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Role of a Laccase in the Degradation of Pentachlorophenol

A. Ricotta,[1] R. F. Unz,[2] J.-M. Bollag[1]

[1]Laboratory of Soil Biochemistry and [2]Department of Civil and Environmental Engineering, Center for Bioremediation and Detoxification, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

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White rot fungi, which are capable of degrading lignin present in wood, are able to degrade many structurally diverse organic pollutants. The organism often used as a model is *Phanerochaete chrysosporium,* although the ability of *Trametes* (*Coriolus*) *versicolor* to degrade xenobiotic chemicals has also been investigated. The ability to mineralize organic pollutants *in vitro* is generally enhanced under culture conditions favorable to the mineralization of the lignin model compounds which suggests that the lignin-degrading system may be involved in the transformation of anthropogenic compounds (Bumpus and Aust 1986).

Fungal extracellular enzymes, including lignin peroxidases (LiPs) and laccases, are implicated in the degradation of lignin. Researchers have proposed that the Lips of *P. chrysosporium* are key enzymes in the fungal degradation of xenobiotic compounds (Kirk and Shimada 1985). Sanglard et al. (1986) demonstrated that LiPs catalyzed the initial oxidation of benzo(a)pyrene [B(a)P] by this fungus. Hammel et al. (1992) suggested that anthraquinone, produced in an LiP-catalyzed reaction, was an intermediate in the mineralization of anthracene by *P. chrysosporium.*

Both laccases and lignin peroxidases act via a nonspecific free radical mechanism, are secreted extracellularly, and are implicated in lignin degradation. Although LiPs have been considered as catalysts in the mineralization of xenobiotic compounds by white rot fungi, the laccases have not. The oxidation of many *o*-and *p*-diphenols and of some *m*-diphenols and monophenols is catalyzed by laccases (Bendall and Gregory 1963). An isolated laccase of *T. versicolor* oxidized PCP, producing the benzoquinones, *p*-chloranil and *o*-chloranil (Konishi and lnoue 1972).

Although the purified laccases and lignin peroxidases oxidize a variety of chemicals, it remains uncertain whether they are integral catalysts in the mineralization pathways of xenobiotics by white rot fungi. An attempt was made in the present investigation to clarify the role of laccase in the

Correspondence to: J.-M. Bollag

transformation of PCP during growth of a fungus, *T. versicolor.* The temporal relationship between the mineralization of ^{14}C-PCP and the formation of extracellular laccase by the fungus was determined. The ability of an isolated extracellular laccase to increase the rate of PCP mineralization by *T. versicolor* was assessed. Finally, products resulting from the transformation of PCP by the fungal cultures and by the isolated laccase were characterized and compared.

MATERIALS AND METHODS

All chemicals used were reagent grade. Pentachlorophenol (PCP), *p*-chloranil (2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione), *o*-chloranil (3,4,5,6-tetrachloro-3,5-cyclohexadiene-1,2-dione), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate; ABTS) and ring-labeled ^{14}C-PCP with a specific activity of 5.8 mCi/mmol (purity >98%) were purchased from Sigma Chemical Co., St. Louis, MO. 3,4-Dimethoxybenzyl alcohol (veratryl alcohol) was obtained from Aldrich Chemical Co., Milwaukee, WI.

T. versicolor was extant in the collection of the Laboratory of Soil Biochemistry and maintained on potato dextrose agar slants at 4°C. Unless otherwise stated, the culture medium employed in experiments contained (g/l): KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.45; Na_2HPO_4, 0.17; $NaNO_3$ 0.15; sucrose, 10; *l*-asparagine, 0.125 and the following micronutrients (mg/l): $MnCl_2 \cdot 4H_2O$, 10; $FeCl_3 \cdot 6H_2O$, 5; $ZnCl_2$, 2; $CaCl_2 \cdot 2H_2O$, 73.5; $CuSO_4 \cdot 5H_2O$, 4; $NaB_4O_7.10H_2O$, 2; $NaMoO_4 \cdot 2H_2O$, 4; thiamine HCl, 0.05 (Leonowicz et al. 1984). Final pH was 5.5. Experiments were initiated by transferring disks (5 mm) of *T. versicolor,* obtained from 6-8 day-old agar plates with the aid of a sterile number 2 cork borer, to the surface of 20 ml of culture medium contained in 125-ml Erlenmeyer flasks. Incubation was carried out statically at 28°C in the dark. All experiments were performed in triplicate.

Evidence of PCP transformation by $^{14}CO_2$ evolution was sought in fun al cultures to which PCP (final concentration, 3.5 µM which included ^{14}C-PCP [1.5 x 10^4 Bq] in ethanol) was added to 16 to 24-hour old *T. versicolor* cultures (referred to as zero time with respect to PCP addition). The culture medium was directly analyzed over the course of the incubation for radiolabeled carbon at the times indicated in Table 1. In some experiments, laccase (10% v/v) and PCP were simultaneously added to 24-hour old *T. versicolor* cultures. Autoclaved fungal cultures, supplemented with active laccase and laccase inactivated by autoclaving, served as controls. The laccase was isolated from *T. versicolor* according to the procedure of Leonowicz et al. (1984). Laccase activity was determined by the oxidation of ABTS (Bourbonnais and Paice 1992). The pH of the reaction mixture was modified to pH 3.5. Lignin peroxidase

activity was measured by the oxidation of 3,4-dimethoxybenzyl alcohol (Tien and Kirk 1984). Evolved CO_2 was trapped in 0.5 N NaOH contained in vials suspended within sealed flasks and measured by liquid scintillation counting (Beta Trac 6895 liquid scintillation counter; Tracer Analytic, Elk Grove Village, IL). $^{14}CO_2$ was verified using a barium chloride precipitation step (Smith and Aubin 1991). Samples (2 ml) of culture medium were removed periodically, acidified to pH 2 with HCl and the PCP was extracted with hexane. At the conclusion of each experiment, the mycelia were filtered from the culture medium, washed three times in distilled H_2O, homogenized in a tissue grinder, and an aliquot measured by LSC. Spectra of PCP and degradation products were measured in the range of 260 to 500 nm with a Spectronic 2000 spectrophotometer (Baush and Lomb, Inc., Rochester, NY).

In order to recover and characterize the products of PCP transformation by *T. versicolor,* PCP (final concentration, 50 µM) in 75 ml ethanol was added to approximately 7-day old fungus cultures exhibiting nearly confluent mycelial mats in Roux flasks containing 150 ml of medium. A similar volume of ethanol without PCP was added to control cultures. After 1,3,5,7,10, and 14 days of incubation, mycelia were collected by filtration using glass wool. The filtrate was acidified to pH 2 with concentrated HCl and three-30 ml portions of ethyl acetate were used to extract metabolites. The extracts were pooled, dried over Na_2SO_4 and filtered. Ethyl acetate was removed in a vacuum evaporator and the residue was redissolved in ethyl acetate (5 ml) for chromatographic analysis.

Products of sole laccase activity on PCP were sought by analyzing samples (2 ml) taken at 0, 1, 2, 4, 8, and 24 h from a reaction mixture consisting of 100 mM PCP in citrate-phosphate buffer (pH 5.5) and laccase (5 U/ml). Samples were analyzed by UV spectroscopy and extracted for the recovery of PCP and PCP oxidation products as earlier described. Both hexane and ethyl acetate extracts were analyzed by gas (GC). GC was performed on a 5890A chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a capillary column (RTx-5, 30 m x 0.32 mm ID; Restek, Bellefonte, PA). The injector and flame ionization detector were set at 250°C. The column was programmed to increase from 150°C to 230° C at 5°C/min. TLC was carried out on reversed-phase, octadecyl silica (C18) plates (200 µm layer thickness) with a fluorescent indicator (J. T. Baker, Inc., Phillipsburg, NJ). Following GC analysis and UV spectroscopy, ethyl acetate extracts were concentrated to about 1 ml for TLC analysis. The plates were developed in two different solvent systems: solvent A consisted of methanol:water:acetic acid (30:20:1, v/v) in 18 mM ammonium acetate and solvent B consisted of acetonitrile:methanol:2-propanol (1:1:3, v/v). Plates were examined under a short wavelength UV lamp.

RESULTS AND DISCUSSION

PCP was rapidly degraded by *T. versicolor.* Significant mineralization began when approximately 80% of the PCP had been transformed. $^{14}CO_2$ was released continuously throughout the incubation period and approximately 21% of the PCP was mineralized after 17 days (Figure 1). During the first two weeks of incubation, the level of extracellular laccase formed by *T. versicolor* was generally less than 0.1 U/ml, however, levels of the enzyme subsequently rose to 0.2 to 0.8 U/ml during the following week (Figure 1). Lignin peroxidase activity was not detected at any time. The isolated extracellular laccase (2.4 U/ml) when added to *T. versicolor* cultures increased the rate of PCP transformation, as measured following hexane extraction of residual PCP from the culture medium (Table 1). The evolution of $^{14}CO_2$ from laccase-supplemented cultures began immediately and 5.1% of the initially applied radioactivity was collected as $^{14}CO_2$ by day 4, compared to 1.7% $^{14}CO_2$ detected in the control culture. By day 17, however, the extent of mineralization in laccase-unsupplemented cultures (21.1%) surpassed that of laccase-supplemented cultures (15.8%; Table 1).

During incubation of PCP with laccase alone, disappearance of PCP was accompanied by the formation of *p*-chloranil and *o*-chloranil as determined by UV spectra of the reaction mixture and the comparative retention times for samples and authentic standards in GC analysis. These results confirmed the observations of Konishi and Inoue (1972). Several unidentified metabolites were produced in culture fluids of the fungus as demonstrated by TLC and GC analyses and two products detected by TLC after one day of incubation had R_f values equal to those of *p*-chloranil and *o*-chloranil. However, after 3, 5, and 7 days of incubation only the metabolite corresponding to *p*-chloranil was detected. After 10 to 14 days of incubation, neither of the benzoquinones was observed. Results were verified by developing the TLC plates with the alternate solvent system. Mineralization of PCP occurred with 9.2% of the initial radioactivity present as $^{14}CO_2$ after 9 days of incubation.

Extracellular laccase (0.03 U/ml) was present in 6-day-old cultures which received PCP and it is probable that the laccase fortuitously catalyzes the oxidation of PCP. However, although we succeeded in detecting *p*-chloranil and *o*-chloranil in the medium, putative formation of the two benzoquinones does not appear to be essential in the metabolic pathway leading to mineralization of PCP. First, the marked increase in the rate of $^{14}CO_2$ release at day 4 was not accompanied by a large increase in extracellular laccase activity as would be expected if a cause and effect relationship existed between the enzyme and $^{14}CO_2$ evolution (Figure 1). In fact, the largest increase in laccase activity occurred as the rate of $^{14}CO_2$ release began to decrease at day 11 (Figure 1). Second, the

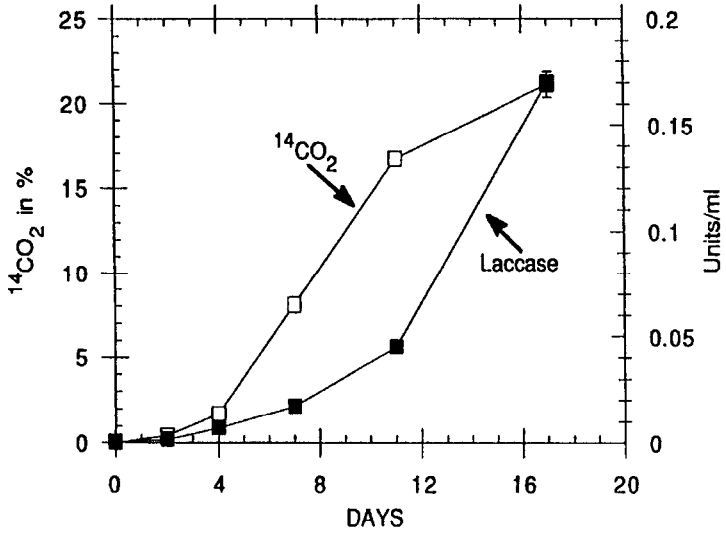

Figure 1. Mineralization of ^{14}C-pentachlorophenol and the formation of extracellular laccase by *Trametes versicolor.* Zero days on the time scale represents the point of addition of PCP to a mature fungal culture. Triplicate values were within 10%.

addition of the laccase to the fungi decreased the extent of mineralization of PCP (Table 1). This is in contrast to an increase in the initial rate of $^{14}CO_2$ release upon laccase addition.

The oxidation of PCP to *p*-chloranil and *o*-chloranil catalyzed by the added laccase may have detoxified PCP. Konishi and Inoue (1972) reported that the antifungal activity of PCP was at least five times more than that of the *p*-chloranil and *o*-chloranil that resulted from its oxidation. Lyr (1963) also reported that laccase secreted by basidiomycetes detoxified PCP in the growth medium. Therefore, if *T. versicolor* is able to mineralize *o*-chloranil and *p*-chloranil, the initial rate of $^{14}CO_2$ release should be greater from cultures receiving laccase than cultures receiving denatured laccase. On the other hand, the presence of excess laccase in the cultures could catalyze the coupling of PCP with its oxidation products (Konishi and Inoue 1972). These dimers may possibly be more resistant to ring cleavage resulting in less mineralization.

The conclusion that laccase does not play an integral role in the mineralization of PCP by *T. versicolor* is supported by the lack of temporal correlation between extracellular laccase formation and the release of $^{14}CO_2$ during PCP metabolism. That the addition of the laccase to

Table 1. Distribution of radioactivity after incubation of ^{14}C-PCP (3.5 *mM*) with *T. versicolor* and *T. versicolor* with simultaneous amendment of an extracellular laccase (2.4 U/ml).

			% OF INITIALLY APPLIED RADIOACTIVITY			
	DAY	$^{14}CO_2$	GROWTH MEDIUM	EXTRACTION OF GROWTH MEDIUM WITH HEXANE	BIOMASS	WASHES*
	0	0	100	98.9 ± .21	ND	ND
	2	0.4 ± .06	82.5 ± .74	60.1 ± 2.4	ND	ND
T. versicolor	4	1.7 ± .14	70.0 ± 1.7	13.6 ± .35	ND	ND
	7	8.1 ± .69	64.8 ± .71	0.80 ± .02	ND	ND
	11	16.7 ± .43	49.4 ± 1.1	0.49 ± .12	ND	ND
	17	21.1 ± .83	46.8 ± 1.7	0.42 ± .23	4.8 ± .56	15.6 ± .53

			% OF INITIALLY APPLIED RADIOACTIVITY			
	DAY	$^{14}CO_2$	GROWTH MEDIUM	EXTRACTION OF GROWTH MEDIUM WITH HEXANE	BIOMASS	WASHES
	0	0	100	98.6 ± .30	ND**	ND
T. versicolor	2	3.3 ± .10	60.4 ± 1.2	21.1 ± 1.6	ND	ND
+	4	5.1 ± .10	52.5 ± 1.1	8.5 ± 2.1	ND	ND
laccase	7	8.8 ± .36	47.7 ± .84	3.9 ± .62	ND	ND
	11	13.5 ± .31	45.8 ± .25	1.7 ± .20	ND	ND
	17	15.8 ± .07	44.2 ± .51	0.6 ± .34	20.8 ± 1.1	10.2 ± .72

* Values are the mean ± standard error of 3 replicate cultures.

** ND = not determined

growing *T. versicolor* did not cause increased $^{14}CO_2$ release from PCP also supports this premise. These findings contrast with the conclusions of Sanglard et al. (1986) which indicate that LiP catalyzes the initial oxidation of B(a)P during mineralization by *P. chrysosporium.* These workers found that the pattern of LiP production in *P. chysosporium* mineralizing ^{14}C-B(a)P paralleled the pattern of release of $^{14}CO_2$. Other investigators, however, have concluded that LiP is not essential in the degradation of xenobiotic compounds by *P. chrysosporium* (Kohler et al. 1988; Yadav and Reddy 1992). The involvement of phenol-oxidizing

enzymes in the mineralization of organic pollutants by white rot fungi requires further investigation.

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