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Cloning and Sequencing of a Laccase Gene from the Lignin-Degrading Basidiomycete *Pleurotus ostreatus*

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The gene (*pox1*) encoding a phenol oxidase from *Pleurotus ostreatus*, a lignin-degrading basidiomycete, was cloned and sequenced, and the corresponding *pox1* cDNA was also synthesized and sequenced. The isolated gene consists of 2,592 bp, with the coding sequence being interrupted by 19 introns and flanked by an upstream region in which putative CAAT and TATA consensus sequences could be identified at positions −174 and −84, respectively. The isolation of a second cDNA (*pox2* cDNA), showing 84% similarity, and of the corresponding truncated genomic clones demonstrated the existence of a multigene family coding for isoforms of laccase in *P. ostreatus*. PCR amplifications of specific regions on the DNA of isolated monokaryons proved that the two genes are not allelic forms. The POX1 amino acid sequence deduced was compared with those of other known laccases from different fungi.

Phenol oxidase (benzenediol O_2:oxidoreductase [EC 1.10.3.2], namely, laccase) catalyzes the oxidation of various aromatic compounds (mono-, di-, and polyphenols, aminophenols, and diamines) by reducing molecular oxygen to water through an oxidoreductive multicopper system (25). This enzyme is probably involved in the metabolism of lignin (14, 17), a complex three-dimensional polymer composed of phenylpropane subunits interconnected by different carbon-carbon and carbon-oxygen-carbon bonds. The proposed physiological role of this enzyme, biosynthetic in plants (3, 31) or biodegradative in fungi (5, 15), is not definitively clarified and is still an object of investigation.

The probable catabolic role of fungal phenol oxidases in lignin biodegradation has attracted considerable interest for potential applications not only in the pulp- and paper-making processes but also in the industrially interesting bioconversions of many xenobiotic aromatic compounds with lignin-like structures (4, 26). The functional role of laccases in fungal physiology and metabolism has been comprehensively reviewed by Thurston (32).

We have studied the enzymes involved in lignin biodegradation produced by the white rot basidiomycete *Pleurotus ostreatus*. This fungus excretes at least three different phenol oxidases, one of which has been purified and extensively characterized (22), and an aryl alcohol oxidase (30), which can fulfill an "ancillary" support for the degrading action of laccases (19).

To understand the molecular basis of enzymatic catalysis and the regulatory mechanism controlling the production of different isoforms of this enzyme, one of the genes coding for phenol oxidases in *P. ostreatus* and the corresponding cDNA were cloned and sequenced. Furthermore, it was demonstrated that at least two genes were present and were not allelic forms.

MATERIALS AND METHODS

Organisms, plasmids, enzymes, and chemicals. *P. ostreatus* Florida mycelia were grown at 28°C in shaken flasks (100 rpm) containing potato dextrose broth (Difco, Detroit, Mich.) with 0.5% yeast extract (Difco) (22); *Escherichia coli* BO 3310 was used as a library propagating host strain.

Plasmids pGEM7Zf(+) and pGEM5Zf(+) were obtained from Promega Biotec, Madison, Wis. pUC18 vector and restriction and modification enzymes were purchased from Boehringer GmbH, Mannheim, Germany, or from Promega Biotec. The Sequenase version 2.0 dideoxy sequencing kit was purchased from U.S. Biochemicals, Cleveland, Ohio. Radioactive materials were obtained from Amersham International, Amersham, United Kingdom.

Oligonucleotides, probes, and primers. Oligonucleotides Mix1 (Omix1, GCG AAGCCGTC$^{G}_{T}$GG$^{G}_{T}$$^{G}_{C}T^{G}_{T}$ACGTCTTCGTTGAC$^{G}_{T}$ATGTACATGTT) based on the amino-terminal sequence of purified laccase (N-ter*Po*, amino acids 7 to 21 [22]) from *P. ostreatus* and oligonucleotides Mix2 (Omix2, GTT$^{G}_{T}$AT$^{G}_{T}$ CC$^{G}_{T}$CC$^{G}_{T}$ACGAA$^{G}_{T}$CC$^{G}_{T}$GT) constructed against the sequence of a tryptic peptide (T1*Po*, amino acids 8 to 15 [22]) purified from the same enzyme were synthesized at Beckman Italia, Milan, Italy. Amplification primers described in the following paragraphs and all other primers used were purchased from Beckman. Oligo-dT- Not I primer-adapter was purchased from Promega Biotec.

Construction of the genomic DNA library. Chromosomal high-molecular-weight DNA from *P. ostreatus* was prepared as described by Raeder and Broda (24). DNA was partially digested with *Sau*3AI (1-h incubation with 0.03 U of enzyme per μg of DNA), and fragments in the molecular size range 2,000 to 4,000 bp were isolated by means of polyacrylamide gel electrophoresis and electroelution. Partial filling in with *E. coli* DNA polymerase Klenow fragment, dGTP, and dATP was performed on size-selected DNA.

The DNA was cloned into the plasmid vector pGEM7Zf(+), previously made end compatible to the DNA fragments by linearization with *Xho*I and partially filled in with Klenow fragment, dCTP, and dTTP.

Propagation and amplification of the library were performed by transforming *E. coli* BO 3310 competent cells (hexamminecobalt chloride procedure [28]) with the ligation mixture and growing the transformants for 4 h in selection medium containing 100 μg of ampicillin per ml.

Southern blot hybridization. Oligonucleotide probes were 5′-end labeled with ^{32}P to a specific activity of 1.0×10^6 cpm/pmol, using [γ-^{32}P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase (28).

The genomic DNA, digested with *Eco*RI, *Bam*HI, and *Eco*RI-*Bam*HI, was electrophoresed in a 0.8% agarose gel and transferred to Hybond N membrane (Amersham). The hybridizations were carried out in 5× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (28). Optimal hybridization temperatures were determined as 55°C for Omix1 and 37°C for Omix2. The nylon membrane was autoradiographed with RX X-ray film (Fuji Film, Tokyo, Japan).

Isolation and sequencing of laccase clones. The colony hybridization experiments were performed under the same conditions described for the Southern blot analysis. The clones were selected with the oligonucleotide mixture Omix1 and purified after a second colony hybridization. Purified plasmid DNAs of the selected clones were also probed with Omix2. Suitable restriction fragments from the positive clones were subcloned into pUC18 vector and sequenced in duplicate and on both strands by the dideoxy-chain termination method (29) with alkali-denatured plasmid templates (13).

mRNA isolation. *P. ostreatus* mycelia were harvested from the culture after incubation at 28°C for 4 days. Total RNA was extracted from the lyophilized mycelia as described by Lucas et al. (18). Poly(A)-containing RNAs were purified by oligo(dT)-cellulose chromatography as described previously (28).

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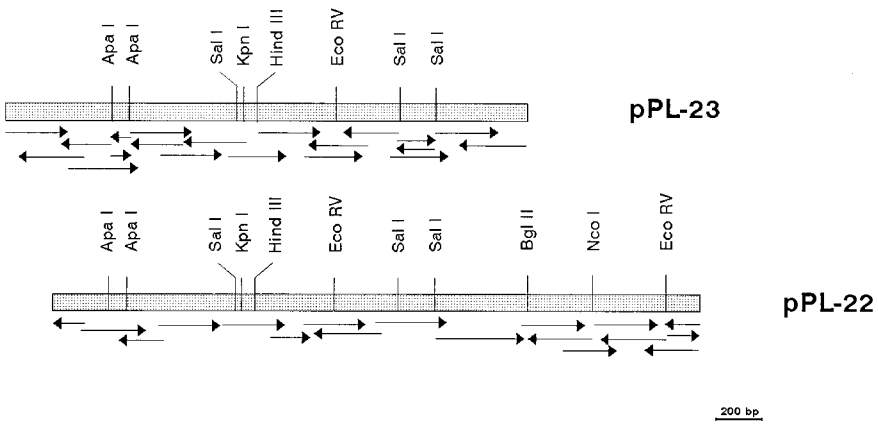

FIG. 1. Restriction maps of pPL-22 and pPL-23 and sequence strategies.

cDNA synthesis and cloning. cDNA synthesis was performed by rapid amplification of cDNA ends under the same experimental conditions described by Frohman et al. (9) and with the oligo O4 as the primer for reverse transcription. The amplification experiments were carried out at 50°C (annealing temperature). For the amplification, the following primers were used:

O1: (1) 5′-ATGTTTCCAGGCGCACGG-3′ (18)
O2: (1516) 5′-C | CAGTCGATCCCAAGTTGGG-3′ (1444)
O3: (1437) 5′-CGCAGATCCCAACTTGGGATCGACTG | GCTT-3′ (1519)
O4: 5′-AATTCGCGGCCGCTTTTTTTTTTTTTTT-3′

The first three primers were derived from the nucleotide sequence determined for the *pox1* gene (numbers in parentheses refer to the numbers used in Fig. 2; vertical bars indicate intron interruptions). Sequence O2 corresponds to the lower strand.

Isolation of monokaryons and PCR gene amplification analysis. *P. ostreatus* mycelia were grown on millet grains in darkness for 1 month, and the fruiting bodies were then obtained after 3 days of exposure to light. Different dilutions of basidiospores, collected from the sterile plastic bag in which the fungus was wrapped, were spread onto PDY agar plates (potato-dextrose agar, 0.5% yeast extract) and incubated at 37°C overnight. After 1 week of incubation at 28°C, visible germinated spores were transferred onto separate plates.

DNA was extracted from monokaryons by the procedure described by Cenis (6). Specific amplifications were performed with, as primers, the oligonucleotide couple O5 (644-GCT GGC ACG TTC TGT AAG-662) and O6 (1214-TCT GCC TCG ATG ACC TGC-1196) for *pox1* and O7 (GCT GGA ACG TTT TGT AAG) and O8 (TCT GCT TCA ATG ACG AGC) for *pox2*, with numbers referring to the numbers used in Fig. 2. Annealing temperatures were 60 and 55°C, respectively.

Search for and analysis of sequence similarity. A computer search was made to find laccases from different sources with sequences similar to the protein sequence derived from the isolated gene, using data from the EMBL and the Swiss Prot Data Bank. Gene and protein sequences were analyzed with CLUSTAL, PALIGN, and NALIGN PC GENE programs (Intelligenetics Inc., Mountain View, Calif.).

Nucleotide sequence accession numbers. The *pox1* and cDNA*pox1* nucleotide sequences reported in this paper have been entered in the EMBL Data Library-GenBank and assigned accession numbers Z22591 and Z34847, respectively.

RESULTS

P. ostreatus genomic DNA was partially digested to obtain fragments with molecular weights suitable to contain complete fungal genes. The cloning strategy described in Materials and Methods was used to set up a representative gene library in the pGEM7Zf(+) plasmid vector to efficiently obtain a single insertion of DNA fragment from the *P. ostreatus* genome per vector molecule. A library containing 1.8×10^6 independent clones was obtained. This number is far larger than that (6.7×10^4) required to represent the whole genome, taking into account the genome size of related fungi (4.4×10^7 bp for *Phanerochaete chrysosporium* [24]).

The library was screened by hybridization with the ^{32}P-labeled oligonucleotide mixture Omix1. To determine the optimal stringency conditions, genomic Southern hybridization experiments were performed and specific hybridization signals were obtained. The screening of 180,000 transformants produced six hybridization-positive clones. These clones were further probed with the ^{32}P-labeled oligonucleotide Omix2. This probe was designed from a reverse translation of a *P. ostreatus* laccase tryptic peptide (T1*Po*), which was located, by homology comparison with *Coriolus hirsutus* laccase (16), in a central position of the protein sequence.

Two of six clones, pPL-22 and pPL-23, were selected, since they hybridized not only with Omix1 but also with Omix2 and contained inserts up to 2,500 bp. As revealed by restriction analysis, more than 80% of the sequence of these clones overlapped (Fig. 1). Both clones were completely sequenced, and it was confirmed that the common core region contained identical sequences. From these data, a nucleotide sequence of 3,155 bp was determined (Fig. 2).

First-strand cDNAs were reverse transcribed from mRNA of a 4-day-old mycelium culture. Two different amplification experiments were performed with O1 and O2 or O3 and O4 oligonucleotides as primers. Two fragments, 900 bp (5′-*pox1* cDNA) and 750 bp (3′-*pox1* cDNA), which together accounted for the entire *pox1* cDNA, were produced. Sequence analysis of these fragments, together with the previously determined nucleotide sequence, led to the definition of the gene (*pox1*) structure and the complete sequence of the encoded protein (POX1), as well as extended upstream and downstream untranslated regions (Fig. 2). Nineteen introns interrupted the coding sequence, and putative CAAT and TATA consensus sequences could be seen in positions −174 and −84, respectively, of the 5′-flanking region.

The isolated gene codes for a protein of 529 amino acids. The translation of 69 bp starting at the initiation codon ATG resulted in a 23-amino-acid putative signal peptide, which shows the typical structure for the sorting of secreted proteins in eukaryotes (33). Potential N-glycosylation sites (Asn-Xxx-Ser/Thr) were found in positions 57, 239, 282, 372, and 465 of the protein, with Asn-372 having a low probability of being glycosylated because of a proline at the C-terminal side of serine (10).

To confirm the identity of *pox1* as a laccase gene, the amino acid sequence deduced was compared with those of other known laccases (Fig. 3). In fact, the comparison with *Coriolus hirsutus*, basidiomycete PM1 (CECT 2971), *Phlebia radiata*, and *Agaricus bisporus* laccases (8, 16, 23, 27) showed a high

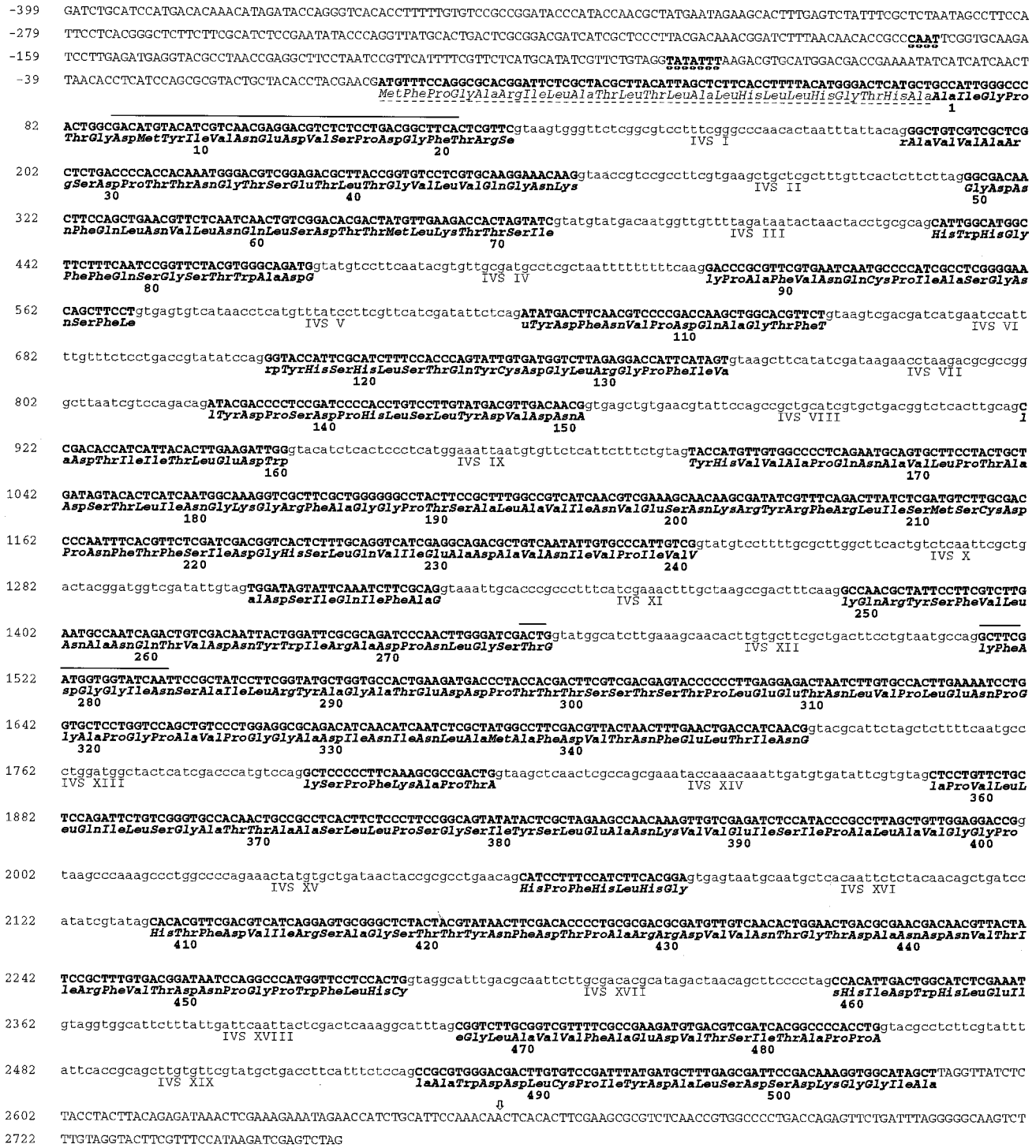

FIG. 2. Nucleotide sequence of the *P. ostreatus pox1* gene with the deduced amino acid sequence of POX1. Putative TATA and CAAT boxes are underlined (°°°°°°°). Introns are shown in lowercase type and indicated by IVS followed by roman numerals. The putative signal peptide is underlined (– – – – –). The polyadenylation site observed is shown by an arrow (⇩). Oligonucleotides Omix1 and Omix2, used for the screening of the genomic library, are overlined.

degree of similarity (63, 61, 59, and 45%, respectively). A much lower score was obtained when the analysis was performed in relation to the ascomycetes *Neurospora crassa* (27%), *Aspergillus nidulans* (20%), and *Cryphonectria parasitica* (26%) laccases (1, 7, 11).

Despite the good match between the amino acid sequences of N-ter*Po* and T1*Po* and the sequence encoded by the *pox1* gene, some discrepancies were noted, as shown in Fig. 4. Furthermore, in the amplification experiments with O1 and O2 as primers, six of eight clones (5′-*pox1* cDNA) were proved to correspond exactly to the gene sequence, while there were significant differences (84% of sequence identity [data not

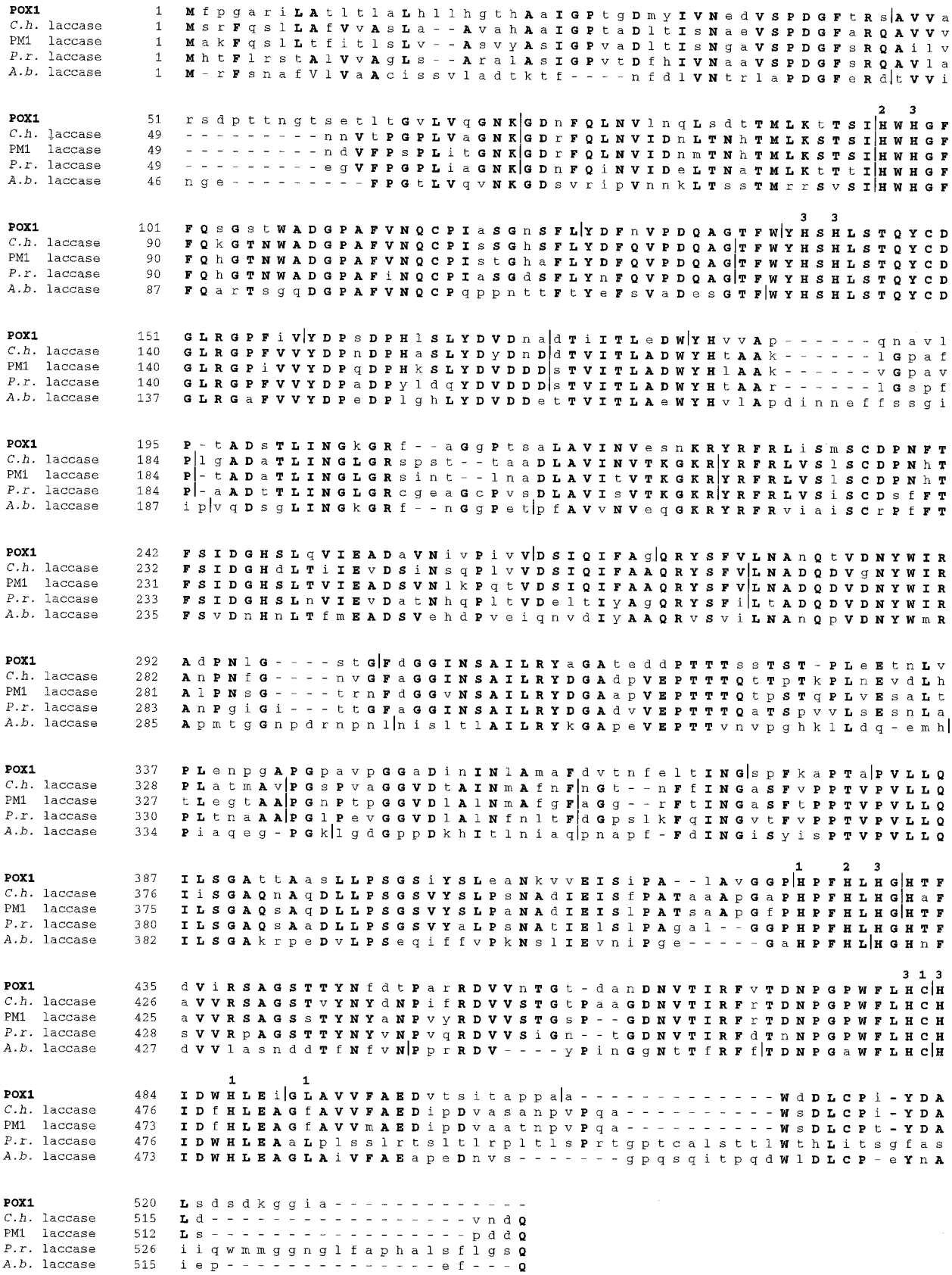

FIG. 3. Alignment of POX1 with *Coriolus hirsutus* (*C.h.*), basidiomycete PM1, *Phlebia radiata* (*P.r.*), and *Agaricus bisporus* (*A.b.*) laccases. Vertical bars indicate intron positions. Possible copper-binding amino acids are indicated by the number corresponding to the copper type.

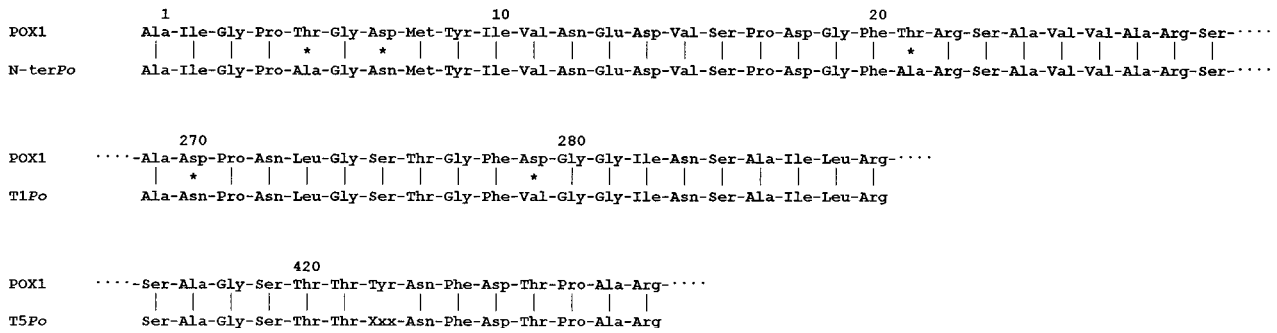

FIG. 4. Comparison of N-terminal (N-ter*Po*) and two tryptic peptide (T1*Po* and T5*Po*) sequences with the corresponding POX1 sequences. Discrepancies are indicated by *.

shown]) with the sequences of the other two clones (5′-*pox2* cDNA).

It is worth noting that the N-terminal sequence of the protein and the sequence of the tryptic peptide T1*Po* were encoded exactly by the *pox2* cDNA, while the corresponding amino acid sequence, derived from *pox1* cDNA, showed five amino acid substitutions. Therefore, it can be concluded that *pox2* cDNA, and not *pox1*, encodes the laccase previously isolated and characterized (22). Furthermore, three genomic clones, positive to Omix1 hybridization, were found to contain differently truncated 5′ portions of *pox2*, whose complete sequence analysis is in progress.

To establish whether *pox1* and *pox2* were alleles or represented distinct members of a gene family, two separate PCR amplifications were performed on the genomic DNAs of six haploid isolates. One of the two different oligonucleotide couples was specific for the *pox1* gene sequence, and the other was designed on the basis of the sequence of the genomic clone pPL-42, corresponding to the *pox2* gene (data not shown). The two primer sets were located at the same position on both gene sequences and allowed the amplification of 570-bp fragments. However, it was possible to distinguish them on the basis of restriction analysis. In fact, the presence of a unique *Hin*dIII site in the *pox1* fragment and a single *Bam*HI site in the *pox2* fragment confirmed the specificity of the amplification. Figure 5 shows the restriction analysis for the dikaryon and one of the six monokaryons. Identical patterns were obtained for all the other monokaryotic isolates. The selective cleavages of the two specifically amplified fragments on the haploid genomic DNA demonstrate that the two genes segregate together into monokaryons and hence are not allelic forms.

DISCUSSION

A gene coding for a laccase from *P. ostreatus* was cloned and sequenced; the structure of the gene was inferred from sequence information of cDNA synthesized by PCR amplification of the specific retrotranscript. The isolated gene (Fig. 2) consists of 2,592 bp interrupted by 19 introns, 47 to 64 bp long. All the introns have a GT(a/g)NG(c/t) consensus sequence at the 5′ splicing site and a (c/t)N(c/t)AG consensus sequence at the 3′ splicing site, both similar to the canonical sequences of splicing in other eukaryotes (2). Within 7 to 21 bp upstream from the 3′ splicing site, an internal consensus sequence ($G_{47\%}/A_{31\%}C_{68\%}/T_{32\%}T_{79\%}/C_{21\%}G_{63\%}/A_{26\%}AC_{58\%}/T_{42\%}$) for the lariat formation can also be located. The number of introns is unusually large compared with other fungal genes, particularly the laccase genes from *Phlebia radiata* (9 introns), *Coriolus hirsutus* (10 introns), PM1 (10 introns), and *Agaricus bisporus* (14 introns) (8, 16, 23, 27).

The high degree of similarity rendered possible an alignment of the *P. ostreatus* deduced protein sequence with those from the basidiomycetes *Coriolus*, PM1, *Phlebia*, and *Agaricus* species. The cysteine residues, probably involved in disulfide bridge formation and found in all the basidiomycetic sequences compared, were also present in POX1 (Cys-117 to Cys-514 and Cys-149 to Cys-236). A putative N-glycosylation site (Thr-465 in POX1) is present in all the compared basidiomycetic proteins and is located in a highly conserved region of the proteins. Similarly, the amino acid regions thought to contain the copper coordination sites (20) are highly conserved in all the sequences analyzed, including those from ascomycetes. This result essentially agreed with the comparative analysis performed by Coll et al. (8).

Many isoforms of enzymes involved in lignin degradation have been demonstrated, to date, to be encoded by more than one gene. Some of these genes were shown to be only allelic counterparts of sister chromosomes (12, 16), while others are

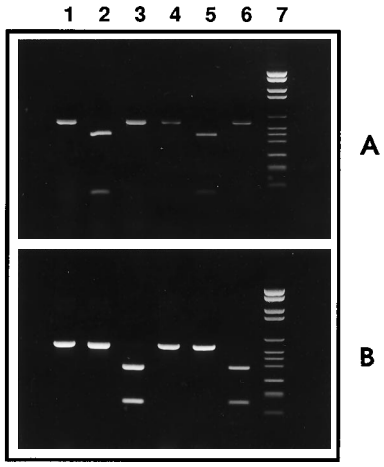

FIG. 5. Restriction analysis of monokaryotic DNAs. Two separate PCR amplifications were performed on the genomic DNAs of six haploid isolates, using two different oligonucleotide couples specific for *pox1* (A) or *pox2* (B). The figure shows the analysis performed on the dikaryon (lanes 1 to 3) and one of the monokaryons (lanes 4 to 6). The amplified fragments (lanes 1 and 4, undigested) in the two different experiments were digested with *Hin*dIII (lanes 2 and 5, unique site in *pox1*, absent in the *pox2* fragment) and *Bam*HI (lanes 4 and 6, unique site in *pox2*, absent in the *pox1* fragment). Molecular size markers (2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp) are shown in lane 7 (DNA molecular weight marker VI; Boehringer).

distinct genes belonging to multigene families (23). Also in *P. ostreatus*, the existence of two distinct genes coding for laccases is demonstrated by the isolation of different genomic and cDNA sequences. The coding regions of these sequences showed a very high degree of similarity, although substantial and significant substitutions were present.

Furthermore, a noticeable diversity in the restriction maps of the two genes (data not shown) suggested the duplication of an ancestor gene. PCR experiments on the haploid isolates definitively demonstrated that the two genes were not allelic but doubled on the same genome. The two genes might just have evolved independently and therefore code for two distinct isoenzymes. The detection of at least three phenol oxidases with quite different substrate specificities (22) in the culture medium of *P. ostreatus* mycelia strongly supports these results.

It would be interesting to investigate the physiological role and the structure-function relationships of these isoenzymes. The location of different laccase forms, not only in the extracellular environment but also in different intracellular compartments, has been ascertained from in vivo immunostaining experiments performed on other fungi (21). The synthesis of these enzymes can also be modulated to differing extents in response to different cell stimuli and/or by specific inducers. These observations suggest the possibility of a cell-sorting-dependent difference in the various laccase isoforms. With this aim, comparative studies are in progress on gene regulation as well as on growth conditions able to differentially induce isoenzyme expression.

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