Identification of two laccase genes in the cultivated mushroom
Agaricus bisporus

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A cDNA library was constructed in λgt11 using mRNA from 11-d-old mycelium of Agaricus bisporus. Three clones containing laccase sequence were identified using an affinity-purified anti-laccase antibody. From one of these clones, a 333 bp sequence was used to identify further cDNA clones (including one which is close to full length) and a genomic clone. The coding sequences found were of two similar but not identical versions with differences at 36 out of 520 residues of deduced amino acid sequence. The laccase genes each encode a sequence expressed as a 2.3 kb mRNA, specifying a 520 residue polypeptide including a 19 amino acid residue signal peptide that is absent from the N terminus of the mature (extracellular) protein. The coding sequence of lcc1 is interrupted by 14 short introns. The lcc1 and lcc2 genes are not allelic as they do not segregate in uninucleate spores derived from a four-spored basidium. Comparison of the deduced amino acid sequences with that of the other fungal laccases that have been cloned, and with the very similar ascorbate oxidases from higher plants shows that whilst some sequence is absolutely conserved at and around the amino acid residues involved in copper binding, the overall sequence similarities are low.

Introduction

Laccase (polyphenol oxidase: EC 1.10.3.2) is produced very abundantly by Agaricus bisporus, constituting 2% of mycelial protein during vegetative growth (Wood, 1980a). The enzyme is found predominantly in the medium and is a glycoprotein of 65 kDa apparent molecular mass that may exist as a dimer (Wood, 1980a; Perry et al., 1993). Although the 65 kDa ‘main polypeptide’ is the form of the enzyme first revealed by in vivo labelling and also constitutes a large fraction of the purified enzyme, smaller species generated by proteolytic cleavage are always present (Perry et al., 1993). Heterogeneity may also be a consequence of variation in the extent of glycosylation of individual laccase molecules, but this has not been verified experimentally. In vitro translation and immunoprecipitation have been used to show that laccase is formed from a nascent polypeptide of 57 kDa apparent molecular mass (Perry et al., 1993).

During recent years the laccase gene and/or cDNA sequence has been described from four sources of which the first to be reported was the ascomycete fungus Neurospora crassa (Germann & Lerch, 1986; Germann et al., 1988). Subsequently the sequences from another ascomycete, Aspergillus nidulans (Aramayo & Timberlake, 1990) and two basidiomycetes, Coriolus hirsutus (Kojima et al., 1990) and Phlebia radiata (Saloheimo et al., 1991) have been published. These sequences show a common pattern, in that they all encode polypeptides of about 550 amino acid residues including an N-terminal signal peptide. In addition, the one cysteine and 10 histidine residues involved in binding the four copper atoms found in each laccase molecule are conserved, together with a small amount of sequence around the

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers L10664 for lcc1 and L10663 for lcc2.
four regions in which the copper ligands are clustered. It is also evident that there is some basic similarity amongst all the large blue copper proteins, that is the laccases, the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Germann et al., 1988; Messerschmidt & Huber, 1990).

Studies of laccase at the molecular level in Ag. bisporus have been undertaken, not only because it is such an abundant protein in this organism, but also because its regulation of expression and activity are (at least at the physiological level) closely integrated with fruit body development (Wood & Goodenough, 1977; Wood, 1980b; Smith et al., 1989).

Methods

Organism. Agaricus bisporus strain D649 was maintained on malt agar at 25 °C and subcultured at monthly intervals. Homokaryon strains B237, B238, B239 and B240 (Loftus et al., 1988) were the complete tetrad progeny from a (rare) four-spored basidium, isolated by Elliott (1972). Cultures for mRNA or DNA isolation were grown in malt-extract liquid medium as described previously (Perry et al., 1993).

Host-vector systems. cDNA library construction employed λgt11 in Escherichia coli Y1090 (Young & Davis, 1983). A genomic clone was isolated from a partial genomic library of Ag. bisporus D649 DNA (Loftus et al., 1988) in λEMBL3 in E. coli NM392 (Frischauf et al., 1983). Subcloned sequences were manipulated in pBluescript KS (Strategene) in E. coli XL1Blue (Strategene).

cDNA cloning. Poly(A)-containing RNA was isolated from the mycelium of 11-d-old Ag. bisporus cultures in malt-extract liquid medium as described previously (Perry et al., 1993). cDNA synthesis employing AMV reverse transcriptase was performed essentially as described previously (McBride & Thurston, 1983). Double stranded cDNA was blunt-ended and ligated to EcoRI adaptors (Pharmacia) for ligation to λgt11 arms (Amersham).

Isolation of laccase cDNA clones. Plaque lifts were screened with an affinity-purified antibody, raised against pure laccase main polypeptide (Perry et al., 1993), using phosphatase-labelled goat anti-rabbit IgG as second antibody, as described by Huynh et al. (1983).

Isolation of clones by hybridization. A fragment from one expressing clone (plcc5a, see Results and Discussion) was used as a random hexamer-primed radiolabelled probe to isolate further (non-expressing) cDNA clones and a genomic clone (λcc1g, see Results and Discussion) from the partial genomic DNA library made by Loftus et al. (1988) by hybridization to plaque lifts using standard methods (Sambrook et al., 1989).

Northern blot analysis. Glyoxylated RNA was analysed by electrophoresis and hybridization with plcc5a as a radiolabelled probe as described previously (Pollard et al., 1988; Thurston et al., 1988).

Sub-cloning and sequencing. Sub-cloning into pBluescript, DNA preparation and restriction analysis were performed by standard methods following Sambrook et al. (1989). Sequencing by the dideoxynucleotide method (Sanger et al., 1977) was carried out on double stranded templates using Sequence version 2 (USB) with specific chemically synthesized oligonucleotide primers where necessary. All sequence reported was determined on both strands.

PCR. Complete inserts of the cDNA clones in λgt11 were amplified using commercially available primers (Promega) complementary to sequence either side of the cloning site in this vector with Taq polymerase (M186, Promega), for 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 3 min at 72 °C, followed by one 99 min period of extension at 72 °C.

Amplification of a fragment from the genomic DNA of Ag. bisporus D649 was for 39 cycles of 1 min at 95 °C, 1 min at 60 °C and 3 min at 72 °C, followed by one 99 min period of extension at 72 °C. The primers for this reaction were complementary to a subterminal region of the λccg1 sequence (5’-3’, residues 2627–2645) and complementary to a subterminal region of plcc6 (3’-5’, residues 2981–2964, as numbered in Fig. 1 in both cases).

All PCR products were isolated by electrophoresis, blunt-ended by filling in with T4 DNA polymerase and cloned into the Smal site of pBluescript. Sequence reported from PCR-derived clones was obtained from three independently amplified DNA fragments giving identical sequence in every case.

Sequence analysis. Sequence data were assembled using IBI Pustell MacVector software. Sequence comparison and alignment was performed using GCG programs (Devereux et al., 1984).

Results and Discussion

Isolation of laccase cDNA clones

cDNA from mRNA of 11-d-old mycelium of Ag. bisporus was used to generate a library of about 107 recombinants in λgt11, using EcoRI adaptors. When 106 plaques were screened with affinity-purified anti-laccase IgG, three positively reacting plaques were isolated and purified (λlcc411, λlcc511 and λlcc611). All three recombinants were found to synthesize immunoreactive fusion proteins when converted to lysogens (data not shown). The λlcc511 insert was subcloned as two EcoRI fragments, plcc5a (333 bp) and plcc5b (110 bp). The plcc5a fragment contained an ORF, in-frame with the β-galactosidase coding sequence of the vector, that encoded the deduced amino acid sequence (residues 408–518 in Fig. 2), including the readily recognizable conserved laccase copper-binding motifs HisProPheHisLeuHisGlyHis(417–424) and HisCysHisAspTrpHis(470–476). When the sequence of plcc5b was joined to the plcc5a sequence at the end that did not match the adaptor sequence used, the ORF was continued for two amino acid residues, followed by a stop codon and 100 bp of 3’-non-coding sequence. The relative orientation of these two subclones (and their contiguity) was subsequently confirmed by PCR amplification and sequencing of the complete insert of λlcc511. The insert of λlcc411 was 109 bp entirely included within the sequence of plcc5a (encoding amino acid residues 434 to 469 in Fig. 2, also in-frame with the vector β-galactosidase sequence). The third independently isolated cDNA clone λlcc611, yielded a single EcoRI fragment on subcloning (plcc6, residues 2754–2841 and 2907–3008 in Fig. 1) that encoded the same two amino acid residues and stop
CTACGTCTTGACAGTATGTCTCTGAAGCACATTGTTAATCATGAGC~AA~CTAAGGCCGATTTAGCTACGGTGA~TC~T~ACCAAC~TC
CAGTGGCAAAGCGCTGGTTTTAGTATTAAATCACACAAACTG~CCACAGTACAACGCCTGCAAG~ACGCGCGTGATGTTCAATACAAACG
TTGAACATCTATGCGCCTGTAACGGGGAGAGAAACCTCGTGAAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATT
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AGGATCAACA AGT AGG CGT TCC AAC GCT GAT GTA TTA GTA TTA GTC GCC GCA TGC ATT TCG AGT GTA GTT GCG AAA
Met Arg Leu Ser Asn Ala Leu Val Leu Val Ala Ala Cys Ile Ser Ser Val Val Arg Val Ala Lys 10

ACC AGA ACC TTC GAC TATG GAT ACC TGC GCT TATG GAT ACC GAG TATG GTA GAT GTA GAT GTA GAT GTA GAT GTA
Thr Arg Thr Phe Asp Phe Asp Leu Val Asp Thr Arg Ala Pro Asp Gly Phe Glu Arg Asp Asp Asp Asp Asp Asp Asp
30

Fig. 1. For legend see p. 1212.
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Fig. 1. The nucleotide sequence of the laccase gene lccl from Ag. bisporus. Intron sequences are shown in lower case. Putative 'CAAT' and 'TATA' motifs in the 5' non-coding region are boxed. Other motifs in the 5' non-coding region are: an inverted repeat between residues 153 and 199 (underlined); a 13 bp sequence homologous to residues 87-75 upstream of an ORF in the La France disease dsRNA MI of Ag. bisporus (overlined 'a', residues 117-129, cf. Harmsen et al., 1991); a 10 bp sequence homologous to residues 59-50 of the N. crassa laccase gene 5' region (overlined 'b', residues 177-186, cf. Germann et al., 1988); a 10 bp sequence homologous to residues 163 to 172 of the C. hirsutus 5' region (overlined 'c', residues 297-306, cf. Kojima et al., 1990).

Possible N-glycosylation sites in the deduced amino acid sequence are shown with an asterisk. Lys₁ is the N-terminal residue of the mature extracellular protein.

Codon found at the 5' end of plcc5b, but was not identical with much of the 3'-non-coding sequence in that clone. PCR amplification of the complete insert in lcc611 showed the presence of an additional insert sequence encoding the seven amino acid residues upstream of the two in the subcloned fragment (i.e. residues 512-518 in Figs 1 and 2) again in-frame with the vector ORF.

The plasmid subclone plcc5a was used as a probe to
isolate further clones from the cDNA library. A cDNA clone lcc451 (one of several apparently identical isolates) gave a 1014 bp EcoRI fragment (encoding residues 180–518 in Fig. 2). PCR amplification of the complete insert of lcc451 showed the presence of four further EcoRI fragments. This sequence begins 5 bp from the start of the Met codon shown as the first residue in Fig. 2 and includes 76 bp of the 3' non-coding sequence derived from plcc5b (1634 bp in all). Within this sequence two regions align with amino acid sequence determined chemically from pure laccase protein. The N terminus of the mature protein is identical with the sequence from Asp., for the 60 residues determined (Fig. 2). The sequence from His., is identical to the N-terminal sequence of a cyanogen bromide fragment of pure laccase for 20 residues (Fig. 2).

Laccase mRNA

Northern blot analysis of both total RNA (Fig. 3) and the poly(A)–containing RNA fraction (not shown) from malt extract-grown Ag. bisporus mycelium showed a single band of mRNA for laccase when probed with a laccase cDNA sequence (plcc5a). Laccase mRNA was estimated to be 2.3 ± 0.15 kb.

Isolation of a genomic laccase sequence

The plasmid subclone plcc5a was also used to probe a partial genomic library of the same strain of Ag. bisporus (Loftus et al., 1988), from which a single reacting clone lccg1 was obtained. From subclones obtained as KpnI, EcoRI and SalI fragments, the sequence shown in Fig. 1.
was located by hybridization with 32P-labelled plcc5a DNA (lane 2).

The laccase probe also hybridized to some bands of marker HindIII-by electrophoresis. After transfer to nitrocellulose, the laccase mRNA digested was determined, as far as residue d-old is 2.3 kb.

Fig. 3. Northern blot analysis of laccase mRNA. Total RNA from 7-
d-old Ag. bisporus mycelium was denatured with glyoxal and separated by electrophoresis. After transfer to nitrocellulose, the laccase mRNA was located by hybridization with 32P-labelled plcc5a DNA (lane 2). The laccase probe also hybridized to some bands of marker HindIII-digested λ phage DNA (lane 1). The most heavily labelled marker band is 2.3 kb.

was determined, as far as residue 2701. The remaining sequence (2702–3008) was obtained by PCR amplification from Ag. bisporus D649 DNA (see Methods).

Existence of two laccase genes

The deduced coding sequence of the overlapping set of cDNA clones (lccl511, lccl411 and lccl451) differed from the cDNA clone lccl1 and the sequence found as exons in the genomic sequence at 36 out of 520 residues. The genomic sequence is named lccl and is shown in Fig. 1. The sequence that we have described only in cDNA is named lccl2 and its deduced coding sequence is shown in Fig. 2, together with indication of those residues that differ in the coding sequence of lccl (at the nucleic acid level, lccl1 and lccl2 differ at 135 of 1563 bp or 8.6% of sequence).

Ag. bisporus strain D649 is a heterokaryon carrying at least two nuclear types, such that the two laccase sequences we have detected could be alleles at the same genetic locus. That this is not so has been established by analysis of a tetrad of homokaryotic strains, isolated by Elliott (1972) from a single four-spored basidium. These four haploid strains all show the same pattern of bands in Southern blot analysis with a laccase probe that binds both lccl and lccl2 sequence (Fig. 4). If the two genes were allelic, two distinct patterns would have segregated 2:2 amongst the four strains, as shown with the control probe λAb22 (Loftus et al., 1988). Further, in a cosmid library of genomic DNA from Ag. bisporus strain C-54-carb-8 (a fungicide-resistant mutant; Loftus et al., 1988), restriction mapping using the same laccase probe showed the presence of more than one gene sequence in several independently isolated clones (Sodhi, 1992).

Structure of the lccl gene

The coding sequence of lccl is interrupted by 14 short introns (48–60 bp) that start with the consensus sequence GTNN(G/T) and end with (T/A/C)A(G/T). Eleven of the 14 have an internal ACT sequence 10–20 bp from 3’ splicing site. These structures are generally in accord with the intron structure of the cell gene and glycer-aldehyde-3-phosphate dehydrogenase genes of Ag. bisporus (Raguz et al., 1992; Harmsen et al., 1992). The fifteenth intron (66 bp) is in the 3’ non-coding sequence and does not obviously exhibit any of the above consensus features.

Upstream of the coding sequence, seven sequence motifs of possible significance have been noticed. First, two possible TATA box and one CAAT element are present (boxed in Fig. 1). Secondly, there are three sequences showing homology to putative promoter region sequence found elsewhere. Two are short regions of homology to promoter region sequence of other fungal laccase genes (see Fig. 1) and the third is a region of homology to sequence 5’ to an ORF in the M1 dsRNA associated with La France disease of Ag. bisporus (Harmsen et al., 1991). Thirdly, the sequence 153–200 contains an inverted repeat of 16 out of 19 residues. Within the 5’ non-coding region there are nine further matches to transcription factor recognition sequences of vertebrates (Faisst & Meyer, 1992) or higher plants (Katagiri & Chua, 1992) if comparison of 6 or 7 bp sequences is made, but no significance can presently be attributed to any such homology. We have not identified any homology with the promoter region of the cell1 gene or the glycer-aldehyde-3-phosphate dehydrogenase genes of Ag. bisporus (Raguz et al., 1992; Harmsen et al., 1992), other than that these genes also have putative TATA and CAAT box sequences.

The deduced amino acid sequence of lccl1 and lccl2

The coding sequence of lccl is 520 amino acid residues of which the first 19 are signal peptide cleaved during export. The N-terminal sequence of the purified extra-
Fig. 4. Southern blot analysis of DNA from a set of homokaryotic strains of *Ag. bisporus*, derived from a single four-spored basidium, probed with a laccase genomic DNA fragment (a) and an anonymous *Ag. bisporus* RFLP probe *zAb22* (b). Lanes 1–4 contain DNA from strains B237, B238, B239 and B240, respectively. In (a) DNA was restricted with *EcoRI* (E), *SalI* (S) and *HindIII* (H). In (b), digests were with *EcoRI* only. The laccase probe gives identical patterns for all four strains, whereas the control probe *zAb22* gives two distinct patterns (1 and 3 are different from 2 and 4 in b).
cellular protein matches the sequence of lcc2 and not lcc1 at all seven positions that differ within the region for which amino acid sequence was chemically determined (Fig. 2). Either the mature protein translated from lcc1 is N-terminally blocked or the extent of expression of lcc1 is significantly less than that of lcc2, although some expression of lcc1 is expected as the sequence was detected as cDNA.

The encoded polypeptides have calculated M, values of 58118.8 Da (lcc1) and 57827.4 (lcc2) and isoelectric points of 5-79 and 4-72, respectively. The size of the nascent polypeptide obtained by in vitro translation and immunoprecipitation was estimated to be 57 kDa (Perry et al., 1993) which is in accord with the above calculated mass values. The calculated isoelectric pH of the products of lcc1 and lcc2 differ by a readily detectable amount, but as extracellular laccase focuses as a diffuse band at pH 3.4-4.0 (Wood, 1980a), the glycosylated enzyme is significantly less negatively charged than calculated for either of the non-glycosylated polypeptide(s). Both lcc1 and lcc2 products have five potential N-glycosylation sites (Fig. 1). Interestingly, the substitutions Ser,259 and Val,341 in the lcc1 product compared with the lcc2 product, corrupt the potential glycosylation site at Asn,329 and create an alternative site at Asp,341.

Codon usage in both genes is similar showing a strong preference for third position C (40.5 and 42.2%), marked preference against third position G (14.8 and 14.6%) and against third position A (16.9 and 16.3%; data for lcc1 and lcc2, respectively). This codon usage is similar to that found for the transcribed Ag. bisporus glyceraldehyde-3-phosphate dehydrogenase gene, in preference for third position C and in preference against G and A (Harmsen et al., 1992). The cel1 gene of Ag. bisporus has comparable preference against G and A, but not the same preference toward C as T is the preferred third base in this gene (Raguz et al., 1992).

Homology of lcc1 and lcc2 with other laccase and ascorbate oxidase sequences

Laccase sequence from Ag. bisporus was compared with laccase from Neurospora crassa (Germann et al., 1988), Aspergillus nidulans (Arayamo & Timberlake, 1990), Coriolus hirsutus (Kojima et al., 1990) and Phlebia radiata (Saloheimo et al., 1991), together with sequence of ascorbate oxidase from cucumber (Ohkawa et al., 1989) and squash (Messerschmidt et al., 1989). Pairwise alignments of all combinations of the above sequences both as amino acid sequence similarity and as nucleic acid sequence for exons of the coding sequences are shown in Table 1. It is evident that the three basidiomycete laccases are more similar than any other combination of sequences, except for the close similarity of the two ascorbate oxidases. It is of note that the two ascomycete laccases are not closely similar to each other and are not any more similar to the other fungal laccases than are the plant ascorbate oxidases. Of the five potential N-glycosylation sites in Ag. bisporus laccase, only one (Asn,423) is conserved in the sequence of laccase from P. radiata and C. hirsutus. All the fungal laccases except that of Asp. nidulans conserve the two pairs of cysteines involved in intra-subunit disulphide bond formation in the ascorbate oxidases (Cys,105-Cys,309 and Cys,135-Cys,233 in the A. bisporus laccase, see Fig. 2) and multiple alignment analysis (not shown) identifies the Asp. nidulans laccase as the least similar of the sequences.

One feature of the laccase sequences that completely defies explanation with current knowledge is the variability in presence of introns from N. crassa with one, Asp. nidulans with five, P. radiata with nine, C. hirsutus with 11 and Ag. bisporus with 15. Exceptionally large numbers of small introns may be a characteristic feature of Ag. bisporus, as its glyceraldehyde-3-phosphate dehydrogenase genes contain nine introns whereas the

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Nucleic acid sequence alignment

Table 1. Percentage similarity of laccase and ascorbate oxidase sequences

Sequence similarity was derived using the GCG program PILEUP, with which gaps are allowed in aligned sequences. The nucleic acid alignments were for exon sequence only. For comparison of deduced amino acid sequence, the amino acids regarded as similar were the default set in the GCG file Simplify.Txt. Asc, Ascorbate.
equivalent genes in Schizophyllum commune and Phanerochaete chrysosporium contain only five and six introns, respectively (Harmsen et al., 1992).

As the primary structure of the N. crassa and P. radiata laccases are both consistent with a β-barrel structure as found crystallographically for ascoborate oxidase (Messerschmidt & Huber, 1990; Saloheimo et al., 1991), it is probable that all these enzymes have a similar three-dimensional architecture, notwithstanding their relatively low similarity of primary structure. Whether the basidiomycete enzymes are more similar because of more recent evolutionary divergence or because their functions are more similar remains unresolved.

Although allelic sequence differences have been demonstrated for laccase genes both in N. crassa (Germann et al., 1988) and C. hirsutus (Kojima et al., 1990), this is the first report of a gene family for laccases, although multiplicity of laccase enzymes at the biochemical level has already been observed widely in the fungi (e.g., Esser & Minuth, 1970; Marbach et al., 1984; Rehman & Thurston, 1992). Within the genome of Ag. bisporus, two tandemly linked glyceraldehyde-3-phosphate dehydrogenase genes have been identified (of which one is apparently a non-transcribed pseudogene; Harmsen et al., 1992); these and our report of the cell gene (Raguz et al., 1992) are the only other published Ag. bisporus nuclear gene sequences at present.

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