: 1, the recipient OJ1078; 2, ChTF5-2(CYP1A1); 3, ChTF5-4(CYP1A1); 4, ChTF5-6(CYP1A1).

Figure 3 (Right). Immunoblot analysis of the microsomal proteins extracted from the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. (A) SYPRO Ruby staining of the microsomal proteins after SDS-PAGE. (B) The microsomal proteins separated on SDS-PAGE were transferred to a PVDF membrane. The blot was incubated with anti-rat CYP1A1 antibody and the signal was made visible by chemilluminescence. Lanes: 1, the recipient OJ1078; 2, ChTF5-2(CYP1A1); 3, ChTF5-4(CYP1A1); 4, ChTF5-6(CYP1A1).

2.2. *Production of the rat* cyp1a1 *cDNA product, CYP1A1 by recombinant* C. hirsutus *strains*

The production and localization of rat CYP1A1 protein in ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were analyzed. In fungi (as in other eukaryotes) the majority of P450 protein has been reported to be present in microsomes [endoplasmic reticulum (ER); Akiyama et al. 2004; Oeda et al. 1985; Van den Brink 1998]. The total protein contained in the microsomal fractions of ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF-5-6(CYP1A1), and OJ1078 was separated by SDS-PAGE (Fig. 3A) and transferred to a PVDF membrane. The protein-transferred membrane was analyzed by immunoblotting using anti-rat CYP1A1 antibody. As shown in Fig. 3B, a single signal was detected at the predicted position (62 kDa) for the microsomal protein-blots of ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1) (lane 3), and ChTF5-6(CYP1A1)(lane 4), and their intensities were similar. No signal was detected in the case of OJ1078 (lane 1). These results showed that similar amounts of the rat CYP1A1 protein were produced in ChTF5-2(CYP1A1), ChTF5-4(CYP1A1) and ChTF5-6(CYP1A1), and transferred to microsomes (ER).

2.3. *Transformation of 2,7/2,8-DCDDs by the rat CYP1A1-producing* C. hirsutus *strains*

We used 2,7/2,8-DCDDs for the experiment. To examine the transformation activity of 2,7/2,8-DCDDs, ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1), and OJ1078 were cultivated in 10 ml MYGC medium containing 10 μg 2,7/2,8-DCDDs in an L-shaped tube at 30 ˚C for 5 days with shaking. We chose the 5-day cultivation from the following reason. *C. hirsutus* monokaryotic strain produces only limited amounts of lignin-degrading enzymes (LiP and MnP), which also transform 2,7/2,8-DCDDs (Yamazaki et al. 2004a; Yamazaki et al. 2004b), thus allowing rat CYP1A1-catalyzed transformation of 2,7/2,8-DCDDs to be easily assessed. Hexane extracts of whole cell cultures were subjected to gas chromatography (GC) and the total amount of 2,7/2,8-DCDDs remaining, both inside the mycelial cells and in the culture medium, was determined (Fig. 4).

2,7-DCDD and 2,8-DCDD give a single peak in GC. First, the recovery (%) by hexane extraction of 2,7/2,8-DCDDs from the whole cell culture was analyzed. The standard amount (10 μg) of 2,7/2,8-DCDDs was added to 10 ml of a 5-day preculture of OJ1078, and extracted with hexane immediately. The peak of 2,7/2,8-DCDDs recovered (Fig. 4B) was compared with that of 10 μg 2,7/2,8-DCDDs subjected directly to GC (Fig. 4A), indicating that 93% of the 2,7/2,8-DCDDs added to the preculture was recovered. The peaks of the 5 day-cultivations of ChTF5-2(CYP1A1) (Fig. 4D), ChTF5-4(CYP1A1) (Fig. 4E), and ChTF5-6(CYP1A1) (Fig. 4F) showed that the three strains transformed 2,7/2,8-DCDDs much more efficiently than OJ1078 (Fig. 4C). The relative transformation of 2,7/2,8-DCDDs (%) was calculated by the peak of Fig. 4B being taken as 100%. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were indicated to transform 71.7%, 69.8%, and 69.4% of 2,7/2,8-DCDDs respectively, while recipient OJ1078 was shown to transform only 11.8% of 2,7/2,8-DCDDs (Fig. 5). The control Arg^+ transformant obtained by introduction of *C. hirsutus arg1*-carrying pUCR1 alone showed a level of transformation of 2,7/2,8-DCDDs similar to that of OJ1078 (data not shown). The results strongly suggest that, at 5-days of cultivation, about 58.5 (70.3-11.8)% of 2,7- and 2,8-DCDD molecules added to the culture medium were transported into the mycelial cells and transformed by the rat CYP1A1 enzyme within them.

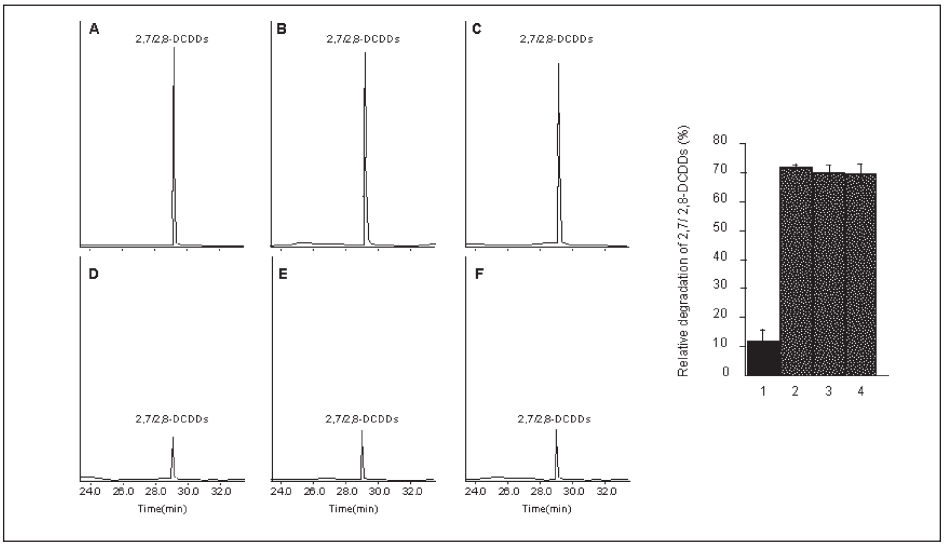

Figure 4 (Left) Gas chromatograms showing an efficient transformation of 2,7/2,8-DCDDs during cultivations of the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. Ten μg each of 2,7/2,8-DCDDs was added to the culture media of ChTF5-2(CYP1A1) (D), ChTF5-4(CYP1A1)(E), ChTF-5-6(CYP1A1) (F), and the recipient OJ1078 (C) before the start of cultivations. After 5 days, the whole cell cultures were extracted with hexane and the resulting extracts were analyzed by gas chromotography (GC). The hexane extract obtained immediately after addition of 10 μg of 2,7/2,8-DCDDs to the 5-day preculture of OJ1078 (B), and 10 μg of the 2,7/2,8-DCDDs alone (A) were also analyzed.

Figure 5 (Right). Transformation of 2,7/2,8-DCDDs during cultivations of the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. The remaining amounts of 2,7/2,8-DCDDs after 5-day cultivations of ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1) (lane 3), ChTF5-6(CYP1A1) (lane 4), and the recipient OJ1078 (lane 1) were estimated by analyzing the peaks of gas chromatograms shown in Fig. 4B-F. The amount of 2,7/2,8-DCDDs shown in Fig. 4B was taken as 100%. Error bars indicate the standard deviations of three replicates

We next examined the level of transformation of 2,7/2,8-DCDDs in a prolonged cultivation. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and OJ1078 were cultivated at 30 ˚C for 16 days, when the production of LiP and MnP reaches a maximum level (Yamazaki et al. 2004a; Yamazaki et al. 2004b). Although a constant recovery (%) of 2,7/2,8-DCDDs from the whole cell culture was not obtained, probably owing to the much larger mass of mycelial cells, roughly about 85-90% of 2,7/2,8-DCDDs was considered to be transformed by ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1), while about 30-35% was presumably transformed by OJ1078 (data not shown).

3. Discusion

Our result suggests that, after 5 days cultivation, only a fraction (11.8%) of 2,7- and 2,8-DCDD molecules were transformed by the extracellular lignin-degrading enzymes LiP and MnP, and that the majority (58.5%) of the 2,7- and 2,8-DCDD molecules, having escaped transformation by LiP and MnP, were transported into mycelial cells and transformed by the rat CYP1A1 enzyme within them. Although their transformation activities towards 2,7/2,8-DCDDs have not yet been examined, the CYPs of the white-rot basidiomycete *P. lindtneri* have been implied to transform non-chlorinated DD into a mono-hydroxylated form (Mori and Kondo 2002). Even if *C. hirsutus* OJ1078 produces CYPs that catalyze the transformation of chlorinated DD as well as non-chlorinated DD, their contribution to transformation of 2,7/2,8-DCDDs may not be significant; the relative transformation of 2,7/2,8-DCDDs (%) by such endogenous CYPs is clearly less than 11.8%.

Although varying slightly depending on the amount of 2,7/2,8-DCDDs added to culture medium, the degree of transformation of 2,7/2,8-DCDDs by the rat CYP1A1-producing *C. hirsutus* strains were very similar (approx. 70%). The activity of cytochrome P450 enzyme is regulated by NADPH-P450 reductase. Thus, it is possible that the amount of NADPH-P450 reductase produced in the recombinant *C. hirsutus* cells is insufficient compared with the amount of P450 enzyme, thereby regulating the latter at a constant lower level. To confirm this, introduction of a *C. hirsutus* NADPH-P450 reductase-expression cassette into the rat CYP1A1-producing *C. hirsutus* strains will be required. As mentioned in the Introduction, a much more efficient transformation (degradation) of TriCDDs as well as 2,7/2,8-DCDDs is thought to be achieved by co-cultivation of the *C. hirsutus* strains producing rat CYP1A1 and those producing large amounts of LiP and MnP.

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