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Characterization and Structural Analysis of the Laccase I Gene from the Newly Isolated Ligninolytic Basidiomycete PM1 (CECT 2971)

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We have isolated and characterized the cDNA and genomic DNA coding for a phenoloxidase, laccase I, previously purified from culture supernatant of the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). A cDNA library from basidiomycete PM1 was constructed, and laccase-encoding cDNAs were identified by screening with antiserum raised against the purified enzyme. The *lac1* gene coding for the laccase was identified in a partial genomic library by using the isolated cDNA as a probe. Nucleotide sequence determination of the full-length cDNA revealed an open reading frame of 1,551 bp encoding a polypeptide of 517 amino acid residues with a putative signal peptide of 21 amino acid residues. Ten small introns interrupted the genomic DNA. A single 1.8-kb transcript mRNA was detected by Northern (RNA) blot analysis, and its 5′ end maps to a position 51 bp upstream from the site of initiation of protein synthesis. Eukaryotic regulatory sequences, CAAT and TATA, were observed in the 5′ flanking region, which also contains sequences similar to those of copper-regulated proteins. Comparative analysis of the predicted amino acid sequence showed that basidiomycete PM1 laccase I had great similarity to the laccases from *Coriolus versicolor*, *Coriolus hirsutus*, and *Phlebia radiata*.

Lignin is a complex phenylpropanoid polymer extremely resistant to degradation. White-rot fungi constitute the only group of microorganisms shown to be effective in the biological degradation of this polymer. Extracellular fungal oxidases, particularly ligninases (peroxidases) and laccases (phenoloxidases), are supposed to begin a nonspecific oxidation process by removing an electron from a subunit of lignin (22). Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing phenoloxidase catalyzing the four-electron reduction of O_2 to water with the concomitant oxidation of phenolic substrates. Laccases are widespread in nature; they are produced by plants and fungi (29) and can oxidize, demethylate, polymerize, or depolymerize phenolic compounds. In plants, laccases have been associated with lignification (4). In fungi, besides lignin degradation (5), they have been involved in different biological processes such as sporulation (25), pigment production during fruit body development (8, 41), and plant pathogenesis (14, 28) in which laccase could potentially contribute to pathogen-mediated degradation of lignified zones (26). Studies of the structure and regulation of laccase-coding genes may help in the elucidation of the roles and enzymatic mechanisms of the different laccases in specific physiological processes.

Three reports on laccase sequences from the white-rot ligninolytic fungi *Coriolus hirsutus* (23), *Phlebia radiata* (38), and *Coriolus versicolor* (21) have been published. Other laccase genes have also been described in nonligninolytic fungi such as *Neurospora crassa* (16), *Aspergillus nidulans* (1), the chestnut blight fungus *Cryphonectria parasitica* (6), and *Agaricus bisporus* (34).

In a previous paper (9), we reported the isolation of the new lignin-degrading basidiomycete PM1 (CECT 2971) and the purification and characterization of an extracellular laccase from culture supernatants of this fungus. The laccase I enzyme is a monomeric glycoprotein with an apparent M_r of 64,000 and a carbohydrate content of 6.5% (9). Like other laccases (30), it contains four copper atoms per molecule. The N-terminal sequence of PM1 laccase I displayed a high degree of similarity to the laccases from *C. hirsutus* and *P. radiata*, while it was very different from other reported laccases, such as those from *A. bisporus* or *N. crassa*.

We describe in the current paper the cloning and sequencing of the cDNA and genomic DNA coding for this laccase and the comparison of the putative laccase I amino acid sequence with other known laccases. The results presented here on the basidiomycete PM1 laccase gene extend the observations on fungal laccase gene structure and indicate a phylogenetic proximity between this newly isolated fungus and the genus *Coriolus*.

MATERIALS AND METHODS

Microorganisms. Basidiomycete PM1 (deposited in the Spanish culture type collection, CECT 2971) was isolated from waste water from the paper mill of the Empresa Nacional de Celulosa, Miranda de Ebro, Burgos, Spain. *C. versicolor* was a present from F. Laborda, *Phanerochaete chrysosporium* BKM-F 1767 (ATCC 24725) was a gift from T. K. Kirk, *Pleurotus eryngii* (A180), and *Heterobasidion annosum* (A198) and *Fomes fomentarius* (A166) were a present from A. Martínez.

Escherichia coli MC1061 was purchased from Amersham (Amersham, United Kingdom) together with the plasmid pUEX1. *E. coli* MV1190 was purchased from Bio-Rad (Richmond, Calif.). Bluescript plasmid was obtained from Stratagene (La Jolla, Calif.).

Culture conditions. Basidiomycete PM1 stock cultures were maintained on solid potato dextrose medium (GIBCO)

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and Czapek Dox medium (composition per liter was as follows: sucrose, 30 g; $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and agar, 20 g). Plates were incubated at 28°C for several days. The liquid medium used for laccase production was GAE (composition per liter was glucose, 10 g; asparagine, 1 g; yeast extract, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g). As an inoculum, we used agar cubes cut from colonized potato dextrose agar plates (9). Static cultures were incubated at 37°C for 45 days, and agitated cultures were incubated at 37°C for 15 days.

P. chrysosporium and *C. versicolor* were grown as described elsewhere (11, 32).

Purification of extracellular laccase I from basidiomycete PM1. Purification of extracellular laccase I was carried out as described previously (9). Enzyme purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein and laccase activity were determined as described previously (9, 27).

PAGE of proteins. SDS-PAGE on 12% (wt/vol) polyacrylamide gels was performed by the method of Laemmli (24). Samples were treated with 1% SDS and 2% dithiothreitol and boiled at 100°C for 5 min. Proteins were revealed by silver staining (31). Nondenaturing electrophoresis was performed under the same conditions, except that SDS and dithiothreitol were omitted and the samples were not boiled. In nondenaturing gels, laccase activity was detected by incubating the gels with 2 mM guaiacol in 50 mM sodium acetate buffer, pH 4.5. After several minutes, brown enzymatic bands appeared as a result of the polymerization of guaiacol.

Antiserum generation. Purified laccase (250 μg), either native or denatured and deglycosylated, was mixed with Freund complete adjuvant (1:1, vol/vol) and injected intradermally into two male New Zealand White rabbits weighing 2 to 3 kg each. Two more doses of 125 μg of protein and Freund incomplete adjuvant (1:1, vol/vol) were applied subcutaneously on days 32 and 90 after the first immunization. Blood extraction was performed on days 30, 45, 50, 55, 100, 110, and 120. Serum titration was done by dot blot.

Immunoblot analysis. Sample proteins were fractionated by SDS-PAGE, transferred to an Immobilon P membrane (Millipore Inc., Bedford, Mass.) electrophoretically, and immunologically detected according to the procedure recommended by the ProtoBlot Western blot AP system (Promega, Wis.). The antiserum was incubated at room temperature for 30 to 60 min with the Immobilon P membrane at a dilution of 1:3,000. Detection of the bound antibodies was performed by using a 1:7,500 dilution of goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase. The antibodies were revealed by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$.

RNA isolation and cDNA library construction. All manipulations of DNA and RNA were performed by established molecular biological methods (2, 39). Fungal mycelia were harvested from cultures of the fungus after incubation at 37°C in a shaker for 4 days in GAE medium, when the phenoloxidase activity was 2 U/ml. Total RNA was extracted from the frozen mycelia as previously described (20). Poly(A)-containing RNAs were purified by oligo(dT)-cellulose chromatography, according to the method of Clemens (7). Double-stranded cDNAs were synthesized by reverse transcription of these RNA templates with oligo(dT) as a primer and by following the procedure recommended by the Amersham cDNA synthesis kit supplier. The cDNAs and the *Bam*HI-digested plasmid pUEX1 were ligated by using adapters consisting of 20 bp of overlapping sequence with a blunt end for ligating to cDNA and a *Bam*HI-cohesive end for ligation to the plasmid. This vector produced fusion proteins of β-galactosidase with the peptide coded by the inserted DNA. The cDNA library was used to transform *E. coli* MC1061, and the transformant colonies were transferred to nitrocellulose filters and SDS lysed. Screening of the library was done by using polyclonal antilaccase antibodies and a second goat anti-rabbit antibody bound to alkaline phosphatase. A second selection of positive clones was carried out by immunoblotting of the cell extracts.

DNA sequencing. The cDNA inserts of positive *E. coli* transformants were subcloned into appropriately digested Bluescript vectors. Nucleotide sequencing of subcloned DNA fragments was carried out on both strands by the dideoxy chain termination method (40) using single-chain templates.

The cDNA sequence was translated to the predicted amino acid sequence by using the DNAsis program. The amino acid sequence was then compared with the sequences available in the EMBL data bases by using the FASTA program. Alignment of the sequences was carried out with the CLUSTAL program.

Genomic DNA isolation and library construction. High-molecular-weight genomic DNA was isolated from frozen mycelia according to the procedure described by Raeder and Broda (35). The genomic DNA was digested with several restriction enzymes, and the fragments were hybridized with the laccase cDNA previously cloned and labeled with [α-^{32}P]ATP (3,000 Ci/mmol) by nick translation (36). *Hin*dIII fragments of 4 to 6 kb were isolated and ligated into Bluescript plasmid. *E. coli* MC1061 was transformed with this partial genomic library, and the screening of the clones was performed by using the *lac1* gene cDNA as a probe.

Analysis of mRNA 5′ termini. mRNA 5′-terminal analysis was carried out by primer extension essentially as described previously (39). Briefly, a synthetic oligonucleotide (sequence 5′ACCATCGGGACTGACAGCAC3′) complementary to nucleotide positions +120 to +101 of the laccase I sequence was 5′ end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol) and polynucleotide kinase. Poly(A) RNA was isolated from basidiomycete PM1 and after denaturing was hybridized with an excess of the labeled complementary oligonucleotide. Reverse transcriptase RAV-2 (Amersham) was then used to extend the primer, producing a labeled cDNA complementary to the RNA template. The radioactive cDNA was analyzed by electrophoresis on a 6% acrylamide–8.3 M urea sequencing gel adjacent to the sequencing reactions of the laccase I gene primed with the same oligonucleotide.

Nucleotide sequence accession number. The sequence of the basidiomycete PM1 (CECT 2971) laccase I gene reported in this paper has been assigned EMBL Data Library accession number Z12156.

RESULTS

Cloning of the cDNA from the basidiomycete PM1 laccase I gene. Rabbit antiserum obtained against purified laccase I was able to specifically recognize this protein (Fig. 1B, lane 3). Immunoblot analysis by SDS-PAGE of the basidiomycete PM1 culture supernatants revealed a single band of the same size as the purified laccase I in static culture supernatant (Fig. 1B, lane 2). Two more bands were detected in shaken culture supernatants (Fig. 1B, lane 1).

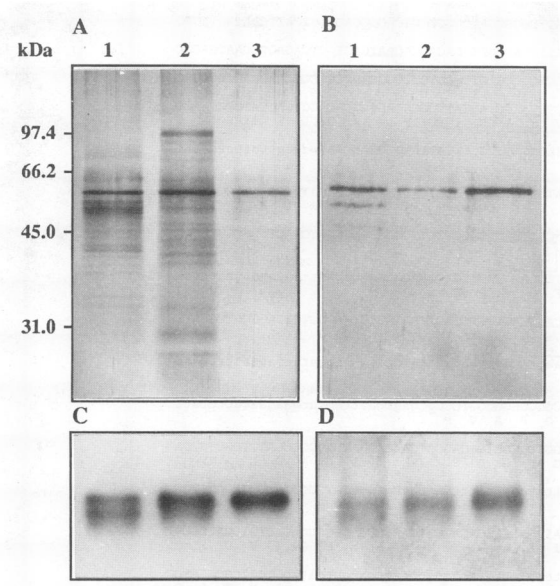

FIG. 1. Basidiomycete PM1 supernatants of agitated (lane 1) or static (lane 2) cultures and purified laccase I (lane 3) were analyzed by SDS-PAGE revealed by silver staining (A) or native electrophoresis developed by activity against guaiacol (C), followed by Western blot analysis (B and D) with anti-laccase I rabbit antiserum.

When the immunoblot was performed after native electrophoresis of culture supernatants, the same laccase activity bands revealed with 2 mM guaiacol were detected with the anti-laccase I antibodies (Fig. 1C and D).

A cDNA library was prepared by using polyadenylated RNAs from basidiomycete PM1 laccase-producing mycelia. The cDNAs obtained were cloned into the pUEX plasmid, and 30,000 colonies were screened with the rabbit anti-laccase I antibodies. Four positively reacting clones were selected and further analyzed by SDS-PAGE followed by Western blotting (immunoblotting) of the bacterial extracts (Fig. 2A, lanes 3 to 6). Restriction mapping and hybridization with the cDNA of the smallest insert as a probe showed that all of them were derived from the same mRNA (data not shown). A schematic representation of the inserts contained in the plasmids from the four selected clones is shown in the Fig. 2B. Clone 6 contained the longest cDNA insert of about 1.8 kb. Since this size could correspond to the whole laccase I coding region, the cDNA was sequenced. It had an open reading frame of 1,551 bp coding for a 517-amino-acid polypeptide. The amino acid sequence deduced from the DNA sequence was compared with the previously described amino acid sequence of the purified laccase I N terminus determined by Edman degradation (9). The results indicated the presence of 21 extra amino acid residues preceding the protein N terminus in the cDNA (boxed in Fig. 3), which have the typical characteristics of a signal peptide (42), with many hydrophobic amino acids and the alanine and serine residues at the cleavage site. The calculated molecular mass for the mature protein was 53.23 kDa.

Structure and organization of the genomic basidiomycete PM1 *lac1* gene. Southern hybridization of *Hin*dIII-digested genomic DNA with the laccase I cDNA as a probe allowed the isolation of the genomic *lac1* gene encoding the laccase I enzyme. The sequence of *lac1* is shown in Fig. 3. Comparison of the genomic DNA sequence with that of the cDNA showed the presence of 10 small introns (51 to 62 bp) in the

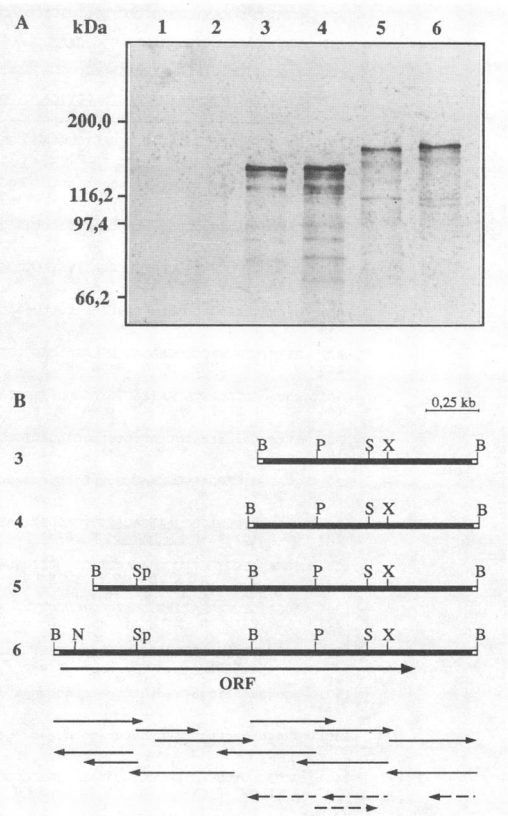

FIG. 2. (A) Western blot analysis of cellular extracts from *E. coli* MC1061 (lane 1), transformed with pUEX1 (lane 2) and laccase I-positive transformant clones from the basidiomycete PM1 cDNA library (lanes 3, 4, 5, and 6). (B) Schematic representation and restriction map of the inserts contained in the four positive clones. The sequencing strategy used for the determination of the nucleotide sequence of the fragment containing the laccase I-coding cDNA is indicated. The arrows show the direction and the extent to which the sequence was determined by the enzymatic dideoxy chain elongation. Dashed arrows indicate that *Taq* polymerase was used for sequencing. The open reading frame (ORF) is indicated as a thicker arrow. Restriction endonuclease sites: B, *Bam*HI; N, *Nhe*I; S, *Sma*I; Sp, *Sph*I; P, *Pst*I; X, *Xho*I.

lac1 gene. Consensus sequences for 5′ splicing GT(AG)(AT) GT in higher eukaryotes exist in all introns except the first (GCGAGT). The 3′ splicing sequence (CT)AG is present in all of them. The predicted peptide carried four potential *N*-glycosylation sites at amino acids 72, 75, 228, and 454. The possible amino acid residues acting as copper ligands were determined by comparison of the coded sequence with that of *Cucumis sativus* ascorbate oxidase (33). The four sequences His-*X*-His act as ligands of type 2 copper and the two type 3 copper atoms, and two His, a Cys, and a Phe act as ligands for type 1 copper (Fig. 3). Four Cys residues corresponding to the Cys-forming disulfide bonds in *C. sativus* ascorbate oxidase are also indicated. A putative consensus polyadenylation signal, AATACA (10), is present 222 bp downstream of the stop codon, and polyadenylation occurs 13 bp after that signal.

Analysis of the 5′ flanking region of the genomic coding sequence revealed the presence of structural features considered important for gene transcription: a TATA box-like sequence (TATAAA) found 83 bp upstream from the presumptive translation start (ATG GCC) and three CAAT

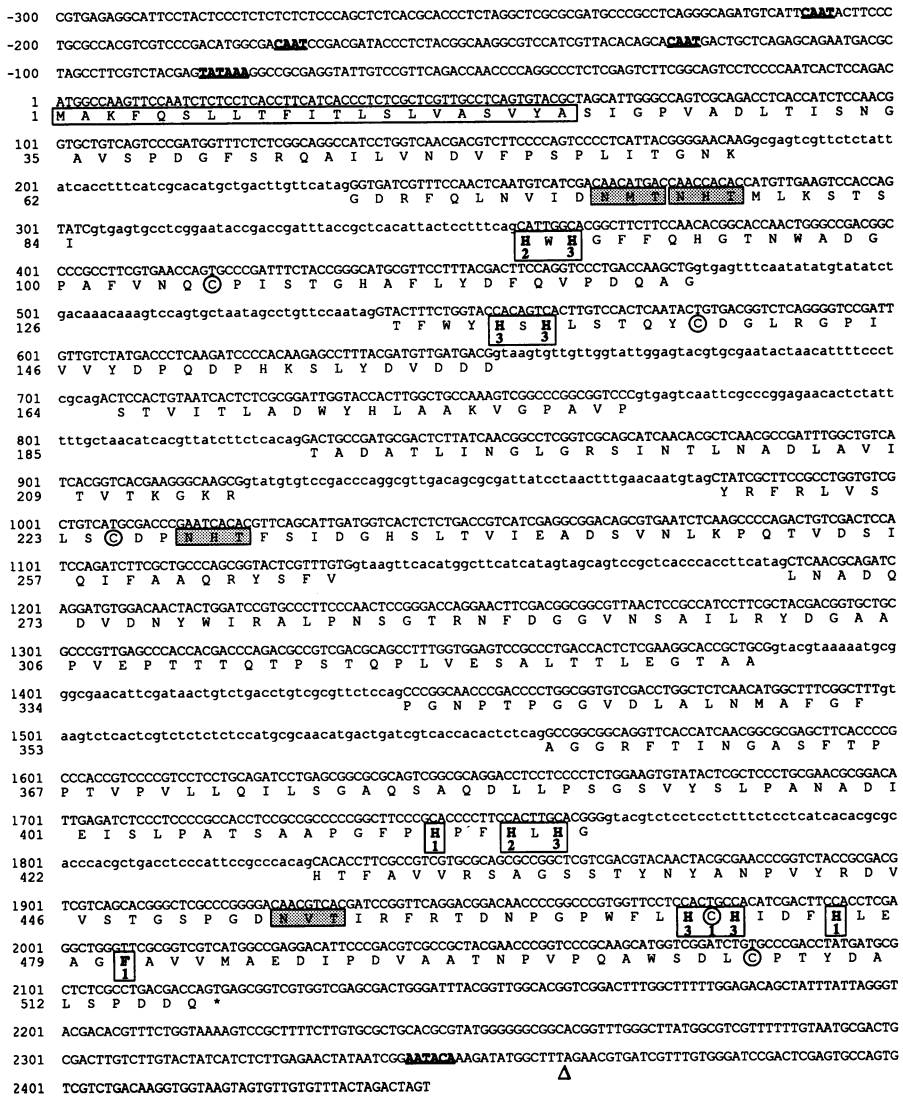

FIG. 3. Nucleotide sequence of the basidiomycete PM1 (CECT 2971) *lac1* gene. Numbering starts at the translational initiation site of the laccase I gene and refers to the nucleotide sequence and the predicted amino acid sequence of the genomic clone. The signal peptide is boxed. The 10 introns are in lower case. The putative *N*-glycosylation sites are shaded. 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 CAAT and TATA promoter elements are in bold underlined letters. A possible polyadenylation sequence at the 3′ end is underlined and the polyadenylation site is indicated (Δ).

elements located at 128, 174, and 211 bp upstream from the ATG. In addition, the *lac1* gene contains at −119 bp a 32-bp sequence which shares a close similarity to a sequence present in the 5′ flanking region of the *Saccharomyces cerevisiae SOD1* gene coding for a Cu-Zn superoxide dismutase (17). This gene is regulated by the levels of copper in the culture medium. The 32-bp sequence, shown in Fig. 4, is the binding site for the copper-regulated transcription factor ACE1, which induces the transcription of the *SOD1* gene.

Mapping the 5′ terminus of the *lac1* gene mRNA. Mycelia of basidiomycete PM1 growing in GAE medium and producing laccase were used to isolate polyadenylated mRNA, and Northern hybridization with the laccase I cDNA as a probe was performed. A single transcript of about 1.8 kb was detected (Fig. 5A). The transcriptional starting point was determined by primer extension of a 20-bp oligonucleotide complementary to the nucleotides +101 to +120 downstream from the ATG of the open reading frame in the sequence. A single transcription initiation site was detected 51 bp upstream from the ATG (Fig. 5B). This site is located 26 bp downstream of the TATAA sequence.

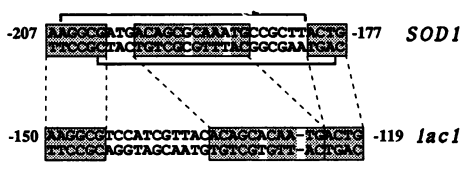

FIG. 4. Nucleotide sequence corresponding to the binding site for the ACE1 transcription factor in the *SOD1* gene from *S. cerevisiae* and the homologous sequence found in the 5′ flanking region of the *lac1* gene from basidiomycete PM1. Identical nucleotides are shaded.

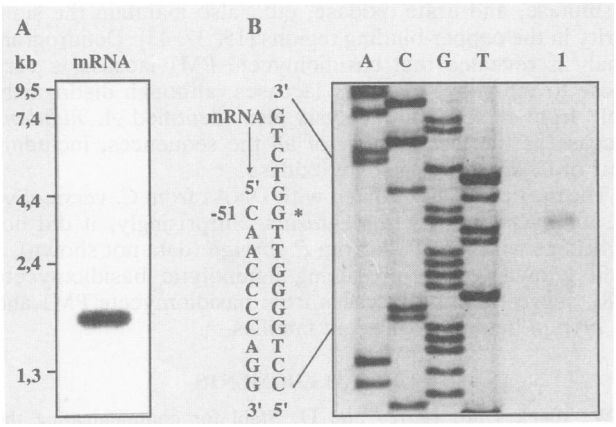

FIG. 5. Northern blot analysis (A) and determination of 5′ end (B) of the *lac1* transcript. The sequence shown corresponds to the minus strand of the genomic DNA, and the primer used for the analysis was the same oligonucleotide used to extend the mRNA with reverse transcriptase. *, transcription start site.

Comparison of the amino acid sequence encoded by the *lac1* gene with other laccases. The deduced amino acid sequence of the *lac1* gene from basidiomycete PM1 was compared with other reported laccases in order to analyze the structural relationships among them. Four blocks of maximal similarity that correspond to the regions around the sequences His-*X*-His were detected. These regions, shown in Fig. 6, are thought to be the coordination sites for the four copper ions of three different types which form the laccase redox center. The sequences of the white-rot fungal laccases in these regions are practically identical.

There is a great similarity along the whole protein sequence between basidiomycete PM1 laccase I and the laccases from other basidiomycetes such as *C. hirsutus* (23), *C. versicolor* (21), *P. radiata* (38), and *A. bisporus* (34). Similarity to those laccases was 84.1, 82.9, 71.4, and 57.6%, respectively. The percent amino acid identity was calculated for pairwise combinations of all the known fungal laccases and the ascorbate oxidase from *C. sativus* and presented as a dendrogram (Fig. 7). Laccases from basidiomycete PM1 and the two *Coriolus* species belonged to the same group, indicating a phylogenetic proximity between basidiomycete PM1 and this genus, although the amino acid identity between *C. hirsutus* and *C. versicolor* laccases was higher (89.2%). By contrast, the laccase of *A. nidulans* is less closely related than the ascorbate oxidase from *C. sativus* to the other laccases.

In Southern blot hybridization analysis of the *lac1* gene with genomic DNAs from several ligninolytic basidiomycetes, strong hybridization bands were detected in the *C. versicolor*, *H. annosum*, and *F. fomentarius* DNAs. Basidiomycete PM1 DNA showed two bands besides the band corresponding to the *lac1* gene, which were less intense and may correspond to other laccase-related genes (data not shown).

DISCUSSION

In this paper, we have described the cloning and structural analysis of the gene coding for the laccase I enzyme from basidiomycete PM1. In the agitated culture supernatants of the fungus, two bands with laccase activity were detected (9) and the antilaccase antibodies were able to recognize three

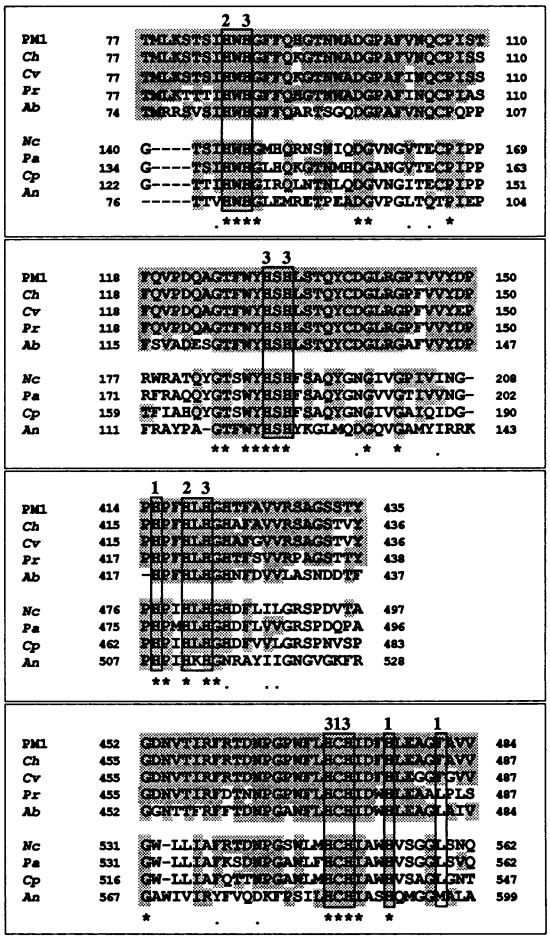

FIG. 6. Alignment of the *lac1* predicted amino acid sequence with those of other known laccase genes at the four regions of maximum similarity. Gaps were introduced where necessary to optimize the alignment. Identical (*) or conserved (.) amino acid residues in all the laccases are indicated. *Ch*, *C. hirsutus*; *Cv*, *C. versicolor*; *Pr*, *P. radiata*; *Ab*, *A. bisporus lcc2*; *Nc*, *N. crassa*; *Pa*, *Podospora anserina*; *Cp*, *C. parasitica*; *An*, *A. nidulans*.

different proteins in those supernatants according to SDS-PAGE and immunoblot analysis. Therefore, several laccase isozymes might exist in basidiomycete PM1, as in *C. versicolor* (21). Whether they are coded by different genes, as in *C. versicolor*, or come from the same gene is not known. All the positively selected clones from the cDNA library carried inserts derived from the *lac1* gene, whereas by using the same cloning strategy (34), cDNAs from *A. bisporus* corresponding to two different laccase genes were cloned. Moreover, Northern (RNA) blot analysis with a *lac1* probe showed a single hybridization band. On the other hand, two less intense bands besides that corresponding to the *lac1* gene were detected by hybridization of the basidiomycete PM1 DNA with the *lac1* probe. If these bands correspond to other laccase genes, the similarity to *lac1* must not be very high, since hybridization with DNAs from other fungi is stronger.

The *lac1* gene coding for the laccase I enzyme from basidiomycete PM1 has the coding region interrupted by 10 introns which are in exactly the same positions as those of the *C. hirsutus* laccase gene. The nine introns of *P. radiata*

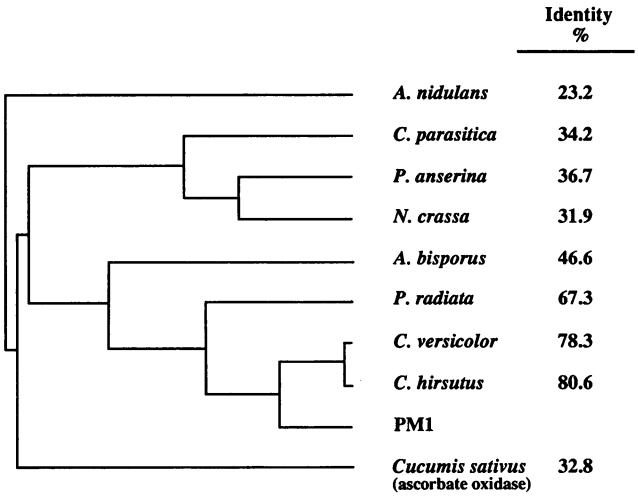

FIG. 7. Dendrogram representation of the similarities between the different laccases and the ascorbate oxidase from *C. sativus*. Analysis was carried out by using the CLUSTAL program. The numbers indicate the percent amino acid identity of the different protein sequences with the deduced amino acid sequence from the basidiomycete PM1 *lac1* gene.

and the eight of *C. versicolor* laccase genes are also located in similar positions (21, 38). Intron and exon functions and splicing signals follow the fungal consensus (3). The open reading frame contains four potential *N*-glycosylation sites. This is in agreement with the endoglycosidase H treatment that revealed the existence of *N*-glycosylation (9). However, there is a difference between the 60,000 M_r of the purified deglycosylated protein and the estimated M_r on the bases of the cDNA sequence (53,230). Therefore, some *O*-glycosylation or other posttranslational modification of the laccase I may also exist.

In the 5′ flanking region of the *lac1* gene, there is a TATA element located 32 bp from the transcription start site. Thirty-six base pairs upstream of this TATA box, there is a 32-bp sequence with a high percent similarity to a sequence contained in the 5′ region of the *S. cerevisiae SOD1* gene, which constitutes the binding site of the copper-regulated transcription factor ACE1 (17). A similar sequence has also been described for the promoter of the metallothionein-coding gene *CUP1* from *S. cerevisiae*, which is regulated by copper (13). It is not known whether the 32-bp sequence located in the promoter region of *lac1* is the binding site for a transcription factor homologous to ACE1. No other regulatory sequences have been described for the laccase or ascorbate oxidase genes reported so far, but *N. crassa* laccase activity is increased when copper is added to the culture medium (19) and there is also an increase of ascorbate oxidase activity in *Cucurbita* sp. cultured cells when copper is added to the medium (12). We could not detect an increase in laccase activity when increasing concentrations of copper were added to minimal medium, since there was no laccase activity in this medium (data not shown).

The laccase I amino acid sequence is very similar to other laccase sequences, mainly in the conserved regions containing the amino acids proposed for the coordination of the three types of copper ions that are present in the enzyme (9). These amino acids are highly conserved in most laccases and other blue copper oxidases (30). Other enzymes using copper as a cofactor such as cytochrome-*c* oxidase, superoxide dismutase, and urate oxidase, etc., also maintain the similarity in the copper-binding regions (15, 37, 43). Dendrogram analysis revealed that basidiomycete PM1 laccase is very close to the genus *Coriolus* laccases, although distinguishable from them. This analysis also identified *A. nidulans* laccase as the least similar of all the sequences, including that of *C. sativus* ascorbate oxidase.

The *lac1* probe hybridized with DNAs from *C. versicolor*, *H. annosum*, and *F. fomentarius*. Surprisingly, it did not hybridize with the DNA from *P. eryngii* (data not shown), a well known laccase-producing ligninolytic basidiomycete (18), suggesting that laccases from basidiomycete PM1 and *P. eryngii* belong to different families.

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