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Identification of a Laccase Gene Family in the New Lignin-Degrading Basidiomycete CECT 20197

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A new lignin-degrading basidiomycete, strain I-62 (CECT 20197), isolated from decayed wood exhibited both a high dephenolization activity and decolorization capacity when tested on effluents from the sugar cane by-product fermentation industry. It has been classified as a member of the *Polyporaceae* family. The major ligninolytic activity detected in culture supernatants of basidiomycete I-62 was a phenoloxidase (laccase), in conjunction with small amounts of manganese peroxidase. No lignin peroxidase was detected. Laccase activity was produced in either defined or complete media. Addition of veratryl alcohol as the inducer, in defined medium, enhanced laccase production 10-fold. The use of fructose instead of glucose as a carbon source resulted in a 100-fold increase in laccase specific activity. Native isoelectrofocusing gels stained with guaiacol revealed the presence of at least seven laccase isozymes, with the most intense band being detected at pI 3. Southern hybridization analysis indicated the presence of a laccase gene family in strain I-62. Three different genes coding for phenoloxidases, *lcc1*, *lcc2*, and *lcc3*, were cloned and characterized. The high degree of homology between laccases from strain I-62 and laccases from *Trametes* species suggests a phylogenetic proximity between this new isolated fungus and the genus *Trametes*.

Lignin is a complex aromatic biopolymer and a major component of the vascular tissue of woody plants and grasses, where it is interspersed with hemicellulose to form lignocellulose. The use of lignocellulosic materials such as wood and straw as a source of fiber, fuel, or food is of major economic and ecological significance. Some of these uses, such as chemical pulping and bleaching in the pulp and papermaking processes, require chemical degradation of lignin and generate great amounts of lignin-derived environmental pollutants (47). Biodegradation of lignin and lignin derivatives might contribute to improved utilization of lignocellulosic materials (7). Some white rot basidiomycetes are unique in their ability to completely degrade all components of lignocellulose (26, 30). In addition, these fungi seem to be the most promising organisms for detoxification of industrial effluents by degrading low-molecular-weight phenolics (6) and have proven to be efficient in the decolorizing of pulp and paper mill wastewaters (4, 52).

Extracellular enzymes from white rot fungi, like lignin and manganese peroxidases (LiP and MnP), have been intensively studied because of their capability of degrading lignin model compounds in vitro (26). Laccases are also very important in lignin degradation, because they are capable of oxidizing and depolymerizing different lignin preparations without the presence of any peroxidase (8), and they are one of the ligninolytic enzymes which have been demonstrated to have a number of potential biotechnological applications (12). Laccases are multicopper enzymes which catalyze the oxidation of phenolic compounds and are found in both plants and fungi (56). In plants, laccases are involved in lignin synthesis (43). In fungi, besides playing a role in delignification, laccases appear to be involved in sporulation (34), pigment production (14, 60), and plant pathogenesis (1, 20, 38). Laccase genes from the non-ligninolytic fungi *Neurospora crassa* (21), *Cryphonectria parasitica* (13), *Aspergillus nidulans* (3), and *Aspergillus terreus* (27) have been cloned. In the ligninolytic fungi *Agaricus bisporus*, *Coriolus hirsutus*, *Pleurotus ostreatus*, *Trametes versicolor*, and *Trametes villosa*, a number of different laccase genes have been cloned (22, 28, 33, 44, 62, 63). While the existence of multiple genes encoding different laccase isozyme activities has been demonstrated, with evidence for at least five laccase genes in *T. villosa* (62, 63) and four in *Rhizoctonia solani* (59), only few molecular studies have been done to understand gene regulation (35, 62) or to achieve heterologous expression of these genes (27, 33, 62).

In the present work, we have selected the lignin-degrading white rot fungus I-62 (CECT 20197) and demonstrated that it produces and secretes several potent isozymes with laccase activity and efficiently decolorizes the effluent of a sugar cane by-product industry. We have cloned and determined the nucleotide sequences of three phenoloxidase genes from this fungus, strongly suggesting the presence of a laccase gene family. The results presented here on the basidiomycete I-62 laccases extend the observations on fungal laccases and indicate a phylogenetic proximity between this newly isolated fungus and the genus *Trametes*/*Coriolus*.

MATERIALS AND METHODS

Microorganisms and culture maintenance. Basidiomycete I-62, which has been deposited in the Spanish Type Culture Collection as strain CECT 20197, was isolated from mycelium growing on decayed wood in Pinar del Río, Cuba, and was a gift from Rafael Castañeda. The strain has been classified as a member of the genus *Trametes* in the *Polyporaceae* family by J.A. Stalper, CBS (Culture Collection), Baarn, The Netherlands. Basidiomycete PM1 (CECT 2971) (15),

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was a gift from Pedro M. Coll. Fungal strains were grown on malt agar plates (2% malt extract, 2% Bacto Agar) at 28°C to produce mycelium and then kept at 4°C. *Escherichia coli* XL1-Blue-MRF′ was purchased from Stratagene.

Culture conditions. Fungal liquid cultures were grown on either a complete or a defined medium. Complete medium (25) contained (per liter) 10 g of glucose, 2 g of ammonium tartrate, 1 g of yeast extract (Difco), 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $KCl \cdot 2H_2O$, and 1 ml of a solution containing (per liter) 0.1 g of $Na_2B_4O_7 \cdot H_2O$, 0.07 g of $ZnSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, and 0.01 g of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$. Defined medium was similar to the medium used for *Phanerochaete chrysosporium* ligninase production (32) and contained (per liter) 10 g of glucose, 0.2 g of ammonium tartrate (1 mM), 0.7 g of $MgSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4, 0.11 g of $KCl \cdot 2H_2O$, 1 mg of thiamine, and 70 ml of a solution containing (per liter) 0.5 g of $MnSO_4 \cdot H_2O$, 1.0 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $CoCl_2$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g of H_3BO_3, 0.01 g of $Na_2MoO_4 \cdot 2H_2O$, and 1.5 g of nitrilotriacetate. Also, 4 mM (final concentration) veratryl alcohol was added to the defined medium. The pH was adjusted to 4.5 with 20 mM dimethylsuccinate. Growth in the defined medium under nonlimiting nitrogen conditions was achieved by adding 10 times more ammonium tartrate (10 mM). When fructose was assayed as the carbon source, the same 1% concentration as for glucose was used. All liquid cultures were performed under agitation (100 rpm) at 28°C.

Inoculum preparation. Eight agar pieces (approximately 1 cm^2) from fully grown plates were placed in 500-ml Erlenmeyer flasks containing 150 ml of either complete or defined medium together with four glass balls 1 cm in diameter, and incubated in a rotary shaker (100 rpm) at 28°C. After 24 h of growth, the cultures were filtered through Miracloth (Calbiochem), and the filtrates were used as the inoculum in a 1/10 (vol/vol) dilution.

Lignin dephenolization assay. To detect ligninolytic activity of different fungal strains on solid medium, the strains were grown on plates containing complete medium supplemented with 0.1% alcalilignin (Induline AT; Sigma). The plates were incubated at 28°C for several days, and a lignin dephenolization test was performed as described by Sundman and Näse (55).

Decolorization assay. A 20% (vol/vol) final dilution of effluent from a sugar cane by-product fermentation industry (alcohol distillery) was added to a 5-day-old culture of basidiomycete I-62 (CECT 20197) in defined medium. Two different experiments were carried out in parallel with either 1% glucose or 1% fructose as the carbon source. The pH (4.5) was kept constant by the addition of 40 mM 2,2-dimethylsuccinate. The color of the effluent was measured periodically at 465 nm by a previously described procedure (2).

Enzymatic activities and protein determinations. Laccase activity (EC 1.10.3.2) in the supernatants of fungal cultures was determined with ABTS (2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate) as the substrate by the method of Wolfenden and Willson (61). Veratryl alcohol was the substrate for the detection of LiP (EC 1.11.1.14) (57). MnP activity (EC 1.11.1.13) was measured by the method described by Pick and Keisare (45) with some modifications, with 0.01% phenol red as the substrate in 100 mM tartrate buffer (pH 5.0) containing 0.1 mM $MnSO_4$ and 0.1 mM H_2O_2. The reaction was stopped by adding 50 μl of 4 M NaOH per ml of the reaction mixture. Enzyme activities were expressed in units, where 1 U is defined as the formation of 1 μmol of product per min.

Protein concentration measurements were performed by the Bradford assay (Bio-Rad) with bovine serum albumin as the standard (9). Colorimetric determinations were performed on a Shimadzu UV 160 recording spectrophotometer. Each assay was done in triplicate.

Glucose concentration determination. The glucose concentrations were determined by the glucose oxidase method (36).

Reducing-substance concentration determination. The concentrations of the reducing substances were measured by the method of Mopper and Gindler (40) with the modifications made by Sinner and Puls (53).

Isoelectric point determination. Analytical isoelectric focusing polyacrylamide gel electrophoresis (24) was performed with a mini-isoelectric focusing cell (model 111; Bio-Rad) by loading 0.2 μg of protein in 5% polyacrylamide gels containing 20% ampholytes (range, 2.5 to 9.0) (Bio-Rad). Laccases were visualized by staining with 2 mM guaiacol in 0.1 M sodium acetate buffer (pH 5.0) (41). The pI of the proteins was determined by comparison with a standard curve obtained by measuring the gel surface pH with a contact electrode. Samples were prepared by precipitating proteins in culture supernatants with ammonium sulfate (85% saturation), resuspending them in 10 mM acetate buffer (pH 5.0), and dialyzing them for 12 h against the same buffer.

Genomic DNA isolation, Southern analysis, and cloning procedures. High-molecular-weight genomic DNA was isolated from frozen mycelia by the procedure described by Raeder and Broda (46). The genomic DNA was digested with several restriction enzymes as specified by the manufacturer (Boehringer Mannheim). DNA fragments were electrophoresed in agarose gels with 1× TAE buffer (0.04 M Tris acetate, 10 mM EDTA) and then transferred to a Hybond N membrane (Amersham). The blots were hybridized under low-stringency conditions at 50°C in 5× SSPE (0.36 M NaCl, 20 mM NaH_2PO_4 [pH 7.7], 2 mM EDTA)–5× Denhardt's solution–0.1% sodium dodecyl sulfate–200 μg of denatured salmon sperm DNA per ml and washed at 62°C in 0.2× SSPE–0.1% sodium dodecyl sulfate. Radioactive DNA probes were prepared with [α-^{32}P]dATP (Amersham) by random priming with an oligolabelling kit (Pharmacia). Pools of genomic DNA fragments corresponding to the hybridization signals were eluted from agarose gels with the Sephaglas BandPrep kit (Pharmacia) and ligated into pUC18 (Pharmacia). *E. coli* XL1-Blue-MRF′ was transformed with these partial genomic libraries, and the clones were screened by colony hybridization. All DNA manipulations were performed by established molecular biology methods (50).

RNA isolation and Northern analysis. RNA isolation and hybridization was carried out by the method of Lockington et al. (37). A 10-μg sample of total RNA was electrophoresed overnight in 1% agarose–formaldehyde gels in 40 mM morpholinepropanesulfonic acid (MOPS; pH 7.0)–10 mM sodium acetate–1 mM EDTA and then transferred to nitrocellulose filters (Schleicher & Schuell) in 20× SSPE. The filters were hybridized in 5× SSPE–50 mM sodium phosphate (pH 6.5)–2.5× Denhardt's solution–10% dextran sulfate–50 μg of single-stranded DNA per ml–50% formamide at 42°C with appropriately labelled probes, washed (the final wash was carried out at 15 min with 0.2× SSPE at 65°C), exposed, and developed. As a control of the amount of RNA, the filter was hybridized with an 0.83-kb *Nco*I-*Kpn*I fragment of the actin gene from *Aspergillus nidulans* (19).

DNA sequencing. Nucleotide sequencing of subcloned DNA fragments was carried out on both strands by the dideoxy chain termination method (51) with the T7 Sequencing kit (Pharmacia). Some nucleotide sequences were determined by *Taq* polymerase cycle sequencing with fluorescently labelled nucleotides, and the reaction mixtures were electrophoresed on an Applied Biosystems automatic DNA sequencer (model 370; version 1.2.0). DNA sequences were translated to the predicted amino acid sequence by using the GCG macro program (17). Amino acid sequences were compared with the sequences available in the EMBL databases by using the FASTA program. Alignment of the sequences was carried out with the CLUSTAL program.

Nucleotide sequence accession numbers. The sequences of the basidiomycete I-62 (CECT 20197) laccase genes *lcc1*, *lcc2*, and *lcc3* reported in this paper have been assigned GenBank Data Library accession no. U65399, U65400 and U65401, respectively.

RESULTS

Ligninolytic activities of basidiomycete I-62. Strain I-62 was chosen, primarily on the basis of vigorous growth, from a variety of basidiomycetes, all of which were growing on decayed wood. A lignin dephenolization test was performed on plates as described by Sundman and Näse (55), with complete medium supplemented with 0.1% Induline (Sigma). After 7 days of growth at 28°C, the dephenolization halo produced by the different fungi tested was compared with the dephenolization pattern produced by the fungus PM1, previously reported as an efficient lignin-degrading basidiomycete (15). Strain I-62 produced the highest degree of dephenolization, even higher than the basidiomycete PM1 (data not shown).

Culture supernatants of strain I-62 were assayed for various ligninolytic activities when the strain was grown for 16 days on both defined and complete media. Maximal MnP and laccase activities are shown in Fig. 1. The highest MnP and laccase activities were achieved in defined medium around day 8 and in complete medium around day 16. The highest laccase activity (4.6 U/ml) achieved in the defined medium was significantly higher than the maximal activity (0.8 U/ml) measured in the complete medium. The same occurred for MnP: 2.5 U/ml in defined medium compared to 1.4 U/ml in complete medium. Not detectable LiP activity was observed under the experimental conditions used.

Supernatant from a 16-day-old culture of basidiomycete grown on defined medium was concentrated and loaded on a native isoelectrofocusing gel. At least seven isozymes with laccase activity (guaiacol staining) were detected (Fig. 2). Estimated pI values for these laccase isozymes were 3 to 4, with the most intense band being detected at pI 3. These are similar to the pI values reported for other fungal laccases (56). The most intense band detected with guaiacol was at pI 3.

Laccase activity of basidiomycete I-62. The highest laccase activity produced by basidiomycete I-62 was obtained when the fungus was grown on defined medium, and some modifications of this medium were used to increase laccase production (Fig. 3). We first checked the effect of veratryl alcohol on laccase

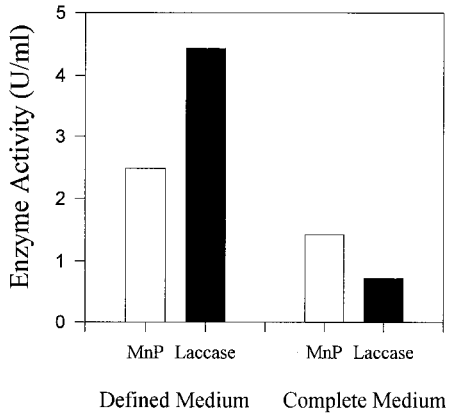

FIG. 1. Maximal laccase and MnP activities measured in supernatants from basidiomycete I-62 cultures grown in defined and complete media for 16 days. The values correspond to day 8 in defined medium and to day 16 in complete medium.

activity. Veratryl alcohol, like 2,5-xylidine and guaiacol, has been described as an inducer of laccase activity (5, 18, 48, 62). Veratryl alcohol significantly increased the production of laccase activity in basidiomycete I-62 with a 10-fold increase in laccase activity in the presence of 4 mM veratryl alcohol (Fig. 3A). This is similar to results previously reported for other fungi (5, 48). The induction was not affected by the use of glucose or fructose as the carbon source. On day 8, the highest levels of laccase activity were 3.3 and 4.5 U/ml with glucose and fructose as the carbon sources, respectively (Fig. 3A).

The high levels of laccase activity detected at the end of the incubation period in the medium with 10 mM ammonium (approximately 3 U/ml) were not due to veratryl alcohol induction, since this substance was absent from the growth medium. Buswell et al. (11) reported that high levels of ammonium (10 mM ammonium tartrate) favor the production of laccase activity in *Lentinus edodes*. In the absence of the inducer, a 10-fold increase in the laccase activity was also detected after 20 days of growth on medium with 10 mM ammonium tartrate (Fig. 3A) compared to the medium with 1 mM

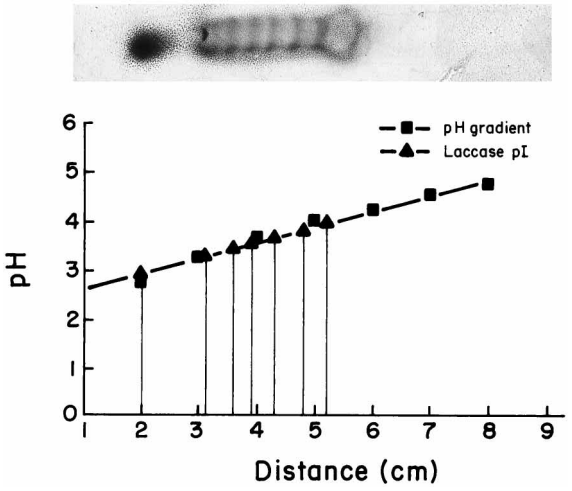

FIG. 2. Isoelectric focusing polyacrylamide gel electrophoresis of concentrated supernatant from a 16-day-old culture of basidiomycete I-62 grown on defined medium; 0.2 μg of protein was loaded. Laccase activity was visualized by guaiacol staining (10 mM guaiacol in 0.1 M sodium acetate buffer [pH 5.0]).

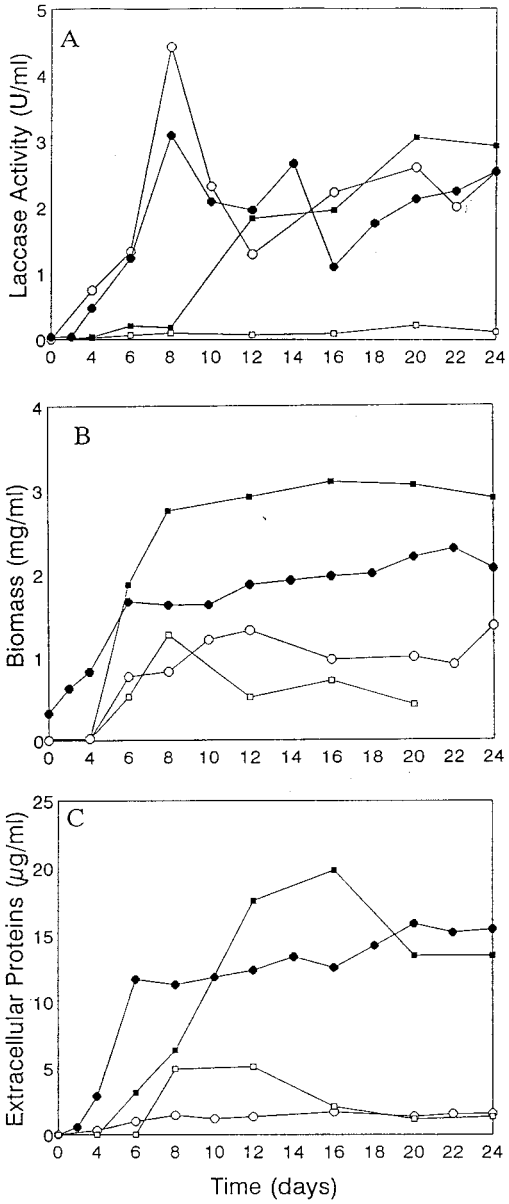

FIG. 3. Laccase activity (A), biomass (mycelial dry weight) (B), and extracellular proteins (C) measured in the cultures of basidiomycete I-62 grown in four different media. The substrate used for measuring the laccase activity was ABTS. The different defined media are 1% glucose–1 mM ammonium tartrate (□), 1% glucose–10 mM ammonium tartrate (■), 1% glucose–1 mM ammonium tartrate–4 mM veratryl alcohol (●), and 1% fructose–1 mM ammonium tartrate–4 mM veratryl alcohol (○). Experimental data are the means of three experiments, and the experimental error was never greater than 5%.

ammonium tartrate. This increase could be the consequence of the higher levels of biomass produced in that medium (3 mg/ml) than those in the medium with 1 mM ammonium tartrate (1 mg/ml) (Fig. 3B). In addition, the highest concentration of extracellular proteins was achieved in the culture medium with a nonlimiting concentration of nitrogen source (10 mM ammonium) (Fig. 3C), demonstrating that it is metabolically more favorable for the fungus.

The substitution of glucose with fructose as a carbon source slightly increased (1.5-fold) the maximal laccase activity on day 8 (Fig. 3A). It is interesting that in the defined medium plus

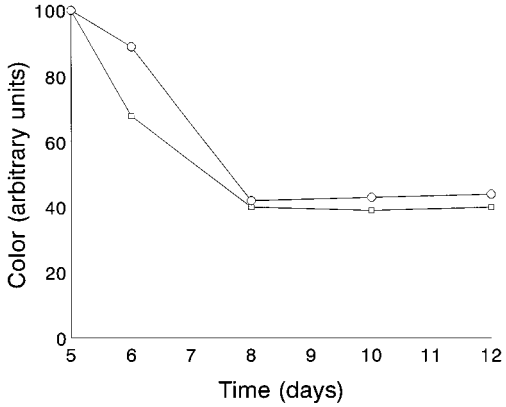

FIG. 4. Decolorization of effluent from the sugar cane by-product fermentation industry in basidiomycete I-62 cultures grown in defined medium, with 1% glucose (□) or fructose (○) as the carbon source. The media were supplemented with 20% (vol/vol) effluent on day 5 of growth.

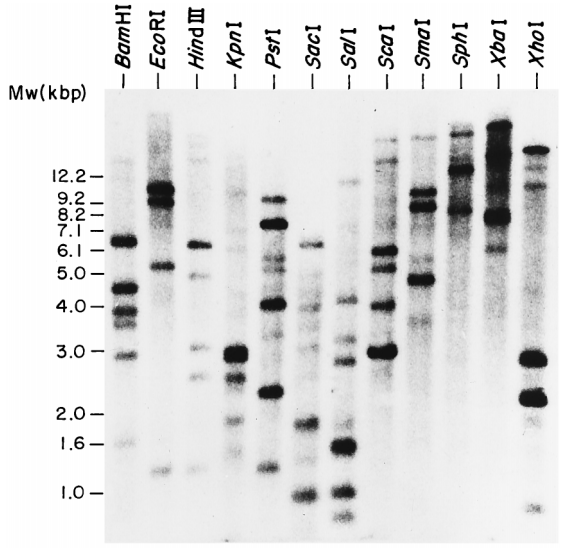

FIG. 5. Southern hybridization of basidiomycete I-62 genomic DNA digested with different restriction enzymes. The *Pst*I-*Xho*I (0.3-kb) fragment from the *lac1* gene of basidiomycete PM1 (16) was used as a probe under low-stringency conditions. Mw, molecular size.

fructose, the levels of biomass and extracellular proteins detected were lower than when glucose was used as the carbon source (Fig. 3B and C), resulting in an increase in laccase specific activity.

Decolorization assays. Decolorization assays were performed using effluents from the sugar cane by-product fermentation industry (alcohol distillery). In media with either 1% glucose or fructose as a carbon source and supplemented at 20% (vol/vol) effluent (final concentration) on day 5, high levels of laccase activity were measured in both media, reaching values of about 6 U/ml on days 6 to 12 (data not shown). Decolorization was around 60% on day 3 following effluent addition (day 8 of the experiment) when either glucose or fructose was used as the carbon source (Fig. 4). No further decolorization was observed following more prolonged periods of incubation. No decolorization was observed after 12 days in uninoculated controls.

Identification and cloning of the laccase genes from the basidiomycete I-62. Digested genomic DNA from basidiomycete I-62 was hybridized with a *Pst*I-*Xho*I (0.3-kb) fragment from the laccase gene *lac1* of the basidiomycete PM1 (CECT 2971), which includes the sequence of a highly conserved region of laccase (16). The multiple bands on the Southern autoradiograph suggested the presence of several laccase genes in strain I-62 (Fig. 5). The presence of at least five bands in all the samples, corresponding to digests with different restriction enzymes, strongly suggested that the basidiomycete I-62 genome contains at least five different laccase genes.

A partial genomic library was constructed, as described in Materials and Methods, to clone the 6.2-kb *Hin*dIII fragment (Fig. 5). The screening of this genomic library, using the same heterologous probe, gave several positive clones, all of which contained a 6.2-kb *Hin*dIII fragment including the first laccase gene, named *lcc1*. By using the same procedure, two different partial genomic libraries were made to obtain the complete *lcc2* genomic DNA sequence. The first was from a pool of *Xho*I-digested genomic DNA containing the 2.0-kb fragment, and the second was from a pool of *Sal*I-digested genomic DNA containing the 1.5-kb fragment (Fig. 5). Finally, we isolated a clone containing the 6.5-kb *Bam*HI fragment, which includes a third laccase gene, named *lcc3* (Fig. 5).

Comparison of the Southern hybridization done with the heterologous probe (Fig. 5) with hybridizations of the same membrane done with specific probes for each of the three laccase genes (*lcc1*, *lcc2*, and *lcc3*) revealed the presence of bands which do not correspond to any of the cloned genes, strongly suggesting the presence of another two laccase genes (data not shown).

Structure and organization of the genomic basidiomycete I-62 *lcc* laccase gene family. The nucleotide and predicted amino acid sequences of the *lcc1*, *lcc2*, and *lcc3* laccase genes are shown in Fig. 6 to 8, respectively. Putative intron positions were deduced on the basis of homology to other fungal laccases and conserved motifs found at the 5′ and 3′ junctions of introns.

The *lcc1* gene contains nine putative introns which range in size from 53 to 64 bp. The predicted precursor protein is 519 amino acids long and contains a signal sequence of 21 amino acids as predicted by the von Heijne rules (58), with cleavage at Ala-Ala. The protein contains eight potential N-glycosylation sites (Fig. 6).

The *lcc2* gene encodes a predicted precursor protein of 520 amino acids which contains a 21-amino-acid signal sequence with cleavage at Ala-Ser. There are nine potential N-glycosylation sites, and the coding region is interrupted by nine putative introns which range in size from 53 to 60 bp (Fig. 7).

The *lcc3* gene encodes a precursor protein of 524 amino acids containing a 25-amino-acid signal sequence, with cleavage between Ala-Ala. The protein contains five potential N-glycosylation sites, and the coding region is interrupted by 10 putative introns, ranging in size from 49 to 63 bp. The estimated molecular masses of the three mature polypeptides are 53.4, 53.5, and 53.5 kDa, respectively (Fig. 8).

Comparison of deduced amino acid sequences of the three *lcc* genes from basidiomycete I-62 with other multicopper blue proteins shows significant homology in the four copper binding regions. These highly homologous regions contain clusters of histidine residues (His-X-His), which constitute the proposed 12 copper binding ligands, as shown in the X-ray crystallographic analysis for *Cucumis sativus* ascorbate oxidase (42).

The predicted mature proteins coded for by *lcc1*, *lcc2*, and *lcc3* are 73.9 to 76.9% identical to one another (Table 1). The percent identities to laccases from other white rot fungi, like

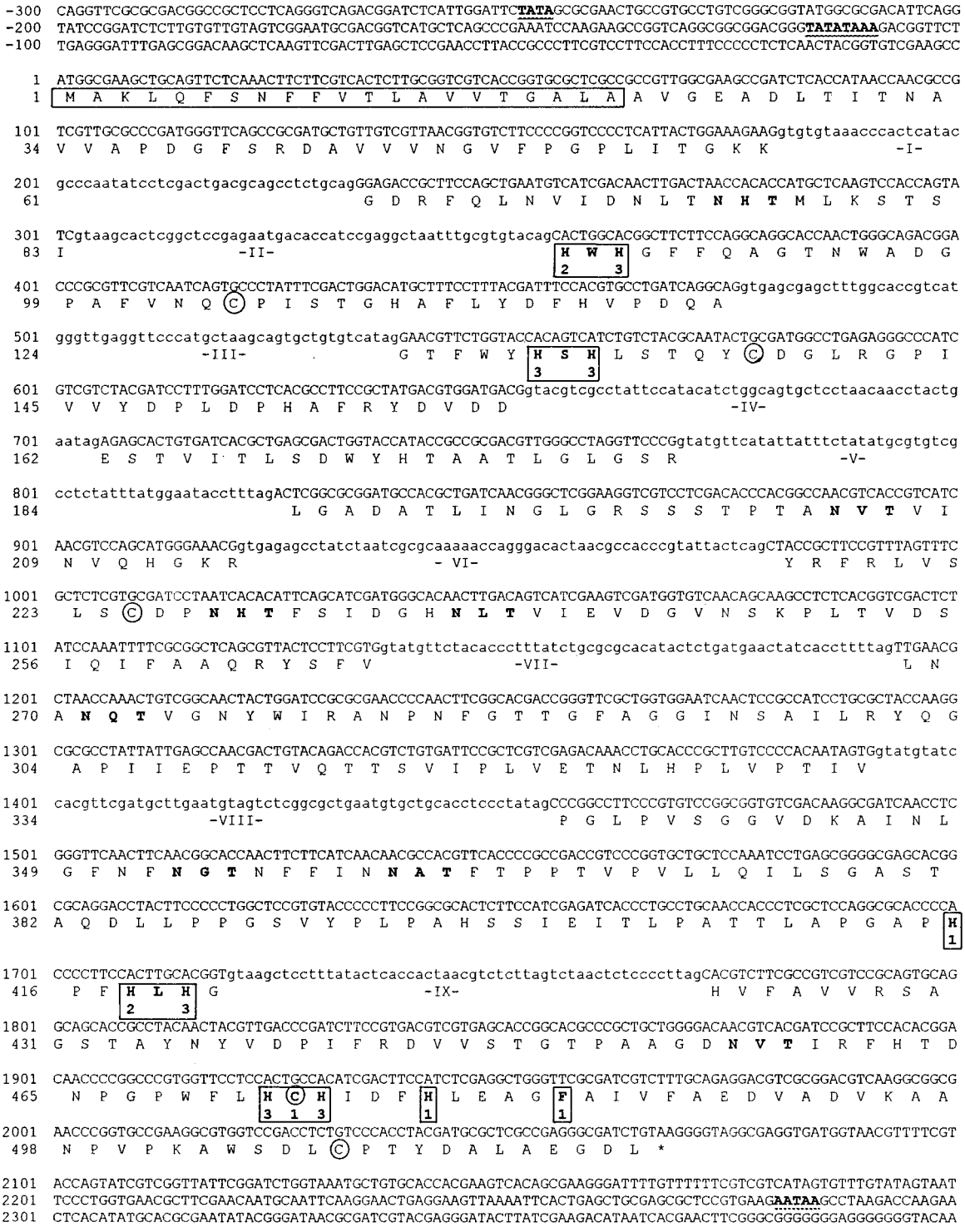

FIG. 6. Nucleotide sequence of the basidiomycete I-62 *lcc1* gene. Numbering starts at the putative translation initiation site of the laccase *lcc1* gene and refers to the nucleotide sequence and the predicted amino acid sequence. The predicted signal peptide is boxed. The nine putative introns are indicated in lowercase type and numbered (I to IX). The putative N-glycosylation sites are in boldface type. The amino acid residues proposed for the coordination of the three types of copper ions are boxed and numbered to indicate the copper configuration. The putative cysteines forming disulfide bridges are circled. Putative TATA promoter elements and a possible polyadenylation sequence at the 3′ end are in boldface type and underlined.

Trametes villosa, *Coriolus hirsutus*, *Trametes versicolor*, basidiomycete PM1, and *Phlebia radiata*, range from 61 to 86%. The percent identities of *lcc1*, *lcc2*, and *lcc3* to genes encoding laccases from *A. bisporus* range from 45.3 to 47.6%.

Transcription of *lcc1* and *lcc2*. Total RNA was isolated from 7 and 16-day-old mycelia from cultures growing on glucose and induced with veratryl alcohol, producing high laccase titers (Fig. 3A). The RNA was hybridized with specific probes for

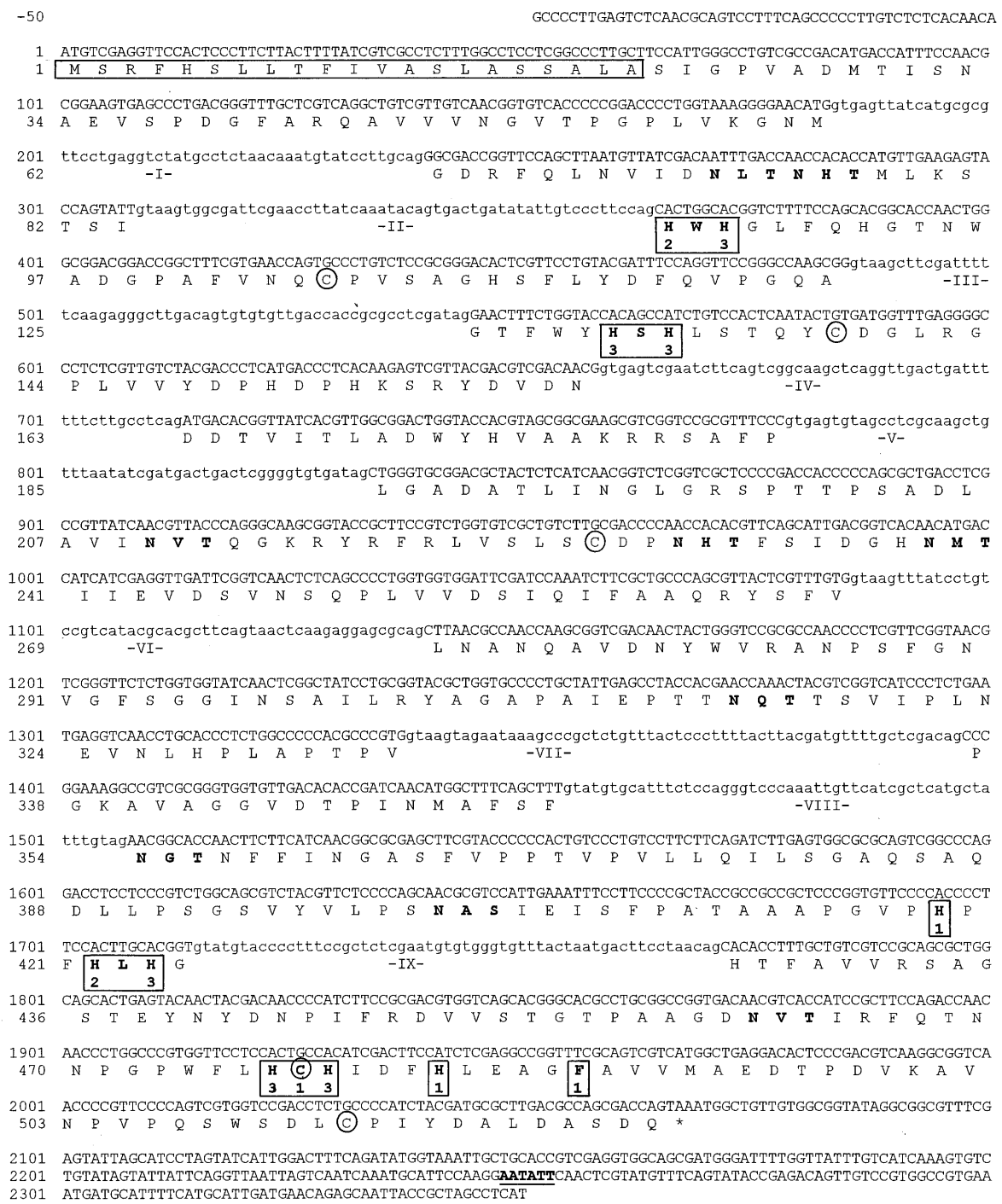

FIG. 7. Nucleotide sequence of the basidiomycete I-62 *lcc2* gene. Numbering starts at the putative translation initiation site of the laccase *lcc2* gene and refers to the nucleotide sequence and to the predicted amino acid sequence. The predicted signal peptide is boxed. The nine putative introns are in lowercase type and are numbered (I to IX). The putative N-glycosylation sites are in boldface type. The amino acid residues proposed for the coordination of the three types of copper ions are boxed and numbered to indicate the copper configuration. The putative cysteines forming disulfide bridges are circled. A possible polyadenylation sequence at the 3′ end is in boldface type and is underlined.

genes *lcc1* and *lcc2*; a gene fragment from positions −60 to 232 in the nucleotide sequence of *lcc1* (Fig. 6) was used. For the detection of the *lcc2* mRNA, a gene fragment from positions 488 to 1157 in the nucleotide sequence (Fig. 7) was used. Transcripts were detected for both genes. Higher levels of *lcc1* transcripts were observed on day 7 (Fig. 9), while *lcc2* transcript levels were highest on day 16, suggesting that *lcc1* and *lcc2* are differentially regulated in I-62 at the transcriptional levels. No transcription for *lcc3* was detected under the conditions tested (data not shown).

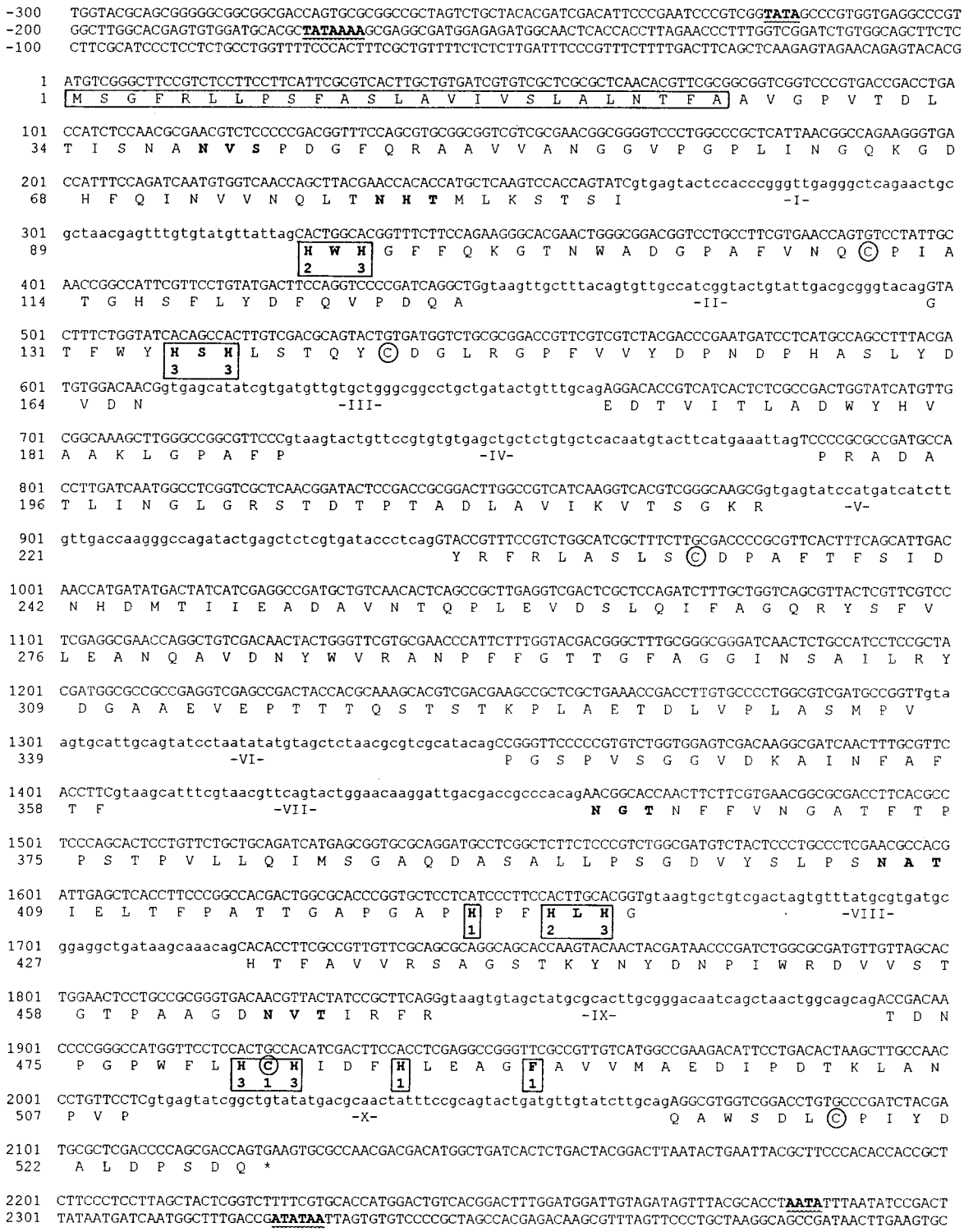

FIG. 8. Nucleotide sequence of the basidiomycete I-62 *lcc3* gene. Numbering starts at the putative translation initiation site of the laccase *lcc3* gene and refers to the nucleotide sequence and to the predicted amino acid sequence. The predicted signal peptide is boxed. The 10 putative introns are in lowercase type and are numbered (I to X). The putative N-glycosylation sites are in boldface type. The amino acid residues proposed for the coordination of the three types of copper ions are boxed and numbered to indicate the copper configuration. The putative cysteines forming disulfide bridges are circled. Putative TATA promoter elements and a possible polyadenylation sequence at the 3′ end are in boldface type and are underlined.

TABLE 1. Percent identity of the predicted *lcc1*, *lcc2*, and *lcc3* product sequences to sequences of other fungal laccases

Fungal laccase[a]	% Identity[b]										
	1	2	3	4	5	6	7	8	9	10	11
1		76.3	73.9	76.9	84.6	77.7	74.6	85.0	46.4	47.6	58.8
2			76.9	84.6	76.5	86.3	77.3	76.9	45.5	47.6	61.3
3				79.8	75.0	80.3	72.7	75.4	45.3	46.4	61.6
4					79.6	91.4	79.6	70.9	43.7	45.2	63.3
5						81.4	73.7	67.7	43.1	44.8	61.5
6							80.2	70.7	44.1	46.6	64.1
7								69.1	44.4	45.6	65.7
8									44.4	44.9	64.3
9										85.2	42.5
10											44.6
11											

[a] 1, *lcc1*; 2, *lcc2*; 3, *lcc3*; 4, *Trametes villosa lcc1* (62); 5, *Trametes villosa lcc2* (62); 6, *Coriolus hirsutus lac1* (33); 7, basidiomycete PM1 (16); 8, *Trametes versicolor* (28); 9, *Agaricus bisporus lac1* (44); 10, *Agaricus bisporus lac2* (44); 11, *Phlebia radiata* (49).
[b] Calculated by the CLUSTAL method.

DISCUSSION

In this work, we describe a new white rot basidiomycete isolate, I-62, which is a member of the genus *Trametes* in the family *Polyporaceae* and displays high extracellular laccase and MnP activity. In laboratory cultures, no LiP activity was detected. Similar observations have previously been made with other white rot fungi which efficiently degrade wood without the production of LiP (18, 23). Basidiomycete I-62 is a particularly good laccase producer, with higher enzyme levels than those described for other white rot fungi such as the basidiomycete PM1 grown under similar physiological conditions (15). A 10-fold increase in laccase activity was observed in the defined medium with veratryl alcohol, which is similar to the induction described for other inducers such as 2,5-xylidine and ferulic acid (18). On the other hand, a twofold increase in biomass production was observed when the fungus was grown in the presence of veratryl alcohol, suggesting that this compound is being used as a carbon source by the fungus (Fig. 3B).

The influence of the ammonium concentration on the production of ligninolytic enzymes has long been a controversial topic. LiP and MnP in *P. chrysosporium* are stimulated by limiting nitrogen concentrations (29, 31, 32). Eggert et al. (18) have reported that laccase activities in culture fluids of *Pycnoporus cinnabarinus* are also dependent on the nitrogen concentration. In *Lentinus edodes*, MnP activity is suppressed by high nitrogen levels while laccase activity is increased under the same conditions (11). In the basidiomycete I-62, laccase production increased at the end of the incubation period following growth on 20 mM ammonium tartrate in the absence of inducer (Fig. 3A). However, both biomass and the concentration of extracellular proteins also increased under these growth conditions (Fig. 3B and C); therefore, no significant increase in laccase specific activity was observed at high nitrogen concentrations.

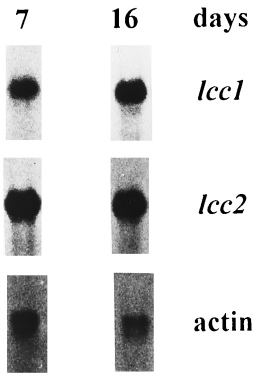

FIG. 9. Northern blot analysis of phenoloxidase transcripts. Nitrocellulose membranes with 10 μg of total RNA were subsequently hybridized with specific probes for *lcc1*, *lcc2*, and actin (see Materials and Methods). The estimated size of *lcc1* and *lcc2* transcripts is 1.8 kb.

In the model white rot fungus *P. chrysosporium*, which until recently was reported to degrade lignin efficiently in the absence of laccase, it has been shown that the production of this enzyme is repressed by glucose (54). Basidiomycete I-62 showed a 1.5-fold increase in laccase activity when grown on fructose as a carbon source, although the most important feature of this production medium was the very low biomass and extracellular protein levels, which resulted in a 100-fold increase in the levels of laccase specific activity. When cultured on sugar cane by-product fermentation industry effluents, the ligninolytic enzymes of basidiomycete I-62 achieved 60% decolorization under the conditions tested (Fig. 4). In fact, slightly higher laccase activity was observed in the media supplemented with effluents. It is possible that this increase in activity is a response developed by the fungus to oxidize the compounds present in the effluent and hence diminish their potential toxic effects (56).

Most molecular studies on ligninolytic enzymes have been done on MnP and LiP, and it has been shown that they are encoded by multiple related genes which appear to be clustered in the *P. chrysosporium* genome (10, 23). The genes coding for them have been shown to be organized in families (10, 23), and all evidence supports the conclusion that laccases are encoded by gene families. In a group of white rot basidiomycetes, the nucleotide sequences for more than two nonallelic laccase genes have been determined (22, 33, 44, 59, 62, 63). Our Southern analysis suggests the presence of at least five genes coding for laccases in basidiomycete I-62. We have cloned three different genes coding for phenoloxidases, *lcc1*, *lcc2*, and *lcc3*, and the differences in the nucleotide sequences and the number and positions of introns indicate that these genes are nonallelic.

The deduced amino acid sequences of *lcc1*, *lcc2*, and *lcc3* predict proteins with the structural characteristics of blue copper oxidases (16, 42). The regions that coordinate Cu^{2+} ions are perfectly conserved, even with blue copper oxidases from higher eukaryotes (39). The laccases encoded by *lcc1*, *lcc2*, and *lcc3* share high degrees of identity with one another (73 to

76%) and with other basidiomycete laccases. The highest identities were found between *lcc1* and the *lacI* gene from *Trametes versicolor* and between *lcc2* and the *lac1* gene from *Coriolus hirsutus* (Table 1). Gene families probably produce closely related proteins that are subtly different in their activities, allowing the transformation of a wider range of substrates or showing differential regulation. It has been shown that two nonallelic laccase genes in *Trametes villosa* and *Rhizoctonia solani* (59, 62) are differentially regulated, as is the case for basidiomycete I-62 *lcc1* and *lcc2* genes (Fig. 9). If gene families encoding laccases in basidiomycetes give this advantage, one would expect a higher degree of homology among laccases from different fungi that have the same function or substrate specificity than among laccases from the same species that have a different substrate specificity. The *lcc1*, *lcc2*, and *lcc3* laccases share the highest degree of homology to other basidiomycete laccases.

The detection by guaiacol staining of various extracellular laccase isozymes in the culture supernatants from the basidiomycete I-62 (Fig. 2), the fact that all the genomic sequences of *lcc* genes have a very high percent identity with other laccase genes from the literature (Table 1), and the fact that they show all the amino acid motifs characterizing laccases, point out that the extracellular phenoloxidases detected are laccases encoded by the *lcc* gene family. We cannot establish a direct relationship between the phenoloxidase activities measured in liquid culture and the three cloned genes, but the fact that *lcc1* and *lcc2* are expressed (Fig. 9) indicates that they are functional genes.

Five laccase genes have been identified and sequenced in *Trametes villosa* (62, 63), and four laccase genes have been cloned in *Rhizoctonia solani* (59). In basidiomycete I-62, the nucleotide sequences of three nonallelic phenoloxidase genes are reported. Current studies are focused on control of gene expression of *lcc1*, *lcc2*, and *lcc3* and purification of the isozymes to achieve a better understanding of the ligninolytic system operating in our strain.

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