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SHORT COMMUNICATION

Biotransformation of polychlorinated dibenzo-*p*-dioxin by *Coprinellus* species

Hiroto Suhara · Ichiro Kamei · Nitaro Maekawa · Ryuichiro Kondo

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Abstract A degradation experiment on dibenzo-*p*-dioxin (DD) and 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) was carried out using basidiomycetous fungi belonging to the genera *Coprinus*, *Coprinellus*, and *Coprinopsis*. Some species showed a high rate of decrease in DD for the 2-week test period. Among them, *Coprinellus disseminatus* showed the highest ability to decrease the DD level, close to 100% by the end of 2 weeks. Further examination showed that *Coprinellus disseminatus* and *Coprinellus micaceus*, belonging to the genus *Coprinellus*, were able to metabolize 2,7-DCDD to a monohydroxylated compound, probably mediated by the P450 system. The metabolism of chlorinated DD by fungi capable of living in soil conditions is reported here for the first time.

Keywords Basidiomycetes · Bioremediation · Cytochrome P450 monooxygenase

Biodegradation of dioxins using white-rot fungi has been widely tested during the past decades. Previous reports have shown that two white-rot fungi belonging to the genus *Phanerochaete* P. Karst., *Phanerochaete chrysosporium* Burd. and *Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden, degraded polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Bumpus et al. 1985; Valli et al. 1992). The most general lignin-degrading enzymes are two closely related heme-containing peroxidases (Martínez 2002; Morgenstern et al. 2008), manganese peroxidase (MnP; EC 1.11.1.13.) and lignin peroxidase (LiP; EC 1.11.1.14.), and a multi-copper-containing phenoloxidase laccase (Lac; EC 1.10.3.2.). Studies on *Phanerochaete chrysosporium* developed a model of 2,7-diclorodibenzo-*p*-dioxin (2,7-DCDD) degradation, which was catalyzed by 1-electron oxidations of LiP (Bumpus et al. 1985; Valli et al. 1992; Joshi and Gold 1994). Takada et al. (1996) also examined the degradation of highly chlorinated dibenzo-*p*-dioxins (DD) and dibenzofurans by *Phanerochaete chrysosporium* and *Phanerochaete sordida* and identified tetrachlorocatechol as one of the metabolites from octachlorodibenzo-*p*-dioxin. In addition, they suggested that these fungi contain another enzyme system to degrade PCDDs and PCDFs. However, the enzymatic system responsible for degradation of dioxins has been unclear.

In recent years, several studies have shown that cytochrome P450 monooxygenase plays an important role in the degradation of dioxins. Cytochrome P450 is a heme protein that is widely distributed in eukaryotes as well as in prokaryotes. The P450 system is involved not only in hydroxylation of organic compounds in biosynthesis pathways but also in detoxification of aromatics and xenobiotics. Hydroxylated metabolites have been obtained from biphenyl, diphenyl ether, polycyclic aromatic hydrocarbons, chloronaphthalene, DDs (nonchlorinated, 2,7-dichloro, 2,3,7-trichloro, and 1,2,6,7- and 1,2,8,9-tetrachloro), and dibenzofurans (nonchlorinated and 2,8-dichloro) treated

H. Suhara (✉) · N. Maekawa
Faculty of Agriculture, Tottori University,
4-101 Koyama-Minami, Tottori 680-8553, Japan
e-mail: h_suhara@muses.tottori-u.ac.jp

I. Kamei
Faculty of Agriculture, Miyazaki University,
1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan

R. Kondo
Laboratory of Systematic Forest and Forest Products Science,
Department of Forestry and Forest Products Sciences,
Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki,
Higashi-ku, Fukuoka 812-8581, Japan

with *Phlebia lindtneri* (Pilát) Parmasto (Mori and Kondo 2002a,b,c; Mori et al. 2003; Kamei and Kondo 2005). Additionally, Kamei et al. (2005) reported that an ability to degrade highly chlorinated dioxins was phylogenetically conserved in a monophyletic clade including several species of the genus *Phlebia* Fr. Among the species of this genus, *Phlebia brevispora* Nakasone showed the highest dioxin-degrading ability and degraded 1,3,6,8-tetrachlorodibenzo-*p*-dioxin. Significant inhibition of metabolism on these substrates was observed by the addition of the cytochrome P450 inhibitors 1-aminobenzotriazole and/or piperonyl butoxide (Mori and Kondo 2002b; Kamei et al. 2005). A similar effect was also observed for the degradation of 2,7-DCDD by the white-rot fungus *Panellus stiptics* (Bull.) P. Karst. (Sato et al. 2002). Thus, these white-rot fungi have been known as effective degraders of dioxins for a long time. However, practical use of white-rot fungi for bioremediation of soil contaminated with dioxins has rarely been carried out because the soil is unsuitable for their mycelial growth. In our preliminary screening of DD degraders that prefer a soil environment as their habitat, we selected several species belonging to the genera *Coprinus* Pers. (Agaricaceae), *Coprinellus* P. Karst., and *Coprinopsis* P. Karst. (Psathyrellacea). In the present study, selection of higher degraders of dioxins was carried out, and their metabolic pathways are discussed.

Dibenzo-*p*-dioxin, phenanthrene, and sodium azide were purchased from Wako Pure Chemicals (Shiga, Japan). 2,7-Dichlorodibenzo-*p*-dioxin was purchased from Accu-Standard (New Haven, CT, USA).

The following 11 isolates of 10 species belonging to *Coprinellus*, *Coprinopsis*, and *Coprinus* were used: *Coprinellus disseminatus* (Pers.) J.E. Lange TUFC11148 and TUFC34534; *Coprinellus ellisii* (P.D. Orton) Redhead, Vilgalys & Moncalv TUFC31678; *Coprinellus micaceus* (Bull.) Vilgalys, Hopple & Jacq. Johnson TUFC30081; *Coprinellus xanthothrix* (Romagn.) Vilgalys, Hopple & Jacq. Johnson TUFC34011; *Coprinopsis atramentaria* (Bull.) Redhead, Vilgalys & Moncalvo TUFC30095; *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo TUFC11574; *Coprinopsis insignis* (Peck) Redhead, Vilgalys & Moncalvo TUFC30864; *Coprinus comatus* (O.F. Müll.) Pers. TUFC11816; *Coprinus quadrifidus* Peck TUFC 32271; and *Coprinus rhizophorus* A. Kawam. TUFC30389. These isolates were deposited in the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University.

Degradation experiments were carried out according to the previous report (Mori and Kondo 2002c), briefly as follows. Two mycelium disks (6 mm in diameter) punched from the edges of mycelium colonies grown on malt extract agar [MA: 1.5% (w/v) malt extract and 1.5% (w/v) Bacto agar; Difco, Detroit, MI, USA] plates were inoculated to 10 ml liquid Kirk HCLN medium [containing 1% (w/v) glucose and 1.2 mM ammonium tartrate] (Tien and Kirk 1988) and adjusted to pH 5.6. Cultures were incubated statically at 25°C for 1 week. After preincubation, 1 μmol DD was added to each culture. Each flask was sealed and then incubated statically at 25°C. As a control, cultures were treated by sodium azide (0.7 mmol/flask) 30 min before adding DD. After an additional incubation period (2 weeks), an internal standard (phenanthrene) was added and homogenized with 20 ml acetone. The cell debris was removed by centrifugation at 3,000 *g* for 15 min. After filtration through a membrane filter (pore size, 0.45 μm), the resulting supernatant was analyzed by high performance liquid chromatography (HPLC) to determine the residual amount of DD in the same manner as in the previous report (Mori and Kondo 2002c).

Five mycelium disks (obtained as above) were inoculated to 10 ml Kirk HCLN medium. After 5 days, 0.1 μmol 2,7-DCDD was added to each culture and incubated statically at 25°C. After additional incubation (2 weeks), cultures were treated by sodium azide. As a control, cultures treated by sodium azide after an initial 5 days of incubation were used. To determine the concentration of 2,7-DCDD, an internal standard (pentachloronitrobenzene) was added, and the cultures were homogenized with 15 ml acetonitrile. After centrifugation (3,000 *g*, 10 min), 1 ml supernatant fluid was extracted by 1 ml *n*-hexane and analyzed by gas chromatography with an electron capture detector (GC/ECD). GC/ECD was performed with the Hewlett-Packard HP6890 GC system. An HP-50+ column [Hewlett-Packard (0.53 mm inside diameter by 15 m length; 1-μm film thickness)] was used, and the oven temperature was programmed to increase from 100° to 290°C at 20°C min^{-1}. Methods for the metabolite detection by GC/MS were carried out according to a previous report (Kamei et al. 2005).

The recovery rates of DD from the fungus tested in the present study were as follows: *Coprinellus disseminatus* TUFC11148 and TUFC34534 were 1.3% and 22.5%, respectively; *Coprinellus ellisii* TUFC31678, 68.6%; *Coprinellus micaceus* TUFC30081, 38.4%; *Coprinellus xanthorix* TUFC34011, 57.8%; *Coprinopsis cinerea* TUFC11574, 45.3%; *Coprinopsis insignis* TUFC30864, 93.3%; *Coprinus comatus* TUFC11816, 68.5%; *Coprinus quadrifidus* TUFC 32271, 18.4%; *Coprinus rhizophorus* TUFC30389, 93.7% (Fig. 1). The test was not carried out for *Coprinopsis atramentaria* TUFC30095 because the fungus did not grow in the liquid culture ($n = 6$). Four strains (two strains of *Coprinellus disseminatus*, *Coprinellus micaceus*, and *Coprinus quadrifidus*), which showed a DD decrease greater than 60%, were selected for the following experiment using 2,7-DCDD. The recovery rate of 2,7-DCDD from the azide-treated control culture with *Coprinellus disseminatus* TUFC34534 was 93.1 ± 3.1%.

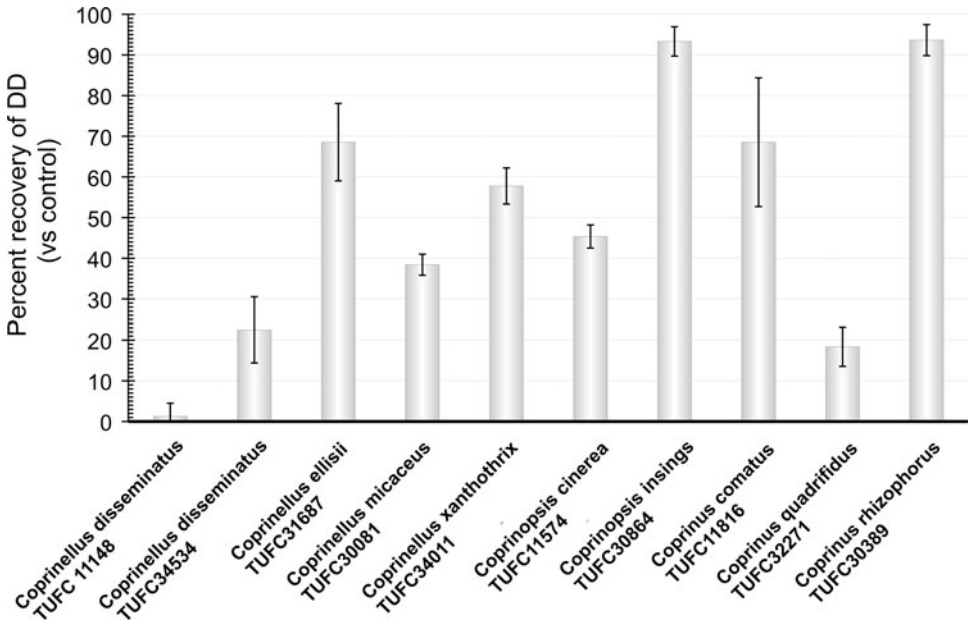

Fig. 1 Degradation experiment of dibenzo-*p*-dioxin (*DD*) by species of *Coprinus* (Agaricaceae), *Coprinellus*, and *Coprinopsis* (Psathyrellaceae) for 14-day incubation period. Values are mean ± SD of triplicate samples

The recovery rates of the 2,7-DCDD from *Coprinellus disseminatus* TUFC11148 and TUFC34534, and *Coprinellus micaceus* TUFC30081, were 85.1% ± 6.6%, 81.8% ± 1.8%, and 48.2% ± 3.5%, respectively. On the other hand, the recovery rate of substrate treated with *Coprinus quadrifidus* TUFC 32271 was 101.3% ± 1.3%.

To confirm the biotransformation of 2,7-DCDD by treatment with *Coprinellus disseminatus* and *Coprinellus micaceus*, organics in the supernatant fluid were analyzed. By the GC/MS analysis, a putative metabolite was detected (retention time, 13.311 min) in the extract from the culture of *Coprinellus micaceus* TUFC30081 (Fig. 2a), which was not found in the azide-treated control (Fig. 2b). As shown in Fig. 2c, the putative metabolite had a parent peak at $m/z = 268$, which correspond to +16 mass (hydroxyl residue) of the 2,7-DCDD parent peak ($m/z = 252$). Also, the existence of two chlorine residues was indicated by the mass at $m/z = 270$ and 272. The sequential fragments were observed at $m/z = 233$ (M^+–Cl) and 205 (M^+–CO, Cl) (Fig. 2c). The future of mass spectrum and retention time of the putative metabolite were the same as that previously reported for monohydroxylated 2,7-DCDD (Mori and Kondo 2002b). A trace amount of metabolite was detected in the methylated extract from the culture with *Coprinellus disseminatus* TUFC11148 and TUFC34534. The metabolite had a mass spectrum showing the following molecular ion results: m/z 282 [molecular mass of 2,7-DCDD(252) +30 mass], 267 (M^+–CH_3), 239 (M^+–$COCH_3$), and 204 (M^+–$COCH_3$, Cl), thus suggesting that the metabolite was a monomethoxy-DCDD. This metabolite was not detected from the extract without methylation. Hence, monohydroxylated 2,7-DCDD was suggested as the metabolite. These results implied that the *Coprinellus* species tested in the present study have the same ability as *Phlebia* species. Monohydroxylation of the aromatic ring of 2,7-DCDD is an important step for biodegradation of dioxins because substitution of a polar residue on aromatic rings may reduce hydrophobicity and increase bioavailability of 2,7-DCDD. The monohydroxylation of chlorinated dioxins by *Phlebia* species suggested that the reaction was mediated by the P450 system as the initial metabolic reaction (Mori and Kondo 2002c; Kamei et al. 2005). The P450 system may also play an important role in the hydroxylation of 2,7-DCDD by *Coprinellus* species. Monomethoxy-DCDD, which is considered as a subsequent metabolite of monohydroxy-DCDD in *Phlebia* species, was not found as a metabolite in the present study. The pathway subsequent to the hydroxylation of 2,7-DCDD by *Coprinellus* species is unclear at this time.

Coprinellus disseminatus and *Coprinellus micaceus*, belonging to the family Psathyrellaceae, were able to degrade 2,7-DCDD, whereas *Coprinus quadrifidus* (Agaricaceae), which showed a comparatively high DD decrease rate, was not able to degrade 2,7-DCDD, which may be caused by the physiological differences between them. The 2,7-DCDD-degrading ability might depend on the particular phylogenetic linage as in the previous report (Kamei et al. 2005), but more related species and strains must be tested. In the case of wood-decay fungi, approximately 50% of 2,7-DCDD was degraded by *Phanerochaete chrysosporium* after a 4-week incubation under nitrogen-limited conditions (Valli et al. 1992). Mori and Kondo (2002b) reported that more than 30% of 2,7-DCDD disappeared during a 14-day treatment with *Phlebia lindtneri*. Kamei et al. (2005) also reported that *Phlebia acerina* Peck and *Phlebia brevispora* degraded 2,7-DCDD

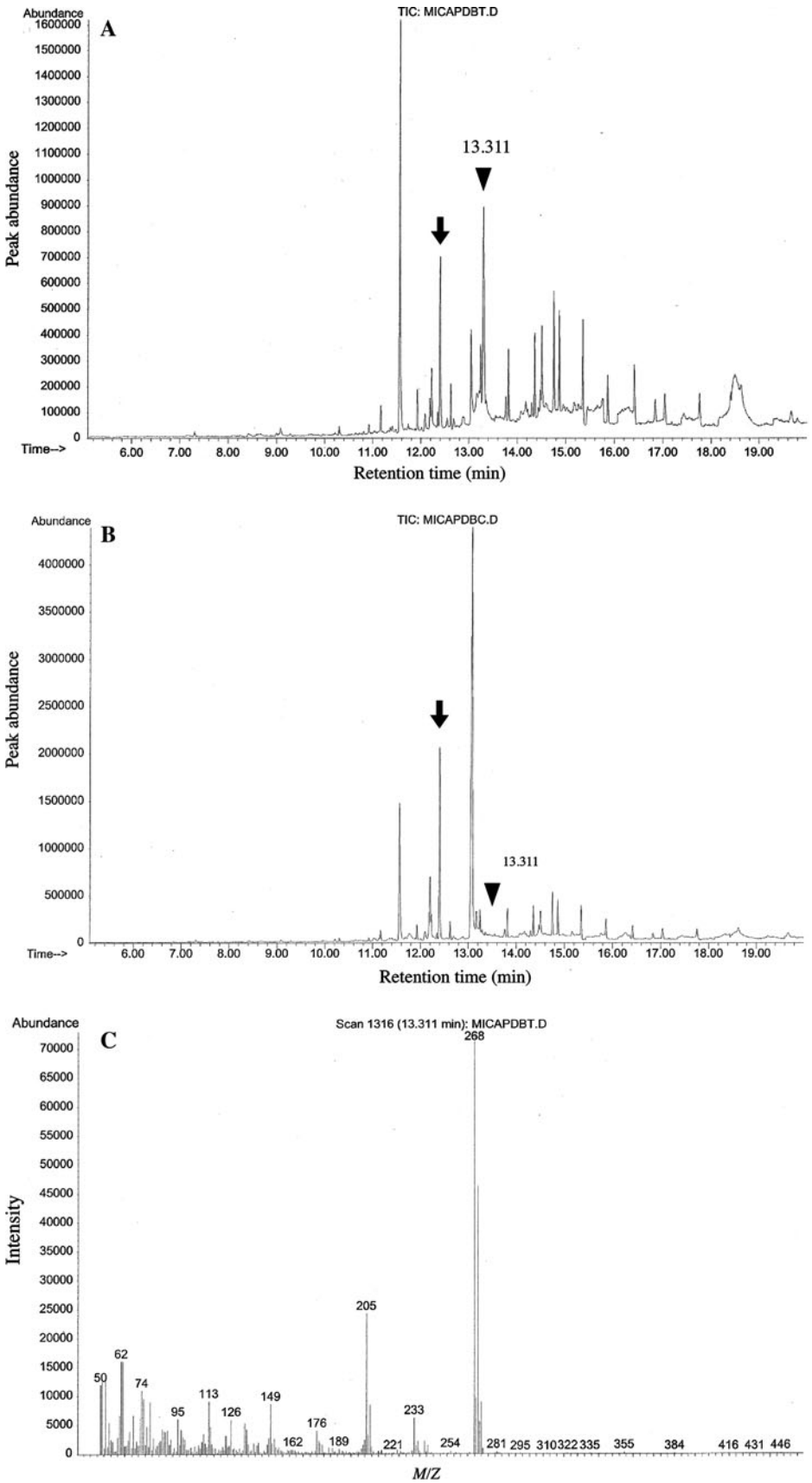

Fig. 2 Total ion chromatogram of the extract from viable (**a**) and azide-treated (**b**) cultures of *Coprinellus micaceus* with 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD). *Arrow* indicates the molecular ion peak of 2,7-DCDD. *Arrowhead* indicates the molecular ion peak of the metabolite, which was not found in the azide-treated control. The retention time of the metabolite is also shown. **c** Mass spectra of the metabolite

approximately 40% and 80% after 14 days of incubation, respectively. The 2,7-DCDD-degrading ability of *C. micaceus* is higher than that of *Phanerochaete chrysosporium*, *Phlebia acerina*, and *Phlebia lindtneri*.

Recently, bioremediation of dioxin-contaminated soil at bench scale was carried out using *Phlebia brevispora* (Kamei et al. 2009). All soils were autoclaved before inoculation of *Phlebia brevispora*, but the dioxin-degrading ability of *Phlebia brevispora* was reduced. In the present study, fungi belonging to the genus *Coprinellus* rather than white-rot fungus were tested for biodegradation of chlorinated DD. *Coprinellus* species mostly prefer a soil environment as their habitat and are found in grasslands and forests. Their main substrates are straw, litter, animal carcasses, excrement, and humus, and they frequently grow on humus-rich soil. Although *Coprinellus disseminatus* and *Coprinellus micaceus* primarily occur on decayed wood, they are also found in soil, leaf litter, wood chips, or decayed plant roots (Bakys et al. 2006; Keirle et al. 2004; Peiris et al. 2008; Türkekul 2003). This trait might give them an advantage for soil remediation, although their ability to degrade dioxins in soil or organic-rich conditions should be investigated.

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