APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1995, p. 3904-3909 Vol. 61. No. 11
0099-2240/95/$04.00+0
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Degradation of 4,4' -Dichlorobiphenyl, 3,3',4,4' -Tetrachlorobiphenyl, and 2,2',4,4',5,5'-Hexachlorobiphenyl by the White Rot Fungus *Phanerochaete chrysosporium*

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Received 22 May 1995/Accepted 29 August 1995

The white rot fungus *Phanerochaete chrysosporium* has demonstrated abilities to degrade many xenobiotic chemicals. In this study, the degradation of three model polychlorinated biphenyl (PCB) congeners (4,4'-dichlorobiphenyl [DCB], 3,3',4,4'-tetrachlorobiphenyl, and 2,2',4,4',5,5'-hexachlorobiphenyl) by *P. chrysosporium* in liquid culture was examined. After 28 days of incubation, ^{14}C partitioning analysis indicated extensve degradation of DCB, including 11% mineralization. In contrast, there was negligible mineralization of the tetrachloro- or hexachlorobiphenyl and little evidence for any significant metabolism. With all of the model PCBs, a large fraction of the ^{14}C was determined to be biomass bound. Results from a time course study done with 4,4'-[^{14}C]DCB to examine ^{14}C partitioning dynamics indicated that the biomass-bound ^{14}C was likely attributable to nonspecific adsorption of the PCBs to the fungal hyphae. In a subsequent isotope trapping experiment, 4-chlorobenzoic acid and 4-chlorobenzyl alcohol were identified as metabolizes produced from 4,4'- [^{14}C] DCB. To the best of our knowledge, this the first report describing intermediates formed by *P. chrysosporium* during PCB degradation. Results from these experiments suggested similarities between *P. chrysosporium* and bacterial systems in terms of effects of congener chlorination degree and pattern on PCB metabolism and intermediates characteristic of the PCB degradation process.

Bacterial degradation of polychlorinated biphenyls (PCBs) has been studied in detail (for reviews, see references 1 and 12). In comparison, relatively little is known about the potential of fungi to degrade various types of PCBs or the metabolic mechanism(s) by which this process might be effected. Degradation of technical PCB mixtures by *Aspergillus* spp. appeared to vary depending on the level of PCB chlorination and the strain being tested (9, 18). For example, *Aspergillus niger* metabolized PCB mixtures with $\leq$ 42% chlorine by weight, but more heavily chlorinated mixtures ($\geq$ 54% chlorine by weight) were not degraded (18). In contrast, *Aspergillus flavus* failed to effect any metabolic changes in PCB mixtures with chlorine concentrations ranging from 32 to 60% by weight (18). In cases in which PCB degradation has been observed, little information concerning the identities of metabolic intermediates that might provide insights into the degradative pathways has been provided. Dmochewitz and Ballschmiter (9) detected trace amounts of hydroxylated trichlorobiphenyls as well as dichloro- and trichloro-benzoic acids during Clophen A 30 (a technical PCB mixture averaging 42% Cl by weight) degradation by *A. niger (9);* isomeric configurations were not specified for any of these compounds. Wallnöfer et al. (22) reported that a soil fungus, *Rhrizopus japonicus,* metabolized 4-chlorobiphenyl and 4,4'-dichlorobiphenyl (DCB) to hydroxylated metabolizes. As with the *A. niger* study, these compounds were detected only in trace amounts and were not conclusively identified.

The white rot fungus *Phanerochaete chrysosporium* has been examined for its ability to degrade many xenobiotic chemicals, including PCBs. Prior investigations have shown that *P. chrysosporium* can degrade some technical PCB mixtures and single congeners (6, 7, 10, 20, 21, 23). In general, the extent of PCB mineralization appears to decrease as the degree of PCB chlorination increases. Thus. mineralization levels determined by Thomas et al. (20) decreased in the following order (with percent mineralization given in parentheses): [^{14}C]biphenyl (up to 23%) > 2-[^{14}C]chlorobiphenyl (up to 16%) >2,2',4,4'-[^{14}C]tetrachlorobiphenyl (TCB) (up to 10%). There is also evidence indicating that PCB degradation by *P. chrysosporium* is influenced by the congeners' chlorination patterns. Thus, in experiments conducted under similar conditions, 10% of 2,2',4,4'-[^{14}C]TCB was mineralized in 32 days, while <1% of 3,3'4,4'-[^{14}C]TCB was mineralized in 30 days (7, 20). The effects of chlorination pattern on PCB degradation are well documented for bacteria and are believed to reflect restrictions on 2,3- or 3,4-dioxygenase attacks (7, 9, 23). A mechanistic interpretation of the chlorination pattern effect in *P. chrysosporium* is prevented by a lack of information regarding the enzymatic basis of PCB degradation by this organism.

The degradation of technical PCB mixtures by *P. chrysosporium* appears to follow the trend expected on the basis of PCB chlorination levels. Thus, mineralization of [^{14}C] Aroclor 1242 (42% chlorine by weight) was reported to be about 20% (6), while that of [^{14}C]Aroclor 1254 (54% chlorine by weight) ranges from 10 to 14% (6, 10). The mineralization patterns of these mixtures presumably reflected the relative availabilities of the more lightly chlorinated congeners, which were preferentially degraded. Consistent with this assumption are the results of Zeddel et al. (23) that indicated that the degradation of a nonspecified PCB mixture by *P chrysosporium* was limited to mono- and dechlorinated congeners. Other reports, however, offer conflicting evidence. For example, the disappearance of highly chlorinated congeners. including a pentachlorinated bi-

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phenyl (50% decrease) and a hexachlorinated biphenyl (53% decrease), was reported to occur in *P. chrysosporium* -inoculated sand cultures (21). In a liquid culture system spiked with [^{14}C]Aroclor 1254, gas chromatography (GC) analysis showed total depletion of the PCBs following growth of *P. chrysosporium (10)*. The ^{14}C partitioning determined for the latter experiment indicated that, while only 8% of the radioactivity was evolved as $^{14}CO_2$, almost 40% was accounted for as either polar products or hypha-bound ^{14}C. The author suggested that the latter fraction could represent PCBs covalently bound to biomass, presumably via some fungus-mediated reaction(s).

The results of these studies illustrate that to adequately evaluate a microbe's abilities to degrade PCBs (or any other organic compound), it is important to assess the occurrence of transformations that may not immediately result in mineralization. Indeed, the best PCB-degrading bacteria yet described would fare poorly in PCB degradation assays that used mineralization as the sole measure of catabolic competence. Yet. the current assessment of PCB degradation by *P. chrysosporium* is largely based on the occurrence and extent of mineralization. Furthermore, to the best of our knowledge, no PCB metabolites that could provide insights into potential degradation pathways utilized by *P. chrysosporium* have been identified. The present study was undertaken to address some of these issues and thereby gain an improved understanding of PCB degradation by *P. chrysosporium*. The objectives were to (i) assess the potential of *P. chrysosporium* to transform model di-, tetra- and hexachlorinated PCBs, (ii) conduct time course studies elucidating the ^{14}C partitioning dynamics of congeners determined to have significant degradation potentials, and (iii) identify metabolizes produced from model PCB congeners.

MATERIALS AND METHODS

Chemicals. Chemicals and reagents used in these studies and their sources were as follows: 4-chlorobenzyl alcohol (4-CBAlc), Aldrich Chemical Company (Milwaukee, Wis.); BSA [*N,O-bis(trimethylsilyl)acetamide*] derivatizing agent, Pierce Chemical Co. (Rockford, Ill.); 4-chlorobenzoic acid (4-CBA), 4,4′-[U-^{14}C]DCB (13.8 mCi/mmol; >98% purity), 3,3′,4,4′-[U-^{14}C]TCB (37.1 mCi/mmol; >95% purity), and 2,2′,4,4′,5,5′-[U-^{14}C]hexachlorobiphenyl (HCB) (9.4 mCi/mmol; >95% purity), Sigma Chemical (St. Louis, Mo.); and 4,4-DCB, Ultra Scientific (North Kingstown, R.I.).

Fungus. *P. chrysosporium* Burd. et Eslyn (BKM F-1767; ATCC 24725) was grown on yeast-malt-peptone-glucose (YMPG) slants at 39°C for 1 week and then stored at 4°C. The YMPG medium was composed of the following (per liter): glucose (10 g), malt extract (10 g), Bacto Peptone (2 g), yeast extract (2 g), asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (1 g), thiamine (1 g), and Bacto Agar (23 g).

PCB transformation assays. The ability of *P. chrysosporium* to degrade 4,4′-DCB, 3,3′,4,4′-TCB, and 2,2′,4,4′,5,5′-HCB in liquid culture containing 20 ml of culture medium inoculated with 2 ml of filtered conidial suspensions (A_{650} = 0.5; 2.5×10^{-6} spores ml^{-1}) obtained from 1-week-old slants was evaluated. Culture medium contained (per liter) glucose (10 g), thiamine-HCl (1 mg), ammonium tartrate (221 mg), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (500 mg), and $Ca(H_2PO_4)_2$ (1 g) and 10 ml of mineral solution (16) in *trans* aconitic acid buffer (1.74 g liter^{-1}, pH 4.5). Five inoculated and four noninoculated cultures for each congener were prepared in 125-ml Erlenmeyer flasks and incubated without shaking at 39°C. Flushing with oxygen (15 min) began 3 days after inoculation and was performed every two days thereafter for the duration of the study (28 days). On day 5, PCBs were added in the following amounts (all in 200 μl of acetone): 4,4′-[^{14}C]DCB (134,396 dpm; 0.68 μg g^{-1}), 3,3′,4,4′-[^{14}C]TCB (153,907 dpm; 1.07 μg g^{-1}), 2,2′,4,4′,5,5′-[^{14}C]HCB (130,360 dpm; 1 μg g^{-1}). Monitoring of mineralization started 2 days after adding the radiolabeled compounds. Mineralization was quantified by measuring the amount of $^{14}CO_2$ evolved.

Culture flasks were designed with inlet-outlet ports to allow for the flushing of headspaces. Oxygen was flushed through inlets packed with sterile glass wool. Outlets were connected to a bubbler with a glass tube in which 200 mg of LC Florisil was packed. The Florisil was stabilized at each end of the tube with pesticide grade glass wool. The outlet end of the glass tube was connected to a bubbler unit containing 10 ml of scintillation fluid (described below) for trapping of CO_2. The Florisil was used to prevent contamination of the CO_2 trap by volatilized [^{14}C]PCBs. In a preliminary experiment, the effectiveness of Florisil was tested by spiking 20 ml of medium with 4,4′-[^{14}C]DCB. The medium was then flushed for 24 h. Florisil's sorption efficiency was evaluated by fitting two consecutive tubes, packed as described above, to the flask outlet. The mass balance analysis showed no ^{14}C breakthrough from the first adsorption tube and an overall ^{14}C recovery of 90%. The scintillation fluid was a mixture of toluene cocktail (4 g of PPO [2,5-diphenyloxazole] liter^{-1} plus 0.1 g of POPOP {*p*-bis-[2-(5-phenyloxazolyl)]benzene} liter^{-1} in toluene), methanol, and ethanolamine (5:4:1, vol/vol/vol). After flushing, the cocktail was transferred to a scintillation vial and the radioactivity was assayed with a 1214 Rackbeta scintillation counter (LKB Wallac, San Francisco, Calif.). At the end of the study, the radioactivity in the volatile traps was determined by combusting the adsorbent in a biological oxidizer (model OX 600; R. J. Harvey Instrument Co., Hillsdale, N.J.). Interior surfaces of all culture glassware were silanized by rinsing with Aquasil (Pierce).

Time course analysis of 4,4′-DCB degradation by *P. chrysosporium*. A time course study to examine partitioning dynamics of 4,4′-[^{14}C]DCB, the only congener determined from the transformation experiments to have significant degradation potential, was conducted. The amounts of ^{14}C mineralized, volatilized, extractable from culture fluid with ethyl acetate, nonextractable with ethyl acetate, hypha bound, and adsorbed to glassware were quantified. Procedures for inoculation and flushing were identical to those employed in the mineralization studies. In this study, 4,4′-[^{14}C]DCB (95,000 dpm in 100 μl of acetone; 0.48 μg g^{-1}) and 4,4′-DCB (10 mg liter^{-1}) were added to the cultures on day 5; flushing began on day 7. Three inoculated and two noninoculated cultures were harvested by the following procedure on days 5, 12, 19, 26, 34, and 40. The culture fluid was separated from the fungal mats by vacuum filtration. After air drying, biomass-bound ^{14}C was determined by combustion as described above. The culture fluid was filtered into culture tubes (25 by 150 mm) that were fitted with Teflon-lined screw caps. The culture fluid was acidified to a pH of <2 with 4 drops of concentrated H_2SO_4 and extracted twice with 20 ml of ethyl acetate. After being shaken by hand 100 times, the ethyl acetate layer was removed with a Pasteur pipette, transferred to a clean culture tube (25 by 150 mm), dried by passing through a column of Na_2SO_4, and collected in a second culture tube. Immediately prior to and after the loading of the ethyl acetate extracts, the Na_2SO_4 columns were rinsed with 5 ml of ethyl acetate. The Na_2SO_4 was prepared by heating in a muffle furnace for 4 h at 400°C. All glassware was heated in a muffle furnace for 1 h at 450°C prior to use. The extracts were concentrated to <1 ml by rotary vacuum evaporation on a Buchi rotavapor and then were concentrated by gentle nitrogen bubbling to achieve a volume of <200 μl. The final volume of the extracts was adjusted to 200 μl with ethyl acetate. The samples were then stored under nitrogen at −20°C in amber vials sealed with Teflon-lined screw caps.

Isotope trapping of 4-CBA. Cultures were prepared as described above, with the following exceptions. For inoculation of cultures, a YMPG agar plate was inoculated with mycelia and spores from a YMPG agar slant of *P. chrysosporium*. The plate was incubated for 5 days at 39°C, and then one 8-mm-diameter agar plug, taken from the actively growing margin of the fungal colony, was used to inoculate each culture. Five days after inoculation, 4,4′-[^{14}C]DCB and 4-CBA were added to the cultures. Five cultures received only 4,4′-[^{14}C]DCB (93,000 dpm in 100 μl of acetone; 0.44 μg g^{-1}). Ten other cultures received both 4,4′-[^{14}C]DCB (93,000 dpm in 100 μl of acetone) and 100 mg of 4-CBA liter^{-1} in 100 μl of acetone. The cultures were incubated at 39°C without shaking, flushed as described above, and harvested 26 days after inoculation. The extraction procedure used in this study was as described earlier, except that each culture was extracted twice with 20 ml of ethyl acetate prior to acidification and then was extracted twice again with 20 ml of ethyl acetate after acidification. For the five cultures that received only 4,4′-[^{14}C]DCB, the ethyl acetate extracts obtained before acidification were pooled as were the ethyl acetate extracts obtained after acidification. Pooling of ethyl acetate extracts was done identically for the cultures that received 4,4′-[^{14}C]DCB and 4-CBA. The pooled extracts were then concentrated to ≤1 ml by rotary vacuum evaporation and then to <200 μl by gentle bubbling with nitrogen. The final volume of each of the pooled, concentrated extracts was then adjusted to 200 μl with ethyl acetate. Extracts were stored at −20°C under nitrogen in amber vials fitted with Teflon-lined screw caps.

Analytical procedures. Ethyl acetate extracts were analyzed and fractionated by high-performance liquid chromatography (HPLC) with a Hewlett Packard 1090 series II liquid chromatograph system equipped with a diode array detector. Separations were achieved on an Adsorbosphere HS silica 5U normal phase column (4.6 by 250 mm) preceded by an Adsorbosphere direct-connect guard column (Alltech, Deerfield, Ill.). Extracts were injected (twice with 20 μl for each extract) and eluted with the following chloroform-acetonitrile gradient (values given for chloroform): 0 to 15 min, 97%; 15 to 20 min, 97 to 70%; 20 to 55 min, 70 to 0%, at a flow rate of 1.5 ml min^{-1}. Column eluant was monitored for A_{254}. Fractions (0.75 ml) were collected with a fraction collector (LKB Wallac) and then transferred to 20-ml scintillation vials containing 10 ml of Poly-Fluor (Packard Instruments, Meridian, Conn.). Radioactivity was assayed as described above. Extracts were reinjected (twice with 20 μl for each extract) to collect the fractions thus identified as having significant radioactivity. The fractions were air dried, resuspended in ethyl acetate (100 μl), and derivatized with 50 μl of *N,O*-bis(trimethylsilyl)acetamide at 60°C for 1 h to produce trimethylsilyl (TMS) derivatives.

The derivatized fractions were analyzed by GC-mass spectrometry (MS) with a Hewlett Packard 5890 series II gas chromatograph equipped with a 7673A autosampler, a split-splitless capillary column injection port, and a model 5972

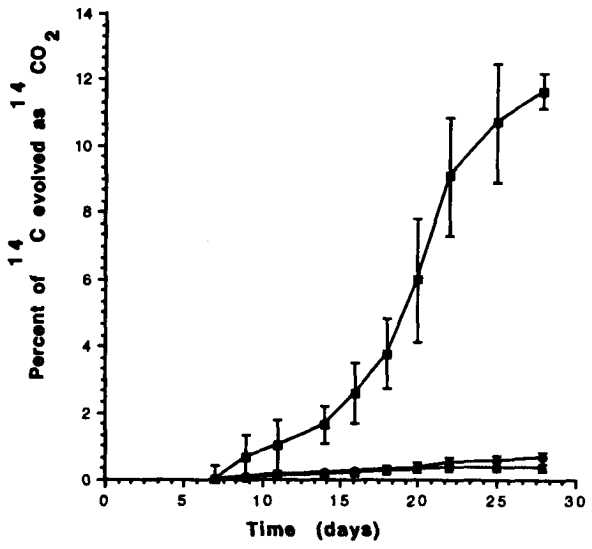

FIG. 1. Mineralization of 4,4'-[^{14}C]DCB (■), 3,3',4,4'-[^{14}C]TCB (●), and 2,2',4,4',5,5'-[^{14}C]HCB (▲) by *P. chrysosporium* in liquid culture. No ^{14}C was recovered in the CO_2 traps from the noninoculated cultures (data not shown).

mass spectrometer detector. Operating parameters for the GC were as follows: injector, 250°C; detector, 300°C; He carrier gas, 0.967 ml min^{-1}; and N_2 makeup gas, 36.1 cm s^{-1}. Separation was achieved with a J & W Scientific (Folsom, Calif.) DB-5 fused silica capillary column (30 m by 0.32 mm; film thickness, 0.25 μm). The temperature program was as follows: 80°C for 2 min; ramp A (80°C to 150°C), 20°C/min; ramp B (150°C to 280°C), 4°C/min; and hold at 280°C, 5 min. The MS interface and ion source temperatures were 300°C. The mass spectrometer was operated in the electron ionization (70 eV) mode, scanning masses of 35 to 550 mass units at 1.6 s decade^{-1}.

TLC and autoradiography. Ethyl acetate extracts from the isotope trapping study were analyzed by thin-layer chromatography (TLC) on Kieselgel 60 gel plates (Alltech) with hexane-ethanol-acetic acid (10:2:1, vol/vol/vol) as the developing solvent mixture. Extracts (5 μl) were loaded onto a plate, developed for 50 min, and then air dried. The plate and a sheet of X-ray film (Eastman Kodak Company, Rochester, N.Y.) were loaded into a cassette and stored at −30°C for 18 days. Radioactive spots on the TLC plates were located by autoradiogram, scraped, and extracted with 400 μl of ethyl acetate. These extracts were concentrated to 100 μl by gentle bubbling with nitrogen. The extracts were then derivatized with 50 μl of *N,O*-bis(trimethylsilyl)acetamide at 60°C for 1 h, and analyzed by GC-MS as described above.

RESULTS

P. chrysosporium mineralized 11.6% of the added 4,4'-[^{14}C]DCB but only negligible amounts of 3,3',4,4'-[^{14}C]TCB and 2,2',4,4',5,5'-[^{14}C]HCB after 28 days of incubation (Fig. 1). No $^{14}CO_2$ was evolved from the noninoculated flasks, indicating that mineralization in the inoculated flasks was caused by the fungus. The rate of mineralization increased up to day 20 and leveled off at day 25 for the DCB, whereas with the other conveners there was no change throughout the 28 days. The amount of ^{14}C volatilized was greatest in flasks containing DCB(ca. 15% in inoculated cultures and 45% in control cultures). Less than l% of the ^{14}C from flasks containing the TCB or HCB volatilized (Table 1). There was a major difference between congeners in amounts of hypha-bound radioactivity: 11% of the ^{14}C in the DCB cultures was biomass bound, whereas >60% of the TCB and HCB congeners was sorbed to the mat. In the DCB cultures, about 9% of the ^{14}C was not extracted with ethyl acetate; with the TCB or HCB, radioactivity in this fraction was negligible.

In the time course study, the mass balance analyses of ^{14}C from 4,4'-[^{14}C]DCB indicated that during the first 12 days, most (up to 60%) of the ^{14}C was adsorbed by the hyphae and the glassware (Table 2). As the experiment progressed, the amounts of ^{14}C converted to $^{14}CO_2$ and volatilized increased. On day 40, mineralization of the DCB appeared to decrease. However, it was noted that from day 14 forward mineralization levels in the three *P. chrysosporium* cultures harvested on the last day lagged behind those for the rest of the cultures (data not shown). The amount of aqueous ^{14}C (i.e., extractable and nonextractable) increased until day 26, after which time the amount of ^{14}C recovered in these fractions remained relatively stable. After an initial increase to 54% during the first week, the amount of hypha-bound ^{14}C decreased to 17% at day 26. Subsequently the amount of radioactivity sorbed to the hyphae did not change appreciably. The amount of ^{14}C associated with the glassware decreased after 1 week (Table 2). However, there was always a significant fraction (i.e., 6 to 11%) of radioactivity adsorbed to the glassware. Total ^{14}C recoveries for the time course study averaged 72% ± 6.47%.

Radioactivity profiles of HPLC fractions from the time course study showed degradation of 4,4'-DCB and formation of metabolizes during the 40-day incubation. The most prominent radioactive peak occurring in all samples was identified as 4,4'-DCB by GC-MS analyses of the relevant fractions. Metabolize accumulation was apparent by day 12, as evidenced by the increasing amounts of radioactivity in 30- to 35-ml fractions. By day 26, a significant radioactive peak was observed and subsequently identified as 4-CBA by GC-MS analysis of its TMS derivative (Fig. 2). The TMS derivative yielded a weak molecular ion at m/z 228 and an abundant ion at *m/z* 213 (·CH_3 loss); this pattern is typical for mass spectra of

TABLE 1. Recoveries[a] of 4,4'-[^{14}C]DCB, 3,3',4,4'-[^{14}C]TCB, and 2,2',4,4',5,5'-[^{14}C]HCB

PCB[b]	Inoculation[c]	^{14}C recovery (mean ± SD [%]) in fraction						
		Mineralized[d]	Volatilized	Extractable[e]	Nonextractable	Wash	Hypha	Total[f]
DCB	+	11.65 ± 2.3	16.90 ± 1.7	20.20 ± 1.07	8.90 ± 1.33	3.37 ± 0.84	11.25 ± 2.39	74.7 ± 3.65
	−	—[g]	46.90 ± 7.0	1.76 ± 0.64	1.63 ± 0.85	4.55 ± 0.21	—	57.3 ± 7.90
TCB	+	—	—	6.22 ± 0.65	—	15.26 ± 1.74	61.10 ± 5.25	84.2 ± 5.38
	−	—	1.62 ± 0.23	3.15 ± 0.89	—	54.25 ± 1.85	—	62.5 ± 6.56
HCB	+	—	—	3.30 ± 0.41	—	20.10 ± 1.49	62.10 ± 8.93	85.0 ± 3.71
	−	—	—	1.29 ± 0.41	—	65.80 ± 5.24	—	63.4 ± 9.03

[a] Percent recoveries represent the means ± standard deviations of five replicates for inoculated cultures and four replicates for noninoculated cultures.
[b] DCB, 4,4'-[^{14}C]DCB; TCB, 3,3',4,4'-[^{14}C]TCB; HCB, 2,2',4,4',5,5'-[^{14}C]HCB.
[c] Inoculation of liquid medium with *P. chrysosporium*.
[d] Evolved and collected as $^{14}CO_2$.
[e] Extracted with ethyl acetate.
[f] Initial amounts of ^{14}C added were 134,396, 153,907, and 130,360 dpm for DCB, TCB, and HCB, respectively.
[g] —, <1% of total ^{14}C added.

TABLE 2. Recoveries[a] of 4,4'-[^{14}C]DCB from liquid medium inoculated with *P. chrysosporium*

Day	^{14}C recovery (mean ± SD [%]) in fraction						
	Mineralized[b]	Volatilized	Extractable[c]	Nonextractable	Wash	Hypha	Total[d]
5	—[e]	—	9.94 ± 1.68	—	20.49 ± 0.45	39.41 ± 2.39	70.77 ± 1.75
12	2.16 ± 0.96	1.55 ± 1.04	10.24 ± 1.04	1.58 ± 0.72	7.33 ± 3.35	54.27 ± 9.27	84.95 ± 3.32
19	5.31 ± 3.07	3.97 ± 1.06	22.08 ± 3.70	4.71 ± 1.26	6.28 ± 1.44	26.86 ± 7.88	69.20 ± 4.07
26	11.33 ± 1.77	2.80 ± 1.39	26.55 ± 2.91	7.26 ± 2.05	9.50 ± 1.46	17.32 ± 1.69	74.76 ± 3.05
34	13.62 ± 0.74	3.35 ± 0.72	22.59 ± 2.94	6.51 ± 1.00	11.39 ± 1.60	14.07 ± 1.11	71.53 ± 2.74
40	7.52 ± 1.41	5.37 ± 1.90	22.84 ± 3.58	5.94 ± 1.50	8.16 ± 0.69	16.65 ± 1.20	66.49 ± 5.56

[a] Percent recoveries represent the means ± standard deviations of three replicates.
[b] Evolved and collected as $^{14}CO_2$.
[c] Extracted with ethyl acetate.
[d] Initial amount of ^{14}C added was 95,000 dpm.
[e] —, <1% of total ^{14}C added.

TMS ethers and esters *(5)*. Also characteristic of a TMS-aromatic acid mass spectrum was the loss of CO_2 following skeletal rearrangement of the silicon atom to the aromatic ring, in this case yielding *m/z 169*. Other diagnostic ions were *m/z* 139 (loss of the silicon-centered analog of acetone from *m/z* 213) and *m/z* 111 (carbon monoxide loss from *m/z* 139). A TMS derivative of an authentic 4-CBA standard gave an identical fragmentation pattern. The radioactive 4-CBA peak was no longer present in samples analyzed on days 34 and 40; at these time points other compounds had formed that eluted much later (i.e., 30- to 40-ml fractions) than did 4-CBA, indicating the conversion of 4,4'-DCB to more polar products.

The presence of hydroxylated metabolizes of 4,4'-DCB in derivatized HPLC fractions was assessed by selected ion monitoring. Hydroxylated 4,4'-DCB transformation products scanned for, but not detected, were dichlorodihydroxy-, dichlorohydroxy-, chlorohydroxy-, and chlorodihydroxy-biphenyls. The ring cleavage product expected to be produced from 4,4'-DCB by the *meta* pathway, 2-hydroxy-3-chloro-6-oxo-(4-chlorophenyl)hexa-2,4-dienoic acid. was also scanned for but not found.

The identification of 4-CBA as a transformation product of 4,4'-DCB was confirmed by the isotope trapping experiment in which 4-CBA was detected in HPLC-fractionated culture extracts by GC-MS analysis and by TLC-autoradiographic analysis of the ethyl acetate extracts. The GC-MS analyses of HPLC-fractionated extracts confirmed the presence of 4-[^{14}C]CBA in both the nonacidified and acidified ethyl acetate extracts from cultures that received only 4,4'-[^{14}C]DCB and in the nonacidified extracts from cultures that received both 4,4'-[^{14}C]DCB and nonlabeled 4-CBA (Fig. 3). In addition, 4-CBAlc was identified by GC-MS in fractions from the latter cultures (Fig. 3). The mass spectrum of the TMS derivative of this compound showed a weak molecular ion at *m/z* 214 and an abundant fragment ion at *m/z* 199 (Fig. 4). As described above, this pattern is consistent with methyl radical loss from the center of TMS derivatives. A skeletal rearrangement similar to that described above for TMS-aromatic acids is also expected for the benzyl-TMS ether; this results in the loss of formaldehyde from the M-CH_3 precursor and gives the ion *m/z* 169. Other diagnostic ions in the mass spectrum were *m/z* 179 (Cl loss from *m/z* 214) and *m/z* 125 (loss of the silicon-centered analog of acetone from *m/z* 199). The mass spectrum of a TMS-derivatized authentic standard of 4-CBAlc was identical to that shown in Fig. 4.

Further confirmation that 4-CBA and 4-CBAlc were 4,4'-DCB metabolizes was provided by the identification of 4-[^{14}C]CBA and 4-[^{14}C]CBAlc in culture extracts. Extracts analyzed by TLC and autoradiography showed that two analytes comigrated with 4-CBA and 4-CBAlc standards. Mass spectra of these compounds were identical to those of TMS-derivatized standards of 4-CBA and 4-CBAlc.

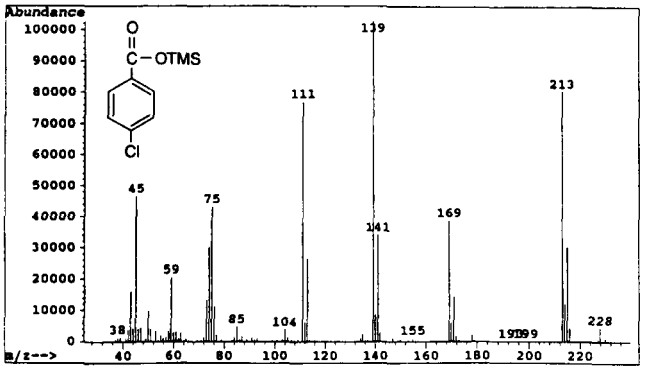

FIG. 2. Mass spectrum of the TMS derivative of 4-CBA identified from the day-26 sample of the time course study in which liquid cultures inoculated with *P. chrysosporium* were spiked with 4,4'-[^{14}C]DCB and 4,4'-DCB.

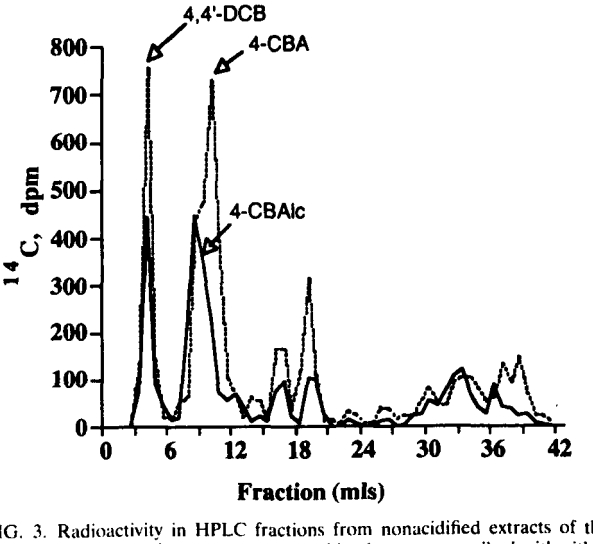

FIG. 3. Radioactivity in HPLC fractions from nonacidified extracts of the isotope trapping study. *P. chrysosporium* liquid cultures were spiked with either 4,4'-[^{14}C]DCB (- - -) or both 4,4'-[^{14}C]DCB and 4-CBA (——) and harvested on day 26.

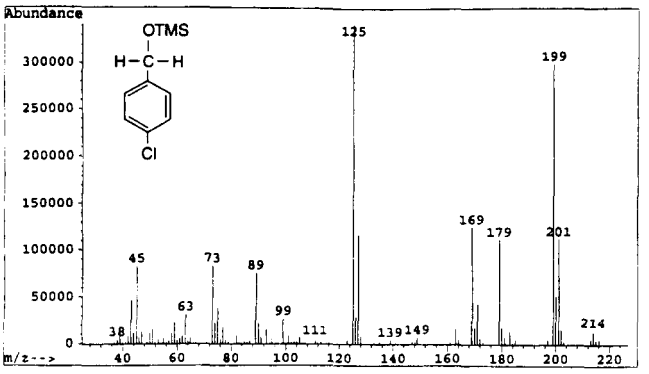

FIG. 4. Mass spectrum of the TMS derivative of 4-CBAlc identified from the isotope trapping study in which both 4,4'-[^{14}C]DCB and 4-CBA were spiked into liquid cultures inoculated with *P. chrysosporium.*

DISCUSSION

Results from the present study were consistent with those of others (7, 20) and indicated that the potential for *P. chrysosporium* to mineralize PCBs decreases as the degree of congener chlorination increases. Moreover, while the ^{14}C partitioning analysis indicated fairly extensive degradation of 4,4'-DCB, there was little evidence for significant metabolism of either 3,3',4,4'-TCB or 2,2',4,4',5,5'-HCB. For the latter two congeners, the greatest difference between the inoculated and noninoculated treatments was the large amount of biomass-bound ^{14}C. As others have suggested (10, 20), this radioactivity could represent PCBs transformed by the fungus into compounds that are covalently bound to cellular macromolecules. While this possibility cannot be ruled out, it seems more likely that, in the present study, biomass uptake of the radioactivity resulted largely from the phase partitioning rather than fungal metabolism of the PCBs. Indirect support for this hypothesis was the finding that in the time course study, ca. 40% of the ^{14}C was sorbed on the same day it was added, and <1 h passed between label addition and biomass harvest. The increase to 54% during the subsequent 7 days could also reflect further partitioning and/or metabolic activity resulting in PCB uptake.

The negligible amount (<1%) of 3,3',4,4'-TCB mineralization determined in this study was consistent with that determined by Bumpus et al. (7). Comparing the lack of 3,3',4,4'-TCB mineralization with the 10% mineralization of 2,2',4,4' -TCB reported by Thomas et al. (20) further substantiated the possibility that the extent of PCB mineralization by *P. chrysosporium* is affected by the congeners' chlorination patterns. The apparent differential susceptibilities of 2,2',4,4'- and 3,3',4,4'-TCB to degradation by *P. chrysosporium* were similar to that of the PCB-degrading bacteria examined by Bedard et al. (4). These investigators hypothesized that the presence of chlorines in the *ortho* positions may have facilitated dioxygenase attack, thus allowing for greater bacterial degradation. Whether or not a similar mechanism could account for differential mineralization of PCBs by *P. chysosporium* remains to be determined.

The importance of congener chlorination patterns in affecting PCB degradation by aerobic bacteria is well established (3, 13). Theories on the effects of chlorination patterns on fungal degradation of PCBs, however, are not as well developed as those for bacteria: the main trend suggested by the literature is a greater resistance of doubly *para* chlorinated congeners to fungal degradation. For example, analysis of Clophen A 30 incubated with *A. niger* indicated that congeners with chlorines in the 4,4' positions showed little, if any, degradation; this phenomenon was referred to as "*para* recalcitrance" (9). Zeddel et al. (23) examined the degradation of a nonspecified technical PCB mixture by three white rot fungi, including *P. chrysosporium;* in all cases 4,4'-DCB was the most recalcitrant DCB congener. However, in the present study, mineralization of 4,4'-DCB was roughly equivalent to that reported by Thomas et al. (20) for 2-chlorobiphenyl, an apparent contradiction of the *para* recalcitrance theory. With aerobic bacteria, the extent to which 4,4'-DCB and most other PCB congeners are degraded can be strain dependent (4, 17). Similar cultural variability could occur with fungi and may partly explain the relatively high level of 4,4'-DCB degradation observed here.

Mass balances from the congener transformation studies showed that a significant fraction (i.e., up to 61%) of the [^{14}C]PCBs added to the cultures was hypha bound. Results from other investigators have also indicated that substantial amounts of PCBs can be sorbed into fungal mycelia. Thomas et al. (20) reported that about 40% of the radioactivity added as 2,2',4,4'-[^{14}C]TCB was hypha associated, whereas Eaton (10) determined that 23% of the [^{14}C]Arockw 1254 incubated with *P. chrysosporium* was mycelium bound. In the latter report, an even greater amount of PCB binding might have been expected given that Aroclor 1254 is composed of tri- through heptachlorinated biphenyls. Collectively, results of the present study and those of other investigators demonstrate that absorption of PCBs by fungal biomass, if not accounted for. may cause PCB biodegradation to be overestimated.

The dynamics of ^{14}C partitioning between the liquid. solid, and gas phases were elucidated by mass balance analysis of the time course study. During the first 12 days, most (40 to 50%) of the radioactivity added as 4,4' -[^{14}C]DCB was associated with the fungal mats. Subsequently, there was a steady decrease in this fraction with concomitant increases in the amounts of ^{14}C recovered in ethyl acetate extracts and as $^{14}C0_2$. Although the nature of the mat-bound ^{14}C is unknown. this radioactivity could be [^{14}C]PCBs or transformation products nonspecifically sorbed to mycelia and/or ^{14}C that has been assimilated into cellular macromolecules. Thomas et al. (20) speculated that the latter possibility probably accounted for the biomass-associated ^{14}C (40% of the radioactivity added as 2,2',4,4'-[^{14}C]TCB) measured at the end of their experiment. In the present study. the progressive decrease in mat-bound ^{14}C could have reflected catabolism of cellular macromolecules containing ^{14}C assimilated from 4,4'-[^{14}C]DCB. Yet. it seems more likely that the progressive decrease in the hypha-bound radioactivity reflected an initial high level of nonspecific sorption of 4,4'-[^{14}C]DCB (or transformation products) followed by degradation to various metabolic intermediates (i.e., as represented by increasing ^{14}C in ethyl acetate-extractable and nonextractable fractions) and $^{14}C0_2$.

Recoveries of ^{14}C in the present study were generally greater than those reported in previous investigations on PCB degradation by *P. chrysosporium.* Eaton (10) reported a 66% recovery of radioactivity from [^{14}C] Aroclor 1254 after a 19-day incubation in liquid culture. In the liquid culture experiments conducted by Thomas et al. (20), ^{14}C recoveries ranged from 35% for 2-[^{14}C]chlorobiphenyl to 53% for 2,2',4,4'-[^{14}C]TCB. By comparison, ^{14}C recoveries in inoculated cultures from the mineralization studies presented here were 57 to 85% and averaged 72% in the time course study with 4,4'-[^{14}C]DCB. Improved ^{14}C balances were obtained by accounting for volatilized radioactivity-and ^{14}C adsorbed to the culture flasks and associated glassware. In this study, glassware adsorption was a significant sink for PCBs; neither Eaton (10) nor Thomas et al. (20), however, reported glassware adsorption. PCBs may also be lost by volatilization during extended incubation, and the

potential for volatile losses increases as the chlorination level decreases (11). In the present study. significant volatile losses of 4,4'-[^{14}C]DCB occurred in both the mineralization and time course experiments but were much greater in the former. While the reason(s) for this variability was not fully determined, isotope dilution effects might have at least partially accounted for the decreased level of volatilization measured in the time course experiment. The results from the present study show that volatile losses can be significant, particularly for the more lightly chlorinated congeners such as 4,4'-DCB. In contrast, Thomas et al. (20) reported negligible volatilization of biphenyl and 2-chlorobiphenyl from noninoculated cultures during a 32-day incubation; more extensive volatilization might have been expected. Eaton (10) did not quantify volatile losses of [^{14}C] Aroclor 1254.

In the present study, 4-CBA and 4-CBAlc were identified as metabolizes produced from 4,4' -DCB by *P. chrysosporium.* To the best of our knowledge, this is the first report providing information on degradation products generated from PCBs by a white rot fungus. We also believe that this is the first report implicating a chlorinated benzylic alcohol as a metabolize produced during PCB degradation. Collectively, results of prior investigators' works and those from the present study suggest that chlorobenzoates may be characteristic intermediates in fungal PCB degradation pathways.

Although chlorobenzoates may be metabolites common to both bacterial and fungal PCB degradation pathways. the mechanisms leading to the formation of these compounds may not necessarily be the same. In bacteria, PCBs are metabolized to chlorobenzoates by the *meta* cleavage pathway (2, 13). Dioxygenases are key enzymes of the *meta* pathway, effecting both the initial 2,3-dihydroxylation and subsequent extradiol (i.e.. *meta)* ring fission. Fungi also appear to transform PCBs to hydroxychlorobiphenyls (9, 22); the enzymes effecting ring hydroxylation, however. have not been identified. The mechanism(s) by which fungi effect PCB ring fission is also unknown. Fungal dioxygenases that mediate intradiol (i.e., *ortho)* cleavage of aromatic compounds like 1,2,4-trihydroxybenzene have been described previously (8, 19). Information concerning the potential of such enzymes to mediate ring cleavage of hydroxychlorobiphenyls is, however, lacking. Phenol oxidases produced by white rot fungi have also been reported to effect ring fission of phenolic substrates (15). Further study is needed to determine which, if any, of these or other mechanisms are utilized by PCB-degrading fungi.

Another issue raised by this study concerns the relation of 4-CBAlc to 4-CBA in the fungal PCB degradation pathway. At least three possibilities can be proposed. First, the PCB ring fission product may first be transformed to 4-CBA, which is then reduced to 4-CBAlc. Second, 4-CBAlc may be formed first and subsequently oxidized to 4-CBA; lignin-degrading fungi are known to produce extracellular aryl alcohol oxidases that mediate such transformations (14). Third, 4-CBA and 4-CBAlc maybe produced independently by parallel metabolic pathways. Additional information is needed to delineate the most likely reaction series.

The present study provides further insights into the degradative activities of *P. chrysosporium* with model PCB congeners. Results from these experiments suggest a number of similarities in PCB biodegradation between *P. chysosporium* and the well-described bacterial systems. Further work is needed, however, to elucidate the mechanisms by which *P. chrysosporium* effects PCB degradation and to develop a framework within which the susceptibilities of various PCBs to degradation by this organism can be rationalized. Mechanistic information would also further bioremediation applications by allowing optimal conditions for PCB degradation by *P. chrysosporium* to be determined.

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