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Purification, Characterization, Molecular Cloning, and Expression of Two Laccase Genes from the White Rot Basidiomycete *Trametes villosa*

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Two laccases have been purified to apparent electrophoretic homogeneity from the extracellular medium of a 2,5-xylidine-induced culture of the white rot basidiomycete *Trametes villosa* (*Polyporus pinsitus* or *Coriolus pinsitus*). These proteins are dimeric, consisting of two subunits of 63 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and have typical blue laccase spectral properties. Under nondenaturing conditions, the two purified laccases have different pIs; purified laccase forms 1 and 3 have pIs of 3.5 and 6 to 6.5, respectively. A third purified laccase form 2 has the same N terminus as that of laccase form 3, but its pI is in the range of 5 to 6. The laccases have optimal activity at pH 5 to 5.5 and pH ≤2.7 with syringaldazine and ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)] as substrates, respectively. The genes *lcc1* and *lcc2* coding for the two purified laccases (forms 1 and 3) have been cloned, and their nucleotide sequences have been determined. The genes for *lcc1* and *lcc2* have 8 and 10 introns, respectively. The predicted proteins are 79% identical at the amino acid level. From Northern (RNA) blots containing total RNA from both induced and uninduced cultures, expression of *lcc1* is highly induced, while the expression of *lcc2* appears to be constitutive. Lcc1 has been expressed in *Aspergillus oryzae*, and the purified recombinant protein has the same pI, spectral properties, stability, and pH profiles as the purified native protein.

Lignin is a structurally complex aromatic biopolymer which is recalcitrant to degradation. The degradation of lignin is an important step in the mineralization of carbon in nature. It has been demonstrated that the extracellular enzymes of some white rot fungi are able to degrade lignin extensively (23). These extracellular enzymes include lignin peroxidases, manganese peroxidases, and laccases. In addition to their industrial application in delignification, laccases are also known to polymerize phenolic compounds. This characteristic of laccases makes them of potential interest for industrial applications involving the polymerization of phenolics in liquids, the oxidation of dyes and dye precursors, and polymerization of lignin and lignosulfonates.

Laccases are multicopper enzymes (EC 10,3.2.2) which catalyze the oxidation of phenolic compounds and are found in both plants and fungi (28). In plants, laccases are involved in lignification (30). In fungi, besides a role in delignification, laccases appear to be involved in sporulation (26), pigment production (10, 38), and plant pathogenesis (17, 27). Laccase genes from the nonlignolytic fungi *Neurospora crassa* (18), *Cryphonectria parasitica* (8), and *Aspergillus nidulans* (2) have been cloned. Laccase genes from the lignolytic fungi *Agaricus bisporus* (32), *Coriolus hirsutus* (24), *Phlebia radiata* (36), *Coriolus versicolor* (21), *Trametes versicolor* (22), and a newly isolated lignolytic basidiomycete, PM1 (11), have been cloned. From *Agaricus bisporus*, two laccase genes that are 93% identical have been cloned. Although biochemically more than one laccase activity has been detected for a few fungi, suggesting the possibility of gene families (4, 29, 42), only one gene has been cloned from all other fungi except *Agaricus bisporus.*

Trametes villosa is a white rot fungus which has lignolytic activity (5). As found for several other fungi, extracellular laccase production in *Trametes villosa* is induced approximately 15-fold by the addition of 2,5-xylidine (4). Our study of the extracellular laccases of *Trametes villosa* was taken as a first step towards the heterologous production of a fungal laccase which would have potential utility in several industrial applications. Attempts to express the fungal laccases from *Phlebia radiata* and *Coriolus hirsutus* in *Trichoderma reesei* (35) and *Saccharomyces cerevisiae* (24), respectively, have resulted in fairly low yields of the enzymes. We report here the purification and characterization of two laccases from an induced culture of *Trametes villosa*, the cloning of the genes coding for these laccases, and the heterologous expression of one of the *Trametes villosa* laccases in *Aspergillus oryzae.*

MATERIALS AND METHODS

Materials. Chemicals used as buffers and substrates were commercial products of at least reagent grade. Chromatography was done with either Pharmacia fast protein liquid chromatography or conventional low-pressure open systems. Spectroscopic assays were conducted with either a spectrophotometer (Shimadzu UV160) with a 1-cm quartz cuvette or a microplate reader (Molecular Devices) with a 96-well plate (Costar; cell culture clusters). Peptide sequencing was done with an ABI 476A sequencer (Applied Biosystems). Triplicate quantitative amino acid analyses with internal standards were done on an HP AminoQuant instrument (Hewlett-Packard). Apparent molecular mass determination by size exclusion gel filtration was done with a Sephacryl S-300 column (diameter, 1 by 112 cm; 88-ml gel) by calibration with blue dextran (2,000 kDa), bovine immunoglobulin G (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and horse heart myoglobin (17 kDa) to calibrate the column.

Laccase purification. Two 1-liter *Trametes villosa* cultures were grown at room temperature in a defined growth medium (14). After 96 h of growth, 2,5-xylidine was added to a final concentration of 1.34 mM, and the cultures were allowed to

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grow for another 24 h. One and one-half liters of culture broth (pH 6; 2.5-mS conductivity) was filtered, centrifuged, and concentrated on a S1Y100 membrane (Amicon; spiral concentrator) at 4°C. The concentrated broth (275 ml) was adjusted to pH 7.7 with NaOH and applied at room temperature to a Q-Sepharose column (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.7). The first active laccase fraction passed through the column during loading and washing with the equilibrating buffer. The second and third active fractions were eluted with a linear gradient of 0 to 0.5 M NaCl. The active fractions named forms 1, 2, and 3 by the elution order were pooled separately, concentrated in Centricon-10 concentrators (Amicon), and then applied to a Superdex-75 column (HR10/30; Pharmacia) equilibrated with 10 mM Tris-HCl–0.15 M NaCl (pH 8). During elution from the Superdex-75 column with the application buffer, apparently pure laccase fractions were eluted at the same elution volume for all three Q-Sepharose fractions.

Laccase assay. Syringaldazine (20 μM) oxidation was performed in 23 mM Na acetate–36 μM $CuSO_4$ (pH 5.5) at 30°C, monitoring the A_{530} change with an extinction coefficient of 65 mM^{-1} cm^{-1} to calculate the rate of oxidation (3). In pH profile studies, assays were done in Britton and Robinson buffers made by mixing 0.5 M NaOH with 0.1 M phosphoric acid–0.1 M acetic acid–0.1 M boric acid to the desired pH. 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) oxidation was done with 1 mM ABTS in Britton and Robinson buffers at 20°C by monitoring the A_{405} change with an extinction coefficient of 35 mM^{-1} cm^{-1} (7). The overlay ABTS oxidase activity assay was done by pouring cooled ABTS-agarose (0.03 to 0.1 g of ABTS, 1 g of agarose, 50 ml of H_2O; heated to dissolve agarose) over a native isoelectric focusing gel and incubating at room temperature. Proteolytic stability was tested with 0.2 μg of laccase per ml and 1 μg of pronase per ml in 10 mM Tris (pH 7.5) at 37°C.

Determination of extent of glycosylation and copper content. Deglycosylation by endo F and *N*-glycosidase F was done as described in the instructions from Boehringer Mannheim. Hydrolysis of protein-bound carbohydrate for monosaccharide compositional analysis was done in duplicate. Lyophilized samples were hydrolyzed in evacuated sealed glass tubes with 100 μl of 2 M trifluoroacetic acid for 1 and 4 h at 100°C.

Monosaccharides were separated by high-performance anion-exchange chromatography with a Dionex CarboPac PA1 column eluted with 16 mM NaOH and detected by pulsed amperometric detection. Because of their different stabilities and release of monosaccharides in 2 M trifluoroacetic acid, the amounts of glucosamine and mannose were determined after 4 h of hydrolysis while the amount of galactose was determined after 1 h of hydrolysis.

Copper content was determined by 2,2′-biquinoline titration (15) and by atomic absorption spectroscopy (kindly carried out by W. Shin and E. I. Solomon of the Department of Chemistry, Stanford University).

Microorganisms. *Escherichia coli* K802 [*e14-*(*mrca*) *mcrB hsdR2 galK2 galT22 supE44 metB1*], *E. coli* XL-1 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZDM15* Tn*10* (Tet^r)]} were purchased from Clonetech and Stratagene, respectively. The *Trametes villosa* (*Polyporus pinsitus* or *Coriolus pinsitus*) strain (CBS 678.70) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *Aspergillus oryzae* A1560 has been described elsewhere (9).

Genomic DNA isolation. Cultures of *Trametes villosa* were grown in 500 ml of YEG (0.5% yeast extract, 2% dextrose) at room temperature for 3 to 4 days. Mycelia were harvested on Mira-cloth, washed twice with Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and frozen quickly in liquid nitrogen. The frozen mycelia were stored at −80°C. DNA was isolated as described previously (40).

RNA preparation. RNA was isolated from mycelia that were harvested from *Trametes villosa* cultures which either had been induced for laccase expression by the addition of 2,5-xylidine or were uninduced. After 96 h of growth at room temperature in a defined growth medium (14), 2,5-xylidine was added to a final concentration of 1.34 mM, and the cultures were allowed to grow for another 24 h. The mycelia were washed and frozen quickly in liquid N_2. RNA was isolated from the mycelia as described previously (40).

For cDNA synthesis, RNA was isolated from 10 g of *Trametes villosa* mycelium grown under xylidine induction for 6.5 h by the guanidinium-CsCl cushion method (37). The polyadenylated RNA was selected on an oligo(dT) column by standard conditions (37).

Preparation of cDNA and genomic libraries. First-strand cDNA was synthesized with reverse transcriptase (Gibco BRL) as described in the supplier's instructions. The cDNA library was constructed with the Librarian IV cDNA kit (Invitrogen). Fifty cDNA pools, each containing approximately 5,000 individual transformants, were obtained, for a total of 250,000 clones.

A library of 7- to 8-kb *Bam*HI-*Eco*RI genomic fragments was constructed in pUC118. Genomic DNA was digested with *Bam*HI and *Eco*RI, and the digest was run on a preparative agarose gel. The region of the gel containing the 7- to 8-kb fragments was excised, and the DNA was extracted from the gel with Geneclean (Bio 101). The isolated DNA was ligated with pUC118 which had been digested with *Bam*HI and *Eco*RI and treated with bacterial alkaline phosphatase (Gibco BRL). Competent *E. coli* XL-1 Blue cells were transformed with the ligation mixture, and the library contained ~8,000 recombinants.

For the preparation of a total genomic library in lambda EMBL4, *Trametes villosa* genomic DNA was partially digested with *Sau*3A. After digestion, the DNA was electrophoresed on a preparative low-melting-point agarose gel, and the region containing 9- to 23-kb-sized DNA was sliced from the gel. The DNA was extracted from the gel with β-agarase (New England Biolabs). The isolated DNA was ligated with EMBL4 arms (Clonetech) as described in the supplier's directions. The ligation was packaged in vitro with a Gigapack II kit (Stratagene). The titer of the library was determined, and the library was amplified with *E. coli* K802 cells. The unamplified library was estimated to contain 35,000 independent recombinants.

PCRs. For PCRs, 100 ng of first-strand cDNA or 100 ng of chromosomal DNA was mixed with primers under standard PCR conditions (37). The first cycle for the reaction was 95°C for 5 min, 45 to 60°C for 1 min, and 72°C for 2 min followed by 30 cycles of 95°C for 1 min, 45 to 60°C for 1 min, and 72°C for 1 to 2 min.

Southern and Northern (RNA) blots. DNA samples were electrophoresed on agarose gels in TAE (0.04 M Tris-acetate, 10 mM EDTA) buffer by standard protocols (37). RNA samples were electrophoresed on agarose gels containing formaldehyde (13). Both DNA and RNA gels were transferred to a Zeta-Probe membrane (Bio-Rad). Blots were hybridized at 65°C in 2× SSPE (0.36 M NaCl, 20 mM NaH_2PO_4 [pH 7.7], 2 mM EDTA), 1% sodium dodecyl sulfate (SDS), 0.5% nonfat dry milk, and 200 μg of denatured salmon sperm DNA per ml and washed at 65°C in 0.2× SSC (30 mM NaCl, 3 mM trisodium citrate), 1% SDS, and 0.1% $NaPP_i$. Radioactive probes were prepared with [α-^{32}P]dCTP (Amersham) and either a nick translation kit (Gibco BRL) or a random primer kit (Gibco BRL).

Quantitation of hybridization signals on Northern blots was performed with the Ambis model 4000 image acquisition and analysis programs.

Library screening. For screening of the size-selected pUC118 libraries, ~500 colonies were plated on 2× YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) medium containing 50 μg of carbenicillin per ml, and the colonies were transferred to Hybond N^+ filters (Amersham) by standard procedures (37). For screening of the genomic bank in lambda EMBL4, appropriate dilutions of the amplified library were plated with *E. coli* K802 cells on NZY plates (0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, 1% NZ amine [pH 7.5]) with 0.7% top agarose. The plaques were transferred to Hybond N^+ membranes (Amersham) by standard procedures (37). Filters were hybridized and washed under stringent conditions at 65°C as mentioned above.

DNA sequencing. Nucleotide sequences were determined by use of *Taq* polymerase cycle-sequencing with fluorescently labeled nucleotides, and reaction mixtures were electrophoresed on an Applied Biosystems automatic DNA sequencer (model 373A; version 1.2.0).

Transformation of *Aspergillus oryzae*. *Aspergillus oryzae* strains were grown for 18 h in YEG at 34°C, and protoplasts were generated and transformed as described previously (9). The protoplasts were transformed with 5 μg each of pDSY2 and a selection plasmid containing the *Aspergillus nidulans amdS* gene. Transformants were selected on minimal medium plates containing 1.0 M sucrose, 10 mM acetamide, and 15 mM CsCl (12, 39).

Screening of laccase transformants. Primary transformants were screened initially on minimal medium plates containing 1% glucose as the carbon source and 1 mM ABTS to test for production of laccase. Transformants that gave green zones on the plates were picked and spore purified before shake flask analysis was done. For the shake flask cultivation of the *Aspergillus oryzae* A1560 laccase transformants, MY51 medium [per liter, 30 g of maltodextrin, 2 g of $MgSO_4$, 10 g of KH_2PO_4, 2 g of K_2SO_4, 2 g of citric acid, 10 g of yeast extract, 0.5 ml of trace metals, 1 g of urea, 2 g of $(NH_4)_2SO_4$ (pH 6.0)] was used. Trace metals contained (per liter) 22 g of $ZnSO_4$, 11 g of H_3BO_3, 5 g of $MnCl_2 \cdot 4H_2O$, 5 g of $FeSO_4 \cdot 7H_2O$, 1.6 g of $CoCl_2 \cdot 5H_2O$, 1.6 g of $CuSO_4 \cdot 5H_2O$, 1.1 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 5 g of Na_4EDTA (pH 6.5). Transformants were grown at 37°C, and samples were centrifuged to clear the broth. The diluted culture broth was assayed with ABTS as a substrate.

Nucleotide sequence accession numbers. The sequences of the *Trametes villosa lcc1* and *lcc2* genes reported in this paper have been assigned GenBank accession numbers L49376 and L49377, respectively.

RESULTS

Purification of laccases from *Trametes villosa* culture broth. During purification, ultrafiltration with a membrane with a molecular mass cutoff of 100 kDa enriched for laccases by 21-fold. The chromatography on Q-Sepharose allowed the separation of three active fractions, and the following gel filtration yielded essentially pure *Trametes villosa* laccase preparations as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Overall, for forms 1, 2, and 3, purification of 3,000-, 2,000-, and 1,000-fold and yields of 28, 3, and 12% were achieved, respectively.

Characterization of the purified laccases. All three purified laccases showed molecular masses of ~60 to 70 kDa by SDS-PAGE (Fig. 1). Size exclusion chromatography of form 1 laccase yielded a molecular mass of 130 kDa, which suggested a

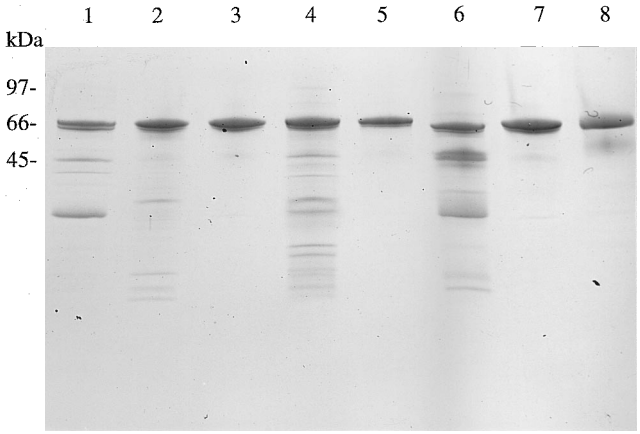

FIG. 1. SDS-PAGE of laccase samples (~2 μg per lane). Lanes: 1, concentrated (136-fold on an SY100 membrane) *Trametes villosa* broth; 2, pooled Q-Sepharose fractions of form 1; 3, purified form 1 after Superdex chromatography; 4, pooled Q-Sepharose fractions of form 2; 5, purified form 2 after Superdex chromatography; 6, pooled Q-Sepharose fractions of form 3; 7, purified form 3 after Superdex chromatography; 8, purified recombinant laccase. The electrophoresis was carried out on a 4 to 15% gel (Bio-Rad), and the staining was done with Coomassie blue (R-250).

dimeric structure of the laccase and which was consistent with the ultrafiltration results on S1Y100 membrane. Although the deglycosylation of form 1 laccase with endo F and *N*-glycosidase F resulted in a mobility increase by SDS-PAGE that corresponds to about 14% loss in mass, total carbohydrate analysis which would detect both N-linked and O-linked oligosaccharides yielded only 0.32-kDa (or 0.5% [wt/wt]) sugars (<1 mol of *N*-acetylglucosamine and 2 mol of mannose per mol of protein).

By native isoelectric focusing, form 1 laccase shows one band at pI 3.5, form 2 laccase shows five bands at pI 5, 6, 6.2, 6.5, and 6.8, and form 3 laccase shows three bands at pI 6.2, 6.5, and 6.8 (data not shown). The ABTS-agarose overlay assay shows that all of the different pI forms are enzymatically active.

Laccase form 1 and form 3 have UV-visible spectra typical of blue laccases, with maxima at 605 and 275 nm and ratios of A_{275}/A_{605} of 23 and 40, respectively. The spectrum for form 2 has a peak at 276 nm and a shoulder around 600 nm. There are 3.6 and 2.2 copper atoms per protein subunit in forms 1 and 3, respectively, as determined by titration with biquinoline.

Laccase form 2 and form 3 have the same amino acid sequences for 29 residues at their N termini, AIGPVAD LVVANAPVSPDGFLRDAIVVNG-, whereas form 1 has a different N terminus of GIGPVADLTITNAAVSPDGF SRQAVVVNG-. All three forms cross-react with polyclonal antibodies raised against form 1 laccase.

With syringaldazine as a substrate, all three forms of laccase have optimal activities at pH 5 to 5.5, 50% of their optimal activities at pH 6, and ≤5% of their optimal activities at pH ≥7. At pH 4, form 3 has 15% of its optimal activity, whereas forms 2 and 1 have 50 to 60% of their optimal activities. At 30°C and at their optimal pH, the specific activities for the laccase forms 1, 2, and 3 are 110, 90, and 38 μmol/min/mg of protein, respectively.

With ABTS as a substrate, forms 2 and 3 have the higher activity at pH 6, while form 1 has the highest activity at the lower limit of the test pH range (pH 2.7). At a pH of ≥7, the activities drop to ≤5%. At a pH of 4, forms 2 and 3 have specific activities of 126 and 253 μmol/min/mg, respectively. At pH 2.7, form 1 has a specific activity of 302 μmol/min/mg.

Cloning of laccase genes. To facilitate the cloning of the gene for laccase form 1, the amino acid sequence of peptides generated by treatment of the purified laccase with trypsin was determined (Fig. 2, underlined peptides). The sequences of these peptides show 75 to 100% identity when compared with the known sequence of the *Coriolus hirsutus* laccase (24). With first-strand cDNA from a 2,5-xylidine culture as a template and degenerate oligonucleotides based on the N terminus of the form 1 laccase (ACCAGNCTAGACACGGGNTGAGATAC TGACGNGAGAGCGGACTTGCTGGTCACTATCTTCGA AGATCTCG) and on the C terminus of the *Coriolus hirsutus* gene (CGCGGCCGCTAGGATCCTCACAATGGCCAAGT CTCTGCCTCGACCTTC), a PCR product of the expected size was obtained. The deduced amino acid sequence of the product as determined from the DNA sequence was consistent with the determined internal peptide sequences of the form 1 laccase.

A cDNA library consisting of approximately 350,000 transformants was prepared from poly(A) RNA isolated from a 2,5-xylidine-induced culture and screened with the PCR product (indicated above) as a probe. More than 100 positive clones were detected. The clones were purified, rescreened, and analyzed by digestion with restriction enzymes. The nucleotide sequence of the longest clone (*LCC* cDNA) was determined on both strands and found to contain a poly(A) track at the 3′ end and to start at amino acid 4 in the mature protein (Fig. 2). The predicted protein sequence codes for a mature protein of 499 amino acid residues, contains six potential N-glycosylation sites (Asn-X-Thr/Ser), and has 90% identity with the sequence of the *Coriolus hirsutus* laccase.

To obtain a genomic clone for the form 1 laccase, Southern blotting was done to determine what size *Bam*HI-*Eco*RI fragments should be selected for a partial genomic library construction. The cDNA clone hybridized under high-stringency conditions to *Bam*HI-*Eco*RI fragments of approximately 7.5 and 4.0 kb and to a *Bam*HI fragment of 5.5 kb (data not shown). On the Southern blot, the cDNA insert also cross-hybridized under high-stringency conditions, with less intensity, to several other genomic bands, suggesting the presence of a gene family. A size-selected library containing 7- to 8-kb *Bam*HI-*Eco*RI genomic fragments was constructed and screened with the cDNA insert as a probe. Several positive clones were purified, rescreened, and analyzed by digestion with *Bam*HI-*Eco*RI. One class of clones (*lcc1*) consisted of two independent clones which had inserts of ~7.2 kb. The nucleotide sequence of *lcc1*, which revealed that the insert contained only a partial open reading frame for the form 1 laccase, was determined. A *Trametes villosa* genomic library in EMBL4 was made and screened with the *lcc1 Bam*HI-*Eco*RI insert as a probe to identify clones containing the entire coding region for the form 1 laccase. One class of clones contained inserts which had *Bam*HI-*Eco*RI fragments of the same size as that of *lcc1*. An 8-kb *Bam*HI fragment was subcloned, and the nucleotide sequence of *lcc1* was determined on both strands by primer walking (Fig. 2). The DNA sequence was identical to that obtained from the 7.2-kb *Bam*HI-*Eco*RI clone and confirmed that *lcc1* is the genomic clone of the *LCC* cDNA.

The *lcc1* gene contains eight introns which range in size from 51 to 57 bp. The precursor protein is 520 amino acids in length and contains a signal sequence of 21 amino acids with cleavage between Ala-Gly. The protein contains six potential N-glycosylation sites.

The gene coding for the form 3 laccase was isolated in the initial attempts to isolate a genomic clone of *LCC* cDNA. A size-selected library of 5- to 6-kb genomic *Bam*HI inserts was constructed, and the library was screened with the *LCC* cDNA

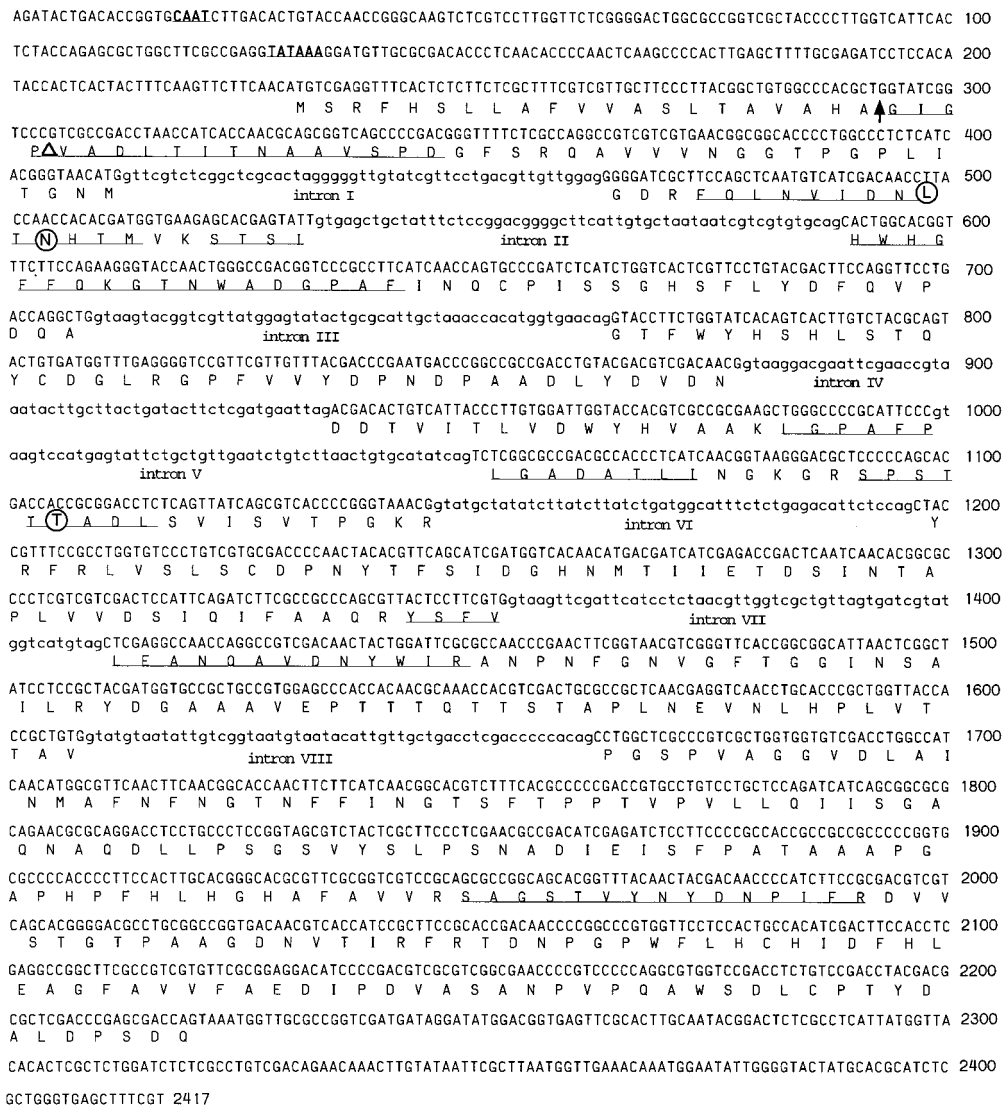

FIG. 2. Nucleotide sequence of *lcc1*. Eight introns are shown in small letters. Putative CAAT and TATA boxes are underlined and in boldface type. The cleavage site of the signal peptide is indicated by an arrow. The 5′ end of *LCC* cDNA is indicated by an empty triangle. The results from the determination of protein sequence are underlined, with differences between the peptide sequence and deduced protein sequence are circled.

insert. Two positive clones were identified and found to contain the same 5.5-kb insert. However, the DNA sequencing analysis revealed that the clone *lcc3* had about 65% identity to the *LCC* cDNA but was not the genomic clone of the cDNA. (The sequence and characterization of *lcc3* will be reported elsewhere.) From the DNA sequence, it was also determined that the *lcc3* insert did not contain the entire coding region for a laccase, and thus it could not be determined if it coded for laccase form 2 or 3. To obtain a genomic DNA fragment containing the entire coding region for *lcc3*, the *Trametes villosa* genomic lambda EMBL4 library was probed with a ~750-bp *Bam*HI-*Stu*I fragment which contained the most-5′ region of *lcc3*. One positive clone, *lcc2*, contained an ~11-kb *Eco*RI fragment which hybridized with the probe. On the basis of a partial DNA sequence, it was confirmed that *lcc2* coded for a third laccase gene. The 11-kb *Eco*RI fragment did not contain the full gene, so a lambda EMBL4 clone containing the full gene was isolated with a 680-bp *Eco*RI-*Pvu*I fragment of the 11-kb *Eco*RI insert as a probe, and a 4-kb *Hin*dIII fragment was subcloned. The complete DNA sequence of the *lcc2* gene was determined for both strands by a primer walking sequencing strategy (Fig. 3).

The *lcc2* gene codes for a precursor protein of 519 amino acids which contains a 20-amino-acid signal sequence as predicted by the von Heijne rules (41). The predicted N terminus of the mature protein is identical to the N-terminal protein sequence determined for the form 2 and 3 purified laccases except for residue 7, which during protein sequencing was identified as an Asp and in the predicted protein sequence is a Ser. During protein sequencing, it was noted that the signal in cycle 7 was weak. The predicted pI of the mature protein coded for by *lcc2* is 5.87. There are six potential N-glycosylation sites, and the coding region is interrupted by 10 introns whose positions were deduced on the basis of homology to other fungal laccases and conserved motifs found at 5′ and 3′ junctions of introns.

The predicted mature proteins coded for by *lcc1* and *lcc2* are 80% identical and have a high degree of identity to laccases

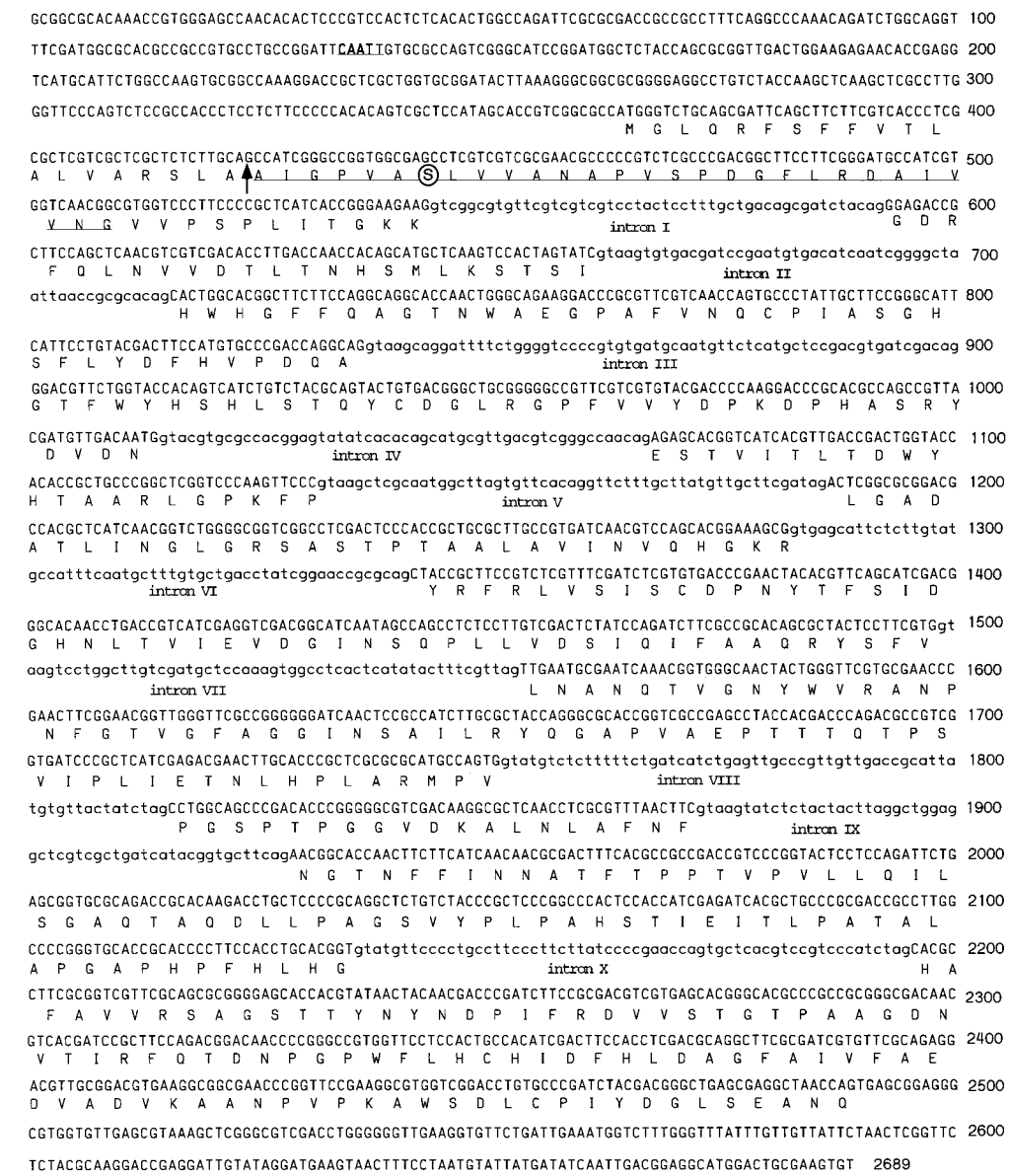

FIG. 3. Nucleotide sequence of *lcc2*. Ten introns are shown in small letters. The putative CAAT box is underlined and in boldface type. The cleavage site of signal peptidase is indicated by an arrow. The results from the determination of protein sequence are underlined, with differences between the peptide sequence and deduced protein sequence circled.

from other lignolytic fungi (Table 1). The percent identities to laccases from other white rot fungi, *Coriolus hirsutus*, *Phlebia radiata*, *Trametes versicolor*, and PM1, range from 62 to 91%. The percent identities of *lcc1* and *lcc2* to laccases from *N. crassa* and *Agaricus bisporus* range from 25 to 44%.

Regulation of *lcc1* and *lcc2* expression. To determine whether *lcc1* and *lcc2* are differentially regulated, total RNA was isolated from either a 2,5-xylidine-induced culture or an uninduced culture. Both cultures were grown in a minimal medium for 4 days, at which time 2,5-xylidine was added to one of the cultures, and both were grown for an additional 24 h. Aliquots of the culture supernatants were assayed for laccase activity with ABTS as a substrate at pH 5. The activities obtained were 0.4 and 8.25 U/ml for the uninduced and induced cultures, respectively (~20-fold induction). Aliquots of total RNA (10 μg) were electrophoresed on a denaturing gel and transferred

TABLE 1. Percent identity of *lcc1* and *lcc2* predicted amino acid sequences to sequences of other fungal laccases

Fungal laccase[a]	% Identity[b]								
	1	2	3	4	5	6	7	8	9
1		79.6	91.4	63.3	79.6	70.9	43.7	25.1	16.0
2			81.4	61.5	73.7	67.7	43.1	23.8	13.0
3				64.1	80.2	70.7	44.1	25.1	13.0
4					65.7	64.3	42.5	23.0	16.7
5						69.1	44.4	24.4	16.0
6							44.4	22.3	16.5
7								25.5	17.0
8									18.4
9									

[a] 1, *LCC1*; 2, *LCC2*; 3, *Coriolus hirsutus*; 4, *Phlebia radiata*; 5, PM1; 6, *Trametes versicolor*; 7, *Agaricus bisporus*; 8, *Neurospora crassa*; 9, *Aspergillus nidulans.*

[b] Calculated by the Clustal method.

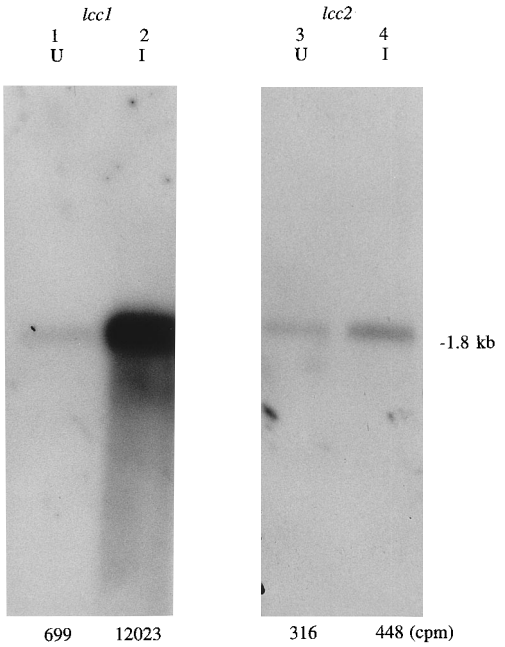

FIG. 4. Northern blot analysis of *lcc1* and *lcc2* transcripts. Total RNA was isolated from both 2,5-xylidine-induced and uninduced cultures of *T. villosa*. Ten-microgram aliquots of RNA were denatured and electrophoresed on a 1% formaldehyde-agarose gel. After transfer to a membrane, *lcc1* and *lcc2* transcripts were detected by hybridization with ^{32}P-labeled probes specific for either gene. Lanes 1 and 3 contain total RNA isolated from an uninduced culture (U). Lanes 2 and 4 contain RNA isolated from an induced culture (I). The autoradiograph containing lanes 1 and 2 was developed after 24 h of exposure. The autoradiograph containing lanes 3 and 4 was developed after 72 h. The size of the transcripts, 1.8 kb, was estimated by comparison with molecular mass standards. The counts per minute per band indicated below the lanes were measured with Ambis model 4000 image acquisition and analysis programs.

to a membrane which was probed with fragments specific for either gene and containing approximately 120 bp upstream of the initiator ATG and the first ~65 to 75 bp of the open reading frame. For the detection of *lcc1* mRNA, a probe fragment from positions 108 to 300 in the nucleotide sequence (Fig. 2) was used, and for the detection of *lcc2* mRNA, a probe fragment from positions 231 to 432 in the nucleotide sequence (Fig. 3) was used. The steady-state levels of mRNA were determined on the basis of the hybridization signal with Ambis model 4000 image acquisition and analysis programs (Fig. 4). Transcription of *lcc1* is induced ~17-fold by the addition of 2,5-xylidine to the culture, and this level of induction is close to the 20-fold increase in laccase activity. In contrast, transcription of *lcc2* is constitutive under the conditions tested. Similar results (data not shown) were obtained with poly(A) RNA.

Expression of *lcc1* in *Aspergillus oryzae*. To produce the *lcc1* gene product in larger quantities for biochemical analysis, the cDNA of *lcc1* was inserted behind the *Aspergillus oryzae* α-amylase gene promoter (9) to obtain the expression vector pDSY2 (Fig. 5). DNA fragments containing the signal sequence, transcription terminator, and polyadenylation signals were also from the α-amylase gene (9). Protoplasts of *Aspergillus oryzae* A1560 were transformed with equal amounts of pDSY2 and a selection plasmid containing the *Aspergillus nidulans amdS* gene (39). Fifty transformants were picked to a minimal medium agar plate containing ABTS, and 21 of the transformants produced green zones indicative of the production of laccase. The laccase-producing transformants were purified twice through conidiospores and inoculated into shake flasks of

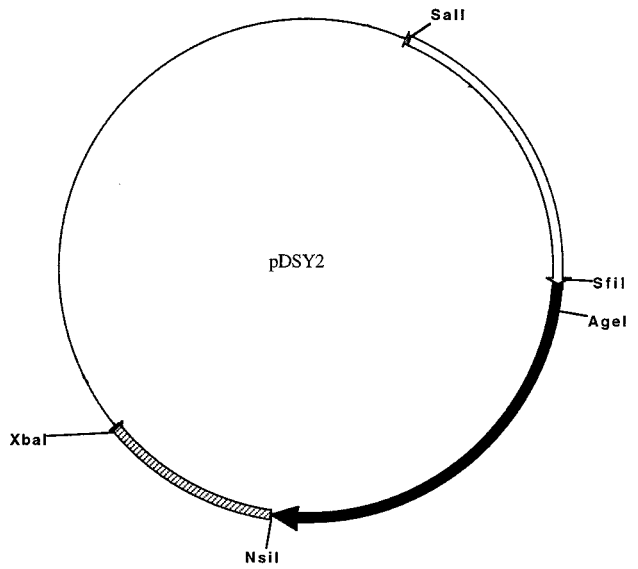

FIG. 5. Vector for *lcc1* expression. TAKA promoter sequences (open bar), *lcc1* sequences (solid bar), TAKA terminator sequences (striped bar), and pUC18 sequences (solid line) are indicated. The vector was used in combination with a selection plasmid to transform *Aspergillus oryzae*.

MY51 medium at 37°C. Samples were taken daily, and the supernatants were assayed for laccase activity with ABTS as the substrate at pH 5. A graph plotting laccase activity versus days of cultivation for a typical transformant is shown in Fig. 6. There was increased production from 48 to 168 h.

The recombinant protein was purified by a protocol similar to that used for the native enzyme (250-fold, 37% yield) and characterized biochemically. The internal protein sequence determined by protein sequencing completely matches the protein as predicted from the DNA sequence. The total mobility of the recombinant laccase is similar to that of native *Trametes*

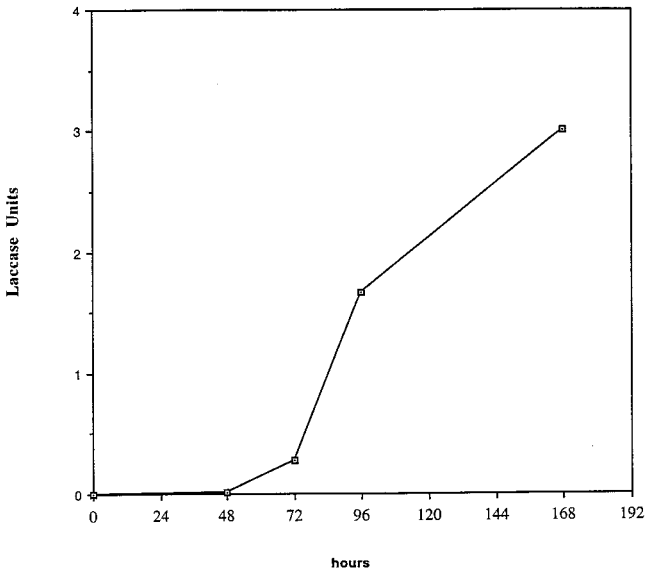

FIG. 6. Levels of laccase produced in an *Aspergillus oryzae* transformant expressing *lcc1*. The transformant was grown in 25 ml of MY51 medium at 37°C, and aliquots of the culture supernatant were assayed on the indicated days with ABTS as a substrate at pH 5.0.

villosa laccase form 1, although the band obtained with SDS-PAGE is more broad, suggesting heterogeneity in glycosylation (Fig. 1, lane 8). However, carbohydrate analysis revealed a 10% glycosylate (10% of total weight; 1 mol of *N*-acetylglucosamine, 16 mol of galactose, and 16 mol of mannose per mol of protein). The recombinant form 1 laccase has a UV-visible spectrum similar to that of the native form, with two maxima at 275 nm (extinction coefficient, 80 mM^{-1} cm^{-1}) and 615 nm (extinction coefficient, 5.3 mM^{-1} cm^{-1}). As determined by both biquinoline titration and atomic absorption spectroscopy, 4.2 copper atoms per subunit protein was found for the recombinant laccase. The recombinant laccase has a pI of 3.5 and the same pH activity profiles and optimal specific activity as the native protein on both ABTS and syringaldazine. The native and recombinant laccases demonstrate similar susceptibilities when assayed with pronase; 2 h of incubation led to 50% residual activity.

DISCUSSION

Trametes villosa has in the past been classified as *Trametes pinsitus*, *Polyporus pinsitus*, and *Coriolus pinsitus*. The existence of multiple laccases in *Trametes* species has been reported previously for *Polyporus versicolor* (4, 29, 42). Under the induction conditions used in our study, three laccase forms have been purified. Form 1 and form 3 have not only different pIs but also different N termini. The cloning of the corresponding genes and the determination of their nucleotide sequences have established the defined difference between the primary structure of form 3, coded for by *lcc2*, and form 1, coded for by *lcc1*. The genes both code for mature proteins which are 499 amino acids in length and have 80% amino acid sequence identity. Form 2 has the same N terminus and subunit molecular mass as form 3 but an experimentally determined pI of 5 to 6. In addition, form 2 does not have a spectral maxima near 600 nm, suggesting that it may have been partially depleted of type I copper during purification. The different pI of form 2 may be due to differential posttranslational modifications such as glycosylation; however, this requires further study. Determination of some of the internal protein sequence of forms 2 and 3 might give further insight into whether the two forms have identical primary protein structures. In our study, only one gene that matches the N terminus has been isolated from the *Trametes villosa* genome; however, an exhaustive search for a second gene with the identical deduced N-terminal protein sequence has not been undertaken.

The purified enzymes have a number of similarities with laccases found in other *Trametes* (*Polyporus*) species. For instance, the subunit molecular mass of 63 kDa, the 14% glycosylation extent, and the ratio of A_{280}/A_{600} equal to 20 are quite similar to that reported for *Polyporus versicolor* laccase (29, 34). Although by SDS-PAGE, as measured by a shift in total mobility upon deglycosylation, the extent of glycosylation is 14% for form 1, a carbohydrate content of 0.5% glycosylates was determined experimentally. In terms of activity, the optimal pH of 5 to 5.5 and the specific activity of 38 to 110 μmol/min/mg with syringaldazine as a substrate found for *Trametes villosa* are also comparable to that observed in *Polyporus anceps* (33), *Polyporus hirsutus* (1), and *Trametes versicolor* (4). In a recent study, two laccases were purified from *Trametes versicolor* (6). The N-terminal amino acid sequences of the *Trametes versicolor* laccases I and II are identical to the N-terminal amino acid sequences we determined for Lcc2 and Lcc1, respectively. Interestingly, the N-terminal amino acid sequences of the *Trametes versicolor* laccases I and II are not identical to the predicted N-terminal amino acids of the *Trametes versicolor* Lcc1 laccase (22), suggesting that there are at least three laccase genes in *Trametes versicolor.*

The deduced protein sequences of the *lcc1* and *lcc2* genes have a high degree of identity (80%) not only with each other but also with the sequences of other basidiomycete laccases, with the highest identity of 90% found between *lcc1* and the *Coriolus hirsutus* laccase gene. The genes also have some identity (25 to 44%) with laccases from the fungi *Agaricus bisporus* and *N. crassa* and have fairly low identity (13 and 16% for *lcc2* and *lcc1*, respectively) with the laccase from *Aspergillus nidulans*. However, there are two highly conserved regions near the carboxyl terminus of all of the laccases mentioned above which are believed to be involved in the coordination of the four copper ions to form a redox center. These regions, HPFHLHGHAF and PWFLHCHIDFHLEAGF, are at positions 393 to 403 and 447 to 463, respectively, within the mature proteins of Lcc1 and Lcc2. In these regions, all of the histidines and cysteines believed to be critical for coordination of the copper ions are strictly conserved.

The cloned *lcc1* and *lcc2* genes have 8 and 10 introns, respectively. In *lcc1*, the positions of the introns are confirmed because we cloned and sequenced the corresponding cDNA *LCC*. The positions of the introns in *lcc2* were deduced on the basis of homology to other fungal laccases and the consensus splicing signals found at 5′ and 3′ intron boundaries (20). The 3′ consensus splice sites (c/t AG) are found in all of the introns; however, the 5′ splice sites of some of the introns do not strictly match the consensus sequence (GTANGT). The variant bases of the 5′ intron splice sites found are in positions 3 and 6, with the most common variant being a C at position 6 as found for introns I, III, V, and VI in *lcc2* and for introns II and VI of *lcc1*. The consensus lariat sequence found internally in fungal introns (PuCTPuAC) is not strictly conserved in the majority of *lcc1* and *lcc2* introns. The size of the introns ranging from 50 to 64 is typical of most fungal introns (20). The positions of the first eight introns in *lcc2* and the introns in *lcc1* are conserved. In addition, the positions of all 10 introns in *lcc2* and the 10 introns in the *Coriolus hirsutus* laccase gene are also conserved.

Within the promoter of *lcc1*, there are putative TATAAA and CAAT motifs found at −102 and −212, respectively. The spacing of these motifs is consistent with those seen for other fungal promoters (20). The promoter of *lcc2* contains a CAAT motif at −230 but does not contain a TATAAA motif. Neither the TATAAA nor CAAT motifs are strictly conserved in the genes of filamentous fungi (20).

In the promoters of filamentous fungal genes, there are conserved nucleotides found near the translation initiation site (20) which agree fairly well with those found in higher eukaryotes, namely, RNN**ATG**G (boldface type indicates the initiator ATG for translation) (25). In filamentous fungi, the −3 position is 90% an A and/or a purine. The −3 positions are A and G in *lcc1* and *lcc2*, respectively.

The expression of *lcc1* is highly induced by the addition of 2,5-xylidine to the culture. In contrast, the expression of *lcc2* is not induced but appears to be constitutive under the conditions tested. The presence of a constitutive laccase activity and an inducible activity in culture broth has been reported previously for *Trametes* species (4).

Heterologous expression of *lcc1* in *Aspergillus oryzae* leads to the production of a recombinant laccase which is similar to the native laccase with respect to UV spectrum, susceptibility to pronase, pI, pH activity profiles, and optimal specific activities. In our studies, the only biochemical difference seen between the recombinant and native forms of laccase is the percent glycosylation, i.e., 0.5 and 10% for the native and recombinant forms, respectively. The addition of more or less carbohydrate

to a protein during heterologous expression is quite common in *Aspergillus oryzae* and *Aspergillus niger*. However, the additional carbohydrate added to the laccase during heterologous expression does not appear to have an effect on its enzymatic activity.

From the Southern blot probed with the *LCC* cDNA insert, it was postulated that there were probably more than two laccase genes in *Trametes villosa*. We have cloned three additional laccase genes (*lcc3*, *lcc4*, and *lcc5*) from *Trametes villosa*, and their sequence, structural organization, regulation, and chromosomal location will be reported elsewhere. Thus, the laccase gene family in *Trametes villosa* appears to consist of at least five genes which appear to be differentially regulated. *Trametes villosa* grows vegetatively as a dikaryon, and we have observed microscopically the presence of clamp connections which are important for the proper distribution of nuclei during mitosis. Because *Trametes villosa* is a dikaryon, we would expect to find alleles of genes. During the cloning of *lcc1* and *lcc2*, we did obtain clones which from partial nucleotide determination appeared to be identical or nearly identical to *lcc1* and *lcc2*. These clones were not further characterized and may indeed be alleles. In other white rot basidiomycetes such as *Phanerochaete chrysosporium*, both manganese peroxidase and lignin peroxidase, two enzymes involved in lignin depolymerization, are also encoded by families of structurally related proteins (16, 19, 31). Why these organisms have gene families for these secreted enzymes is unknown, and systematic genetic analyses are required to elucidate their functions.

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