

Molecular Analysis of a Laccase Gene from the White Rot Fungus *Pycnoporus cinnabarinus*

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It was recently shown that the white rot basidiomycete *Pycnoporus cinnabarinus* secretes an unusual set of phenoloxidases when it is grown under conditions that stimulate ligninolysis (C. Eggert, U. Temp, and K.-E. L. Eriksson, *Appl. Environ. Microbiol.* 62:1151–1158, 1996). In this report we describe the results of a cloning and structural analysis of the laccase-encoding gene (*lcc3-1*) expressed by *P. cinnabarinus* during growth under xyloidine-induced conditions. The coding region of the genomic laccase sequence, which is preceded by the eukaryotic promoter elements TATA and CAATA, spans more than 2,390 bp. The corresponding laccase cDNA was identical to the genomic sequence except for 10 introns that were 50 to 60 bp long. A sequence analysis indicated that the *P. cinnabarinus lcc3-1* product has a Phe residue at a position likely to influence the reduction-oxidation potential of the enzyme's type 1 copper center. The *P. cinnabarinus lcc3-1* sequence was most similar to the sequence encoding a laccase from *Coriolus hirsutus* (level of similarity, 84%).

By definition, laccases (*p*-diphenol:O₂ oxidoreductase; EC 1.10.3.2) catalyze the oxidation of *p*-diphenols and the concurrent reduction of dioxygen to water, although the actual substrate specificities of laccases are often quite broad and vary with the enzyme source (11, 29). Laccases are members of the blue copper oxidase enzyme family characterized by having four cupric (Cu²⁺) ions coordinated such that each of the known magnetic species (type 1, type 2, and type 3) is associated with a single polypeptide chain. The Cu²⁺-binding domains are highly conserved in the blue copper oxidases, and the crystallographic structure of ascorbate oxidase, another member of this enzyme class, has provided a good model for the structure of the laccase active site (30, 31). This model has been supported by the results of numerous studies of the electron transfer reactions that occur between cupric ions during catalysis (35, 39, 40).

In contrast to our understanding of the electron transfer reactions that occur in laccases, relatively little is known about the physiological functions of these enzymes. Laccases have been implicated in pigmentation (1, 9), fruiting body formation (26), and pathogenicity (7, 45), as well as in lignin degradation (41) and biosynthesis (27). Very few of these functions have been experimentally proven, and only because of the availability of multiple gene sequences and crystallographic data has it been possible to speculate about how structure-function relationships may be important in the specific roles played by these enzymes (46). Some of this speculation has involved attempts to address the apparent contradictory functions of laccases in the synthesis and breakdown of lignin (3, 11).

To better understand the role of laccases in lignin degradation by white rot fungi, we studied the ligninolytic system of *Pycnoporus cinnabarinus*, a basidiomycete that produces an unusual set of ligninolytic enzymes. Just a single isoform of laccase, but no lignin peroxidase (LiP) or manganese peroxi-

dase (MnP), was produced by this organism under conditions that stimulated lignin degradation (13). We wanted to determine more completely the pattern of phenoloxidase production in *P. cinnabarinus*, so the primary objective of this study was to analyze the structure of the *P. cinnabarinus* laccase gene and determine whether there are multiple laccase genes in the *P. cinnabarinus* genome.

MATERIALS AND METHODS

Organisms and reagents. *P. cinnabarinus* PB (= ATCC 200478), an isolate recovered from decaying pine wood in the vicinity of Sydney, New South Wales, Australia, was maintained as described previously (13). *Escherichia coli* INV α F' (One Shot competent cells) and the pCR2.1 vector used for direct cloning of PCR products were purchased from Invitrogen (San Diego, Calif.). Unless otherwise indicated, the enzymes used to manipulate DNA or RNA were obtained from Boehringer Mannheim (Indianapolis, Ind.), New England Biolabs (Beverly, Mass.), or Invitrogen (T4 DNA ligase) and were used according to the manufacturers' instructions. All chemicals and reagents were at least analytical grade.

Oligonucleotides, probes, and primers. The sequences of most oligonucleotide primers used in this study are shown in Fig. 1; the exceptions are the sequences of the oligonucleotides used to isolate the *P. cinnabarinus lcc3-1* promoter. A digoxigenin-labeled laccase probe was prepared by using primers P3 and P6 (Fig. 1). The AP oligonucleotide primer was purchased from Life Technologies (Bethesda, Md.). Other primers were synthesized at the Molecular Genetics Instrumentation Facility of the University of Georgia.

RNA isolation. *P. cinnabarinus* cultures grown for 3 days in modified Dodson medium (13) at 30°C on a rotary shaker (135 rpm) were induced with 2,5-xyloidine (10 μ M) as described previously (8). Longer cultivation times led to increased production of extracellular polysaccharides, which strongly interfered with RNA isolation. Fungal mycelia were collected by filtration, and then they were washed twice in sterile phosphate buffer (20 mM, pH 7.0) and frozen in liquid nitrogen before RNA was isolated by the method of Chomczynski and Sacchi (8). For Northern analyses, total RNA (10 μ g) was separated on a 1.4% (wt/vol) agarose gel (32), transferred to Nytran-Plus membranes (Schleicher & Schuell, Keene, N.H.), and hybridized with labeled probes under high-stringency conditions as described below for the Southern blot analysis.

Genomic DNA isolation. Mycelia from *P. cinnabarinus* grown in 250 ml of malt extract medium (15 g/liter, pH 5.0) at 30°C for 4 days were harvested, washed, and frozen in liquid N₂ as described above. High-molecular-weight genomic DNA was isolated from frozen mycelia after grinding by using a plant DNA isolation kit (Boehringer) as recommended by the manufacturer.

cDNA synthesis, 5' anchor ligation PCR, and PCR cloning. Ligation-anchored PCR (42) was used to obtain full-length *P. cinnabarinus* laccase cDNA. Total RNA (1.0 μ g) was primed by using a degenerate oligonucleotide primer (primer P1) designed to complement the third (from the amino terminus) copper-binding domain (domain III) that is conserved in laccases and other blue copper oxidases

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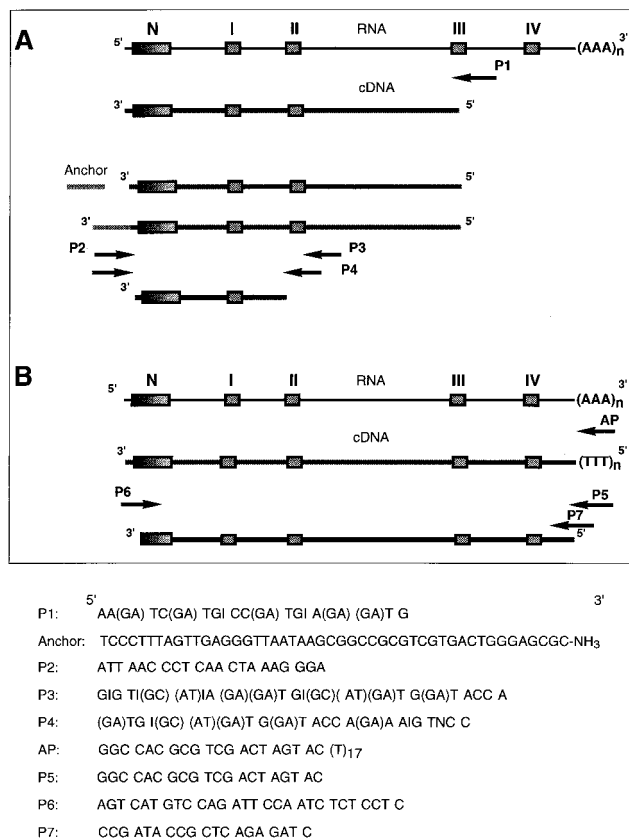


FIG. 1. Strategy used for PCR cloning of the laccase-encoding cDNA from *P. cinnabarinus* and oligonucleotide primer sequences. The boxes indicate the regions encoding the N terminus of the mature protein and the Cu(II)-binding regions of the laccase that are highly conserved in blue copper oxidases. (A) Results of laccase-specific reverse transcription of *P. cinnabarinus* RNA and anchor-ligated PCR used to amplify the 450-bp fragment of the *P. cinnabarinus* laccase cDNA on which the gene-specific primer P6 was based. (B) After primer AP-primed reverse transcription of full-length *P. cinnabarinus* mRNAs, primer P5 was used in conjunction with primer P6 to amplify the laccase-encoding sequence. Primers P6 and P7 were used to clone the genomic laccase sequence.

(Fig. 1A). Subsequent reverse transcription was performed with Superscript reverse transcriptase (Life Technologies), and an oligonucleotide anchor was ligated to the 5' end of the resultant cDNA. Primer P2, which was complementary to the anchor sequence, was used in combination with two degenerate primers, primers P3 and P4, which were synthesized to match the second copper-binding domain (domain II) conserved in laccases, to amplify a 450-bp fragment of the *P. cinnabarinus* laccase gene. For PCR amplification of the anchor-ligated cDNA with primers P2 and P3, an aliquot (2 μ l) of the ligation mixture was used as the template in a 25- μ l reaction mixture containing *Tfl* polymerase (Epicentre Technologies, Madison, Wis.). For half-nested amplification of the first PCR product, a template (1 μ l) was added to a reaction mixture containing primers P2 and P4 and Expand high-fidelity polymerase (Boehringer Mannheim). The resultant product was subcloned into the pCR2.1 vector (Invitrogen), and two clones were sequenced. From the resulting sequence, primer P6, an oligonucleotide primer whose sequence exactly matched the sequence of the 5' untranslated region of the *P. cinnabarinus* laccase mRNA, was synthesized. After reverse transcription of total *P. cinnabarinus* RNA with primer AP, primer P5, which complemented a portion of the primer AP sequence, was used in combination with primer P6 and Expand polymerase to amplify the full-length *P. cinnabarinus* laccase cDNA.

To isolate genomic laccase sequences, exact-match primers P6 and P7 (the sequence of P7 corresponded to the sequence immediately downstream of the stop codon) were used in PCR mixtures (volume, 25 μ l) containing genomic DNA (1 μ g) and Expand polymerase. Amplified products that were approximately 2,100 bp long were cloned into the pCR2.1 vector, and two of the resultant clones were sequenced on both strands.

Isolation of the *P. cinnabarinus* *lcc3-1* promoter region. *P. cinnabarinus* genomic DNA was digested with *Kpn*I, which cuts at positions 1050 and 1296 in the *lcc3-1* gene. The cleavage products were circularized by ligation with T4

DNA ligase. The putative promoter region upstream of the laccase coding sequence was amplified by a two-step inverse PCR process by using primers P_A (5'-CCACAGCGGCAAGAGAGACG-3') and P_B (5'-GAGGACAAAGGAGA GGAGAGATTGG-3') (which were directed in the 5' direction from nucleotides 343 and 319, respectively) and primer P_C (5'-GATCACCCCGTCCTCTCA-3') (which was directed in the 3' direction from nucleotide 457). Sequential PCR amplifications were performed by starting with an aliquot (2 μ l) of the ligation product in a 25- μ l reaction mixture containing Expand polymerase. The resultant 1,400-bp fragment was subcloned into the pCR2.1 vector, and two of the resultant clones were sequenced on both strands.

DNA sequencing. Nucleotide sequences were determined by using *Taq* polymerase cycle sequencing and an automated DNA sequencer (model ABI 377; Perkin-Elmer Corp., Foster City, Calif.) at the Molecular Genetics Instrumentation Facility on the University of Georgia campus. All cloned DNAs were sequenced on both strands, and the encoded amino acid sequences were predicted by using Gene Runner (Hastings Software, Hastings-on-the-Hudson, N.Y.). Sequences were aligned by using the CLUSTAL V algorithm (MegAlign; DNASTAR, Madison, Wis.).

Southern blot analysis. Restriction endonuclease-digested DNA samples (10 μ g) were separated on a 0.8% agarose gel and transferred to Nytran-Plus nylon membranes (Schleicher & Schuell) by using the procedure of Zhou et al. (49). When high-stringency conditions were used, hybridization was performed at 45°C with a DIG Easy Hyb solution (Boehringer Mannheim), the filters were washed in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% sodium dodecyl sulfate (SDS) at 24°C and then in 0.1 \times SSC-0.5% SDS at 68°C. Unless indicated otherwise, when low-stringency conditions were used, hybridization was performed at 42°C with the same hybridization solution, and the filters were washed at 24°C in 2 \times SSC-0.5% SDS and then at 49°C in 0.1 \times SSC-0.5% SDS. Blots were developed by following the manufacturers' instructions for chemiluminescent detection of digoxigenin-labeled probes with alkaline phosphatase-antibody conjugates (Boehringer Mannheim). Digoxigenin-labeled probes for the *P. cinnabarinus* laccase gene were prepared by using primers P2 and P3 to amplify a 450-bp fragment from the cloned cDNA.

Nucleotide sequence accession number. The nucleotide sequence of *P. cinnabarinus* *lcc3-1* reported in this paper has been deposited in the EMBL/GenBank database under accession no. AF025481.

RESULTS

Isolation of the laccase cDNA and characterization of the deduced protein. The longest cDNA clone of the *P. cinnabarinus* laccase gene was 1,828 bp long without the poly(A) tail and contained a 1,554-bp open reading frame. Blots of the transcription products obtained from 3-day-old mycelium probed with a 450-bp digoxigenin-labeled fragment from the cDNA clone revealed that a single transcript about 1,800 bp long was produced (data not shown). Thus, the transcript size was consistent with the length predicted from the cDNA sequence.

G and C accounted for 58% of the nucleotides in the coding sequence, and the proportion of G and C in degenerate codon positions was high (66% GC, with C [46%] \gg A [6%]). A similar or even more pronounced preference for pyrimidine bases has been found in other fungal genes (22, 36). Some examples of extreme codon bias in the *P. cinnabarinus* laccase are Leu (87% C/UUG/C versus 13% CUU), Val (80% GUG/C versus 20% GUA/U), and Phe (90% UUC versus 10% UUU).

The 21-amino-acid N-terminal sequence of the purified *P. cinnabarinus* laccase (13) was identical to residues 22 to 42 predicted from the open reading frame of the cDNA (Fig. 2). The putative 21-amino-acid signal sequence (Fig. 2) was followed by a sequence that could act as a peptidase recognition site as determined by the (-3, -1)-rule (43), which predicts that there is a small, uncharged amino acid residue (Ala) at position -1 relative to the cleavage site. Thus, the cleavage site sequence had the most common pattern, A-X-A, found in the C termini of signal peptides. The core region of the signal peptide is predominantly hydrophobic, which is typical for eukaryotic signal sequences (33).

It was predicted that the mature laccase polypeptide secreted by *P. cinnabarinus* contained 497 amino acids and had a composite molecular mass of 53,871 Da. *P. cinnabarinus* laccase purified from culture supernatants was previously shown

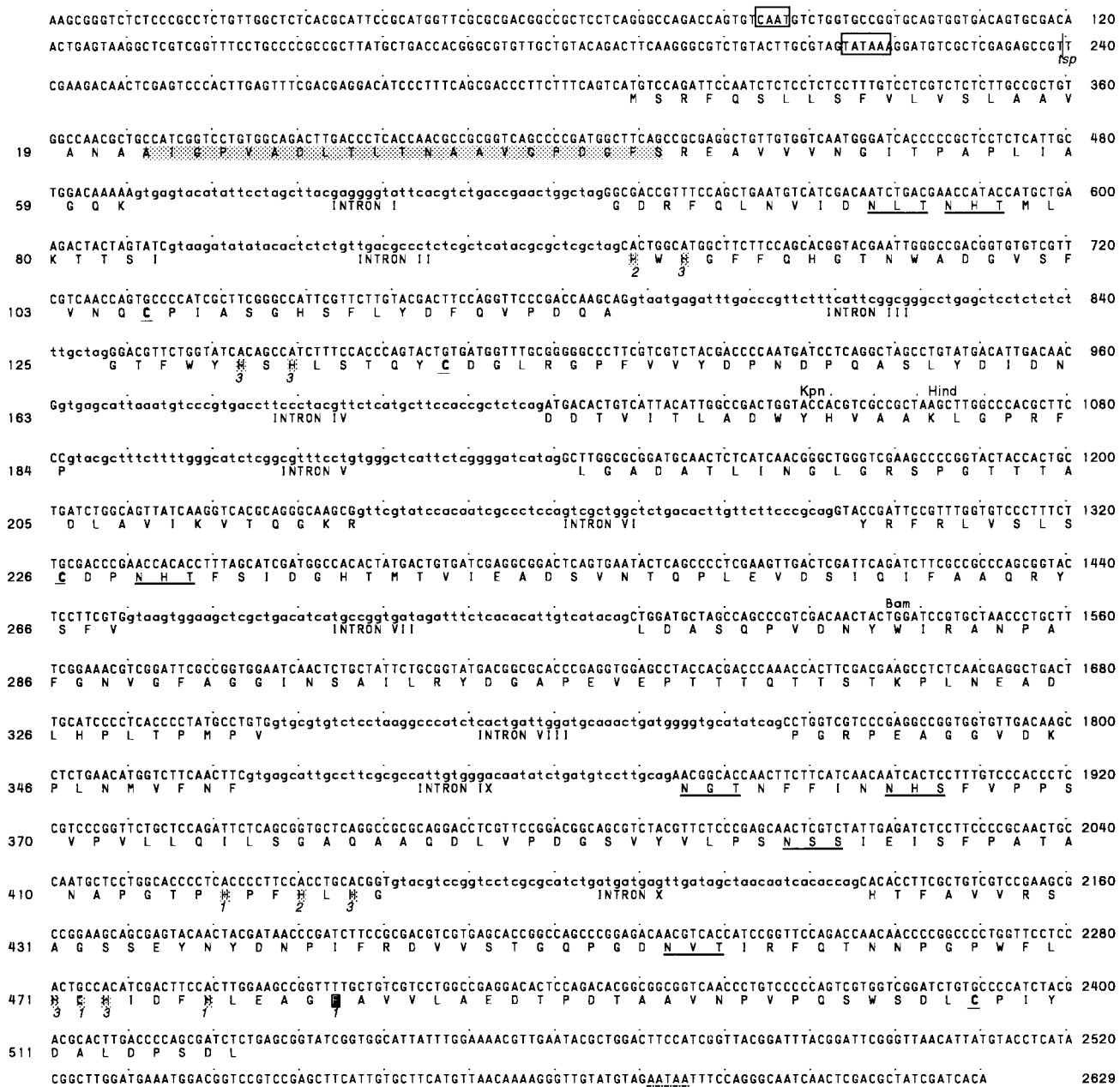


FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *P. cinnabarinus lcc3-1* gene. Putative CAAT and TATA boxes are indicated by open boxes. The N-terminal sequence of the purified laccase from *P. cinnabarinus* (13) is indicated by a shaded box. Possible N-glycosylation sites are underlined. Residues involved in binding Cu(II) ions are marked by single boxes. The numbers in italic type (1, 2, and 3) indicate with which of the three Cu(II) types the residues coordinate. The putative polyadenylation signal is underlined with a dotted line. Recognition sites for restriction endonucleases *Kpn*I, *Hind*III, and *Bam*HI, as well as the most prominent transcriptional start site (*isp*), are indicated.

to have an apparent M_r of ca. 76,000 (as determined by SDS-polyacrylamide gel electrophoresis) or 81,000 (as determined by gel filtration) (13); thus, the observed and predicted M_r values for the deduced protein differed by about 30%. Glycosylation is one form of posttranslational processing that is probably responsible for at least some of the difference (38). The laccase contains six potential N-glycosylation sites (Asn-Xxx-Ser/Thr), at positions 72, 75, 229, 354, 362, and 455 of the deduced protein (Fig. 2), although for steric reasons, it seems unlikely that the sites at positions 72 and 75 are glycosylated simultaneously. On the other hand, the carbohydrate content

reported previously for the secreted protein (13) (9%) is not sufficient to completely explain the differences between the predicted and observed molecular weights. The isoelectric point calculated for the cloned gene product (pI 4.5) also differs from the experimentally determined isoelectric point for the purified laccase (pI 3.7) (13). The amino acid residues that act as Cu^{2+} ligands are highly conserved in all blue copper oxidases, including laccases (31). All of the expected Cu^{2+} ligands (10 His residues and one Cys residue) were present in the *lcc3-1* coding sequence and are numbered in Fig. 2 on the basis of whether they coordinate

with the type 1, type 2, or type 3 Cu^{2+} centers. Another residue (Phe), which is numbered in Fig. 2 to indicate interaction with the type 1 copper center and is located nearest the carboxyl terminus of the protein, varies in laccases from different sources and is considered a residue that is probably important in governing the reduction-oxidation potential of type 1 copper centers (46).

Isolation and structural analysis of the laccase genomic sequence. A portion of the laccase gene was amplified from *P. cinnabarinus* DNA by using oligonucleotide primers whose sequences correspond to sequences identified in the 5' and 3' untranslated regions of the mRNA (Fig. 2). The 2,390-bp coding region was 526 bp longer than the corresponding cDNA sequence, and a comparison of the sequences revealed 10 short introns (length, 50 to 60 bp) in the genomic sequence.

Inverse PCR was used to isolate the laccase gene promoter. Circularized *KpnI* digests of *P. cinnabarinus* genomic DNA were amplified with primers based on the genomic laccase sequence (primers P_A [nucleotide 343], P_B [nucleotide 319], and P_C [nucleotide 457]). This approach yielded an additional 1,400 bp of upstream sequence, and 240 bp of this sequence, including putative CAAT and TATA promoter elements, is shown in Fig. 2. An analysis of 5' RACE (random amplification of cDNA ends)-amplified cDNAs strongly suggested that transcription of the laccase gene starts 68 bp upstream of the translational start site (nucleotide 240). Thus, the location of the putative TATA element in this promoter is similar to the locations found in several other fungal genes, in which the TATA box is generally located 30 to 60 nucleotides upstream from the transcriptional start site (20). Paired TATA and CAAT elements have been identified in other fungal laccase promoters (Tvi [21, 22], Po [19], Ch [24], and Pa [17]); however, although the order of these motifs is conserved, their absolute positions vary. In genes of filamentous fungi, pyrimidine-rich sequences often directly precede the transcriptional start site, particularly in highly expressed genes (2, 20); however, such sequences were not found in the *P. cinnabarinus* laccase promoter. A putative polyadenylation signal, AATAA, which is a slight variation of the consensus polyadenylation signal sequence AATAAA (37), was found 167 bp downstream of the laccase stop codon.

The 10 introns in the laccase gene were in good agreement with respect to the consensus sequence predicted for the 5' splice sites of eukaryotic genes, GT(a/g)NG(c/t) (2). Only introns 6 (T at position 3) and 3 (T at position 5) exhibited slight variations. The consensus sequence for 3' splice sites, (c/t)N(c/t)AG, also matched, except for position 1 in introns 5 and 7 (A) and introns 6 and 9 (G). The overall exon-intron structure of the *lcc3-1* gene was very similar to the structure determined for the *Coriolus hirsutus* laccase gene (24).

Laccase gene family. Southern blot analysis was used to estimate the number of laccase genes in the *P. cinnabarinus* genome. Genomic DNA that had been digested with *EcoRI*, *BamHI*, and *HindIII* was hybridized to a 450-bp digoxigenin-labeled cDNA probe spanning the region from the start of the open reading frame to the second Cu-binding domain. None of these restriction enzymes cut in the probe sequence. In *HindIII* digests, the laccase probe hybridized to a single band of approximately 8.6 kb (Fig. 3), but three *EcoRI* fragments (4.1, 5.5, and 6.7 kb) and four *BamHI* fragments (3.7, 3.9, 6.1, and 8.1 kb) were detected by the probe. Each of the fragments in the latter digests was large enough to contain a complete copy of the laccase gene.

Similarity to other laccase sequences. The results of a comparison of the amino acid sequence of the *P. cinnabarinus* laccase (encoded by the *P. cinnabarinus lcc3-1* gene) with all

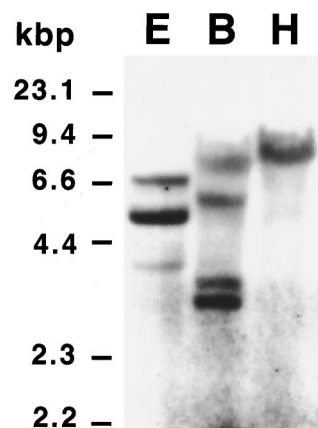


FIG. 3. Southern blot of *P. cinnabarinus* genomic DNA. Total DNA from *P. cinnabarinus* was digested with *EcoRI* (lane E), *BamHI* (lane B), or *HindIII* (lane H). The resultant DNA fragments (10 μg per lane) were resolved by agarose gel electrophoresis and blotted onto a nylon membrane. The filter was probed with a 450-bp digoxigenin-labeled fragment of *P. cinnabarinus lcc3-1*. The relative mobilities of *HindIII*-restricted lambda DNA fragments are indicated on the left.

laccase sequences available in databases are shown in Table 1. *P. cinnabarinus lcc3-1* is most closely related to *C. hirsutus phe1* (84.0% similarity), followed by *Trametes villosa lcc1* (83.0% similarity) and *lac2* of the unidentified basidiomycete strain CECT 20197 (82.6% similarity). The laccases isolated so far from the ligninolytic basidiomycetes are highly conserved (>58% similarity). In general, sequence similarity follows phylogenetic position: basidiomycetous laccases (36 to 84% similarity) > ascomycetous laccases (23 to 25% similarity) > plant laccases (18 to 20% similarity), with the notable exception of the *Aspergillus nidulans* laccase (17% similarity).

DISCUSSION

Structural similarity of laccases. A laccase-encoding gene and its corresponding cDNA were cloned from *P. cinnabarinus*, and the gene product was shown to correspond to a laccase previously isolated from ligninolytic cultures of this organism (13). The single cysteine residue and 10 histidine residues that bind the four catalytic cupric ions in all blue copper oxidases, including laccases, were conserved in the *P. cinnabarinus* gene. Studies of type 1 copper centers have shown that an additional residue 10 amino acids downstream of the conserved cysteine can have a major effect on the redox potential of the cupric ion (6). This residue was found to be a phenylalanine residue in the *P. cinnabarinus* laccase, but leucine and methionine residues have been found in the laccase sequences of other fungi and plants (Fig. 4). The results of site-directed mutagenesis studies performed with azurin (6, 31), as well as work done by Xu et al. (46), support the hypothesis that laccases harboring phenylalanine residues at this position should have type 1 copper centers with high redox potentials, whereas the copper centers of laccases with methionines at this position should have low redox potentials. On the basis of the three known possible residues at the critical position, we categorized the known laccase sequences into classes 1, 2, and 3 in order of postulated increasing redox potential. This classification is also coordinated with the proposed *Lac1* (Met)-*Lac2* (Leu)-*Lac3* (Phe) nomenclature recently submitted by one of us (J.F.D.D.) to the Commission for Plant Gene Nomenclature (CPGN) for plant laccase genes.

TABLE 1. Levels of similarity between *P. cinnabarinus lcc3-1* and other fungal and plant laccase genes^a

| Organism and gene ^b | % Similarity to gene: | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------------------------|-----------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | |
| 1. <i>P.c. lcc3-1</i> | | 84.0 | 83.0 | 75.3 | 60.9 | 67.6 | 64.5 | 68.0 | 73.7 | 82.6 | 78.4 | 76.0 | 62.5 | 58.5 | 58.3 | 43.6 | 42.5 | 37.1 | 36.1 | 35.7 | 25.5 | 25.1 | 23.4 | 20.1 | 17.8 | 21.2 | 18.7 | 19.9 | 18.3 | 17.4 | |
| 2. <i>C.h. pol</i> | | | 91.0 | 79.4 | 60.9 | 68.1 | 64.0 | 68.7 | 77.6 | 86.3 | 80.6 | 79.5 | 63.5 | 61.3 | 61.2 | 44.4 | 42.9 | 36.7 | 34.6 | 35.8 | 23.8 | 21.7 | 24.6 | 20.2 | 20.8 | 22.9 | 22.5 | 21.3 | 19.0 | 17.1 | |
| 3. <i>T.vi. lcc1</i> | | | | 77.1 | 60.3 | 68.3 | 64.0 | 68.8 | 77.1 | 84.6 | 80.0 | 78.3 | 62.5 | 58.1 | 59.8 | 43.8 | 42.7 | 37.5 | 35.4 | 36.9 | 24.8 | 23.1 | 24.2 | 20.8 | 20.6 | 22.1 | 22.5 | 21.9 | 19.8 | 17.1 | |
| 4. <i>T.vi. lcc2</i> | | | | | 62.4 | 65.1 | 62.8 | 65.7 | 84.2 | 76.3 | 75.1 | 72.9 | 60.5 | 59.9 | 59.7 | 43.4 | 42.2 | 36.6 | 35.1 | 39.1 | 23.9 | 23.1 | 23.7 | 20.0 | 21.2 | 23.1 | 22.5 | 20.4 | 20.0 | 12.1 | |
| 5. <i>T.vi. lcc3</i> | | | | | | 59.2 | 52.0 | 59.4 | 63.6 | 60.9 | 61.3 | 59.0 | 59.4 | 51.2 | 52.6 | 43.8 | 43.3 | 40.8 | 38.1 | 37.4 | 22.8 | 22.0 | 23.3 | 19.0 | 19.7 | 20.3 | 20.1 | 21.6 | 19.5 | 17.3 | |
| 6. <i>T.vi. lcc4</i> | | | | | | | 69.4 | 99.4 | 66.3 | 66.3 | 67.3 | 67.3 | 61.9 | 61.2 | 60.0 | 44.4 | 43.3 | 39.8 | 36.9 | 37.3 | 26.0 | 21.7 | 22.5 | 17.9 | 19.0 | 20.8 | 19.8 | 21.7 | 19.6 | 16.2 | |
| 7. <i>T.vi. lcc5</i> | | | | | | | | 69.8 | 63.8 | 63.1 | 63.9 | 64.0 | 57.5 | 52.9 | 48.0 | 40.6 | 39.8 | 33.8 | 33.8 | 33.0 | 22.8 | 22.6 | 21.1 | 15.6 | 19.9 | 19.4 | 20.5 | 20.1 | 15.4 | 14.6 | |
| 8. <i>T.v. lcc1</i> | | | | | | | | | 66.9 | 66.9 | 67.9 | 67.3 | 62.3 | 61.5 | 60.4 | 44.2 | 43.1 | 40.0 | 36.7 | 37.5 | 26.0 | 23.8 | 22.3 | 17.9 | 19.0 | 20.6 | 20.0 | 21.7 | 19.6 | 16.2 | |
| 9. <i>B.20197 lac1</i> | | | | | | | | | | 76.1 | 73.0 | 73.5 | 58.6 | 58.6 | 58.8 | 44.5 | 43.2 | 38.9 | 36.8 | 39.3 | 24.9 | 25.6 | 23.7 | 21.6 | 22.7 | 23.1 | 23.1 | 22.7 | 17.9 | 16.6 | |
| 10. <i>B.20197 lac2</i> | | | | | | | | | | | 77.1 | 76.6 | 61.0 | 60.0 | 59.8 | 44.2 | 42.1 | 36.5 | 35.0 | 36.0 | 22.3 | 24.2 | 23.1 | 21.2 | 21.3 | 22.7 | 22.3 | 21.0 | 18.8 | 15.4 | |
| 11. <i>B.20197 lac3</i> | | | | | | | | | | | | 72.3 | 60.7 | 61.6 | 60.7 | 44.2 | 43.1 | 37.4 | 36.8 | 38.2 | 26.5 | 22.3 | 26.0 | 22.1 | 22.5 | 24.6 | 23.5 | 22.5 | 20.6 | 11.5 | |
| 12. <i>B.PM1 lac1</i> | | | | | | | | | | | | | 64.4 | 60.0 | 59.8 | 43.9 | 43.5 | 40.2 | 36.6 | 37.5 | 23.8 | 24.4 | 23.2 | 19.0 | 22.8 | 23.6 | 23.6 | 20.3 | 18.4 | 14.5 | |
| 13. <i>P.r. lac</i> | | | | | | | | | | | | | | 55.8 | 56.8 | 41.5 | 41.7 | 36.1 | 31.9 | 38.4 | 23.4 | 23.0 | 23.5 | 18.4 | 19.7 | 19.0 | 20.6 | 20.8 | 17.3 | 16.4 | |
| 14. <i>P.o. pox1</i> | | | | | | | | | | | | | | | 89.8 | 43.5 | 42.9 | 35.5 | 35.9 | 37.6 | 22.3 | 21.9 | 23.1 | 18.7 | 18.9 | 17.8 | 17.6 | 18.9 | 19.1 | 14.4 | |
| 15. <i>P.o. pox2</i> | | | | | | | | | | | | | | | | 43.8 | 43.1 | 36.0 | 36.6 | 38.2 | 22.7 | 23.3 | 22.7 | 17.4 | 17.6 | 18.4 | 17.6 | 19.7 | 18.9 | 13.3 | |
| 16. <i>A.b. lcc1</i> | | | | | | | | | | | | | | | | | 93.1 | 39.4 | 39.0 | 41.0 | 24.4 | 22.1 | 25.0 | 19.6 | 18.8 | 20.0 | 19.4 | 18.3 | 19.6 | 14.8 | |
| 17. <i>A.b. lcc2</i> | | | | | | | | | | | | | | | | | | 40.2 | 39.2 | 41.7 | 24.0 | 22.7 | 24.6 | 19.6 | 19.6 | 20.2 | 19.8 | 18.7 | 19.2 | 18.3 | |
| 18. <i>R.s. lcc2</i> | | | | | | | | | | | | | | | | | | | 62.4 | 46.1 | 18.9 | 21.7 | 20.5 | 21.2 | 20.3 | 19.8 | 21.5 | 22.3 | 20.7 | 16.5 | |
| 19. <i>R.s. lcc3</i> | | | | | | | | | | | | | | | | | | | | 42.2 | 23.4 | 23.4 | 21.2 | 19.5 | 19.1 | 18.2 | 18.5 | 21.5 | 19.5 | 15.6 | |
| 20. <i>R.s. lcc4</i> | | | | | | | | | | | | | | | | | | | | | 22.6 | 23.4 | 23.0 | 20.3 | 21.1 | 19.6 | 20.0 | 21.8 | 22.2 | 13.4 | |
| 21. <i>P.a. lac2</i> | | | | | | | | | | | | | | | | | | | | | | 52.6 | 59.8 | 16.8 | 19.0 | 18.9 | 19.7 | 18.9 | 16.1 | 11.5 | |
| 22. <i>C.pa. lac-1</i> | | | | | | | | | | | | | | | | | | | | | | | 53.8 | 19.5 | 18.3 | 17.7 | 19.0 | 21.5 | 18.6 | 14.4 | |
| 23. <i>N.c. lac</i> | | | | | | | | | | | | | | | | | | | | | | | | 16.8 | 16.9 | 20.1 | 20.0 | 16.3 | 15.6 | 16.1 | |
| 24. <i>L.t. Lac2-1</i> | | | | | | | | | | | | | | | | | | | | | | | | | 77.4 | 75.3 | 77.4 | 54.0 | 39.8 | 14.9 | |
| 25. <i>L.t. Lac2-2</i> | | | | | | | | | | | | | | | | | | | | | | | | | | 85.0 | 91.1 | 56.7 | 42.1 | 14.2 | |
| 26. <i>L.t. Lac2-3</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | 88.9 | 56.4 | 42.5 | 14.3 | |
| 27. <i>L.t. Lac2-4</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 57.5 | 42.8 | 15.0 |
| 28. <i>N.t. pTL3</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 46.9 | 15.6 |
| 29. <i>A.p.</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 14.7 |
| 30. <i>A.n. yA</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

^a Calculated by using the CLUSTAL method with data for the inferred proteins.^b Abbreviations (and references from which data were obtained or accession no.): *P.c.*, *Pycnoporus cinnabarinus*; *C.h.*, *Coriolus hirsutus* (24); *T.vi.*, *Trametes villosa* (47); *T.v.*, *Trametes versicolor* (22); B.20197, basidiomycete strain CECT 20197 (28); B.PM1, basidiomycete strain PM1 (10); *P.r.*, *Phlebia radiata* (38); *P.o.*, *Pleurotus ostreatus* (19); *A.b.*, *Agaricus bisporus* (34); *R.s.*, *Rhizoctonia solani* (44); *P.a.*, *Podospira anserina* (17); *C.pa.*, *Cryphonectria parasitica* (7); *N.c.*, *Neurospora crassa* (18); *L.t.*, *Liriodendron tulipifera* (GenBank accession no. U73103-106); *N.t.*, *Nicotiana tabacum* (23); *A.p.*, *Acer pseudoplatanus* (25); *A.n.*, *Aspergillus nidulans* (1).

| | | | | | | | |
|-------------------------------|-----|---------------|---------------|-------------|-----|-----|---------|
| <i>P. cinnabarinus</i> lcc3-1 | 464 | N P G P W F L | H C H I D F H | L E A G F | A V | 584 | CLASS 3 |
| <i>C. hirsutus</i> po1 | 466 | N P G P W F L | H C H I D F H | L E A G F | A V | 486 | |
| <i>T. villosa</i> lcc1 | 466 | N P G P W F L | H C H I D F H | L E A G F | A V | 486 | |
| <i>lcc2</i> | 465 | N P G P W F L | H C H I D F H | L D A G F | A I | 485 | |
| <i>lcc4</i> | 465 | N P G P W F L | H C H I D F H | L E A G F | A I | 485 | |
| <i>lcc5</i> | 472 | N P G P W F L | H C H I D F H | L E A G F | A I | 492 | |
| <i>T. versicolor</i> lcc1 | 466 | N P G P W F L | H C H I D F H | L E A G F | A I | 486 | |
| Bas. 20197 <i>lac1</i> | 465 | N P G P W F L | H C H I D F H | L E A G F | A I | 485 | |
| <i>lac2</i> | 466 | N P G P W F L | H C H I D F H | L E A G F | A V | 486 | |
| <i>lac3</i> | 470 | N P G P W F L | H C H I D F H | L E A G F | A V | 490 | |
| Bas. PM1 <i>lac1</i> | 463 | N P G P W F L | H C H I D F H | L E A G F | A V | 493 | |
| <i>R. solani</i> lcc2 | 542 | N P G P W F L | H C H I D W H | L E E G F | A M | 562 | |
| <i>lcc3</i> | 515 | N P G P W F L | H C H I D W H | L E E G F | A M | 535 | |
| <i>P. radiata</i> lac | 466 | N P G P W F L | H C H I D W H | L E A A L | P L | 486 | CLASS 2 |
| <i>P. ostreatus</i> pox1 | 473 | N P G P W F L | H C H I D W H | L E I G L | A V | 793 | |
| <i>P. ostreatus</i> pox2 | 476 | N P G P W F L | H C H I D W H | L E I G L | A V | 796 | |
| <i>A. bisporus</i> lcc1 | 463 | N P G A W F L | H C H I D W H | L E A G L | A I | 483 | |
| <i>lcc2</i> | 463 | N P G A W F L | H C H I D W H | L E A G L | A I | 483 | |
| <i>T. villosa</i> lcc3 | 419 | N P G P W F L | H C H I D F H | L Q A G L | A I | 459 | |
| <i>P. anserina</i> lac2 | 541 | N P G A W L F | H C H I A W H | V S G G L | S V | 561 | |
| <i>C. parasitica</i> lac-1 | 526 | N P G A W L M | H C H I A W H | V S A G L | G N | 546 | |
| <i>N. crassa</i> lac | 541 | N P G S W L M | H C H I A W H | V S G G L | S N | 561 | |
| <i>L. tulipifera</i> Lac2.1 | 526 | N P G V W F M | H C H F D V H | L S W G L | K M | 546 | |
| Lac2.2 | 541 | N P G V W F M | H C H L E V H | T S W G L | K M | 561 | |
| Lac2.3 | 542 | N P G V W F M | H C H L E V H | T S W G L | K M | 562 | |
| Lac2.4 | 541 | N P G V W F M | H C H L E V H | T S W G L | K M | 561 | |
| <i>N. tabacum</i> pTL3 | 513 | N P G V W F M | H C H L E I H | T T W G L | K M | 533 | |
| <i>A. pseudoplatanus</i> | 519 | N P G V W F L | H C H F E R H | T T W G M | A V | 539 | CLASS 1 |
| <i>A. nidulans</i> yA | 578 | D K F P S I L | H C H I A S H | G M G M A L | | 598 | |

FIG. 4. Alignment of the amino acid sequences constituting the copper-binding domain closest to the carboxyl terminus in laccases from a variety of sources. The residues in boxes are associated with the type 1 and type 3 copper centers, as indicated by the numbers at the bottom. It has been predicted that the residues in the box farthest to the right (M, L, and F) influence the redox characteristics of the enzyme, and so these residues provide the basis for assigning laccases to class 1, 2, or 3. The numbers on either side of the sequences indicate the positions of the amino acids within the laccase polypeptide, starting from the translational start site. Annotated laccase gene sequences that lead to a putative protein sequence with an anomalous Cu-4 center, including the *Cryptococcus neoformans* (45) (GenBank accession no. L22866) and *Rhizoctonia solani* lcc4 (44) (GenBank accession no. Z54277) sequences, are not shown. *T. versicolor*, *Trametes versicolor*; Bas., basidiomycete; *P. anserina*, *Podospora anserina*; *L. tulipifera*, *Liriodendron tulipifera*; *N. tabacum*, *Nicotiana tabacum*; *A. pseudoplatanus*, *Acer pseudoplatanus*.

The *A. nidulans* (class 1) laccase exhibits very low levels of similarity to all other known laccases, including those isolated from plants. This probably reflects the very specialized function of this laccase (i.e., spore morphogenesis) (1). In contrast, the *Botrytis cinerea* laccase, whose nucleotide sequence is not known, has been implicated in the detoxification of phytoalexins (4). These examples highlight the variety of specialized physiological functions for which laccases have evolved in fungi. It remains to be seen, however, whether multiple laccases that have different physiological functions can be isolated from the same organism.

Multiple laccase genes in *P. cinnabarinus*. When grown in liquid culture, *P. cinnabarinus* secreted a single laccase isozyme (13). Stimulation of laccase secretion by adding 2,5-xylidine, a strategy previously shown to induce laccase production in a wide variety of basidiomycetes (5), did not result in production of additional bands in isoelectric focusing gels. Likewise, reverse transcriptase PCR analyses did not reveal any additional laccase transcripts when the fungus was exposed to 2,5-xylidine. The N-terminal sequences deduced for all of the cDNA-encoded laccases isolated were identical to the sequence previously determined for the laccase purified from cultures.

On the other hand, Southern analysis revealed the presence

of a small gene family encoding laccases in *P. cinnabarinus*. Whereas the laccase probe hybridized to four *Bam*HI fragments, it hybridized to only a single 8.6-kb *Hind*III fragment, suggesting that there is a laccase gene cluster in *P. cinnabarinus*. As *P. cinnabarinus* PB is a dikaryon, allelic variants of the laccase gene are expected, and the presence of four nonallelic laccase genes is highly unlikely since all of them would have to be located on the same 8.6-kbp *Hind*III fragment.

The copy numbers of laccase genes vary among fungi. A laccase gene family in which the genes encoding two of five laccases were located on the same chromosome was found in *T. villosa* (47, 48), and three laccase genes were found to be clustered within approximately 11 kb of each another in *Rhizoctonia solani* (44). *Pleurotus ostreatus* and *Agaricus bisporus* each contain at least two different laccase genes (19, 34), while allelic forms of a single laccase gene have been found in *C. hirsutus*, as well as *Neurospora crassa* (18, 24). Single copies of laccase genes were also found in *Phlebia radiata* and *Cryphonectria parasitica* (7, 38).

In *P. cinnabarinus*, the laccase purified from liquid cultures was found to be important not only for lignin degradation (14, 16) but also for synthesis of the phenoxazinone pigments which give the fruiting bodies a red color (15). In fact, a more precise name for the laccase from *P. cinnabarinus* is 3-hydroxyanthranilate:O₂ oxidoreductase. The phenoxazinone pigments and, consequently, the laccase activity can also be linked to the antimicrobial activity of this organism (12). Thus, the product of one laccase gene in *P. cinnabarinus* appears to serve two separate, but interwoven, functions in this fungus. It remains to be determined under what conditions the other laccase genes are expressed and what physiological function(s) they perform under those conditions.

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