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Isolation of Laccase Gene-Specific Sequences from White Rot and Brown Rot Fungi by PCR

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Degenerate primers corresponding to the consensus sequences of the copper-binding regions in the N-terminal domains of known basidiomycete laccases were used to isolate laccase gene-specific sequences from strains representing nine genera of wood rot fungi. All except three gave the expected PCR product of about 200 bp. Computer searches of the databases identified the sequence of each of the PCR products analyzed as a laccase gene sequence, suggesting the specificity of the primers. PCR products of the white rot fungi *Ganoderma lucidum*, *Phlebia brevispora*, and *Trametes versicolor* showed 65 to 74% nucleotide sequence similarity to each other; the similarity in deduced amino acid sequences was 83 to 91%. The PCR products of *Lentinula edodes* and *Lentinus tigrinus*, on the other hand, showed relatively low nucleotide and amino acid similarities (58 to 64 and 62 to 81%, respectively); however, these similarities were still much higher than when compared with the corresponding regions in the laccases of the ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa*. A few of the white rot fungi, as well as *Gloeophyllum trabeum*, a brown rot fungus, gave a 144-bp PCR fragment which had a nucleotide sequence similarity of 60 to 71%. Demonstration of laccase activity in *G. trabeum* and several other brown rot fungi was of particular interest because these organisms were not previously shown to produce laccases.

Wood rot fungi are important degraders of the major plant polymers lignin, cellulose, and hemicellulose in the biosphere (4, 17, 29). White rot fungi completely mineralize these polymers to CO_2, whereas brown rot fungi efficiently decompose cellulose and hemicellulose components of wood but mineralize lignin only to a limited extent (17). Laccases, lignin peroxidases (LIPs), and manganese-dependent peroxidases (MNPs) are three classes of lignin-modifying enzymes that are believed to be important in the fungal degradation of lignin by white rot fungi (4, 15, 17, 29, 34, 35). Laccases, LIPs, and MNPs can oxidize phenolic compounds, thereby creating phenoxy radicals, while nonphenolic compounds are oxidized via cation radicals. Laccases oxidize aromatic compounds with relatively low ionization potentials, whereas compounds with higher ionization potentials are readily oxidized by LIPs (9). Some white rot fungi contain all three classes of the lignin-modifying enzymes, and the others contain only one or two classes of these enzymes (9, 14, 25). In comparison, very little is known about the lignin-modifying enzymes in brown rot fungi except for a couple of reports on LIP and MNP activities (10, 33). Specifically, the presence of laccases in brown rot fungi has not previously been reported.

Laccase, apart from its role in lignin biodegradation (5, 14), also has important applications in the bleaching of wood pulp (6). Biochemically, laccase (*p*-diphenol oxidase [EC 1.10.3.2]) is a blue copper oxidase that catalyzes the one-electron oxidation of organic substrates coupled to the four-electron reduction of molecular oxygen to water (21, 34). The copper content of laccases has been reported to vary between two and four atoms for each enzyme molecule or subunit (34). All the basidiomycete laccases described to date are glycoproteins and contain four copper atoms per molecule. The copper-binding amino acids and their general distribution in the protein are well conserved in the basidiomycete laccases (7, 34).

The structure and function of fungal laccases were recently reviewed by Thurston (34). Laccase gene and cDNA sequences from a number of basidiomycetes (13, 14, 16, 18, 22, 26, 31, 36) and a few ascomycetes (2, 12) have been characterized. Recently, Coll et al. (7) sequenced a laccase gene from strain PM1, which is closely related to *Coriolus versicolor*. The experimental strategies used in these and other recently published (13, 36) studies for the isolation of laccase genes and cDNAs have involved the purification of laccases either to raise antibodies for screening of expression libraries (7, 26, 31) or to design oligonucleotide probes based on the amino acid sequences (2, 12, 13, 16, 18, 22, 36). However, these strategies involve labor-intensive experimentation. In this study, we investigated whether it was possible to rapidly isolate and characterize laccase gene-specific sequences from different wood rot basidiomycetes. We used PCR primers based on conserved sequences around the two pairs of histidines in the N-terminal copper-binding regions of known basidiomycete laccases. Our results indicate that the PCR approach can be used to isolate laccase gene-specific sequences from wood rot fungi and, for the first time, show the presence of laccase gene-specific sequences as well as laccase activity in the brown rot fungus *Gloeophyllum trabeum.*

MATERIALS AND METHODS

Organism and culture conditions. The fungal strains used in this study and their sources are shown in Table 1. Cultures of white rot and brown rot fungi were maintained on slants of 2% malt extract (ME) agar containing 20 g of malt extract per liter and 15 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter.

To test for laccase activity, fungal strains were grown on ME plates for 7 days and mycelial plugs (5 mm in diameter) were inoculated into ME liquid medium (three plugs per 100 ml in 500-ml Erlenmeyer flasks). After incubation for 7 days at room temperature under static conditions, the mycelial growth was separated from the medium by filtration through four layers of sterile cheesecloth. The mycelial mass was washed three times with sterile distilled water (100 ml per wash), and the mycelia were resuspended in dimethyl succinate-buffered low-N

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TABLE 1. Fungal strains used in this study

Strain	Type[a]	Source
Fomes fomentarius (Linn.:Fr.)Kickx.:TJV-93-7-T	WR	FPL[b]
Ganoderma lucidum (Leys.) Kars. 103561	WR	FPL
Ganoderma lucidum (Leys.) Kars. 58537	WR	FPL
Gloeophyllum trabeum (Pers.:Fr.) Murrill FP-105470-Sp	BR	FPL
Gloeophyllum trabeum (Pers.:Fr.) Murrill Madison-539-T	BR	FPL
Gloeophyllum trabeum (Pers.:Fr.) Murrill Madison-617-R	BR	FBL
Grifola frondosa Dick.:Fr.	WR	Lab stock
Lentinula edodes (Berk.) Pegl.:RA-3-2-E	WR	FPL
Lentinus (*Panus*) *tigrinus* Bull.:Fr.	WR	Lab stock
Phlebia brevispora Nakas.:HHB-7099-Sp	WR	FPL
Phlebia tremellosa (Schrad.:Fr.) Nakas. et Burds.:FP-101416-Sp	WR	FPL
Pleurotus ostreatus (Jacq.:Fr.) NRRL 2366	WR	NRRL[c]
Postia balsamea (Peck) Julich RLG-13906-Sp	BR	FPL
Postia placenta (Fr.) M. J. Lars. & Lomb. Mad-575	BR	FPL
Trametes (*Coriolus*) *versicolor* (Linn.:Fr.) ATCC 12679	WR	ATCC[d]
Wolfiporia cocos (Wolf) Gilbn. & Ryv. FP-90850-Sp	BR	FPL

[a] Type of wood decay: WR, white rot; BR, brown rot.
[b] USDA Forest Products Laboratory, Madison, Wis.
[c] Northern Regional Research Center, Agricultural Research Service, USDA, Peoria, Ill.
[d] American Type Culture Collection, Rockville, Md.

(2.4 mM N) or high-N (24 mM N) liquid medium (8) and blended for 5 min on ice with an Omni-mixer (Ivan Sorvall, Inc., Newtown, Conn.). The blended mycelia were inoculated into dimethyl succinate-buffered low-N or high-N medium (1 ml of inoculum per 10 ml of medium in 125-ml Erlenmeyer flasks). The cultures were incubated at room temperature (25°C) under static or shaken (200 rpm) conditions and were flushed with 100% oxygen at the time of inoculation and every third day thereafter. Aliquots (100 μl) were aseptically removed from these cultures at various times during incubation (see Results) and tested for activity.

Isolation of DNA. Two mycelial plugs (5 mm in diameter) of different fungi grown on plates of ME agar were inoculated into ME liquid medium (100 ml in 500-ml Erlenmeyer flasks) containing (per liter) 20 g of malt extract, 20 g of glucose, and 1 g of peptone. The cultures were incubated at room temperature for about 10 days, after which the mycelia were harvested from the culture medium by filtration through four layers of sterile cheesecloth. Genomic DNA was isolated from each fungus by the procedure described by Rao and Reddy (27).

PCR amplification. Laccase contains four copper atoms per molecule or subunit (reviewed in reference 34). Structure-function studies have shown that 1 cysteine and 10 histidine residues are involved in binding the four copper atoms and that the sequences around these residues are highly conserved in different laccases. Two pairs of histidines are located in the N-terminal domain, and the remainder of the histidines are found near the C terminus. Panels A and B of Figure 1 show the conserved sequences (designated I and II) around the two pairs of histidines present in the N-terminal domain. Forward and reverse degenerate PCR primers based on the conserved sequences in the copper-binding regions I and II were constructed (Fig. 1C). Individual genomic DNA isolated from each of the wood rot fungi was used as the template in the PCR. For PCR amplification, 20 μl of template DNA (2 ng/μl) was mixed with 10 μl of 10× *Taq* buffer (GIBCO BRL, Bethesda, Md.), 3 μl of $MgCl_2$ (50 mM), 2 μl of each deoxynucleoside triphosphate (10 mM), 5 μl of each of the primers (10 μM), and 0.5 μl of *Taq* polymerase (5 U/μl) (BRL), adjusted to 100 μl with sterilized deionized distilled water, and overlaid with 100 μl of sterile mineral oil. The DNA amplification was performed in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.) with an initial cycle of denaturation (5 min at 94°C), annealing (2 min at 54°C), and extension (20 min at 72°C) followed by 35 cycles of denaturation (1 min at 94°C), annealing (2 min at 54°C), and extension (5 min at 72°C) and then by a final incubation (10 min at 72°C). After amplification, each reaction mixture was mixed well with 100 μl of chloroform and the aqueous phase was separated from the mineral oil-chloroform phase. The aqueous phase was further purified on a MicroSpin S-400 column (Pharmacia Biotech, Piscataway, N.J.) as specified by the manufacturer. A 10-μl sample from each reaction mixture was loaded on an agarose gel (1% NuSieve, 2% SeaKem GTG; FMC, Rockland, Maine) and electrophoresed in Tris-acetate-EDTA buffer (32) for 3 h at 80 V. The 123-bp DNA size marker set (GIBCO BRL) was run in a separate lane. The gel was stained with ethidium bromide, and DNA bands were visualized under UV light and photographed.

Cloning and sequencing of PCR-amplified products. DNA bands corresponding to the major PCR-amplified products were sliced out of the agarose gels, and the DNA was purified with the Sephaglas BandPrep kit (Pharmacia Biotech, Piscataway, N.J.). Selected PCR-amplified products were cloned into a T-vector system devised from pBluescript (SK^+; Stratagene, La Jolla, Calif.) as described by Marchuk et al. (20). Plasmid DNA, containing the cloned PCR-amplified products, from *Escherichia coli* XL1-Blue cells (Stratagene) was extracted and purified from 5 ml cultures using the FlexiPrep kit (Pharmacia Biotech). Cloned PCR-amplified products were then sequenced with T3 and T7 fluorescent primers and the ABI fluorescent sequencing system (Applied Biosystems, Inc., Foster City, Calif.). The sequencing was done in both directions with the Catalyst Labstation Robot (Applied Biosystems, Inc.), and the products were analyzed on a model 373A sequencer at the Michigan State University DOE-PRL Plant Biochemistry Facility. Nucleotide similarities and translation of the exon sequences were done with the GENEPRO program (Riverside Scientific Enterprises, Seattle, Wash.), and the sequences were aligned visually.

Southern hybridization. Genomic DNA, isolated from *Ganoderma lucidum* FP-58537-Sp, *Gloeophyllum trabeum* Mad-617-R, *Pleurotus ostreatus*, and *Fomes fomentarius*, was digested with *Eco*RI. A Southern blot of the digested DNA, electrophoresed on a 1% agarose gel, was probed with the *Gloeophyllum trabeum* Mad-617-R laccase PCR product, labeled with [α-^{32}P]dCTP by random-prime labeling (32) for 24 h at 42°C, and subjected to post-hybridization washes at medium stringency (32). The hybridized Southern blot was then exposed to an X-ray film for 48 h at −70°C.

Enzyme assays. Laccase activity was determined spectrophotometrically with ABTS [2,2′-azinobis(3-ethylbenzathiazoline-6-sulfonic acid)] as the substrate (23). The reaction mixture (1 ml) consisted of 0.5 ml of culture fluid and 0.5 ml of 50 mM glycine-HCl buffer (pH 3.0) containing 14 μmol of ABTS. The activity was monitored by measuring the change in A_{418} for 5 min. The laccase assays were performed at pH 3.0 with ABTS as the substrate, because this was the optimum pH for laccase activities in both *Gloeophyllum trabeum* and *Ganoderma lucidum* (results not shown). The enzyme activity was expressed as nanokatals or microkatals (nanomoles or micromoles, respectively, of substrate transformed per second) in 1 liter of extracellular culture fluid. The laccase activity in extracellular culture fluids treated with excess catalase (to remove H_2O_2) was similar to that in untreated culture fluids, indicating lack of interference by peroxidase activity. Laccase activity was further confirmed by using syringaldazine as the substrate (19) at the pH optima of 5.5 to 6.0. The extracellular culture fluids were also tested for the presence of tyrosinase (28), LIP, and MNP as previously described (8).

RESULTS

PCR amplification of sequences. The conserved copper-binding regions I and II around the two pairs of histidines in the N-terminal domains of laccases of white rot fungi are shown in Fig. 1. From previously published laccase sequences, the expected size of the PCR-amplified products, obtained with the PCR primers described in Fig. 1, was around 200 bp. Analysis of the PCR-amplified products by agarose gel electrophoresis showed a DNA band of ~200 bp in most of the fungi tested (Fig. 2). *Ganoderma lucidum* 103561, *Grifola frondosa*, *Lentinula edodes*, and *Lentinus tigrinus* (lanes 3, 6, 7, and 8, respectively) showed, in addition to the 200-bp PCR fragment, a 144-bp PCR product. *Grifola frondosa* and *Lentinus tigrinus* also gave several minor products in addition to the 144- and 200-bp bands (lanes 6 and 8, respectively). However, *Gloeophyllum trabeum* Mad-617-R gave a single 144-bp PCR product (lane 5) whereas *Fomes fomentarius* (lane 2) and *Pleurotus ostreatus* (lane 11) gave no PCR products, even though both these organisms are known to produce laccases (see Discussion).

Sequence analysis of the PCR-amplified products. The nucleotide sequences of the ~200 bp PCR products are shown in Fig. 3. The nucleotide sequences of the second, smaller PCR products (144 bp) of *Lentinula edodes*, *Ganoderma lucidum* 103561, *Gloeophyllum trabeum* Mad-617-R, and *Lentinus tigrinus* were also determined to find if these organisms contained laccase-gene specific sequences (Fig. 3). The results (Fig. 3 and 4) showed that both the larger and smaller PCR products contained laccase gene-specific sequences. Computerized com-

[A] Protein sequences

Copper-binding region I							Copper-binding region II						
H	W	H	G	F	F	Q	T	F	W	Y	H	S	H

[B] Nucleotide sequences

Copper-binding region I														Copper-binding region II													
		T			T			T	T	G							T			C	T	C	T				
5′-	C	A	C	T	G	G	C	A	C	G	G	N	T	T	C	T	T	C	A	A	5′-	A	C	N	T	T	C	T	G	G	T	A	T	C	A	C	A	G	T	C	A	C

[C] PCR primers

Primer I (Forward primer)														Primer II (Reverse primer)													
		T			T			T	T							A		G		G		G				A	
5′-	C	A	C	T	G	G	C	A	C	G	G	N	T	T	C	T	T	C	A	5′-	G	T	G	A	C	T	A	T	G	A	T	A	C	C	A	G	A	A	N	G	T

FIG. 1. Design of degenerate PCR primers corresponding to the first two consensus copper-binding regions in the N-terminal domains of laccases from white rot fungi. [A] Conserved amino acid sequences in copper-binding regions I and II. [B] Degenerate nucleotide sequences corresponding to copper-binding regions I and II shown in panel A. For Ser (S), only two (of the possible six) codons were selected, because they were known to be conserved in the lignin-degrading basidiomycetes *Coriolus hirsutus* (18), *Phlebia radiata* (31), and PM1 (7) at that position. [C] Degenerate forward and reverse PCR primers based on the nucleotide sequences given in panel B.

parison of the nucleotide sequences with the nucleotide sequences in gene databases, using the BLASTN program (1), showed that the sequenced basidiomycete PCR products were indeed laccase gene-specific sequences. This conclusion is further supported by the fact that the PCR product of *Trametes versicolor*, a widely studied ligninolytic basidiomycete included in this study, had 93% nucleotide similarity (100% similarity in deduced amino acid sequences) to the corresponding region of the laccase gene from a different strain of *Trametes versicolor* (synonym: *Coriolus versicolor*) previously reported (22). Moreover, the nucleotide sequences as well as the deduced amino acid sequences of *T. versicolor* had a high degree of similarity to the corresponding sequences in the laccase genes of three other basidiomycete species: *Coriolus hirsutus*, *Phlebia radiata*, and strain PM1 (Fig. 3). However, the amino acid similarity to the corresponding sequences in the ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa* and to the litter fungus, *Agaricus bisporus*, was quite low (Fig. 4).

Laccase activity. The data presented in Fig. 3 and 4 strongly support the presence of laccase gene-specific sequences in the brown rot fungus *Gloeophyllum trabeum* Mad-617-R. Since there have been no published reports on the presence of laccase activity in any brown rot fungus, we studied the time course of laccase production in this organism. We found that the organism produces laccase at levels comparable to those reported for a number of white rot fungi (7, 11, 25, 30) and that maximal activity is seen on about day 5 of incubation (Fig. 5A). Although the activity drops after day 5, relatively high residual levels of activity are seen even on day 10. We extended this study by initiating experiments to determine if other brown rot fungi also produced laccases. The results showed that laccases are produced in different amounts by a number of other brown rot fungi (Table 2). *Postia placenta* Mad-575 produced higher levels of laccase (90 nkat/liter) than did the other brown rot fungi. Several other brown rot fungi, including *Antrodia vaillantii* FP-90877-R and FP-104402-Sp, *Fomitopsis pinicola* HHB-14787-T and FP-133890-T, *Gloeophyllum trabeum* FP-125076-Sp, *Piptoporus betulinus* DL-1-T, and *Postia placenta* Mad-698, did not show production of laccases in the three culture media used (data not shown). Further studies showed that high levels of extracellular laccase (47.5 μkat/liter [Fig. 5B]) are produced by *Ganoderma lucidum* when grown in high-nitrogen medium under shaken conditions. This is several orders of magnitude higher than that previously reported for

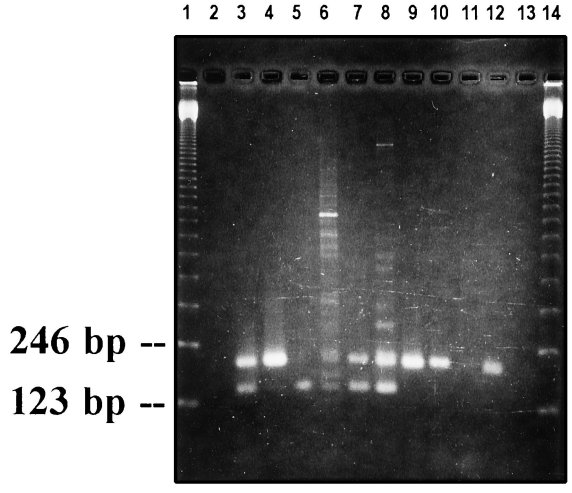

FIG. 2. Analysis of PCR-amplified products by agarose gel electrophoresis. Each PCR mixture was run on a 1% NuSieve GTG–2% SeaKem GTG (FMC) agarose gel for 3 h at 80 V. The gel was stained with ethidium bromide and photographed under UV light. Lanes: 1 and 14, 123-bp DNA ladder (GIBCO BRL); 2, *Fomes fomentarius*; 3, *Ganoderma lucidum* 10356; 4, *Ganoderma lucidum* 58537; 5, *Gloeophyllum trabeum* Mad-617-R; 6, *Grifola frondosa*; 7, *Lentinula edodes*; 8, *Lentinus tigrinus*; 9, *Phlebia brevispora*; 10, *Phlebia tremellosa*; 11, *Pleurotus ostreatus*; 12, *Trametes versicolor*; 13, control reaction mixture without DNA. Note that *Fomes fomentarius* and *Pleurotus ostreatus* showed no bands (lanes 2 and 11).

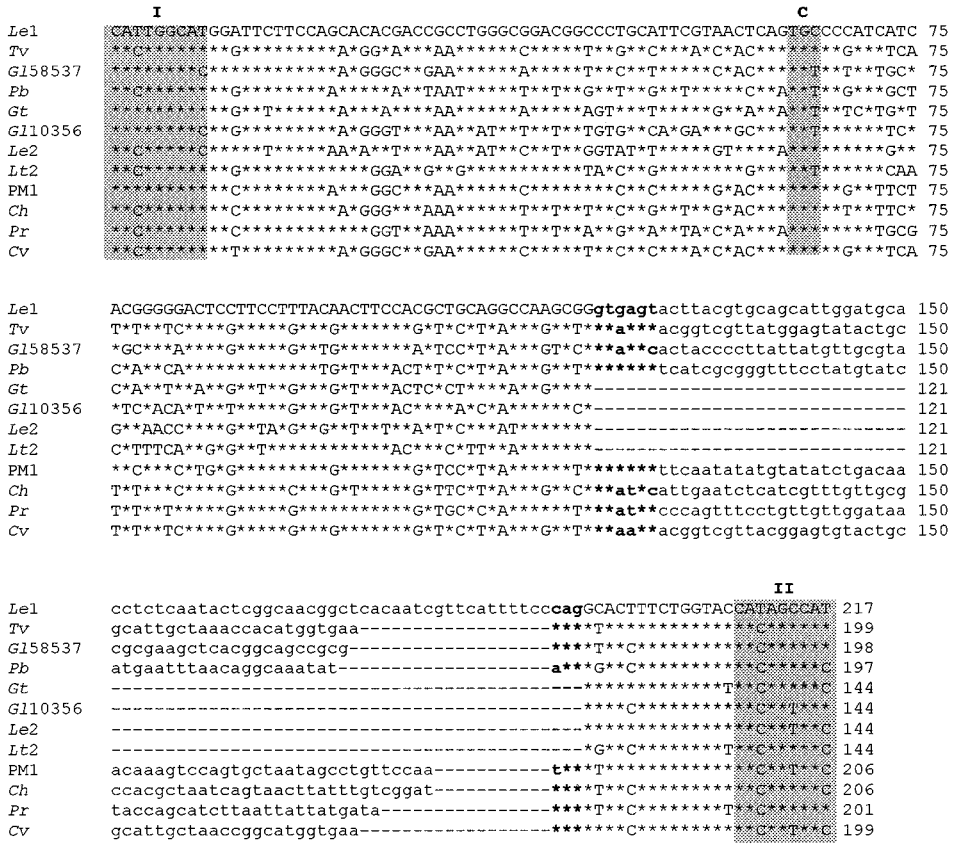

FIG. 3. Alignment of the nucleotide sequences of PCR-amplified products. The region amplified was between copper-binding regions I and II (shown boxed) in the N-terminal domains of the laccases of *Trametes versicolor* (*Tv*), *Phlebia brevispora* (*Pb*), *Ganoderma lucidum* 103561 (*Gl*10356), *G. lucidum* 58537 (*Gl*58537), *Lentinula edodes* (*Le*), *Lentinus tigrinus* (*Lt*), and *Gloeophyllum trabeum* (*Gt*). *Le*1 and *Le*2 are the 217- and 144-bp PCR products, respectively, of *Lentinula edodes*. Corresponding sequences from PM1 (7), *Coriolus hirsutus* (*Ch*) (18), *Coriolus versicolor* (*Cv*) (22), and *Phlebia radiata* (*Pr*) (31) are presented for comparison. Deduced exon and intron sequences are presented as capital and lowercase characters, respectively. Consensus intron splice sites are presented as bold lowercase characters. The intron sequences were based on comparison with the published sequences (7, 18, 22, 31) and consensus sequences for 5′ splicing GT(AG)(AT)GT and 3′ splicing (CT)AG junctions present in filamentous fungi (3). Intron sequences were not aligned because of the low homology between them. Asterisks are used in every position where the sequence matched that of *Le*1; gaps are indicated by hyphens. The nucleotides coding for the conserved putative cysteine (involved in forming a disulfide bridge) are shown in the shaded box marked C.

many of the basidiomycete laccases (23–25, 30), except strain PM1, which was reported to produce very high levels of laccase activity (7). Laccase activity in both *Gloeophyllum trabeum* and *Ganoderma lucidum* was also demonstrated with syringaldazine as the substrate, although, as expected, the activity levels were 7- to 10-fold lower than those seen with ABTS as the substrate (results not shown).

DISCUSSION

Laccases are copper-containing oxidases which catalyze the four-electron oxidation of a variety of phenolic compounds and a simultaneous four-electron reduction of oxygen to water. The PCR strategy used in this study is based on the use of degenerate primers corresponding to the consensus sequences conserved in the copper-binding regions in the N-terminal domains of known basidiomycete laccases (7, 18, 21, 22, 26, 31, 34). The results show that this is a useful and valid approach for screening wood rot fungi for the presence of laccase gene-specific sequences. On the basis of published reports (7, 18, 22, 26, 31), the expected size of the PCR product, with the primers described in Fig. 1, was about 200 bp. In agreement with previous results reporting laccase activity in *Grifola frondosa*, *Ganoderma lucidum*, *Lentinula edodes*, *Lentinus tigrinus*, *Phle-*

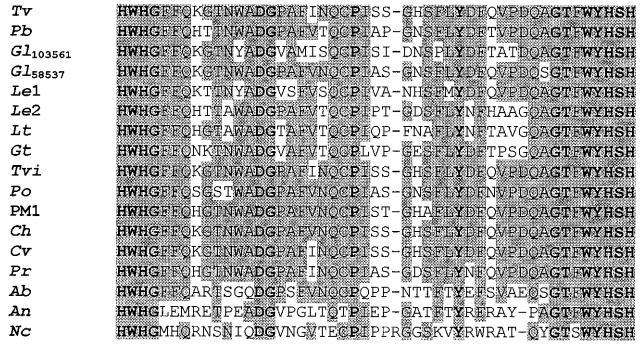

FIG. 4. Alignment of the predicted amino acid sequences of the PCR-amplified products. The sequences are compared with the corresponding sequences from *Trametes villosa* (*Tvi*) (36), *Pleurotus ostreatus* (*Po*) (13), PM1 (7), *Coriolus hirsutus* (*Ch*) (18), *Phlebia radiata* (*Pr*) (31), *Agaricus bisporus* (*Ab*) (26), *Aspergillus nidulans* (*An*) (2), and *Neurospora crassa* (*Nc*) (12). The abbreviations for the other fungal strains are described in Fig. 3. Alignment was done with the GENEPRO program (Riverside Scientific Enterprises). The program introduces gaps where necessary to optimize the alignments. Amino acids with a ≥50% match are highlighted. Invariant amino acids are shown in bold.

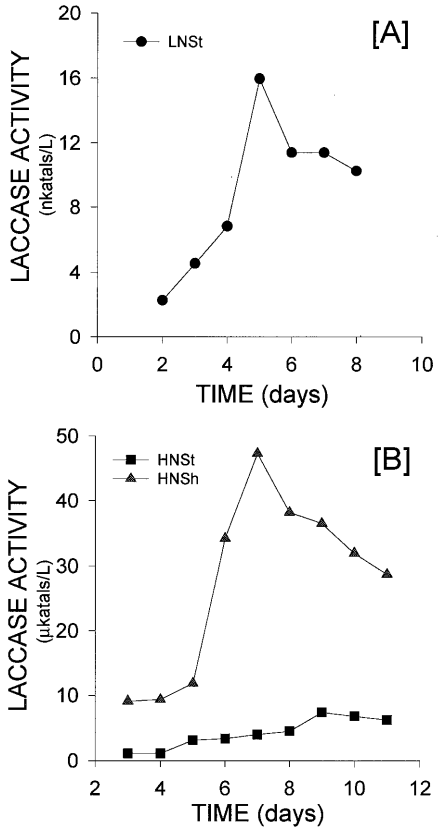

FIG. 5. Production of laccase activity by the brown rot fungus *Gloeophyllum trabeum* Mad-617-R [A] and the white rot fungus *Ganoderma lucidum* 58537 [B]. The cultures were grown in dimethyl succinate-buffered low-nitrogen (2.4 mM N) and high-nitrogen (24 mM N) liquid medium (10-ml volumes in 125-ml Erlenmeyer flasks) as described by Dass and Reddy (8). The cultures were incubated at room temperature under static conditions or on a shaker (220 rpm) and tested for laccase activity on the days shown. Laccase activity was determined spectrophotometrically with ABTS as the substrate (23). LNSt, low-nitrogen static; HNSt, high-nitrogen static; HNSh, high-nitrogen shaken.

bia brevispora, *Phlebia tremellosa*, and *Trametes versicolor*, all these organisms gave the expected 200-bp PCR product (25; reviewed in references 9 and 14). However, *Fomes fomentarius* and *Pleurotus ostreatus*, two white rot fungi previously reported to produce laccases (9), failed to yield laccase PCR products in three separate experiments in which different genomic DNA preparations were used as templates. The reason for this failure could be attributed to the possible presence of an intron in either one or both DNA template regions corresponding to the primers, thereby resulting in low binding of either one or both primers to the DNA template. Consistent with this suggestion, an intron has been reported previously in the copper-binding region II of the laccase gene of *Agaricus bisporus* (26), and Giardina et al. (13) recently showed the presence of an intron in the copper-binding region II of the laccase gene of *Pleurotus ostreatus*. The presence of laccase gene-specific sequences in *Pleurotus ostreatus* and *Fomes fomentarius* is further supported by our Southern hybridization data (Fig. 6), which showed the presence of positive hybridization bands when the *Gloeophyllum trabeum* laccase PCR product was used as the probe (lanes 3 and 4); however, the hybridization bands obtained for *Fomes fomentarius* were weak.

Several white rot fungi, including *Ganoderma lucidum* 10356, *Gloeophyllum trabeum* Mad-617-R, *Grifola frondosa*, *Lentinula edodes*, and *Lentinus tigrinus*, each gave a smaller (~150-bp)

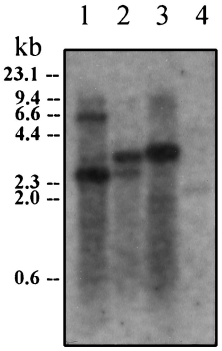

FIG. 6. Southern hybridization with a laccase PCR product as the probe. Shown is a Southern blot of the *Eco*RI-digested genomic DNA of selected wood rot fungi probed with the [α-^{32}P]dCTP-labeled laccase PCR product of *Gloeophyllum trabeum* 617-R. Lanes 1 through 4 contained DNA from *Ganoderma lucidum* 58537, *Gloeophyllum trabeum* Mad-617-R, *Pleurotus ostreatus*, and *Fomes fomentarius*, respectively.

PCR product. All these fungi, except *Gloeophyllum trabeum*, also gave the expected ~200-bp PCR product (Fig. 2). Sequence analysis (Fig. 3) showed that these smaller PCR products have relatively high nucleotide similarities to the corresponding sequences of the larger, 200-bp PCR products, except that they lack the intron (3) present in the larger products, similar to the laccase genes from *Neurospora crassa* OR and TS (12). Our results also showed that the amino acid sequences deduced from the nucleotide sequences of the smaller PCR products have relatively low similarity to those of the larger PCR products from the same organism. For example, the larger, 200-bp PCR product (*Gl*1) and the smaller, 144-bp PCR product (*Gl*2) of *Ganoderma lucidum* had 70% amino acid similarity to each other but *Gl*1 had 91% similarity to the 200-bp PCR product of *Trametes versicolor*. Similarly, the 200-bp PCR product (*Le*1) and the 144-bp PCR product (*Le*2) of *Lentinula edodes* had 66% amino acid similarity but *Le*1 had 79 and 81% amino acid similarity, respectively, to the 200-bp PCR products of *Coriolus versicolor* and *Coriolus hirsutus*. These results suggest that in fungi which yielded two PCR products, the smaller PCR product represents a different laccase gene from that represented by the larger PCR product. Similar laccase gene polymorphism has also been shown in other fungi (12, 16, 18, 26). We cannot completely rule out the possibility that the smaller PCR product represent pseudogenes, although this seems unlikely because only the smaller

TABLE 2. Laccase activity in liquid cultures of selected brown rot fungi[a]

Fungus	Laccase activity (nkat/liter) in:		
	LN	HN	ME
Gloeophyllum trabeum FP-105470-Sp	19[b]	14[b]	0
Gloeophyllum trabeum Mad-539-T	19	14	29
Gloeophyllum trabeum Mad-617-R	29	24	0
Postia balsamea RLG-13906-Sp	19	33	10[b]
Postia placenta Mad-575	90[b]	29	29
Wolfiporia cocos FP-90850-Sp	0	67	0

[a] Brown rot fungi were grown in low-nitrogen (LN), high-nitrogen (HN), and ME liquid media (as described in Materials and Methods) at room temperature under static conditions. The cultures were oxygenated on the day of inoculation and every third day thereafter. Laccase activity is presented as nanokatals per liter of extracellular fluid from cultures harvested on day 6 (peak activity), unless stated otherwise.

[b] In these cultures, peak activity was seen on day 9 after inoculation.

PCR product is amplified from *Gloeophyllum trabeum*, which also has been shown to produce laccase activity (Fig. 5A) (see discussion below).

The *Gloeophyllum trabeum* PCR product showed high similarity (70 to 81%) to the corresponding amino acid sequences deduced from laccase gene sequences of *Trametes versicolor*, *Coriolus hirsutus*, *Coriolus versicolor*, *Lentinula edodes*, *Lentinus tigrinus*, *Phlebia brevispora*, *Phlebia radiata*, PM1, and *Ganoderma lucidum*. There have been very few studies on the production of lignin-modifying enzymes by brown rot fungi barring a couple of reports on the production of MNP (33) and LIP (10). Also, the production of laccase activity by brown rot fungi has not been previously reported. In this study, we have demonstrated for the first time the presence of laccase activity in the brown rot fungus *Gloeophyllum trabeum* Mad-617-R. To rule out the possibility of interference by peroxidase, the ABTS assays were repeated by adding excess catalase to the extracellular culture fluids to remove H_2O_2. These catalase-treated culture fluids gave activities almost identical to the untreated culture fluids (data not shown). Also, the presence of laccase activity was demonstrated with syringaldazine as the substrate. Furthermore, the culture fluids were negative for MNP, LIP, and tyrosinase. Moreover, we have recently extended the presence of laccase activity to several other brown rot fungi (Table 2).

The results indicate that the PCR primers used in this study are useful in isolating laccase gene sequences from previously uncharacterized white rot and brown rot basidiomycetes. That the PCR products represent laccase gene fragments is suggested by the following lines of evidence: (i) the expected ~200-bp PCR amplified product was obtained in 8 out of the 11 wood rot fungi tested; (ii) the sequences of the PCR products analyzed (including some smaller PCR products) had a high degree of similarity to corresponding regions of previously published laccase gene sequences (7, 13, 18, 22, 26, 31, 36); and (iii) the PCR products were identified as laccase gene fragments by the BLASTN computerized analysis program (1). The strategy outlined in this study should also be useful in isolating the individual laccase genes and cDNAs from different wood rot fungi with the PCR products as probes. This approach is being used for isolation and characterization of laccase genes from selected wood rot basidiomycetes included in this study.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Aramayo, R., and W. E. Timberlake.** 1990. Sequence and molecular structure of the *Aspergillus nidulans yA* (laccase I) gene. Nucleic Acids Res. **18:**3415.
3. **Ballance, D. J.** 1986. Sequences important for gene expression in filamentous fungi. Yeast **2:**229–236.
4. **Boominathan, K., and C. A. Reddy.** 1992. Fungal degradation of lignin: biotechnological applications, p. 763–822. *In* D. K. Arora, R. P. Elander, and K. G. Mukerji (ed.), Handbook of applied mycology, vol. 4. Fungal biotechnology. Marcel Dekker, Inc., New York.
5. **Bourbonnais, R., and M. G. Paice.** 1990. Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Lett. **267:**99–102.
6. **Bourbonnais, R., and M. G. Paice.** 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. **36:**823–827.
7. **Coll, P. M., C. Tabernero, R. Santamaría, and P. Pérez.** 1993. Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. **59:**4129–4135.
8. **Dass, S. B., and C. A. Reddy.** 1990. Characterization of extracellular peroxidases produced by acetate-buffered cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **69:**221–224.
9. **de Jong, E., J. A. Field, and J. A. M. de Bont.** 1994. Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiol. Rev. **13:**153–188.
10. **Dey, S., T. K. Maiti, and B. C. Bhattacharyya.** 1991. Lignin peroxidase production by a brown-rot fungus *Polyporus ostreiformis*. J. Ferment. Bioeng. **72:**402–404.
11. **Fukushima, Y., and T. K. Kirk.** 1995. Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl. Environ. Microbiol. **61:**872–876.
12. **Germann, U. A., G. Müller, P. E. Hunziker, and K. Lerch.** 1988. Characterization of two allelic forms of *Neurospora crassa* laccase. J. Biol. Chem. **263:**885–896.
13. **Giardina, P., R. Cannio, L. Martirani, L. Marzullo, G. Palmieri, and G. Sannia.** 1995. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. Appl. Environ. Microbiol. **61:** 2408–2413.
14. **Hatakka, A.** 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:**125–135.
15. **Higuchi, T.** 1993. Biodegradation mechanism of lignin by white-rot basidiomycetes. J. Biotechnol. **30:**1–8.
16. **Kawai, S., Y. Iimura, K. Takenouchi, Y. Katayama, and N. Morohoshi.** 1993. Cloning and sequence analysis of laccase genes and construction of a host-vector system in *Coriolus versicolor*. J. Cell. Biochem. Suppl. **17(C):**192.
17. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
18. **Kojima, Y., Y. Tsukuda, Y. Kawai, A. Tsukamoto, J. Sugiura, M. Sakaino, and Y. Kita.** 1990. Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J. Biol. Chem. **265:**15224–15230.
19. **Leonowicz, A., and K. Grzywnowicz.** 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microb. Technol. **3:**55–58.
20. **Marchuk, D., M. Drumm, A. Saulino, and F. S. Collins.** 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. **19:**1154.
21. **Messerschmidt, A., and R. Huber.** 1990. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Eur. J. Biochem. **187:**341–352.
22. **Morohoshi, N.** 1993. Construction of high activity lignin-degrading basidiomycetes. Ph.D. dissertation. Tokyo University of Agriculture and Technology, Tokyo, Japan. (In Japanese.)
23. **Niku-Paavola, M.-L., E. Karhunen, P. Salola, and V. Raunio.** 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. **254:**877–884.
24. **Orth, A. B., D. J. Royse, and M. Tien.** 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl. Environ. Microbiol. **59:**4017–4023.
25. **Peláez, F., M. J. Martínez, and A. T. Martínez.** 1995. Screening of 68 species of basidiomycetes involved in lignin degradation. Mycol. Res. **99:**37–42.
26. **Perry, C. R., M. Smith, C. H. Britnell, D. A. Wood, and C. F. Thurston.** 1993. Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. J. Gen. Microbiol. **139:**1209–1218.
27. **Rao, T. R., and C. A. Reddy.** 1984. DNA sequences from a ligninolytic filamentous fungus *Phanerochaete chrysosporium* capable of autonomous replication in yeast. Biochem. Biophys. Res. Commun. **118:**821–827.
28. **Ratcliffe, B., W. H. Flurkey, J. Kuglin, and R. Dawley.** 1994. Tyrosinase, laccase, and peroxidase in mushrooms (Agaricus, Crimini, Oyster, and Shiitake). J. Food Sci. **59:**824–827.
29. **Reddy, C. A., and T. M. D'Souza.** 1994. Physiology and molecular biology of the lignin peroxidases of *Phanaerochaete chrysosporium*. FEMS Microbiol. Rev. **13:**137–152.
30. **Rüttimann, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuña.** 1992. Ligninolytic enzymes of the white-rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Appl. Biochem. Biotechnol. **16:**64–76.
31. **Saloheimo, M., M.-L. Niku-Paavola, and J. K. C. Knowles.** 1991. Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J. Gen. Microbiol. **137:**1537–1544.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Szklarz, G. D., R. K. Antibus, R. L. Sinsabaugh, and A. E. Linkins.** 1989. Production of phenol oxidases and peroxidases by wood-rotting fungi. Mycologia **81:**234–240.
34. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
35. **Yaropolov, A. I., O. V. Skorobogat'ko, S. S. Vartanov, and S. D. Varfolomeyev.** 1994. Laccase: properties, catalytic mechanism, and applicability. Appl. Biochem. Biotechnol. **49:**257–280.
36. **Yaver, D. S., F. Xu, E. J. Golightly, K. M. Brown, S. H. Brown, M. W. Rey, P. Schneider, T. Halkier, K. Mondorf, and H. Dalbøge.** 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl. Environ. Microbiol. **62:**834–841.