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Laccase and Its Role in Production of Extracellular Reactive Oxygen Species during Wood Decay by the Brown Rot Basidiomycete *Postia placenta*▽†

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Brown rot basidiomycetes initiate wood decay by producing extracellular reactive oxygen species that depolymerize the structural polysaccharides of lignocellulose. Secreted fungal hydroquinones are considered one contributor because they have been shown to reduce Fe^{3+}, thus generating perhydroxyl radicals and Fe^{2+}, which subsequently react further to produce biodegradative hydroxyl radicals. However, many brown rot fungi also secrete high levels of oxalate, which chelates Fe^{3+} tightly, making it unreactive with hydroquinones. For hydroquinone-driven hydroxyl radical production to contribute in this environment, an alternative mechanism to oxidize hydroquinones is required. We show here that aspen wood undergoing decay by the oxalate producer *Postia placenta* contained both 2,5-dimethoxyhydroquinone and laccase activity. Mass spectrometric analysis of proteins extracted from the wood identified a putative laccase (Joint Genome Institute *P. placenta* protein identification number 111314), and heterologous expression of the corresponding gene confirmed this assignment. Ultrafiltration experiments with liquid pressed from the biodegrading wood showed that a high-molecular-weight component was required for it to oxidize 2,5-dimethoxyhydroquinone rapidly and that this component was replaceable by *P. placenta* laccase. The purified laccase oxidized 2,5-dimethoxyhydroquinone with a second-order rate constant near 10^4 M^{-1} s^{-1}, and measurements of the H_2O_2 produced indicated that approximately one perhydroxyl radical was generated per hydroquinone supplied. Using these values and a previously developed computer model, we estimate that the quantity of reactive oxygen species produced by *P. placenta* laccase in wood is large enough that it likely contributes to incipient decay.

Brown rot basidiomycetes are the principal recyclers of woody biomass in coniferous forest ecosystems and also the chief cause of decay in wooden structures (8, 41). Unlike the closely related white rot fungi, they degrade the cellulose and hemicellulose in wood while mineralizing little of the lignin that shields these structural polysaccharides from enzymatic attack. As a result, extensively brown-rotted wood consists primarily of an oxidized, partially cleaved residue derived from the original lignin (7, 16, 21, 22, 40). This failure to remove lignin efficiently suggests that brown rot systems contain fewer components than white rot systems and, in agreement, the recently published genome sequence of *Postia placenta* shows that this brown rot fungus lacks the ligninolytic peroxidases generally thought important for white rot (26). Instead, brown rot fungi appear to rely, at least during incipient decay, on small agents that can penetrate the lignin to access the polysaccharides (10, 14).

A better understanding of the biodegradative agents produced by *P. placenta* may provide clues about what constitutes a minimally effective system for the microbial deconstruction of lignocellulose. One potential low-molecular-weight contributor is an extracellular metabolite, 2,5-dimethoxyhydroquinone (2,5-DMHQ), which has been found in cultures of *P. placenta* and other brown rot fungi and also shown to reduce Fe^{3+} with concomitant H_2O_2 production, thus producing hydroxyl radicals ($^{\cdot}OH$) via the Fenton reaction (Fig. 1, reaction 6) (4, 17, 20, 29, 34, 36). Past work has shown that the chemical changes introduced by brown rot fungi into wood, cellulose, and other polymers are consistent with attack by reactive oxygen species (ROS) such as $^{\cdot}OH$ (4, 7, 19, 21–23). A second small agent with a proposed role is extracellular oxalic acid, which *P. placenta* produces in sufficient quantity to acidify colonized wood to pH 2 to 4. Assays *in vitro* have shown that cellulose is slowly hydrolyzed at these acidities (11).

However, there is an apparent contradiction between these two mechanisms: oxalate is a strong chelator of Fe^{3+}, and the resulting Fe^{3+} trioxalate complex has too negative a reduction potential to react readily with methoxyhydroquinones such as 2,5-DMHQ (28, 37). In considering this problem, we noted the surprising finding that the *P. placenta* genome encodes two putative laccases, enzymes that are considered atypical of brown rot fungi (26). Laccases oxidize methoxyhydroquinones to semiquinone radicals, which generally have more negative reduction potentials than their parent hydroquinones (38), and are therefore expected to be better reductants of Fe^{3+}. In addition, methoxysemiquinones reduce O_2 to generate perhydroxyl radicals ($HOO^{\cdot}$) and their conjugate base superoxide ($O_2^{\cdot -}$), which dismutate to produce H_2O_2. Furthermore,

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$$4H_2Q + O_2 \xrightarrow{\text{laccase}} 4HQ^{\bullet} + 2H_2O \quad (1)$$

$$HQ^{\bullet} + Fe^{3+} \rightleftharpoons Q + Fe^{2+} + H^{+} \quad (2)$$

$$HQ^{\bullet} + O_2 \rightleftharpoons Q + HOO^{\bullet} \quad (3)$$

$$HOO^{\bullet} + Fe^{3+} \rightleftharpoons O_2 + Fe^{2+} + H^{+} \quad (4)$$

$$HOO^{\bullet} + Fe^{2+} + H^{+} \rightleftharpoons H_2O_2 + Fe^{3+} \quad (5)$$

$$H_2O_2 + Fe^{2+} + H^{+} \longrightarrow HO^{\bullet} + Fe^{3+} + H_2O \quad (6)$$

$$2HOO^{\bullet} \longrightarrow H_2O_2 + O_2 \quad (7)$$

$$H_2Q + Q \rightleftharpoons 2HQ^{\bullet} \quad (8)$$

FIG. 1. Chemical reactions discussed in the text. For simplicity, the $HOO^{\cdot}/O_2^{\cdot -}$ acid/base pair is shown only as $HOO^{\cdot}$. H_2Q, hydroquinone; $HQ^{\cdot}$, semiquinone; Q, quinone.

$HOO^{\cdot}/O_2^{\cdot -}$ can reduce some Fe^{3+} chelates to generate additional Fe^{2+} and can oxidize some Fe^{2+} chelates to generate additional H_2O_2 (9, 13, 33, 38). By these routes, a *P. placenta* laccase could bypass the requirement for the hydroquinone to react directly with Fe^{3+} and could thus generate a complete Fenton system (Fig. 1, reactions 1 to 7).

Here we have expressed one of the *P. placenta* putative laccase genes heterologously and thus demonstrate that it encodes a typical laccase. In addition, we show that laccase activity and this particular enzyme are present in wood undergoing decay by *P. placenta*. Furthermore, we report that 2,5-DMHQ is present in the biodegrading wood, that it is a substrate for the *P. placenta* laccase, and that its oxidation during incipient wood decay requires a macromolecular component that is replaceable by *P. placenta* laccase. Finally, we show that the oxidation of 2,5-DMHQ by the *P. placenta* laccase results in significant H_2O_2 production, and we estimate that the quantity of ROS produced by this route is large enough that it probably contributes to incipient brown rot.

MATERIALS AND METHODS

Reagents. 2,5-DMHQ was prepared from 2,5-dimethoxy-1,4-benzoquinone (2,5-DMBQ; TCI America, Portland, OR), recrystallized, and stored at −20°C under argon as previously described (20). A fresh stock solution of 2,5-DMHQ (25 mM) was prepared in argon-purged dimethyl sulfoxide and used immediately for each reaction performed. Recombinant enzymes were purchased from New England Biolabs (Ipswich, MA), and nucleic acid extraction kits were purchased from Qiagen (Valencia, CA). All other chemicals were of analytical grade and, unless otherwise specified, were purchased from Sigma-Aldrich (Milwaukee, WI).

Organism and culture conditions. *P. placenta* strain MAD-698-R was obtained from the USDA Forest Mycology Center (Madison, WI). The fungus was maintained on potato dextrose agar at 28°C, and confluent plates were stored at 4°C.

For wood decay experiments, aspen wafers (20 by 10 by 1 mm, 117 ± 17 mg [dry weight], cut with the grain parallel to the 10-mm axis) were autoclaved twice and placed in one layer on the surface of nutrient agar plates (1.5% malt extract, 0.2% yeast extract, 1.5% agar) that were already fully covered by actively growing *P. placenta* mycelium. Two weeks later, a second layer of twice-autoclaved wafers was placed atop the thoroughly colonized first layer in each plate. Wafers from the top layer were then harvested weekly for the experiments described below.

Assessment of wood decay. Stiffness loss in wafers was measured perpendicular to the wood fibers, before and after fungal colonization, using a Rheometrics DMTA V dynamic mechanical analyzer (TA Instruments, New Castle, DE). The procedure was as described previously (36), except that an 8-mm span and 0.049% strain were used. Dry weights of aspen wafers were measured after vacuum-drying for 72 h at 50°C.

Quantification of metabolites and iron in the liquid fraction of colonized wood. To quantify 2,5-DMHQ in the aqueous phase of decaying wood, we stacked 8 to 10 wafers from each harvest in a folded polypropylene sheet and crushed them rapidly in a large vise. The liquid pressed out (henceforth referred to as "squeezate") was immediately analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described previously (17, 36).

For oxalate analysis, H_2SO_4 was added to measured portions of the squeezates to give a final concentration of 0.1 N, after which the samples were stored at 4°C overnight, centrifuged (21,000 × *g*, 10 min), and filtered through 0.45-μm-pore size membranes. The oxalate in each sample was then quantified by ion exclusion HPLC as described previously (15).

To determine total dissolved iron, squeezates from 50 colonized wafers were pooled for each harvest time and analyzed by inductively coupled plasma atomic emission spectrometry on a Jobin Yvon-Ultima instrument. The pH values of squeezates were also determined on these pooled samples.

Transcript analysis. For total RNA purification, 500 mg of colonized wafers was ground with a mortar and pestle in liquid nitrogen. RNA was then extracted with an RNeasy minikit (Qiagen, Valencia, CA) with the following modifications to the manufacturer's protocol: the sample was suspended in 5 ml of extraction buffer, treated for 10 min at ambient temperature, and then centrifuged for 10 min at ambient temperature at 3,500 × *g*, after which the supernatant fraction was filtered to remove insolubles. Subsequent steps were conducted as specified in the kit. The resulting RNA samples were then treated with RNase-free DNase (Qiagen, Valencia, CA) as outlined by the manufacturer. RNA concentration was measured by microspectrophotometry with a Nanodrop NT1000 instrument (Thermo Fisher Scientific, Waltham, MA).

First-strand cDNA was synthesized by using SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions with 2 μg of RNA and oligo(dT) in 25-μl reactions. The mixtures were then diluted 1:10 with RNase-free, distilled, deionized water and used for reverse transcription-PCR (RT-PCR) analyses using the indicated primers (Table 1). The PCRs (25 μl) contained 12.5 μl of *Taq* polymerase mixture (Promega), 10 pmol of each primer, and 1 μl of cDNA. PCR conditions were as follows: a denaturation step at 94°C for 3 min, followed by 30 cycles that consisted of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and finally an extension step at 72°C for 7 min. Samples (5 μl) were electrophoresed on a 0.8% agarose gel, and the bands were visualized with ethidium bromide. The amplified cDNA fragments were then eluted and cloned, and their identities were confirmed by sequencing.

Identification of a laccase in colonized wood. Biodegrading wafers (10 g original dry weight) were harvested after 2 weeks and stirred overnight at 4°C in 500 ml of Bis-Tris buffer (50 mM, pH 6.0) containing 1 M NaCl to extract extracellular proteins. The liquid phase was separated from the solids by vacuum filtra-

TABLE 1. Laccase-specific primers used in this study

Protein	Primer		Usage
	Orientation	Sequence (5′→3′)	
111314	Forward	GGGGTACCATGACACTAGTATCTATC	Full-length cDNA cloning
111314	Reverse	AACGCGGCCGCTCAGTGGTCGATTC	Full-length cDNA cloning
111314	Forward	TCCTGCGGCTCCGCCATTC	RT-PCR
111314	Reverse	TCTGCAGTGCCATTGATGTC	RT-PCR
62097	Forward	TCTCCTTCACCAACCCTAAG	RT-PCR
62097	Reverse	TGGGGCTAGTAATCGGACAC	RT-PCR

TABLE 2. Properties of wood and of wood squeezates after colonization of aspen by *P. placenta*

Time (wk)	Wood property (mean ± SD)[a]		Squeezate component or property			
	Wt loss (%)	Stiffness loss (%)	Oxalate (mM) ± SD[b]	pH[c]	Fe (μM)[c]	2,5-DMHQ (μM) ± SD[b]
1	0.6 ± 0.1	2 ± 1	21.7 ± 4.6	2.9	39	342 ± 6
2	2.8 ± 0.5	7 ± 2	11.8 ± 5.3	4.0	36	215 ± 6
3	5.4 ± 0.9	56 ± 5	1.6 ± 0.5	4.5	38	97 ± 16
4	12.9 ± 1.9	69 ± 6	–	–	–	31 ± 5
5	30.1 ± 3.1	–[d]	–	–	–	–
6	51.5 ± 4.9	–	–	–	–	–

[a] $n = 5$ samples. Measurements are versus uninoculated controls.
[b] $n = 3$ samples. Fungal metabolites were undetectable in uninoculated controls.
[c] Values for pooled squeezates from 50 colonized wafers.
[d] –, not determined.

tion through Whatman no. 1 paper, after which additional liquid was obtained by placing portions of the extracted wafers in polyethylene bags, crushing them in a large vise, and filtering the resulting squeezates. The crushed wafers were then stirred in buffer again overnight as just described to give additional extract, which was also collected by vacuum filtration. The pooled extracts (~1 liter) were dialyzed by repeated cycles of Bis-Tris buffer addition (20 mM, pH 5.5) and concentration in an ultrafiltration cell fitted with a 10-kDa cutoff membrane, after which the sample was concentrated to a final volume of 1 ml in the same apparatus.

A 15-μl portion of the concentrated sample was loaded onto a sodium dodecyl sulfate (SDS) Ready-Gel (4 to 15% polyacrylamide gradient; Bio-Rad, Hercules, CA) without mercaptoethanol addition or boiling and was electrophoresed for 1.5 h at 150 mV. The gel was then soaked in glycine-HCl buffer (50 mM, pH 3.0) for 5 min, after which ABTS [2,2′azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] stock solution was added to a final concentration of 0.5 mM. After 5 min, the dark green, activity-stained region was excised and submitted to the University of Wisconsin Biotechnology Center for "in-gel" tryptic digestion as described on the Center's website (http://www.biotech.wisc.edu/ServicesResearch/MassSpec/ingel.htm). The peptides were analyzed at the Center by nanobore liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described in the supplemental material.

Heterologous expression of the laccase. Recombinant *P. placenta* laccase (protein ID 111314) was expressed in *Pichia pastoris* strain GS115 using an Easy Select *Pichia* expression kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The forward and reverse primers (Table 1) were designed based on the reading frame of the laccase and used with *Taq* polymerase mixture (Promega) to amplify full-length cDNA from the total RNA that had been obtained from colonized wood as described above. The PCR products were separated by electrophoresis on a 1.0% agarose gel, and a 1.6-kp product was isolated by using a DNA gel extraction kit (Qiagen). The purified product was digested with KpnI and NotI, inserted into a pPICZ B vector (Invitrogen) and then transformed into *Escherichia coli* DH5α competent cells. Positive clones were verified by PCR and DNA sequence analysis.

Transformations into *P. pastoris* were carried out by electroporation according to the manufacturer's instructions. Putative transformants were transferred onto minimal methanol plates containing 0.2 mM $CuSO_4$ and 0.2 mM ABTS, and laccase-producing clones were identified after 2 days incubation at 28°C by the presence of a dark green color around the colonies. One positive transformant was grown in buffered minimal glycerol medium, and the resulting cell pellets were then resuspended in buffered minimal methanol medium according to the manufacturer's instructions, except that 0.2 mM $CuSO_4$ was also included. The cultures (100 ml) were grown at 18°C in the presence of 0.5% methanol with shaking at 250 rpm. They were assayed daily for laccase activity, using ABTS as the substrate, as described below.

After 8 days, 1 liter of medium was harvested by centrifugation at 3,500 × *g* for 10 min and then concentrated and dialyzed against sodium phosphate buffer (25 mM, pH 6.5) by repeated ultrafiltration through a 10-kDa cutoff membrane. Portions (2 ml) of this crude enzyme preparation were applied to a HiTrap Q FF anion-exchange cartridge (Amersham, Piscataway, NJ) preequilibrated with the same buffer. Proteins were eluted at 1 ml min^{-1} and ambient temperature using a 0 to 400 mM gradient of NaCl in the buffer over 60 min. The laccase activity eluted at ~150 mM NaCl. These fractions were pooled, concentrated using a 10-kDa cutoff membrane, and subjected to gel permeation chromatography (Superdex 75 10/30 column; Amersham) in 25 mM sodium phosphate buffer (pH 6.5) containing 200 mM NaCl at an ambient temperature and a flow rate of 0.5 ml min^{-1}. The fractions containing laccase activity were pooled and concentrated as described above and then dialyzed against 25 mM sodium phosphate buffer (pH 6.5). Portions (1 ml) were applied to an anion-exchange column (Mono-Q HR 5/5; Amersham) that had been preequilibrated with the same buffer. Proteins were eluted at 0.5 ml min^{-1} and ambient temperature using a 0 to 500 mM gradient of NaCl in the buffer over 60 min. The laccase-containing fractions were pooled, concentrated, dialyzed against deionized, distilled water, and stored at 4°C.

Characterization of the laccase. The molecular mass of the purified laccase was estimated by denaturing SDS-PAGE on a 4 to 15% gradient gel as described above for nondenaturing PAGE. The gel was calibrated with prestained molecular mass markers (EZ-Run; Fisher Scientific, Hampton, NH). The laccase band was stained with Coomassie blue R-250.

Oxidation of ABTS, 2,6-dimethoxyphenol (DMP), and syringaldazine by the recombinant laccase was monitored spectrophotometrically at 420 nm (ε = 36,000 M^{-1} cm^{-1}), 477 nm (ε = 14,800 M^{-1} cm^{-1}), and 525 nm (ε = 65,000 M^{-1} cm^{-1}), respectively (35). The pH optima for these oxidations were determined in 100 mM citrate-phosphate buffer (pH 2.5 to 8.5). The specific activity of the purified recombinant laccase on ABTS (0.5 mM) at 23°C and pH 2.6 was 945 U mg^{-1}, where 1 U represents the oxidation of 1 μmol of ABTS to its cation radical in 1 min.

Initial rate kinetics of the *P. placenta* laccase for the above substrates were determined spectrophotometrically at 23°C and the optimum pH values as just described, using 0.011 μM enzyme and a substrate concentration range of 0.025 to 0.500 mM for ABTS, 0.022 μM enzyme and a substrate concentration range of 0.025 to 0.250 mM for 2,6-DMP, and 0.033 μM enzyme and a substrate concentration range of 0.020 to 0.400 mM for syringaldazine. Kinetic parameters were obtained by nonlinear regression using a least-squares minimization (Solver function; Microsoft Excel, Redmond, WA). The confidence intervals were estimated by using an F-test formalism to determine the increase in the sum of the error squares that is statistically significant at the 95% level (2).

The oxidation of 2,5-DMHQ by the *P. placenta* laccase was assayed in sodium oxalate buffer (15 mM, pH 3.0, 23°C) that had been prepared with iron-depleted water (pretreated with Chelex resin; Bio-Rad) to minimize the extent of Fenton chemistry. The reactions (0.8 ml) contained 0.044 μM laccase and were initiated with 2,5-DMHQ. The 2,5-DMBQ produced was identified by reversed-phase HPLC as described previously (17, 36). The H_2O_2 produced was determined by adding *Aspergillus niger* catalase (10 U; Merck, Darmstadt, Germany) to the reaction mixtures after 2,5-DMHQ oxidation was complete and then measuring the O_2 evolution that occurred ($2H_2O_2 \rightarrow O_2 + 2H_2O$) using a FOXY-OR125-G oxygen sensor (Ocean Optics, Dunedin, FL). The initial rate kinetics of 2,5-DMHQ oxidation were determined by monitoring rates of O_2 uptake immediately after 2,5-DMHQ addition (0.050 to 0.400 mM), assuming that the initial O_2 concentration was 250 μM and that the stoichiometry of hydroquinone oxidation by laccase was $4H_2Q + 3O_2 \rightarrow 4Q + 2H_2O_2 + 2H_2O$ (Fig. 1, balanced sum of reactions 1, 3, and 7). The kinetics were analyzed by nonlinear regression as outlined above.

RESULTS

Decay of colonized wood. We found that *P. placenta* produced a typical brown rot when grown on aspen wafers over nutrient agar. That is, the colonized wood first lost structural

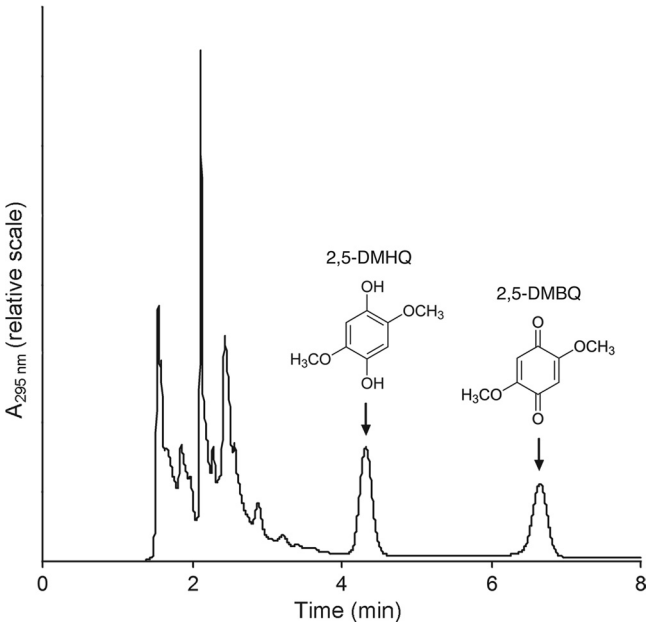

FIG. 2. Reversed-phase HPLC analysis of a typical week 2 squeezate from colonized wood.

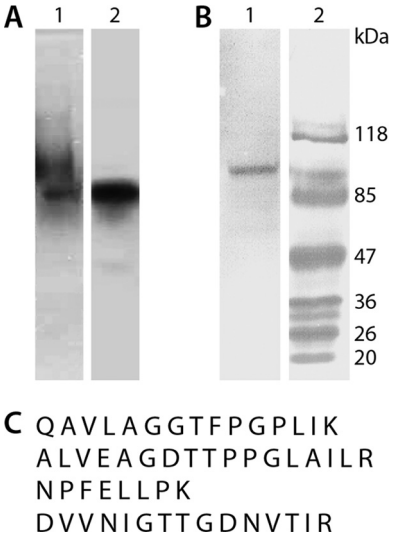

FIG. 3. Analyses of the *P. placenta* laccase. (A) nondenaturing SDS-PAGE of concentrated squeezate (1 μg of protein, lane 1) and of the purified, heterologously expressed laccase (1 μg, lane 2) after staining for activity with ABTS. (B) Denaturing SDS-PAGE of the purified laccase (1 μg, lane 1) and of molecular mass markers (lane 2). (C) Peptides detected by LC-MS/MS analysis of a tryptic digest of the activity-stained bands cut from gel A, lane 1.

integrity, as shown by rapid stiffness loss between weeks 2 and 3, before the most active phase of weight loss, which commenced between weeks 3 and 4 (Table 2). This classical feature of brown rot is generally thought to reflect an accumulation of scissions in the cellulose and hemicelluloses that occurs prior to significant removal of these structural polysaccharides (5). Based on these results, we looked for potentially interesting substances that might have a role during the first 2 weeks of decay.

2,5-DMHQ and its oxidation in colonized wood. We crushed the biodegrading aspen wafers to extract representative samples of their aqueous phase, and immediately analyzed these squeezates by reversed-phase HPLC (Fig. 2). In addition, we assayed the squeezates for total iron by plasma emission spectroscopy and for oxalate by ion exclusion HPLC. The results showed that the samples contained both 2,5-DMHQ and iron, but during the first 2 weeks they also contained about 10 to 20 mM oxalate (Table 2), which is sufficient to inhibit the direct reaction between 2,5-DMHQ and Fe^{3+} almost completely (Table 3, assay 5). Nevertheless, we found that all of the 2,5-DMHQ originally present was oxidized to 2,5-DMBQ within an hour after the squeezates were separated from the colonized wood (data not shown). This result indicated that some additional oxidant besides Fe^{3+} was present in the colonized wood and was able to oxidize 2,5-DMHQ.

TABLE 3. 2,5-DMHQ-dependent O_2 uptake rates in preoxidized squeezate and in model reactions

Assay	Ingredient(s)	Initial O_2 uptake rate ($\mu M\ min^{-1}$)
1	Squeezate	0.0
2	Squeezate, 200 μM 2,5-DMHQ	16.7
3	Ultrafiltered squeezate, 200 μM 2,5-DMHQ	3.2
4	Ultrafiltered squeezate, 200 μM 2,5-DMHQ, 5 U of *P. placenta* laccase ml^{-1}	17.8
5	15 mM sodium oxalate (pH 3.0), 30 μM Fe^{3+}, 200 μM 2,5-DMHQ	0.4
6	2 mM sodium oxalate (pH 3.0), 30 μM Fe^{3+}, 200 μM 2,5-DMHQ	5.5
7	15 mM sodium oxalate (pH 3.0), 200 μM 2,5-DMHQ, 200 μM 2,5-DMBQ	2.8

Laccase in colonized wood. For at least the first 3 weeks of colonization, the biodegrading wood contained RNA transcripts encoding both putative laccases (gene model e_gw1.174.29.1, protein model 62097; and gene model estExt_Genewise1Plus.C_300114, protein model 111314; see the *P. placenta* genome database at http://genome.jgi-psf.org/Pospl1/Pospl1.home.html), as shown by RT-PCR analysis and sequencing of the resulting cDNAs (data not shown). Squeezates from colonized wood oxidized the laccase substrates ABTS and 2,6-DMP, and ultrafiltration of these samples through 10-kDa cutoff membranes showed that the substance responsible for the activity was macromolecular. The oxidizing activity toward ABTS was not enhanced by the addition of H_2O_2, and the addition of H_2O_2 alone did not result in detectable O_2 production. That is, neither peroxidase nor catalase activity was detectable in the squeezates (data not shown).

Nondenaturing SDS-PAGE of concentrated squeezates then revealed that they contained a complex mixture of proteins, among which the laccase activity migrated as two incompletely resolved bands that stained for activity with ABTS (Fig. 3A). The entire activity-stained region from one gel was excised, and a tryptic digest was analyzed by LC-LC/MS with a search of the resulting data against the *P. placenta* protein database. No peptides were found that matched putative laccase model 62097, and therefore we decided not to pursue further work with it, although the negative MS result does not

rule out the possibility that the protein was actually present. However, four peptides with unambiguous sequence tags gave unique matches, with 10% coverage, to protein model 111314 (Fig. 3C), which is 58% identical to a previously described laccase from the white rot fungus *Trametes villosa* (National Center for Biotechnology Information accession no. JC5355) (39).

Properties of the *P. placenta* laccase. We expressed protein 111314 in *P. pastoris* and purified it to apparent homogeneity by anion exchange and gel permeation chromatography. When analyzed by nondenaturing SDS-PAGE, it comigrated with one of the laccase bands found in squeezates and also stained with ABTS (Fig. 3A). Denaturing SDS-PAGE of the heterologously expressed laccase indicated that it had a molecular mass of 95 kDa (Fig. 3B), which suggests that its extent of glycosylation was ca. 40%, considering that the predicted molecular mass of the mature protein 111314 is 57 kDa.

The purified laccase oxidized the secreted fungal metabolite 2,5-DMHQ, giving 2,5-DMBQ as the sole organic product detectable by HPLC analysis. In addition, this reaction produced H_2O_2, as shown by the evolution of O_2 after the addition of catalase to the mixtures after 2,5-DMHQ oxidation was complete. The calculated yield of H_2O_2 from reactions initially containing 200 μM 2,5-DMHQ was 113 ± 21 μM ($n = 3$). This result is a strong indication that the reactions produced $HOO^{\cdot}/O_2^{\cdot -}$ (Fig. 1, reaction 3) and, given the stoichiometry of the dismutation reaction for this radical (Fig. 1, reaction 7), we infer that approximately one $HOO^{\cdot}/O_2^{\cdot -}$ was produced per 2,5-DMHQ supplied. The oxidation of 2,5-DMHQ by the *P. placenta* laccase showed no evidence of saturation kinetics, in agreement with earlier work on laccase-catalyzed hydroquinone oxidations (30). Instead, the oxidation is best described as a second-order reaction between enzyme and substrate that exhibits an apparent $k = 1.08 \times 10^4\ M^{-1}\ s^{-1}$ at a physiological pH of 3.0 (95% confidence interval = 1.04×10^4 to $1.12 \times 10^4\ M^{-1}\ s^{-1}$).

The *P. placenta* laccase also oxidized the standard laccase substrates syringaldazine (pH optimum, 5.0), 2,6-DMP (pH optimum, 4.8), and ABTS (pH optimum, 2.6). Syringaldazine oxidation did not show saturation kinetics, instead exhibiting second-order kinetics with an apparent $k = 1.90 \times 10^5\ M^{-1}\ s^{-1}$ (95% confidence interval = 1.86×10^5 to $1.94 \times 10^5\ M^{-1}\ s^{-1}$). ABTS oxidation showed typical Michaelis-Menten kinetics, with a K_m of 41 μM (95% confidence interval = 29 to 57 μM) and a k_{cat} of 1280 s^{-1} (95% confidence interval = 1,200 to 1,370 s^{-1}). The oxidation of 2,6-DMP also exhibited saturation kinetics, with a K_m of 58 μM (95% confidence interval = 41 to 81 μM) and a k_{cat} of 1,970 s^{-1} (95% confidence interval = 1,780 to 2,210 s^{-1}).

Role of laccase in 2,5-DMHQ oxidation. Further work provided evidence that a *P. placenta* laccase was chiefly responsible for the oxidation of 2,5-DMHQ we observed in squeezates from decaying wood. To do these experiments, we pooled squeezates from 100 1- and 2-week-colonized aspen wafers and then allowed the endogenous 2,5-DMHQ they already contained to oxidize completely to 2,5-DMBQ. Assays of this material showed that it contained approximately 15 mM oxalate (pH 3.0), 40 μM Fe, 500 μM 2,5-DMBQ, and 5 ABTS U ml^{-1} of laccase activity. We set aside some of this preoxidized squeezate and filtered the remainder through a 10-kDa cutoff membrane to remove the laccase activity it contained, as well as the unidentified proteins that were present. Some of this ultrafiltered squeezate was also taken aside, after which we added heterologously expressed *P. placenta* laccase to the remainder to bring its total laccase activity back to the level of 5 ABTS U ml^{-1} we observed in the original, unfiltered squeezate. We then compared the initial rates of O_2 uptake that occurred in each sample after 200 μM freshly prepared 2,5-DMHQ was added. The results showed that a high-molecular-weight component was responsible for more than 80% of the 2,5-DMHQ-dependent uptake rate that occurred in unfiltered squeezate and that supplementation of the ultrafiltered squeezate with heterologously expressed *P. placenta* laccase restored its original rate of 2,5-DMHQ-dependent O_2 consumption (Table 3, assays 2 to 4).

In an attempt to identify the source of the slower, nonenzymatic O_2 uptake that occurred in the above experiment (Table 3, assay 3), we first prepared a model squeezate consisting of 15 mM oxalate (pH 3.0), 30 μM Fe^{3+}, and 200 μM 2,5-DMHQ. The resulting rate of O_2 uptake (Table 3, assay 5) was too low to account for the nonenzymatic O_2 uptake rate, which incidentally confirms that a direct, uncatalyzed reaction between 2,5-DMHQ and Fe^{3+} trioxalate is unimportant in the *P. placenta* system. By contrast, this direct reaction proceeded readily in 2 mM oxalate, a concentration close to that found in brown rot fungi that are not oxalate accumulators (Table 3, assay 6) (36).

We then prepared another model squeezate with 200 μM 2,5-DMBQ in place of Fe^{3+} and found that the addition of 200 μM 2,5-DMHQ to this system resulted in an initial rate of O_2 uptake similar to the residual rate we observed in ultrafiltered squeezate (Table 3, assays 3 and 7). These results indicate that the laccase-independent O_2 uptake in the squeezate resulted from disproportionation reactions between 2,5-DMHQ and 2,5-DMBQ (Fig. 1, reaction 8), which apparently provide an alternative, slow route to produce semiquinone radicals that react with O_2 (27). Our experimental design probably exaggerated the contribution of semiquinone disproportionation, because the preoxidized squeezates unavoidably contained higher than physiological levels of 2,5-DMBQ.

DISCUSSION

Laccase and its potential role in brown rot. For many years, brown rot fungi have been identified by the negative result they give in the Bavendamm test, that is, by the failure of their cultured mycelia to give a color reaction with laccase substrates such as gallic acid. By contrast, most white rot fungi give a positive reaction in this test (1, 5). Recent work has shown that the situation is more complicated: a few brown rot fungi have been shown to oxidize gallic acid, to secrete uncharacterized substances that oxidize ABTS, or to harbor laccase-like gene sequences (6, 24). Our results with *P. placenta* now establish that a true laccase is expressed by at least one typical brown rot fungus.

Fungal laccases have been proposed to have two biodegradative roles in wood decay. The first, originally proposed for white rot fungi, is that these enzymes might oxidize low-molecular-weight mediators to diffusible free radicals that can penetrate the wood cell wall and act as direct oxidants of lignocellulose (18, 25). Although many synthetic laccase medi-

ators with this activity have been discovered (31), and some naturally occurring phenols have been shown to promote laccase-catalyzed xenobiotic oxidations *in vitro* (3), no natural ligninolytic mediators have yet been found in wood undergoing fungal decay. Thus far, our HPLC analyses of wood colonized by *P. placenta* have likewise shown no evidence for mediators of this type.

The second mechanism, also originally proposed for white rot fungi (9, 12, 13, 33), is that laccases might oxidize hydroquinones to produce semiquinones that act as reductants of O_2 and Fe^{3+}, thus generating a complete Fenton system (Fig. 1, reactions 1 to 7). There is evidence that this pathway for ROS production is feasible (9, 12, 13, 33), but one reservation is that no white rot fungus has yet been shown to produce hydroquinones, and consequently the proposed mechanism would have to depend on the small quantity of methoxyhydroquinones that might be produced from lignin when it is cleaved. By contrast, previous work has shown that diverse brown rot fungi produce extracellular methoxyhydroquinones (17, 20, 29, 34, 36), and our new results establish that 2,5-DMHQ is a major extracellular metabolite in *P. placenta*-colonized wood. Since it is now clear that *P. placenta* also produces the requisite laccase, we propose that this mechanism for ROS production is most relevant to brown rot.

There are two key points raised by our findings. First, much of the 2,5-DMHQ in the colonized wood was continuously maintained in its reduced form (Fig. 2), even though its natural tendency was to oxidize after the squeezates were removed from the wood. This result indicates that the 2,5-DMHQ pool in the biodegrading wood was not static. Instead, the hydroquinone must have undergone continuous oxidation to 2,5-DMBQ with concomitant $HOO^{\cdot}/O_2^{\cdot -}$ production, while being simultaneously regenerated so that it was present in a dynamic steady state. One possibility is that the 2,5-DMBQ produced in the wood is continuously reduced back to 2,5-DMHQ by *P. placenta* (20). Alternatively or in addition, the fungus may continuously produce the hydroquinone *de novo* to replace what is lost via biodegradation.

Second, this continuous turnover of 2,5-DMHQ required a high-molecular-weight component that was replaceable by *P. placenta* laccase (Table 3). In fungi that produce little oxalate, laccases are not essential because the secreted hydroquinones can reduce Fe^{3+} directly, thus generating not only Fe^{2+}, but also the $HOO^{\cdot}/O_2^{\cdot -}$ that is one of the components needed to initiate Fenton chemistry (17, 20, 37). However, this route cannot operate efficiently in high oxalate producers such as *P. placenta* because hydroquinones ($E^{\circ} \approx +1{,}000$ mV) are poor reductants of Fe^{3+} trioxalate ($E^{\circ} = -120$ mV) (28, 37, 38). In this case, laccases have an important role because they provide an iron-independent route to produce $HOO^{\cdot}/O_2^{\cdot -}$.

Relevance of laccase-catalyzed ROS production to incipient wood decay. Although an exact measure of ROS production in biodegrading wood is probably unobtainable, it is possible to make a rough comparison between the amount of ROS needed to account for incipient wood biodeterioration and the amount of ROS that laccase-catalyzed 2,5-DMHQ oxidation is expected to provide. Previous results published by Cowling show there is a correlation between weight loss and decrease in holocellulose viscosity when sweetgum wood is degraded by the strain of *P. placenta* we used (5). Since sweetgum is chemically similar to aspen (32) and there is a direct relationship between holocellulose viscosity and holocellulose molecular weight (42), we were able to use Cowling's data to estimate the number of holocellulose scissions that occur in decaying aspen at 3% weight loss, the value that our cultures achieved in 2 weeks (Table 2). We then used our previously described computer model (36), adjusted to reflect the chemical composition of aspen (32), to estimate the number of $^{\cdot}OH$ necessary to account for these scissions. This approximate calculation indicates that about 14 μmol of $^{\cdot}OH$ per g (dry weight) of wood was produced during the first 2 weeks of decay.

By comparison, our results with the *P. placenta* laccase indicate that approximately one $HOO^{\cdot}/O_2^{\cdot -}$ was produced per 2,5-DMHQ oxidized by the laccase. Given an average steady-state 2,5-DMHQ concentration of 200 μM, an average laccase concentration of 0.06 μM (i.e., 5 ABTS U ml^{-1}), a rate constant of $1.08 \times 10^4\ M^{-1}\ s^{-1}$ for the reaction between these two species, and a water content near 50% (wt/wt) in the wood (our observations), about 160 μmol of $HOO^{\cdot}/O_2^{\cdot -}$ was produced per g (dry weight) of wood during the first 2 weeks of decay. In theory, since the balanced equation for the reaction of $HOO^{\cdot}$ in the presence of iron salts is $3HOO^{\cdot} \rightarrow 2O_2 + H_2O + {}^{\cdot}OH$ (Fig. 1, balanced sum of reactions 4 to 6), the potential quantity of $^{\cdot}OH$ produced by this route during the first 2 weeks was a little over 50 μmol per g (dry weight) of wood, more than enough to account for the holocellulose scission that is indicated by the extent of weight loss in the wood.

However, it remains to be determined how efficiently Fenton chemistry is driven when laccase oxidizes 2,5-DMHQ under physiological conditions. The reduction potential of the 2,5-DMHQ semiquinone is apparently unknown, and might be too positive for this radical to reduce Fe^{3+} trioxalate efficiently. $HOO^{\cdot}/O_2^{\cdot -}$ is reported to be sufficiently reducing (38) but dismutates rapidly (Fig. 1, reaction 7). Accordingly, it is possible that *P. placenta* has other mechanisms to reduce extracellular Fe^{3+} and that the principal role of the laccase/2,5-DMHQ system is to produce, via $HOO^{\cdot}/O_2^{\cdot -}$ dismutation, the H_2O_2 required in the Fenton reaction. The calculations we have outlined above indicate that the amount of H_2O_2 thus produced would be about 80 μmol per g (dry weight) of wood if none of the semiquinones or $HOO^{\cdot}/O_2^{\cdot -}$ reduce Fe^{3+} and if dismutation is the only fate of $HOO^{\cdot}/O_2^{\cdot -}$. This value appears consistent with a biodegradative role for laccase-catalyzed ROS production, considering that only 14 μmol of H_2O_2 per g (dry weight) is required to support the amount of $^{\cdot}OH$ production indicated by the weight loss in the wood.

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