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Biodegradability and biodegradation pathways of chlorinated cyclodiene insecticides by soil fungi

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An aerobic dieldrin-degrading fungus, *Mucor racemosus* strain DDF, and two aerobic endosulfan-degrading fungal strains, *Mortierella* sp. strains W8 and Cm1–45, were isolated from soil contaminated with organochlorine pesticides. Strain DDF degraded more than 90% dieldrin during 10-days of incubation at 25°C and showed the production of a small amount of aldrin trans-diol. Moreover, strain DDF reduced levels of aldrin trans-diol while producing unknown metabolites that were determined to be aldrin trans-diol exo- and endo-phosphates. On the other hand, *Mortierella* sp. strains W8 and Cm1–45 degraded more than 70% and 50% of α and β-endosulfan, respectively, over 28 days at 25°C, in liquid cultures containing initial concentrations of 8.2 µM of each substance. Only a small amount of endosulfan sulfate, a persistent metabolite, was detected in the both cultures, while these strains could not degrade endosulfan sulfate when this compound was provided as the initial substrate. Both strains generate endosulfan diol as a first step in the degradation of endosulfan, then undergo further conversion to endosulfan lactone.

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Keywords: zygomycete, mucor, mortierella, dieldrin, endosulfan, persistent organic pollutants.

Introduction

Organochlorine pesticides, including aldrin, dieldrin, endrin, dichloro-diphenyl-trichloroethane (DDT), endosulfan, heptachlor, hexachlorocyclohexanes (HCHs), and mirex, are synthetic chemicals. Some of these compounds have been used extensively to control insect and mite vectors of human, and also domestic animal diseases and insect pests that damage agricultural crops. Organochlorine pesticides are bioaccumulative, toxic, and persistent in the environment. Although their use was prohibited by many countries in the 1970s, organochlorine insecticides are still found in the environment.1,2) Endosulfan and endosulfan sulfate were included in the Stockholm Convention list of persistent organic pollutants (POPs) on April 29, 2011. Endosulfan is a broad-spectrum chlorinated cyclodiene insecticide that has been used extensively on a variety of crops for over 30 years.3) This compound is a mixture of two stereoisomers, α- and β-endosulfan, at a ratio of 7:3. Endosulfan is extremely toxic to fish and invertebrates and has increasingly been implicated in mammalian gonadal toxicity, genotoxicity, neurotoxicity4) and endocrine disruption.5) The estimated acute aquatic toxicity of endosulfan toward striped bass (*Morone saxatilis*) and the water flea (*Daphnia magna*) ranges from 0.1 to 166 µg/L.5) Endosulfan is still present in soils because it was widely used until relatively recently and has a half-life in soil of 60 days for α-endosulfan and 800 days for β-endosulfan.6) Moreover, estimated half-lives for combined toxic residues (endosulfan plus endosulfan sulfate) ranging from roughly 9 months to 6 years have been reported.5) Endosulfan can be released into the atmosphere through spray drift, post-application volatilization, and wind erosion of soil particles.

Microbial degradation is a cost-effective and efficient way to remediate contaminated environments. The degradation of organochlorine pesticides using bacteria7,8) and fungi9) has been reported, and filamentous fungi have been shown to be capable of degrading a variety of environmentally persistent organic pollutants, including hexachlorocyclohexane (HCH),10) DDT,11) and endosulfan.12) Matsumura and Boush13) isolated dieldrin-degrading *Trichoderma viride* from soil that had been heavily contaminated with various insecticides. They also suggested that 6,7-trans-dihydroxydihydroaldrin (aldrin trans-diol) might be a major product based on its having an identical Rf value to an authentic control. Moreover, Wedemeyer14) described the conversion of dieldrin *in vitro* by *Aerobacter aerogenes* to a compound chromatographically similar to 6,7-trans-dihydroxydihydroaldrin. Aldrin trans-diol (LD₅₀: 1250 mg kg⁻¹) showed a much lower toxicity to mice than dieldrin (LD₅₀: 65 mg kg⁻¹),
therefore, it is important to distinguish between the toxicity of dieldrin and its metabolites. However, limited information is available regarding metabolites formed by the aerobic biodegradation of dieldrin. Therefore, there is still an urgent need to develop an effective method for bioremediation of aerobic zones polluted with organochlorine insecticides. Phanerochaete chrysosporium is a commonly studied white rot fungus that showed to degrade and mineralize a wide range of industrial and agricultural pollutants, including DDT. Phanerochaete chrysosporium degraded approximately 90% of endosulfan and generated endosulfan sulfate and endosulfan diol as the principal metabolites. Kullman and Matsumura proposed pathways through which the oxidation of diol to hydroxyether was formed and showed that dialdehyde and lactone were end products of the metabolism. Kamei et al. also reported that the white rot fungus Trametes hirsuta degraded more than 90% of endosulfan and approximately 70% of endosulfan sulfate, producing endosulfan diol and endosulfan dimethylene as metabolites. In other studies, Trichoderma harzianum and Mucor thermohyalospora degraded endosulfan, apparently via both the oxidation of endosulfan to endosulfan sulfate and its hydrolysis to endosulfan diol.

The present study reveals soil fungi that can degrade dieldrin and endosulfan and their degradation pathways.

1. Biodegradation of dieldrin

1.1. Degradation experiments

All fungal strains except Trichoderma strains, which were used in this study, were grown on PDA agar medium in Petri dishes at 25°C. Fungal disks (6 mm diameter) were taken from the margins of fungal colonies grown for 14 days. Each fungal disk was transferred into 10 mL of modified Czapek-Dox (MCD) liquid medium (Glucose; 10 g, MgSO 4 ⋅7H 2 O; 0.5 g, NaNO 3 ; 2.0 g, FeSO 4 ⋅4H 2 O; 0.01 g, K 2 HPO 4 ; 1.0 g, Bacto™ yeast extract; 0.5 g per liter) in a sterilized 100 mL Erlenmeyer flask. After pre-incubation for 7 days, 50 µL of dieldrin in acetone was added to each inoculated flask (final concentration: 13.2 µM). To prevent volatilization of dieldrin, the flask was sealed with a glass stopper. The cultures were incubated statically for 14 days at 25°C under dark conditions. As a control, cultures were killed by autoclaving (121°C, 15 min) after 7 days of pre-incubation. Following the incubation period, cultures were homogenized with 15 mL of acetone, after which the residual biomass was removed by centrifugation at 3000 × g for 10 min. An aliquot of 1 mL of supernatant was then transferred into a test tube and extracted with 5 mL of hexane. Next, dieldrin was analyzed by gas chromatography with a 63Ni electron capture detector (GC/ECD), which was performed on an HP 6890 GC system linked to an HP 5890 detector and a 15 m column (HP50+, J&W Scientific, Folsom, CA, USA). The oven temperature was programmed to increase from 150 to 200°C at 20°C/min and 200 to 280°C at 20°C/min. The recovery rate of dieldrin in the control cultures was found to be 90–100%, which indicated that the extraction was efficient and the analysis deviation was small. In the screening assay there were one and three replicates of samples and controls, respectively. Following screening, the time course degradation experiment for dieldrin-degrading fungus was performed in triplicate.

The degradation experiment was conducted using 35 strains of Trichoderma spp. and 36 strains that were isolated from a soil with annual endosulfan applications for more than 10 years. After pre-incubation for 7 days, 50 µL of dieldrin in acetone was added to each inoculated flask (final concentration: 13.2 µM). Among Trichoderma spp., strain 93155 was capable of degrading dieldrin with 19.7% (2.6 µM) degradation after 14 days of incubation. However, a fungus isolated from soil contaminated with endosulfan (strain DDF) degraded 95.8% of dieldrin from an initial concentration of 13.2 µM. A subsequent time course degradation experiment revealed strain DDF degraded dieldrin from 14.3 to 1.01 µM during 10-days of incubation at 25°C (Fig. 1).

The ITS rRNA sequence of strain DDF (471 nucleotides, GenBank accession no. AB536702) was compared with those of the fungal sequences in the GenBank database. Strain DDF exhibited a high sequence similarity to those of Mucoraceae fungi as indicated by the constructed phylogenetic dendrogram. The highest sequence identity (100%) was found with Mucor racemosus f. racemosus (GenBank accession no. AY213659); therefore, strain DDF was designated as Mucor racemosus strain DDF. These findings are in accordance with those of Anderson et al., who reported that Mucor alternans degraded dieldrin and DDT.

1.2. Detection of the metabolites produced during dieldrin degradation

The dieldrin-degrading fungus was grown in 500 mL of MCD medium containing 13.2 µM dieldrin for 5 days, after which the culture was homogenized and separated into a mycelial fraction and a fluid fraction by centrifugation. The fluid fraction was subsequently acidified to pH 2.0 with 0.1 N HCl and extracted three times with ethyl acetate. The mycelial fraction was also extracted three times with ethyl acetate. The ethyl acetate ex-
tracts were then mixed, dried with anhydrous sodium sulfate, and evaporated to dryness. The concentrate was subsequently re-suspended into acetone/hexane (1:1) and derivatized with bis (trimethylsilyl) trifluoroacetamide (BSTFA). Aldrin trans-diol in the BSTFA-derivatized concentrate was detected by GC/ECD. The oven temperature was programmed to increase from 150 to 200°C at 20°C/min and then 200 to 280°C at 20°C/min, while the inlet temperature was set at 180°C.

Gas chromatography-mass spectrometry (GC/MS) analysis for confirmation of aldrin trans-diol was conducted using a HP6890 series high resolution gas chromatograph (HRGC) with a 60 m column (DB5-MS, J&W Scientific, Folsom, CA, USA) interfaced to a high-resolution mass selective detector (HRMS) (Micromass Autospec-Ultima) to detect low concentrations of the material. The oven temperature was programmed to increase from 140 to 200°C at 10°C/min, followed by 200 to 270°C at 2°C/min and then 270 to 300°C at 30°C/min. The inlet temperature was set at 180°C. The full scan mass spectrum of aldrin trans-diol derivatized with BSTFA is shown in Fig. 2a. The product generated a molecular ion peak at m/z 542, as well as remarkable fragment ions at m/z 506.8763 and 508.8708. Aldrin trans-diol as a metabolite was confirmed by monitoring of the chlorination pattern using the selective ion monitoring (SIM) mode of HRGC-HRMS. The mass spectra m/z 414.8248, 416.8241, 506.8763 and 508.8708 of aldrin trans-diol were selected. In addition, a lock-mass m/z (430.9728) from perfluorokerosene (PFK) was used for drift correction. The relative intensities of the target fragment ions of the metabolite were 39.3 (414.8248), 70.2 (416.8241), 100 (506.8763) and 54.0 (508.8708), the metabolite was detected at a retention time of 27.95 min, and its retention time and the relative inten-

Fig. 2. (a) A mass spectrum of aldrin trans-diol derivatized with BSTFA. The base ion peak m/z 73 was omitted because its abundance was too high. Monitoring ions were selected for the comparatively high intensity chlorination pattern. The chemical structure of aldrin trans-diol derivatized with BSTFA is also indicated. (b) The retention time of aldrin trans-diol derivatized with BSTFA detected by GC/MS analysis with a HRMS-HRGC, and the set of individual intensities of the target ion based on the chlorination pattern.
A degradation test was performed by adding 50 µL of an aldrin trans-diol stock solution (1000 mg L\(^{-1}\), dissolved in acetone) to each culture (final aldrin trans-diol concentration: 5 mg L\(^{-1}\)) following a pre-incubation period. The cultures were acidified to pH 2.0 with 0.1 M aqueous HCl following the degradation test, then extracted with ethyl acetate. The ethyl acetate extracts were evaporated, after which the residues were dissolved in acetonitrile:H\(_2\)O (1:1). The presence of any metabolites in the supernatants was determined periodically by UPLC-ESI (−)-MS and HR-FAB (−)-MS.

Inspection of the UPLC-ESI (−)-MS spectra for degradation of aldrin trans-diol by strain DDF indicated that the concentration of aldrin trans-diol had been reduced and a new peak with an earlier retention time than that of aldrin trans-diol had been detected. This new peak had a \(m/z\) value of 476.8 in UPLC-ESI (−)-mode (Fig. 3b). Moreover, on the basis of the isotopic fragmentation pattern, the compound possessed six chlorine atoms (Fig. 3b). These findings suggested that the new product was not formed as a result of an elimination reaction involving the chlorine atoms of the host compound. Additionally, the levels of aldrin trans-diol in the liquid culture containing strain DDF were decreased by 78% after 14 days, and the metabolite signal at \(m/z\) 476.8 gradually increased with time (Fig. 3a). In an attempt to identify the metabolites, chemical derivatizations of aldrin trans-diol were performed. Trial sulfations of the aldrin trans-diol were conducted according to standard conditions (SO\(_3\)-pyridine in DMF and/or pyridine at 20–60°C); however, the reactions were unsuccessful, possibly because of hydrogen bonding between the two hydroxy groups. Eventually, the reaction was achieved by microwave treatment using the method described by Kiyota et al.\(^{22}\) Briefly, the crude product was purified by reversed-phase TLC to give a 1:1 mixture of the sodium salts of the 6-exo- and 7-endo-sulfates. This mixture was further purified to give an 83:17 mixture of the 6-exo- and 7-endo isomers. The stereochemistry of the product was determined by a nuclear Overhauser effect spectroscopy (NOESY) experiment. UPLC-ESI (−)-MS analysis indicated that the retention times...
of the synthetic sulfates were 4.32 and 4.71 min in the 6-exo- and 7-endo-sulfates, respectively. However, the retention time (2.38 min) of the natural metabolite was earlier than that of the synthetic sulfates (4.32 and 4.71 min) (data not shown). Based on these results, the possibility of the metabolites being the corresponding phosphate moiety with the same mass number \( (m/z = 476.8 \ [M-H]^-) \) was considered. Therefore, 6-exo-phosphate was prepared by condensation of aldrin trans-diol with phosphoric acid using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI), after which the stereochemistry was determined by a NOESY experiment. Analysis by HPLC revealed that the retention time of the synthetic phosphorylated aldrin trans-diol was in agreement with that of the natural metabolite (data not shown). Furthermore, the natural extract was subjected to HR-FAB (−)-MS analysis, which revealed that the \( [M-H]^- \) value \( (m/z = 474.8400) \) was very similar to the value calculated for phosphate \( (m/z = 474.8397 \text{ for } C_{12}H_{10}^{35}\text{Cl}_{6}O_{5}P) \), but not that of sulfate \( (m/z = 474.8302 \text{ for } C_{12}H_{9}^{35}\text{Cl}_{6}O_{5}S) \). The mass accuracy for the natural metabolite \((\text{measured mass } / \text{theoretical exact mass})\) was 0.63 ppm when compared with aldrin trans-diol phosphate. The identification criterion was based on that of Pihlström,\(^{23}\) who stipulated a mass accuracy value of less than 5 ppm. Analysis by HR-MS was conducted to evaluate identification of metabolites in the environment\(^{23}\) because the target metabolites were in trace amounts and unstable. When the metabolite was analyzed in UPLC-SIM-ESI (−)-mode using a mobile phase composed of acetonitrile and a 0.2% acetic acid solution (30:70 v/v), the peak signal for the metabolite consisted of two resolved peaks (data not shown). These results indicated that the identities of the natural metabolites were aldrin trans-diol 6-exo- and 7-endo-phosphates. The overall proposed pathway of degradation by strain DDF is shown in Fig. 4.

2. Biodegradation of endosulfan

2.1. Microorganisms and isolation of fungi from soil

Soil dilution (soils A, B and C) and soil washing (soil D) were conducted to isolate the soil fungi from the soil as follows: 1 g of each soil sample was mixed with 20 mL of sterile distilled water using a Universal Homogenizer (Nihon Seiki Seisakusho, Tokyo, Japan), then subjected to 10\(^{-2}\), 10\(^{-3}\) and 10\(^{-4}\) dilutions. Aliquots of 100 \( \mu \text{L} \) of the resulting soil solutions were subsequently placed onto a plate of Martin agar medium containing chloramphenicol (0.25 g L\(^{-1}\)), after which the plates were incubated at 25°C for 5 days. The fungal colonies were subsequently classified into several groups according to their morphology, growth rate and color, after which representatives of each group were picked up with a sterile needle and sub-cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). Soil washing was then conducted according to Gams and Domsch.\(^ {19} \) Briefly, a 10 g aliquot of soil sample was placed on the rough sieve of the sterilized soil washing apparatus, 90 mL of sterilized water were added and the apparatus was sealed. The apparatus was subsequently shaken vertically for 1 min, after which the water suspension was discarded through the drain tube (washing procedure). The washing procedure was repeated 20 times. Soil ag-
gregates that were obtained on a fine sieve following the washing procedure were aseptically transferred onto a plate of Martin agar medium and then incubated at 25°C for 5 days. The fungal colonies obtained were classified into several groups according to their morphology, growth rate and color, and representatives of each group were picked up with a sterile needle and subcultured on PDA.

The degradation experiment was performed using 35 strains of *Trichoderma* spp. and 133 strains (Kagoshima: 48, Ehime: 38, and Niigata: 47) of soil isolates. There was no endosulfan-degrading *Trichoderma* used in this study. However, two fungi (strains W8 and Cm1-45) isolated from soil D, which was contaminated with organochlorine pesticides, degraded α and β-endosulfan. Strain Cm1-45 degraded 47.2% of α-endosulfan and 25.1% of β-endosulfan during 14 days of incubation, while strain W8 degraded 53.3% of α-endosulfan and 11.1% of β-endosulfan. A time course degradation using strains W8 and Cm1-45 was then performed, during which both strains degraded more than 70 and 50% of α and β-endosulfan, respectively, over 28 days at 25°C (Fig. 5). However, only 3.2 and 1.2 µM endosulfan sulfate was detected in the cultures of strains W8 and Cm1-45, respectively. Endosulfan sulfate as an initial substrate was not degraded by strains W8 and Cm1-45, and the recovery of this compound was found to be approximately 100%.

2.2. Identification of endosulfan-degrading fungi

The ITS rRNA gene sequences of strain W8 (501 nucleotides, GenBank accession no. AB542930) and Cm1-45 (501 nucleotides, GenBank accession no. AB542931) were compared with those of the fungal sequences in GenBank. Strains W8 and Cm1-45 exhibited high sequence similarities to those of *Mortierellaceae* fungi. The highest sequence similarity (100%) was found with *Mortierella verticillata* (GenBank accession no. AY997063). Strains W8 and Cm1-45 were closely related to *Mortierella verticillata*.

2.3. Detection of metabolites in endosulfan degradation

In the ethyl acetate extracts of cultures from strains W8 and Cm1-45, four different peaks with retention times of 6.57, 5.69, 7.93 and 9.86 min were observed upon GC/ECD analysis of α and β-endosulfan. These peaks were assigned as endosulfan diol, endosulfan ether, endosulfan lactone and endosulfan sulfate based on their having the same retention time as authentic standards. The time courses of endosulfan degradation and endosulfan diol production by strains W8 and Cm1-45 are shown in Fig. 5. When endosulfan was added to the MCD cultures, it decreased with accompanying growth of strains W8 and Cm1-45 and endosulfan diol was produced. In contrast, no production of endosulfan diol, endosulfan ether or endosulfan lactone was detected in the autoclaved control cultures. Endosulfan diol, endosulfan ether, endosulfan lactone and endosulfan sulfate generated molecular ion peaks at *m/z* 360, 342, 358 and 422, respectively, and the relative intensity of the chlorination pattern matched the isotope ratio of Cl6 in all detected metabolites. The metabolites were detected at retention times of 15.1, 11.4, 13.3 and 15.8 min in endosulfan diol, endosulfan ether, endosulfan lactone and endosulfan sulfate, respectively, and these retention times and mass spectral patterns matched those of the individual standards. The proposed pathway of degradation by strain W8 or Cm1-45 is shown in Fig. 6.

**Concluding remarks**

Dieldrin- and endosulfan-degrading fungi were screened from soil contaminated with endosulfan and their degradation abilities were demonstrated. *Mucor racemosus* strain DDE decreased dieldrin by converting it to aldrin trans-diol phosphate via al-
Mortierella sp. strains W8 and Cm1–45 generate endosulfan diol as a first step in the degradation of endosulfan, then undergo further conversion to endosulfan lactone.

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