Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*

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Received 4 March 1997

Abstract The white-rot fungus, *Pycnoporus cinnabarinus*, provides an excellent model organism to elucidate the controversial role of laccase in lignin degradation. *P. cinnabarinus* produces laccase in one isoform as the predominant phenoloxidase in ligninolytic cultures, and neither LiP nor MnP are secreted. Yet, *P. cinnabarinus* degrades lignin very efficiently. In the present work, we show that laccase-less mutants of *P. cinnabarinus* were greatly reduced in their ability to metabolize ^{14}C ring-labeled DHP. However, $^{14}CO_2$ evolution in these mutant cultures could be restored to levels comparable to those of the wild-type cultures by addition of purified *P. cinnabarinus* laccase. This clearly indicates that laccase is absolutely essential for lignin degradation by *P. cinnabarinus*.

Key words: Lignin degradation; White-rot; Laccase; Laccase-less mutant; *Pycnoporus cinnabarinus*

1. Introduction

The only microorganisms which to any extent can degrade lignins, are the white-rot fungi. The basidiomycete *Phanerochaete chrysosporium* has served as a model organism for studies of lignin biodegradation and most of our knowledge of the enzyme mechanisms involved in lignin degradation stems from studies with this fungus [1]. Lignin degradation by *P. chrysosporium* correlates with the secretion of two peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) [2–4]. However, recent studies suggest that in white-rot fungi the combination of laccase (EC 1.10.3.2) with either LiP and/or MnP is a much more common combination of phenoloxidases than the LiP/MnP pattern found in *P. chrysosporium* [5].

Very recently, even *Phanerochaete* species were found to produce a laccase [6,7]. The contribution of laccase to ligninolysis has been less clear than that of LiP or MnP. For a long time, laccase was not considered as an enzyme suitable for the complete oxidation of lignin because of the low redox potential of the enzyme that did not allow for oxidation of non-phenolic lignin structures. However, Bourbonnais and Paice [8] showed that the artificial laccase substrate ABTS could act as a redox mediator which enabled laccase to oxidize also non-phenolic lignin compounds. We have recently demonstrated [9] that *P. cinnabarinus* produces a metabolite, 3-HAA, that mediates the oxidation of non-phenolic substrates by laccase and also allows for its depolymerization of DHP. This was the first description of how laccase might function in a biological system for the complete degradation of lignin.

The possibility to explain the mechanisms for lignin degradation through the production of phenoloxidase-less mutants of white-rot fungi has been tried. However, these studies could not give clear answers to the question whether laccase is essential for lignin degradation, since the white-rot fungi used for this purpose secrete other phenoloxidases in addition to laccase [10–12].

With *P. cinnabarinus* we have found an ideal model organism to study the possible role of laccase in lignin degradation since laccase is the predominant phenoloxidase secreted under ligninolytic conditions [13]. Despite the lack of LiP and MnP production, the rate of lignin degradation by *P. cinnabarinus* is comparable to that of *P. chrysosporium* [14]. The simple extracellular phenoloxidase system of *P. cinnabarinus* has enabled us to use mutagenesis to produce Lac^- strains to precisely define the role of laccase in lignin degradation by this fungus.

2. Materials and methods

2.1. Chemicals

All reagents were at least of analytical grade and purchased from either Sigma or Aldrich. ^{14}C-ring-labeled DHP of coniferyl alcohol was prepared by the 'Zutropfverfahren' (Odier and Heckman, INRA, France) and previously characterized as described in [1]. Laccase was purified from *P. cinnabarinus* cultures as described elsewhere [13].

2.2. Organisms and culture conditions

The *P. cinnabarinus* strains, PB wild-type and two laccase-less mutants, used in this study, were maintained on 2% (w/v) malt extract agar plates grown at 24°C and kept at 4°C. All cultivation steps were carried out under sterile conditions. For UV-mutagenesis of the *P. cinnabarinus* wild-type, conidiospore suspensions were prepared by cultivating the fungus on rice according to Eggert et al. [13]. For the DHP mineralization study, 25 agar blocks (0.5 cm × 0.5 cm) of the wild-type strain and the laccase-less mutants growing on malt extract agar plates were transferred into 250 ml Erlenmeyer flasks containing 50 ml 2% malt extract (w/v) medium (pH 5.5). These precultures were incubated for six days at 30°C without shaking. Mycelia of precultures were washed aseptically with phosphate buffer (pH 6.0) over a strainer, homogenized in a blender, and used as inoculum for the ^{14}C-ring-labeled-DHP mineralization experiments.

2.3. Assays for enzyme activities

After removing the mycelium by centrifugation (10 min at 5000 × *g*), laccase activity was determined by monitoring the oxidation at 420 nm of 500 μM ABTS (2,2′-azino-bis-[3-ethylthiazoline-6-sulfonate]) buffered with 50 mM sodium tartrate buffer (pH 4.0). Enzyme

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Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate); DHP, dehydrogenative polymer; DMF, dimethylformamide; 3-HAA, 3-hydroxyanthranilate; LiP, lignin peroxidase; MnP, manganese-dependent peroxidase; Lac^- strain, laccase-less mutant; Pc wt, *Pycnoporus cinnabarinus* wild-type

PII S0014-5793(97)00301-3

units are given in mol of product formed per min ($\varepsilon_{max}=3.6\times10^4\times M^{-1}\times cm^{-1}$). Peroxidase activity was measured by adding H_2O_2 to 100 μM final concentration to the laccase assay solution (pH 3.5) and subtracting the previously determined laccase activity. All values represent the mean from duplicate measurements with a maximal sample mean deviation of ±11% of the measured values.

2.4. Mutagenesis and screening for laccase-less mutants

For mutagenesis, 7 ml spore suspension of the *P. cinnabarinus* wild-type (5×10^5/ml) were transferred into petri dishes (5 cm diameter) and irradiated with UV light, under conditions sufficient to give a 1% survival rate (3 min; 30 W lamp; 8 cm distance from the light source, under shaking on a slowly rocking table). Irradiated spores were plated onto 2% malt extract (w/v) agar plates containing 0.02% Na-deoxycholate (pH 5.5) for induction of colonial growth [15] and guaiacol (10 mM), as the laccase substrate, and incubated for 4 days at 24°C. Laccase-positive colonies showed brownish-red zones due to the oxidation of guaiacol. Colonies lacking colored zones were transferred to fresh malt extract agar plates containing deoxycholate and guaiacol and rescreened under the same conditions. The stability of these putative laccase-less mutants was further tested by cultivating the strains in liquid malt extract (2% w/v) medium (pH 5.5) as well as liquid basal medium and monitoring the production of laccase over 7 days. Basal liquid medium contained the following (per liter): glucose, 3.0 g; KH_2PO_4, 1.0 g; NaH_2PO_4, 0.26 g; $(NH_4)_2SO_4$ (2.4 mM), 0.137 g; $MgSO_4\cdot7H_2O$, 0.5 g; $CuSO_4\cdot7H_2O$, 0.5 g; 2,2-dimethylsuccinic acid, 2.2 g; $CaCl_2\cdot2H_2O$, 74 mg; $ZnSO_4\cdot7H_2O$, 6 mg; $FeSO_4\cdot7H_2O$, 5 mg; $MnSO_4\cdot4H_2O$, 5 mg; $CoCl_2\cdot6H_2O$, 1 mg; vitamin solution, 500 μl [16]. The pH of the medium was adjusted to 4.6 with 1 N NaOH. For the laccase induction study, the laccase-less mutants were cultivated in liquid basal medium (pH 4.6) for 10 days and 2,5-xylidine (15 μM) was added to the culture flasks on day 3.

2.5. Detection of 3-hydroxyanthranilate

The Pc*lac1* and Pc*lac2* mutants were cultivated in liquid malt extract (2% w/v) medium (pH 5.5) as well as liquid basal medium (pH 4.6), and the production of 3-hydroxyanthranilate in the culture supernatants was monitored over 7 days by HPLC analysis using a C-18 reversed phase column [17].

2.6. Mineralization of ^{14}C-ring-labeled DHP

250 ml Erlenmeyer flasks containing 25 ml basal liquid medium (pH 4.6) were inoculated with 1 ml of homogenized mycelium from precultures of *P. cinnabarinus* wild-type and two laccase-less mutants, Pc*lac1* and Pc*lac2*, and incubated at 30°C without shaking. All incubations were done in triplicate. On day 4 of the cultivation, 8 μl of ^{14}C-ring-labeled DHPs in DMF (40 000 dpm) were added to the flasks. The flasks were tightly closed with rubber stoppers in which small glass cups were inserted containing 1 ml of 1 N NaOH to absorb released $^{14}CO_2$. Culture flasks were not flushed with oxygen, but shaken for 10 min without stoppers under sterile conditions each time samples were taken. Evolution of $^{14}CO_2$ was measured over a period of 38 days. At regular intervals, the cups containing NaOH and trapped $^{14}CO_2$ were emptied into 10 ml of liquid scintillation fluid (Ultima Gold, Packard Instr. Co., Downers Grove, IL, USA) and replaced with fresh 1 N NaOH. The radioactivity was quantified using a liquid scintillation counter (Tri-Carb 1900CA, Packard Instr. Co.). All values represent the mean from three independent cultures.

For the laccase complementation experiments, purified *P. cinnabarinus* laccase was added to cultures of mutants Pc*lac1* and Pc*lac2* on day 16, 22, 28, and 31 to give approximately the same laccase activity as in *P. cinnabarinus* wild-type cultures. Glucose concentrations of the cultures containing glucose as the carbon source were monitored (Chemstrip bG, Boehringer Mannheim). On day 16 and 22, when glucose levels reached concentrations of less than 200 mg/l, glucose was added to the culture flasks to give a final concentration of 3 g/l.

Uninoculated experiments were run in triplicate containing 25 ml of liquid basal medium (pH 4.6) and radio-labeled DHPs (40 000 dpm) only, and with addition of purified *P. cinnabarinus* laccase (0.6 U/ml), respectively. Samples were taken as described above.

At the end of the experiment the biomass of each flask was recovered by filtration over an ultrafiltration membrane (0.45 μm pore size, Millipore). After washing the mycelium with phosphate buffer (pH 6.0) the dry weight of the mycelium was quantified by weighing the dried filters.

2.7. Weight loss in rotting of wood blocks

Sterile sapwood blocks of pine (*Pinus taeda*) and yellow poplar (*Liriodendron tulipifera*) 25×20×100 mm were used in this study. Inoculation and rotting of the wood blocks (triplicate samples) was carried out as described by Yu and Eriksson [18].

3. Results and discussion

Lac$^-$ strains of *P. cinnabarinus* were produced by UV-radiation of conidiospores of the wild-type strain (Pc wt). The screening for Lac$^-$ strains on malt agar plates using guaiacol as an indicator for laccase activity resulted in the isolation of 25 different mutant strains with strongly reduced laccase activity. In contrast to the Pc wt, mutants showed very low or no laccase activity when cultivated on malt extract agar plates or in liquid basal medium. These results were confirmed by a second cultivation in liquid basal medium. To investigate whether the production of laccase in the laccase-less mutants was inducible, 2,5-xylidine, which strongly enhances laccase production by the Pc wt [13], was added to liquid cultures with basal medium to a final concentration of 15 μM. 2,5-Xylidine had an enhancing effect on the production of laccase in some of the tested Lac$^-$ strains whereas in most of the strains laccase levels remained unchanged. As for the Pc wt, no peroxidase activity was detectable in the mutant strains.

For the [^{14}C]DHP mineralization study, two *P. cinnabarinus* Lac$^-$ strains, Pc*lac1* and Pc*lac2* were chosen. The morphology of these two mutants was identical to the wild-type of *P. cinnabarinus*. With and without addition of 2,5-xylidine, laccase activities of the two mutants, Pc*lac1* and Pc*lac2* were lower than 0.01 U/ml corresponding to only 0.5% of the laccase activity of Pc wt in liquid basal medium and 0.1% of the laccase activity in 2,5-xylidine-induced cultures. Therefore, Pc*lac1* and Pc*lac2* were promising candidates to study whether laccase is essential for lignin degradation by *P. cinnabarinus*.

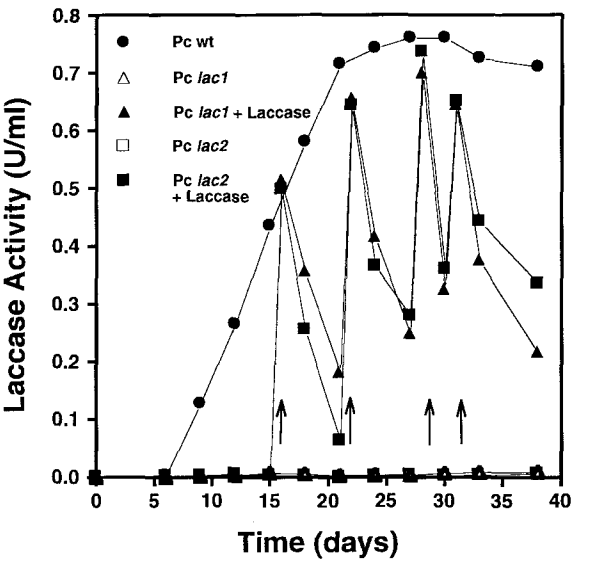

Fig. 1. Laccase production by *P. cinnabarinus* wild-type and Lac$^-$ strain Pc*lac1* and Pc*lac2*. On day 16, 22, 28, and 31, laccase purified from Pc wt was added to Pc*lac1* and Pc*lac2* cultures to approximately the same laccase levels as those produced by Pc wt.

The role played by laccase in lignin degradation has remained obscure since the low redox potential of this enzyme appeared to make it incapable of oxidizing non-phenolic lignin constituents [19]. In *P. cinnabarinus* the lack of LiP does not require compensation by a laccase having an unusually high redox potential [13]. As shown previously in in vitro studies, the *P. cinnabarinus* laccase and 3-HAA, a metabolite produced by the fungus [17], could be used in combination to oxidize non-phenolic substrates and even depolymerize radiolabeled DHP [9]. Because of the strong evidence that 3-HAA is a necessary component of the *P. cinnabarinus* ligninolytic system, we tested whether production of 3-HAA was affected in the mutant strains. Accumulation of 3-HAA was, in both mutants, comparable to the Pc wt, reaching concentrations of about 20 μM on the second day of cultivation. However, Pc*lac1* and Pc*lac2* did not show accumulation of the red pigment, cinnabarinic acid, typical for Pc wt cultures. This result supports our previous finding that in *P. cinnabarinus* laccase catalyzes the oxidation of 3-HAA to cinnabarinic acid [17]. Addition of purified laccase to the culture supernatant of Pc*lac1* and Pc*lac2* led to formation of cinnabarinic acid, which could be followed photospectroscopically [17].

To investigate whether laccase-less mutants of *P. cinnabarinus* lack the ability to degrade lignin, *P. cinnabarinus* wild-type and the Lac$^-$ strains were cultivated in liquid basal medium containing ^{14}C-ring-labeled DHP (40 000 dpm). Release of $^{14}CO_2$ as well as laccase and peroxidase activities were monitored for 38 days by taking samples every third and every fifth day, respectively. The intention of this experiment was the exact comparison of ligninolytic activities between *P. cinnabarinus* wild-type and laccase-less mutants rather than optimization of lignin degradation rates. Therefore, oxygen was not supplied by flushing of the cultures at regular intervals. Since oxygen has been shown to have a positive effect on the mineralization of lignin, evolution of $^{14}CO_2$ from radiolabeled DHPs by Pc wt was lower than under higher oxygen atmosphere (Eggert, unpublished results).

In accordance with our previous observations, only very low laccase activities (less than 0.01 U/ml) were produced in cultures of Pc*lac1* and Pc*lac2* and no peroxidase activity was detectable. No inducing effect of DHPs on laccase production was observed (Fig. 1). These almost neglectible laccase activities correlated with very low $^{14}CO_2$ evolution of the mutant cultures. In wild-type cultures, 10.0% of the DHPs were mineralized to $^{14}CO_2$ and a maximal laccase activity of 0.76 U/ml was reached. Only 0.4% and 0.61% of radio-labeled DHP was mineralized to $^{14}CO_2$ after 38 days of growth in basal medium (Fig. 2), corresponding to 4.0% and 6.0% of the total $^{14}CO_2$ released in cultures of Pc wt, respectively. Since growth of Pc wt and both mutants was comparable (yielding about 150 mg (± 12%) mycelial dry weight), these results demonstrate that mutants of *P. cinnabarinus* lacking laccase show a drastically reduced ability to mineralize synthetic lignin.

To further confirm the importance of laccase for lignino-

Table 1
Weight loss (%) caused by Pc wt, Pc*lac1* and Pc*lac2* in wood blocks inoculated with the respective strains for 12 weeks

	Softwood	Hardwood
Pc wt	42 ± 4	58 ± 5
Pc*lac1*	8 ± 2	6 ± 3
Pc*lac2*	6 ± 4	7 ± 2

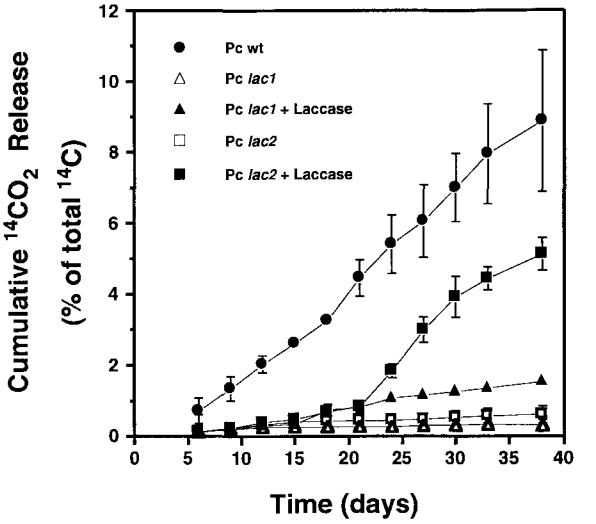

Fig. 2. Metabolism (evolution of $^{14}CO_2$) of $^{14}CO_2$-ring-labeled DHP by *P. cinnabarinus* wild-type, Pc*lac1*, Pc*lac2*, Pc*lac1*+laccase, and Pc*lac2*+laccase.

lytic activity in *P. cinnabarinus,* we studied whether the ligninolytic capacity of the Lac$^-$ strains, Pc*lac1* and Pc*lac2*, could be restored by supplementing laccase. Purified Pc wt laccase was added to cultures of Pc*lac1* and Pc*lac2* on day 16, 22, 28, and 31 to approximately the same laccase activity levels as in wild-type cultures (Fig. 1, indicated by arrows). Addition of purified laccase to Pc*lac1* and Pc*lac2* resulted in a significant increase in $^{14}CO_2$ evolution. Cumulative release of $^{14}CO_2$ was about 4-fold higher for Pc*lac1*, and 8.4-fold higher for Pc*lac2* compared to cultures of both mutants to which no laccase was added (Fig. 2).

After the first addition of laccase on day 16, no immediate effect was observed. However, after a short delay, $^{14}CO_2$ evolution rates of Pc*lac2* increased drastically, reaching about 137 dpm/day. These rates were comparable or even slightly higher than those of the Pc wt (122 dpm/day from day 22 to 30). Even though Pc*lac1* did not reach the mineralization rates of Pc wt or Pc*lac2* complemented with laccase, addition of laccase had an enhancing effect also on the mineralization of the lignin substrate by Pc*lac1*. In uninoculated control flasks, with and without laccase, release of $^{14}CO_2$ was not detectable (data not shown). As shown in this complementation study, the ability of Pc*lac2* to mineralize synthetic lignin could be restored to approximately the same or even a higher $^{14}CO_2$ release rate than the wild-type. Also in Pc*lac1*, laccase addition resulted in a slightly enhanced release of $^{14}CO_2$ compared to the culture without laccase addition. It is not clear yet whether pleiotropic effects caused by the undefined nature of UV- induced mutations might have contributed to the difference between Pc*lac1* and Pc*lac2*.

To study the impact of laccase deletion on the ability to degrade wood, both softwood and hardwood blocks were inoculated for 12 weeks with Pc wt, Pc*lac1*, and Pc*lac2* respectively. Table 1 shows that Pc wt caused a considerable weight loss in both the softwood and hardwood blocks while the weight losses caused by the Lac$^-$ strains were minimal. Controls with uninoculated wood blocks did not show any weight reduction.

It has earlier been demonstrated that laccase-less mutants from two basidiomycete species, i.e. *Pleurotus sajor-caju* and

Pleurotus eryngii lost the ability of the wild-type to decolorize the polymeric dyes, carminic acid, and Remazol brilliant blue [11]. However, most *Pleurotus* species produce other phenol-oxidases in addition to laccase and such a system is not as suitable as the *P. cinnabarinus* system to demonstrate the importance of laccase. Similar studies have been performed with mutants of *Trametes versicolor* that lost the ability of the wild-type strain to bleach hardwood kraft pulp [12]. These mutants produced very low amounts of laccase as well as MnP and could therefore not give a clear answer to the question whether laccase is necessary for lignin degradation.

Here we have demonstrated that the two *P. cinnabarinus* laccase-less strains could not metabolize ^{14}C-ring-labeled DHP to $^{14}CO_2$. Addition of purified laccase to the mutants increased $^{14}CO_2$ evolution to give rates comparable to the Pc wt. These results clearly indicate that laccase is absolutely essential for lignin degradation in *P. cinnabarinus*.

Acknowledgements: We thank Ms. Lori McClanhan for her help with production and screening of the laccase-less mutants that were used here. Financial support for this work was, in part, a fellowship from the Alexander von Humboldt-Stiftung to C.E., National Science Foundation Grant, MCB-9507331, and the University of Georgia Biotechnology Grant Program.

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