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Degradation of chlorinated pesticide DDT by litter-decomposing basidiomycetes

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Abstract One hundred and two basidiomycete strains (93 species in 41 genera) that prefer a soil environment were examined for screening of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) biodegradation. Three strains within two litter-decomposing genera, *Agrocybe* and *Marasmiellus*, were selected for their DDT biotransformation capacity. Eight metabolites; 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), two monohydroxy-DDTs, monohydroxy-DDD, 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol, putative 2,2-bis(4-chlorophenyl)ethanol and two unidentified compounds were detected from the culture with *Marasmiellus* sp. TUFC10101. A P450 inhibitor, 1-ABT, inhibited the formation of monohydroxy-DDTs and monohydroxy-DDD from DDT and DDD, respectively. These results indicated that oxidative pathway which was catalyzed by P450 monooxygenase exist beside reductive dechlorination of DDT. Monohydroxylation of the aromatic rings of DDT (and DDD) by fungal P450 is reported here for the first time.

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Introduction

1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) has been widely used for pest control since the 1940s because of its low cost, easy synthesis, broad-spectrum activity, and long operative activity (Turusov et al. 2002). However, it was banned in many industrialized countries during the 1970s because of its harmful effects on wildlife and human health via incorporation into the food chain (Foght et al. 2001; Jones and de Voogt 1999). DDT is still used in several developing countries for preventing malaria, although, it is mostly carcinogenic and is also known endocrine disruptors that increase environmental estrogen. This pesticide is a semi-volatile organic compound and has a propensity to be persistent and widely transported within the environment (Simonich and Hites 1995). Therefore, even in countries where it has been banned, it survives in soil, air, water and food.

Bioremediation using bacteria and fungi has been recognized as a useful method of degrading various recalcitrant pesticides, including DDT, because bioremediation has little harmful effect on natural environments. Enhancement of the biodegradation or mineralization of DDT by microorganisms has been

demonstrated both in vitro and in situ (Pfaender and Alexander 1972; Subba-Rao and Alexander 1977; Bumpus and Aust 1987; Wedmeyer 1996; Aislable et al. 1997; Hay and Focht 1998; Kamanavalli and Ninnekar 2004). Attention has been focused on the bioremedial potential of basidiomycete white-rot fungi such as *Phanerochaete chrysosporium* (Bumpus and Aust 1987; Fernando et al. 1989; Bumpus et al. 1993). 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethane (dicofol), 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol (FW-152), and 4,4′-dichlorobenzophenone (DBP) have been detected as metabolites of DDT (Aislable et al. 1997). DDT biodegradation has been assumed to be dependent on a lignin-degrading system (Bumpus and Aust 1987; Bumpus et al. 1993). Recently, the degradation of DDT by brown-rot fungi via the Fenton reaction has been reported (Purnomo 2008). However, the question remains whether wood-rotting fungi, primarily living in woody substrates, can be used for soil remediation. An alternative approach using ectomycorrhizal basidiomycetes has been reported (Huang et al. 2007). This study showed that some ectomycorrhizal basidiomycetes that exist naturally in soil are capable of degrading DDT through a similar pathway to that of the white-rot fungi.

We chose to investigate basidiomycete fungi for bioremediation of DDT-contaminated soil, focusing on those collected from woody debris on soil, and to clarify their metabolic pathways for the degradation of DDT.

Materials and methods

Chemicals

p,p′-DDT and *p,p′*-DDD were purchased from Tokyo Chemical Industry (Tokyo, Japan). Trimethylsilyldiazomethane (TMS-diazomethane) was purchased from GL Science Inc. (Tokyo, Japan). 1-aminobenzotriazole (1-ABT) and *p,p′*-DDE were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Fungal isolates

The fungal isolates examined were obtained from basidiomata mostly collected from leaf-litter, decayed branches, humus and various kinds of woody debris on the forest floor. Species were identified by morphological observation of basidiomata using a stereomicroscope, and by genetic analyses of isolates. All fungal isolates were deposited in the Fungus/Mushroom Resource and Research Center (TUFC), Tottori University, Japan. Species names and strain numbers are shown with the experimental results in Table 1.

Genetic analyses

Genetic analyses were carried out using internal transcribed spacer region (ITS) and/or partial 25S regions of ribosomal DNA. ITS1-F and ITS4-B (Gardes and Bruns 1993) primers were used for ITS and LR0R and LR5 (Moncalvo et al. 2000) primers were used for 25S analyses. Polymerase chain reactions were performed as previously reported (Maekawa et al. 2005). Sequencing was performed on an ABI 3130 (Life Technologies Japan, Tokyo, Japan) genetic analyzer according to the manufacturer's protocols. The obtained sequences were subjected to a blast search via the DNA Data Bank of Japan to infer phylogenetic position. For some strains, phylogenetic analysis was also carried for 25S sequences by Neigbor-joining methods in MEGA4. The design of 25S datasets was guided by blast searches and drew together with previously published data (Binder et al. 2005). Additional sequences for phylogenetic analysis were obtained from the International Nucleotide Sequence Database Collaboration.

Culture conditions

Isolates were grown on malt extract agar [MA; 1.5 g malt extract (Difco, Detroit, MI, USA) and 1.5 g Bacto agar (Difco) per l] at 25°C in the dark. Five mycelium disks (5 mm in diameter), punched from colony edges, were inoculated into 10 ml of liquid modified Melin-Norkans medium [MMN; 0.5 g malt extract, 1.0 g glucose, 0.035 g ammonium tartrate, 0.05 g KH_2PO_4, 0.015 g $MgSO_4 \cdot 7H_2O$, 0.0025 g NaCl, 1.2 ml of 1 g l^{-1} $FeCl_3$ solution and 0.1 mg thiamin hydrochloride per l; pH 5.3] in 100 ml Erlenmeyer flasks. The cultures were incubated statically at 25°C in an ambient atmosphere.

Degradation experiments

Each compound (DDT or DDD) dissolved in *N,N*-dimethylformamide (6.25 mmol l^{-1}) was added,

without evaporation, to 2 week old cultures (25 nmol ml^{-1} at final concentration). Each flask was sealed with a glass stopper and sealing tape, and then incubated statically at 25°C. As a control, three cultures were treated with sodium azide (70 μmol ml^{-1} at final concentration) at 30 min before the substrate was added; this killed the fungal culture. After an additional incubation period (2 weeks, or 4 weeks in some experiments), phenanthrene (50 nmol ml^{-1} at final concentration) was added to cultures as a surrogate. Cultures were homogenized with 20 ml of acetone using an homogenizer HG-200 (Hsiangtai Machinery Industry Co., Ltd, Taipei, Taiwan), and then cell debris were removed by centrifugation at $3000 \times g$ for 15 min. After filtration through a membrane filter (pore size 0.45 μm, Millex-LH; Advantec Toyo Kaisha, Ltd., Tokyo, Japan), the resulting supernatant was analyzed by high performance liquid chromatography (HPLC) to determine recovery of DDT (or DDD). HPLC was carried out using a Hitachi L-2130 pump (Hitachi Ltd., Tokyo, Japan) with a Hitachi L-2420 UV/BIS detector (at 235 nm) fitted with an Inertsil ODS-3 column (4.6 mm inner diameter, 15 mm long; GL Science Inc.). The supernatant was eluted with acetonitrile +1 ml l^{-1} trifluoroacetic acid (3 + 1 by volume) at a flow rate of 1 ml min^{-1}. Retention times of DDT, DDD and DDE were 17.5, 10.9 and 22.8 min, respectively. Percentage recovery of DDT and DDD was calculated by comparison with a standard curve. The standard curve was prepared by HPLC analysis using a mixture of standard DDT (or DDD, 0, 0.0625, 0.125, 0.1875 and 0.25 μmol) and standard phenanthrene (0.5 μmol) dissolved in 20 ml of acetone + 10 ml H_2O. The recovery of surrogate was evaluated by absolute calibration curve of phenanthrene.

To identify metabolic products, the supernatant was acidified with HCl to pH 2 and extracted once with 40 ml of ethyl acetate and then twice with 40 ml of *n*-hexane. These extracts were combined and dried over anhydrous sodium sulfate, then evaporated until dry. Residues were dissolved in *n*-hexane and purified using a silica gel cartridge column (Bond Elute Glass, GL Science Inc.). The silica gel column was pre-eluted with 10 ml *n*-hexane and then eluted with 10 ml *n*-hexane + ethyl acetate (8 + 2 by volume). The latter fraction was analyzed using gas chromatography/mass spectrometry (GC/MS) following evaporation. GC/MS was performed on a GC-17A gas chromatograph (Shimadzu, Tokyo, Japan) linked to a JMS-Automass sun mass spectrometer (JEOL, Tokyo, Japan) and a 30 m fused HP-5 column (0.32 mm inner diameter, 0.25 μm film thickness; Agilent Technologies, Tokyo, Japan). The column was operated with helium as the carrier gas at a constant flow rate of 1.0 ml per min. The splitless injectors were maintained at 250°C. The oven temperature was programmed at 90–310°C, increasing at 10°C min^{-1}. Initial and end temperatures were held for 6 min. Monitoring of mass was carried out using the electron ionization mode at 70 eV (*m/z* 100–400 with 300 ms). Derivatization analyses of metabolites were performed using TMS-diazomethane according to the manufacturer's protocol, with an overnight reaction.

Cytochrome P450 inhibitor experiments

Fungal cultures were incubated as described above except that Difco™ potato dextrose broth (PDB) (Becton, Dickinson and Company, NJ, USA) was used as the growing medium. The cytochrome P450 inhibitor 1-ABT (final concentration at 0, 0.01, 0.1 and 1.0 mmol l^{-1}) was added to the 2 week old cultures 30 min before addition of 0.25 μmol DDT (or DDD). Cultures were incubated for 2 weeks, and percentage recovery of compounds and metabolites was analyzed as described above. To statistically confirm results of the P450 inhibitory test, one-way analysis of variance (ANOVA) was carried out. The statistic assessment is shown by $F(x, y) = F$-ratio, significance probability. x and y values in parentheses show between-group degrees of freedom and within-group degrees of freedom, respectively.

Extracellular enzyme activities

To determine ligninolytic enzyme activities and DDT conversion activity, extracellular enzyme activities were measured. After incubation for 14 days, extracellular fluid was collected and filtrated through a 0.2 μm membrane filter. Manganese peroxidase (MnP) activity was determined by monitoring the oxidation of Mn^{2+} at 270 nm in 50 mmol l^{-1} malonate buffer (pH 4.5) with 1 mmol l^{-1} manganese sulfate. Lignin peroxidase (LiP) activity was determined by measuring the formation of veratryl aldehyde from veratryl alcohol (0.5 mmol l^{-1} final concentration) at 310 nm in 20 mmol l^{-1} succinate

buffer (pH 3.0). Laccase activity was estimated by measuring the oxidation of 2,6-dimethoxyphenol at 460 nm in 50 mmol l^{-1} malonate buffer (pH 5.0). To determine MnP and LiP activity, 0.2 mmol l^{-1} hydrogen peroxide (H_2O_2) was added to the reaction mixture. The DDT conversion activities of extracellular enzymes were tested using 10 ml extracellular fluid. Degradation testing was carried out as described above.

Results

Screening experiment

One hundred and two basidiomycete strains (93 species in 41 genera) were examined for DDT biotransformation ability. Percentage recovery of DDT for each strain is shown in Table 1. In present experiment, the percentage recoveries of phenanthrene and DDT from azid-treated control culture with *Marasmiellus chamaecyparidis* TUFC11832 were 96.3 ± 1.4% and 96.4 ± 0.2%, respectively. Seven strains in five genera showed more than 10% DDT disappearance ($P < 0.05$). Strains and percentage disappearance were as follows; *Cerinomyces* sp.1 TUFC10803, 26.2%; *Marasmiellus* sp.1 TUFC10101, 21.3%; *Tremella* sp.1 TUFC11347, 18.2%; *Tremella foliacea* TUFC10801, 17.2%; *Dacrymyces* sp.1 TUFC10104, 14.6%; *Agrocybe media* TUFC34444, 14.1%; *M. chamaecyparidis* TUFC11832, 10.2%. These fungi belong to three orders; Tremellales, Dacrymycetales and Agaricales.

No metabolite was observed from *T. foliacea* TUFC10801 or *Tremella* sp.1 TUFC11347. Low percentage recovery of DDT was observed for some *Tremella* strains. These strains grow as yeasts in liquid media, and it is difficult to crush cells with a homogenizer. Hence, cell debris of *Tremella* spp. were crushed in a mortar using sea sand and liquid nitrogen, and a large amount of DDT was recovered without any metabolite. Decrease of DDT in the samples from these strains was possibly caused by uptake of DDT into cells and nonspecific adsorption to cell surfaces. DDD was detected from *Cerinomyces* sp.1 TUFC10803 and *Dacrymyces* sp.1 TUFC10104 (Dacrymycetales), and no other metabolite was found. Formation of DDD was confirmed by comparing with standard DDD by HPLC and/or GC/MS analyses. Further decrease of DDT was not observed when the treatment period was extended to 4 weeks for *Dacrymyces* sp.1 TUFC10104 (14.6 ± 3.82% for 2 weeks, 11.2 ± 3.31% for 4 weeks). It was inferred that DDD is the end metabolic product for these Dacrymycetales strains. Hence, *Cerinomyces* sp.1 TUFC10803 and *Dacrymyces* sp.1 TUFC10104 were not further used in the study. DDD was also observed from *A. media* TUFC34444, *M. chamaecyparidis* TUFC11832 and *Marasmiellus* sp.1 TUFC10101, and some other metabolites were also observed in these samples.

DDD was reported as a major metabolite of DDT (Aislabie et al. 1997; Foght et al. 2001; Macalady et al. 1987; Menzer and Nelson 1991; Rochkind et al. 1986), and acts as an analogue of DDT. Thus, a DDD degradation experiment was carried out using the Agaricales group selected above. Results of DDT and DDD degradation experiments using these fungi are shown in Fig. 1. All selected strains were capable of decreasing DDD. Percentages of DDD degraded were as follows: *Marasmiellus* sp.1 TUFC10101, 19.9%; *A. media* TUFC34444, 17.9%; *M. chamaecyparidis* TUFC11832, 6.0%. Following these results, *Marasmiellus* sp.1 TUFC10101 was selected for subsequent experimentation and analysis.

Metabolic products

GC-MS analysis of organic solvent extracts from cultures of *Marasmiellus* sp.1 TUFC10101 with DDT yielded seven chlorinated metabolites not found in the azide-treated control (Table 2). Retention times (r_t) of them were 13.03, 13.88, 18.92, 20.55, 21.90, 22.41 and 22.62 min. The retention time and the mass spectrum of the metabolite at 20.55 min were identical to that of the authentic DDD.

Identification of monohydroxy-DDT and -DDD

For DDT and related compounds, intensity of the parent ions was very low compared with the base peaks when analyzed by electron ionization because the chlorinated methyl at the carbon bridge (C1) easily fragments. The parent ions of authentic DDT and DDD were observed at $m/z = 354$ and 320, respectively, but in trace amounts (Table 2).

The mass spectra of peaks detected at $r_t = 21.90$, 22.41 and 22.62 min were very similar and are

Table 1 Fungal strains tested in the present study and DDT disappearance rate

Family	Species	Strain (TUFC no.)	Percentage recovery (%)[a]		Percent disappearance[b]
			Treatment	Control	
Agaricaceae	*Agaricus* sp.1	11262	113.2 ± 5.41	110.1 ± 1.57	–
	Agaricus sp.2	11106	99.2 ± 2.21	100.6 ± 0.15	1.4
	Agaricus sp.3	11091	108.2 ± 7.63	102.2 ± 2.49	–
	Calvatia craniiformis	11114	101.6 ± 0.66	104 ± 3.23	2.3
	Coprinus comatus	11273	95.1 ± 8.27	100.1 ± 1.66	5
	Coprinus quadrifidus	32271	115 ± 6.33	116.9 ± 7.1	1.9
	Coprinus rhizophorus	30389	106 ± 2.64	105.8 ± 2.31	–
	Coprinus sp. 1	10979	105 ± 4.29	104.7 ± 2.44	–
	Lycoperdon perlatum	11377	101.9 ± 2.73	101.5 ± 1.68	–
Auriculariaceae	*Auricularia polytricha*	10349	104.6 ± 1.57	105.4 ± 4.36	0.8
	Basidiodendron sp.	10656	83.5 ± 0.82	88.7 ± 1.5	5.2*
	Protodaedalea hispida	10609			No growth
	Pseudohydnum gelatinosum	11805	105.2 ± 2.9	107.5 ± 2.1	2.3[c]
Calostomataceae	*Calostoma* sp.	11806	94 ± 4.71	92.4 ± 7.8	–
Cantharellaceae	*Cantharellus luteocomus*	11807			No growth
Clavulinaceae	*Multiclavula clara*	11158	99.3 ± 2.79	98.2 ± 6.57	–
	Multiclavula sp.	11808			No growth
Dacrymycetaceae	*Cerinomyces pallidus*	10694	84.9 ± 5.11	90.7 ± 4.55	5.7
	Cerinomyces sp. 1	10803	69.3 ± 9.94	95.5 ± 5.1	26.2*
	Dacrymyces sp. 1	10104	83 ± 1.46	97.6 ± 3.83	14.6*
Geastraceae	*Gaestrum triplex*	11759			No growth
Hydnaceae	*Hydnum* sp.	10351	107.9 ± 2.71	109 ± 3.26	1.03
Hydnangiaceae	*Laccaria amethystea*	11809	97.4 ± 5.23	91.4 ± 8.66	–
Lyophyllaceae	*Lyophyllum shimeji*	35015	104.8 ± 1.57	101.5 ± 2.47	–
	Lyophyllum shimeji	35016	92.9 ± 1.55	101.1 ± 0.71	8.2*
	Lyophyllum shimeji	35019	98 ± 1.96	101.5 ± 2.49	2.5
Marasmiaceae	*Gymnopus confluens*	34244	87.5 ± 3.41	89 ± 3.44	1.5
	Gymnopus confluens	33623	86 ± 2.3	94.7 ± 0.31	8.7
	Gymnopus confluens	11823	91.9 ± 1.53	93.7 ± 5.16	1.8
	Gymnopus dryophilus	11824	83 ± 2.52	89.2 ± 0.53	6.2*
	Gymnopus peronatus	33271	94.3 ± 4	89.7 ± 7.18	–
	Gymnopus peronatus	30623	92.3 ± 1.16	89.6 ± 1.28	–
	Gymnopus peronatus	11834	89.5 ± 4.13	88.8 ± 3.12	–
	Gymnpuus sp. 1	10102	94.2 ± 9.34	102.8 ± 2.01	8.6
	Gymnopus sp. 2	11826	93.6 ± 1.5	98.2 ± 2.47	4.6*
	Gymnopus sp. 3	11827	91.1 ± 0.85	91.2 ± 3.67	0.1
	Gymnopus sp. 4	11828	93.2 ± 2.69	94.7 ± 1.66	1.4
	Gymnopus sp. 5	11822	93.4 ± 1.26	95.9 ± 0.97	2.5*
	Gymnopus sp. 6	11519	88.4 ± 5.44	86.9 ± 0.15	–
	Gymnopus sp. 7	11831	84.5 ± 4.55	90.7 ± 8	6.2
	Gymnopus sp. 8	11830	88.2 ± 0.05	90.2 ± 0.04	2
	Gymnopus sp. 9	11833	92.5 ± 3.45	94.7 ± 0.36	2.2

Table 1 continued

Family	Species	Strain (TUFC no.)	Percentage recovery (%)[a]		Percent disappearance[b]
			Treatment	Control	
Marasmiaceae	*Marasmiellus chamaecyparidis*	11832	88 ± 6.27	98.2 ± 0.22	10.2*
	Marasmiellus sp. 1	10101	73.3 ± 9.66	94.6 ± 1.37	21.3*
	Marasmius pulcherripes	11108	102.7 ± 0.57	100.1 ± 1.63	–
	Marasmius sp. 1	11025	92.6 ± 3.71	101.7 ± 1.45	9.1
	Marasmius sp. 2	11817	90.9 ± 9.76	102.1 ± 9.39	11.2
	Marasmius sp. 3	11107	102.8 ± 4.16	100.1 ± 4.14	–
	Marasmius sp. 4	11818	109.5 ± 1.49	112.6 ± 9.37	3.1
	Marasmius sp. 5	11829	89.4 ± 6.45	97.2 ± 4.74	7.8
	Rhodocollybia butyracea	11109			No growth
	Rhodocollybia butyracea	33635	97.1 ± 3.89	99.7 ± 2.54	2.6
	Rhodocollybia maculata	30352	87.7 ± 1.5	93.4 ± 1.81	5.7*
	Rhodocollybia prolixa	33289	91.9 ± 1.88	94.2 ± 0.5	2.3
Mycenaceae	*Mycena chlorophos*	10927	104 ± 0.86	103.6 ± 0.9	–
	Mycena crocata	11093	106.3 ± 0.47	104.7 ± 0.95	–
	Mycena haematopus	11265	101.5 ± 2.32	104.7 ± 1.94	3.2
	Mycena polygramma	11820	111.5 ± 1.54	108.7 ± 0.03	–
	Mycena sp. 1	11154			No growth
	Mycena sp. 2	11052	104.4 ± 1.15	105.6 ± 2.31	1.2
	Mycena sp. 3	11155			No growth
Pluteaceae	*Volvariella* sp.	10920	100.7 ± 2.62	101.6 ± 0.22	0.9
Physalacriaceae	*Strobilurus ohshimae*	10567	102 ± 1.49	99.7 ± 0.64	–
Psathyrellaceae	*Coprinellus disseminatus*	10974	52.5 ± 1.16	54.3 ± 0.4	1.8
	Coprinellus ellisii	31678	111.4 ± 3.23	114.5 ± 2.6	3.1
	Coprinellus micaceus	30081	111.5 ± 3.62	109.8 ± 3.5	–
	Coprinellus sp. 1	10976			No growth
	Lacrymaria velutina	11810	98.6 ± 1.03	98.8 ± 2.06	0.2
	Psathyrella piluforms	11268	102.7 ± 3.49	104.3 ± 5.37	1.6
	Psathyrella sp. 1	11226	106.1 ± 2.83	109.8 ± 2.41	3.7
	Psathyrella sp. 2	11149	102.4 ± 2.47	106.6 ± 6.33	4.1
	Psathyrella sp. 3	11811	102.1 ± 5.67	104 ± 3.8	1.9
	Psathyrella sp. 4	11812	94 ± 4.17	92.4 ± 7.83	–
	Psathyrella sp. 5	11001	99.4 ± 3.1	105.8 ± 1.28	6.4*
Rhizopogonaceae	*Rhizopogon roseolus*	10011			No growth
	Rhizopogon roseolus	10007	93.6 ± 7.31	102.6 ± 1.67	9
Septobasidiaceae	*Septobasidium* sp.	11813			No growth
Sirobasidiaceae	*Sirobasidium* sp.	10953	85.4 ± 4.57	87.6 ± 3.35	–
Strophariaceae	*Agrocybe arvalis*	11825	86.8 ± 7.32	95.7 ± 1.46	8.9
	Agrocybe farinacea	11449	96.4 ± 7.1	102.4 ± 1.45	6
	Agrocybe media	34444	80.2 ± 7.14	94.3 ± 1.86	14.1*
	Hypholoma sp.	11814	107.3 ± 1.04	105 ± 0.64	–
	Pholiota sp.	11815	93.7 ± 3.6	96.1 ± 7.3	2.4
Suillaceae	*Suillus granulatus*	10624			No growth

Table 1 continued

Family	Species	Strain (TUFC no.)	Percentage recovery (%)[a]		Percent disappearance[b]
			Treatment	Control	
Tremellaceae	*Holtermannia corniformis*	11115	101.2 ± 2.37	97.8 ± 1.51	–
	Tremella folicacea	10801	79.3 ± 0.9	96.5 ± 4.3	17.2*
	Tremella fuciformis	11038	66.8 ± 9	76 ± 8.48	9.2
	Tremella fuciformis	11046	58.6 ± 4.08	59.2 ± 11.1	0.6[c]
	Tremella mesenterica	11211	101.7 ± 6.32	104.9 ± 0.43	3.2[c]
	Tremella sp. 1	11347	69.2 ± 6.2	87.5 ± 1.7	18.2[c]*
	Tremella sp. 2	11217	57 ± 19.6	75.9 ± 3.95	18.8[c]
	Tremella sp. 3	10955	101.9 ± 4.37	104.3 ± 3.55	2.4[c]
	Tremella sp. 4	11224	60.5 ± 8.01	56.2 ± 4.19	–[c]
Tricholomataceae	*Clytocybe* sp.	11345	96.7 ± 8.6	105.4 ± 0.16	8.7
	Collybia confluens	11000	95.5 ± 0.92	101.6 ± 6.27	6.1
	Collybia sp. 1	11593	94.9 ± 4.11	95.1 ± 3.72	0.2
	Collybia sp. 2	10103	84.5 ± 10.4	97.1 ± 4.45	12.6
	Collybia sp. 3	34247	90.2 ± 3.31	93.3 ± 2.28	3.1
	Lepista sp.	11269	100.6 ± 2.7	103.3 ± 1.08	2.7
	Leucopaxillus giganteus	10661	102.1 ± 0.52	98 ± 4.62	–
	Pleurocybella porrigens	11801	107.2 ± 3.5	105.9 ± 0.8	–
	Tricholoma muscarium	10954			No growth

* $P < 0.05$

[a] Values given are the means ± standard deviations for triplicate cultures

[b] Percent disappearance = percent recovery in control minus percent recovery in treatment. A dash (-) shows a negative percent

[c] 4 days treatment

considered to represent different isomers of monohydroxylated-DDT or -DDD. The intensity of these compounds was low, especially the compound at $r_t = 21.90$ min. The metabolite detected at $r_t = 22.41$ min had a parent peak at $m/z = 370$, that corresponded to +16 mass (hydroxyl residue) of the DDT parent peak ($m/z = 354$). Since the intensity of the peaks detected at $r_t = 21.90$ and 22.62 was too low, no parent peaks were observed for these. These three compounds ($r_t = 21.90$, 22.41 and 22.62) had a base peak ion at $m/z = 251$. The existence of two chlorine residues was indicated by the masses at $m/z = 253$ and $m/z = 255$ that have relative peak intensities toward $m/z = 251$ with 0.65 (with one ^{35}Cl and one ^{37}Cl) and 0.1 (with two ^{37}Cl), respectively (Table 2). Since DDT and DDD have a base peak ion at $m/z = 235$ (M^+–CCl_3 or –$CHCl_2$), the base peak ion at $m/z = 251$ corresponds to the substitution of the hydroxyl residue (+16 mass). The loss of CCl_3 (or $CHCl_2$) from the parent ion gives rise to these fragments. The sequential fragments at $m/z = 181$ and 152 likely arise from the loss of both chlorines and the further loss of CHO from the base peaks, respectively. These characteristics were previously reported during description of the fragment ions in the mass spectra of monohydroxy-DDT isomers (Nadeau et al. 1994) and monohydroxy-DDD isomers (Hay and Focht 2000).

With methyl derivatization analysis, these three compounds disappeared and gave rise to two new compounds at $r_t = 22.33$ and 22.93 min. The new peaks had a base peak at $m/z = 265$ (M^+–CCl_3 or –$CHCl_2$). The existence of two chlorine residues was indicated by masses at $m/z = 267$ and 269 that had relative peak intensities toward $m/z = 265$ with 0.65 and 0.1, respectively. The sequential ionization peaks at $m/z = 215$ correspond to the loss of CH_3 and Cl, and $m/z = 195$ correspond to the loss of 2Cl from the base peak. This indicates hydroxyl residue was substituted to methoxyl residue. The one remaining methylated metabolite was not observed. The reason may be the small volumes of the original metabolites

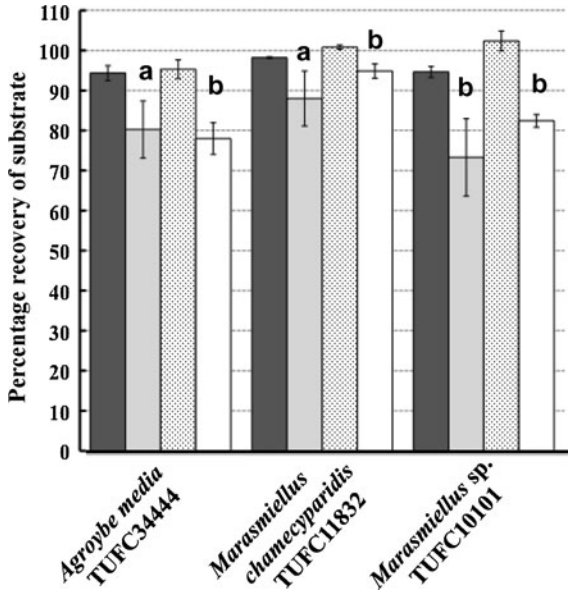

Fig. 1 Percentage recovery of DDT and DDD after treatment with a selected fungus and incubation for 14 days. *Dark gray columns* (viable cultures) and *light gray columns* (azide-treated controls) represent percentage recovery of DDT. *Dotted columns* (viable cultures) and *blank columns* (azide-treated controls) represent percentage recovery of DDD. Each value is the mean ± SD of triplicate samples. *(a)* $P < 0.05$ *(b)* $P < 0.01$

(before methylation). It is also possible that derivatization masked the positional effect of the different hydroxyl isomers on retention time. Thus, the two different hydroxy isomers could be comigrated. The metabolite at $r_t = 22.41$ and 22.62 min was not detected when DDD was degraded by *Marasmiellus* sp.1 TUFC10101, but a trace amount of the peak at $r_t = 21.90$ was detected. With methylation, the metabolite at $r_t = 21.90$ disappeared and gave rise to a new compound at $r_t = 22.33$ min.

These results indicate that the metabolite detected at $r_t = 21.90$ min was monohydroxy–DDD, and the metabolites detected at $r_t = 22.41$ and 22.62 min were monohydroxy-DDT isomers.

Other metabolites

The metabolite at $r_t = 18.92$ min, tentatively named putative metabolite #1, had a base peak ion at $m/z = 199$, and two ionization peaks at $m/z = 234$ and 165, that may correspond to $[M–CH_2OH, H, Cl]^+$, $[M–CH_2OH, H]^+$ and $[M–CH_2OH, 2Cl]^+$. Methylation of this metabolite caused loss of the peak, and gave a new peak at $r_t = 19.40$ min. The newly detected peak had a base peak ion at $m/z = 235$ ($M^+–CH_2OCH_3$), and two sequential ionization peaks at $m/z = 199$ ($M^+–CH_2OCH_3$, H, Cl) and 165 ($M^+–CH_2OCH_3$, 2Cl). This metabolite was also obtained when DDD treated with *Marasmiellus* sp.1 TUFC10101. This metabolite was considered to be 2,2-bis(4-chlorophenyl)ethanol (DDOH).

The structures of metabolites detected at $r_t = 13.03$ and 13.88 min, tentatively named putative metabolites #2 and #3, have not yet been determined.

From cultures of *Marasmiellus* sp.1 TUFC10101 with DDD, monohydroxy-DDD, putative metabolite #1, and an additional metabolite ($r_t = 21.42$) were detected. The additional metabolite had a ionization peak at $m/z = 251$ ($M–CHCl_2$), as do monohydroxy-DDD and DDT. An ionization peak at $m/z = 251$ suggests the existence of a hydroxyl residue corresponding to the mass of monohydroxy compounds. However, unlike monohydroxy compounds, the metabolite had sequential peaks at $m/z = 139$ and 111, that may correspond to $[M–CHCl_2, C_6H_4Cl, H]^+$ and $[C_6H_4Cl]^+$. No effect was observed when derivatization with TMS–diazomethane was carried out for this metabolite. Steric hindrance between the hydroxyl residue and adjacent aromatic rings may inhibit methylation. In addition, the mass spectrum of this metabolite is similar to that of authentic 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol (dicofol). These results suggest that the metabolite detected at $r_t = 21.42$ min was 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol (FW152).

Inhibition effects of P450 monooxygenase

No significant inhibitory effect on DDT degradation was observed ($F(3, 15) = 3.29$, $P > 0.05$) in cultures incubated with DDT and 1-ABT (Fig. 2), and DDD was observed as a metabolite without other metabolite. However, significant dose-dependent inhibition of DDD degradation was observed (F (3,8) = 4.07, $P < 0.05$) when cultures incubated with DDD and 1-ABT (Fig. 2), and only a trace amount of FW-152 was detected. There was no visible effect on mycelial growth from toxicity due to the low concentrations of inhibitors used in this experiment. The DDT with 1-ABT experiments also indicated no significant effect on fungal growth at the concentration used.

Table 2 Mass spectra and GC retention times of standard compounds and metabolite products (and its derivatives)

Substrate or metabolite	GC retention time (min)	Mass spectrum *m/z* (relative intensity)
DDT (standard)	21.23	356 (*), 354 (*), 239 (10.5), 237 (63.5), 235 (100), 199 (11.6), 165 (30.3)
DDD (standard)	20.55	322 (*), 320 (*), 239 (9.8), 237 (62.1), 235 (100), 199 (12.2), 165 (25.4)
DDE (standard)	19.7	320 (2.1), 318 (4.4), 316 (3.2), 250 (10.8), 248 (63.8), 246 (100), 210 (20.2), 176 (39.0)
Monohydroxy-DDT	22.41	372 (*), 370 (3.5), 255 (11.1), 253 (64), 251 (100), 181 (28.1), 152 (16.8)
Monohydroxy-DDT	22.62	255 (9.4), 253 (65.3), 251 (100), 181 (31.3), 152 (18.7)
(Monomethoxy-DDT)	22.93	269 (6), 267 (64.2), 265 (100), 215 (12.5), 195 (25.6)
Monohydroxy-DDD	21.9	255 (*), 253 (67.4), 251 (100), 181 (44.3), 152 (17.2)
(Monomethoxy-DDD)	22.33	269 (13.9), 267 (59.2), 265 (100), 215 (10.4), 195 (12.6)
Putative metabolite #1 (DDOH?)	18.92	238 (10.5), 236 (50.8), 234 (75.3), 199 (100), 165 (48.1)
(Monomethoxy-DDOH?)	19.4	239 (28.7), 237 (46.9), 235 (100), 199 (22.3), 165 (45.3)
Putative metabolite #2	13.88	244 (5.8), 242 (30), 240 (48.6), 229 (20.3), 227 (96.1), 225 (100), 187 (77.6), 147 (46.8)
Putative metabolite #3	13.03	208 (44.1), 206 (79.5), 195 (9), 193 (76.8), 191 (100), 141 (50.1), 113 (78.6)
FW-152	21.42	255 (3.3), 253 (48.7), 251 (75.3), 141 (43.4), 139 (100), 111 (45)
Dicofol (standard)	17.07	254 (2.4), 252 (14.51), 250 (22.8), 217 (3.5), 215 (10.8), 141 (33.6), 139 (100), 111 (35.3)

* Trace amount

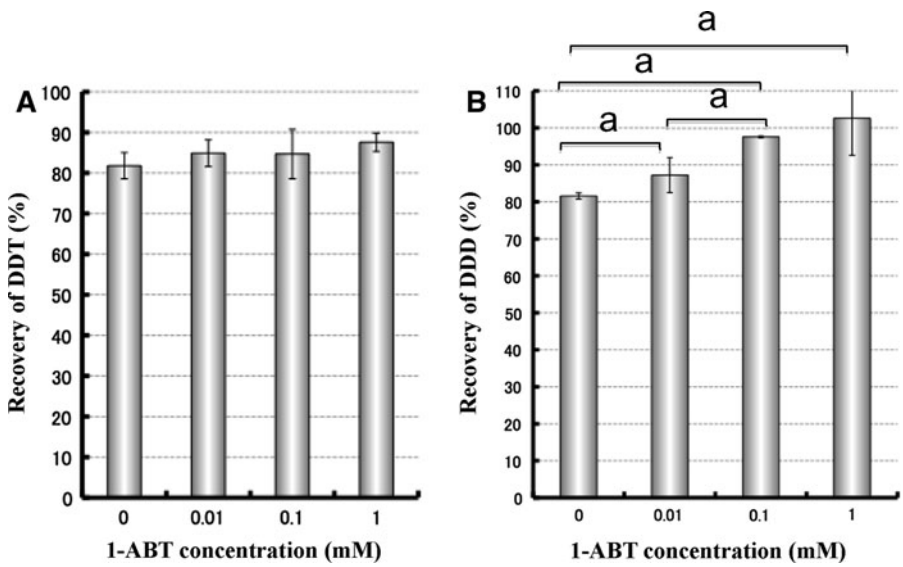

Fig. 2 Effect of 1-aminobenzotriazole concentrations on the degradation of DDT (**a**) and DDD (**b**) by *Marasmiellus* sp.1 TUFC10101 after 14 days incubation. Each value is the mean ± SD of triplicate samples. *(a)* $P < 0.05$

Discussion

Although, a range of basidiomycetes has been reported as capable of degrading DDT, we present here degradation of DDT by basidiomycetes isolated from leaf-litter on forest soil. Monohydroxylation of the aromatic rings of DDT and DDD is also reported.

In 2 weeks incubation, 21.3% of DDT was degraded by *Marasmiellus* sp.1 TUFC10101. Other basidiomycetes, *Boletus edulis* (an ectomycorrhizal fungus), *Gloeophyllum trabeum* (a brown-rot fungus) and *P. chrysosporium* (a white-rot fungus), can degrade DDT by approximately, 50% (over 15 days), 35% (over 14 days) and 50% (over 30 days),

respectively (Bumpus and Aust 1987; Huan et al. 2007; Purnomo et al. 2008). Compared with these previous reports, the DDT degradation ability of *Marasmiellus* sp.1 TUFC10101 is low; however, further degradation occurred when using longer treatment periods of 21 days (29%) and 28 days (35%). Application of wood-rotting fungi for bioremediation of DDT-contaminated soil has been difficult because soil is unsuitable for their mycelial growth (Lang et al. 1997, Martens and Zadrazil 1998). Alternatively, Ectomycorrhizal fungi may be suitable for remediation of DDT-contaminated soil, but these need live symbiotic host plants. Since *Marasmiellus* sp.1 TUFC10101 colonizes soil environments including humic acids, leaf-litter, decayed branches and other woody debris, the fungus appears to be adaptive for soil remediation. To research practicalities for bioremediation, DDT degradation experiments using model soil must be performed.

In the experiments using *Marasmiellus* sp.1 TUFC10101 and DDT, the formation of hydroxylated DDT and DDD suggests the involvement of oxidative enzymes. The involvement of lignin-degrading enzymes or related oxidases for DDT degradation by basidiomycetes has been suggested previously (Bumpus and Aust 1987; Huan et al. 2007). Thus, we measured the activities of the ligninolytic enzymes, LiP, MnP and laccase. All measured enzyme activities were quite low (data not shown). DDT degradation by culture filtrate was also tested, but no significant decrease in DDT was observed nor any metabolite found. These results suggest that the initial metabolism of DDT by *Marasmiellus* sp.1 TUFC10101 is not performed by extracellular ligninolytic enzymes.

Subsequently, we examined the involvement of the P450 system on DDT and DDD degradation. A P450 inhibitor 1-ABT inhibited the formation of monohydroxy-DDT from DDT, although the formation of DDD was not inhibited. It is strongly suggested that the production of monohydroxy-DDT is performed by P450 monooxygenase, and that conversion from DDT to DDD is not dependent on the P450 system.

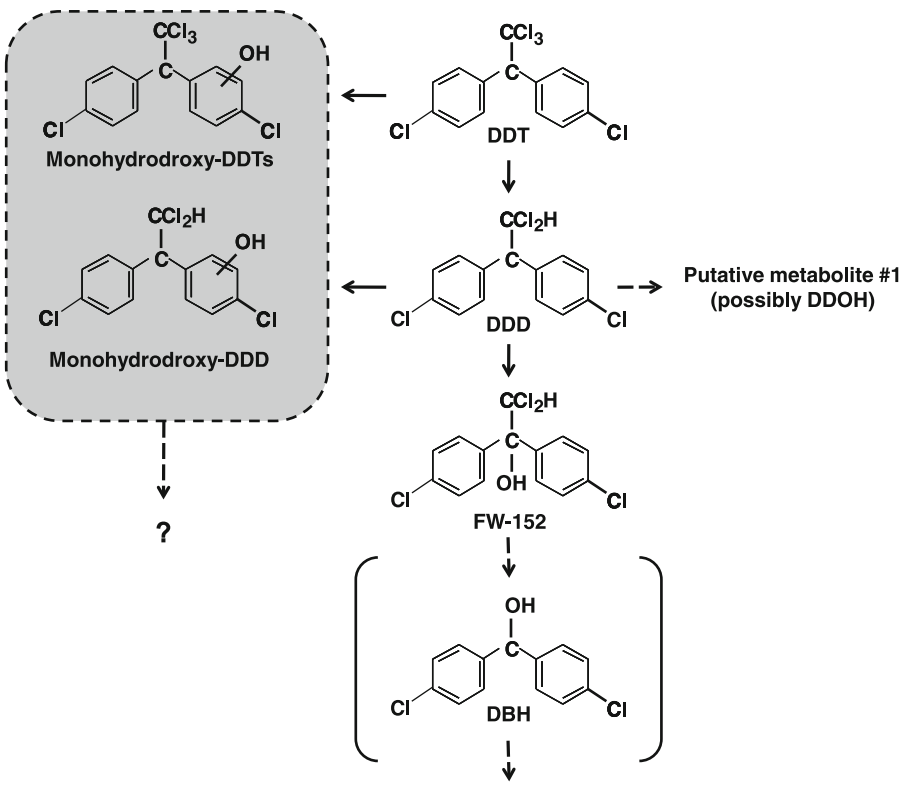

Fig. 3 Proposed pathway for the biotransformation of DDT by *Marasmiellus* sp.1 TUFC10101. Parts shown in *gray* indicate the novel DDT metabolic pathways proposed here. Structures in *parentheses* and pathways shown by *dotted lines* are unidentified

Hence, as shown in Fig. 3, the existence of two initial metabolic pathways for DDT degradation by *Marasmiellus* sp.1 TUFC10101 is suggested.

The first pathway could be mediated by the P450 system that produces monohydroxy-DDTs (Fig. 3). This pathway seems to be minor pathway, since 1-ABT did not statistically reduce DDT disappearance. A similar pathway was reported for the degradation of dioxins by white-rot fungi (Mori and Kondo 2002; Kamei and Kondo 2005). Monohydroxy-DDT, and -DDD were found in the DDT (DDD)-degrading metabolic pathway of *Ralstonia eutropha* strain A5 (Nadeau et al. 1994, Hay and Focht 2000). However, the monohydroxy-DDT (-DDD) was generated from acid dehydration of 2,3-dihydrodiol-DDT (-DDD). Formation of 2,3-dihydrodiol-DDT (-DDD) was mediated by dioxygenase (Nadeau et al. 1994, Hay and Focht 2000).

In the second pathway, DDT is first dechlorinated to DDD by an unidentified system (not P450). Some of the DDD undergoes monohydroxylation, which produce monohydroxy-DDD. Some of the residual DDD was oxidized to FW-152 and putative metabolite #1 (possibly DDOH) (Fig. 3). Monohydroxy-DDD and putative metabolite #1 were not found in the presence of 1-ABT, thus involvement of P450 system is proposed. FW-152 may be transformed by sequential steps into 4,4′-dichlorobenzhydrol (DBH). Although, we did not find DBH as metabolites of DDD from *Marasmiellus* sp.1 TUFC10101, DBH was obtained as a metabolite from *M. chamaecyparidis* TUFC11832. FW-152 and DBH were obtained as metabolites of DDT during previous research using basidiomycetes (Bumpus and Aust 1987; Huang et al. 2007; Purnomo et al. 2008). Thus, the existence of a metabolic pathway from FW-152 to DBH is suggested. For *A. media* and *M. chamaecyparidis*, the DDT degradation pathway may be similar to that of *Marasmiellus* sp.1 TUFC10101, because they are phylogenetically close to the latter, and several metabolites were the same as for *Marasmiellus* sp.1 TUFC10101.

In the present study, we provide the evidence about DDT (DDD) monohydroxylation of aromatic ring mediated by P450 as a new metabolic pathway. The proposed pathway we present may not be the full DDT biodegradation pathway, as the identities of a number of additional metabolites remain to be determined.

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