Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*

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A cDNA library was constructed in λ gt11 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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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 λ gt11 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 λ EMBL3 in *E. coli* NM392 (Frischauf *et al.*, 1983). Subcloned sequences were manipulated in pBluescript KS (Stratagene) in *E. coli* XL1Blue (Stratagene).

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 *Eco*RI adaptors (Pharmacia) for ligation to λ gt11 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 (λ lccg1, 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 dideoxy-chain termination method (Sanger *et al.*, 1977) was carried out on double stranded templates using Sequenase 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 λ gt11 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 9.9 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 9.9 min period of extension at 72 °C. The primers for this reaction were complementary to a subterminal region of the λ lccg1 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 *Sma*I 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 10^7 recombinants in λ gt11, using *Eco*RI adaptors. When 10^5 plaques were screened with affinity-purified anti-laccase IgG, three positively reacting plaques were isolated and purified (λ lcc411, λ lcc511 and λ lcc611). All three recombinants were found to synthesize immunoreactive fusion proteins when converted to lysogens (data not shown). The λ lcc511 insert was subcloned as two *Eco*RI 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 HisCysHisIleAspTrpHis(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 λ lcc511. The insert of λ lcc411 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 λ lcc611, yielded a single *Eco*RI fragment on subcloning (plcc6, residues 2754-2841 and 2907-3008 in Fig. 1) that encoded the same two amino acid residues and stop

CTACGTCTTGACAGTATGTCTCTGAAGCACATTGTTAATCATGAGCGAACCTAAGGCCGATTAGCTACGGTGAGTCGTGACCAACGTC	89
CAGTGGCAAAGCGCTGGTTTTAGTATTAAATCACAACTGAACCACAGTACAACGCCCTGCAAGACACGCGCTGATGTTCAATACAAACG	179
TTGAACATCTATGCGCCTGTAACGGGGAGAGAAAACCTCGTGAAACTGCCCTGGATGATAAATTTTCATCAGCTTCGAATGTCAGTGACATT	269
GAATGTTATAAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTTTAGAAAATTTCCGTGGATTGTGGTCTCAGGCAT	359
ATCGCTGGTGGTTTTCTCTTGTGATAAAAAATATGTATCTTCAGGCATAAAATGACGGGGGCAGAGGACCGTGGATTGACTCTCCAGCGGT	449
AGGATCAACA ATG AGG CTG TCC AAC GCT TTA GTA TTG GTC GCC GCA TGC ATT TCG AGT GTA GTT GCG AAA	519
Met Arg Leu Ser Asn Ala Leu Val Leu Val Ala Ala Cys Ile Ser Ser Val Val Ala Lys	
ACC AGA ACC TTC GAC TTC GAC CTA GTC AAT ACC AGG CTC GCG CCT GAT GGG TTT GAA AGG GAgtagtata	590
Thr Arg Thr Phe Asp Phe Asp Leu Val Asn Thr Arg Leu Ala Pro Asp Gly Phe Glu Arg As	
tttctatacagattcatcgtagagtttactgaccttgatttaaactagC ACC GTG GTC ATC AAT GGA GAA TTC CCT GGA	670
INTRON I p Thr Val Val Ile Asn Gly Glu Phe Pro Gly	
ACA CTG ATC CAA GTC AAC AAG GGC GAC AGT GTC CGC ATC CCT CTC CAT AAC AAA CTG ACT AGC CCG ACC	739
Thr Leu Ile Gln Val Asn Lys Gly Asp Ser Val Arg Ile Pro Leu His Asn Lys Leu Thr Ser Pro Thr	
ATG AGG CGA AGT GTA TCC ATTgtgagttactttttgagcccagattgacttgactatataacacaaattagCAT TGG CAT	819
Met Arg Arg Ser Val Ser Ile INTRON II His Trp His	
GGA TTT TTC CAA GCG AGA ACT TCT GGT CAG GAC GGG CCT TCC TTC GTG AAT CAG TGC CCT CAG CCA CCC	888
Gly Phe Phe Gln Ala Arg Thr Ser Gly Gln Asp Gly Pro Ser Phe Val Asn Gln Cys Pro Gln Pro Pro	
AAC ACA ACC TTC ACC TAT GAA TTC AGC GTG GCG GAG CAG TCT GGA ACG TTCgtctctgatcccttaaatacatc	961
Asn Thr Thr Phe Thr Tyr Glu Phe Ser Val Ala Glu Gln Ser Gly Thr Phe	
* ttcagcgtaaattgacattttcttcaagttatTGG TAC CAT TCA CAC TTG TCC ACC CAA TAC TGT GAT GGT CTT CGC	1037
INTRON III Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg	
GGT GCA TTC ATC GTT TAC GgtaattccttctcttgcatgcaagatatattttgccgactttcggttagAC CCA CGT GAT	1117
Gly Ala Phe Ile Val Tyr A INTRON IV sp Pro Arg Asp	
CCA CTC AGA CAT CTC TAT GAT GTT GAT GAC GAA AGT ACC GTC ATC ACT CTA GCT GAA TGG TAT CAC ATC	1186
Pro Leu Arg His Leu Tyr Asp Val Asp Asp Glu Ser Thr Val Ile Thr Leu Ala Glu Trp Tyr His Ile	
CTC GCA CCC GAT GCC ACT AAC GAG TTT TTC AGC TCT GGC ATT ATA CCgtaagcttcttaaggaatatttattcat	1262
Leu Ala Pro Asp Ala Thr Asn Glu Phe Phe Ser Ser Gly Ile Ile Pr INTRON V	
tttcataacccaattcctgcagC GTG CAA GAT TCT GGC TTG ATC AAC GGT AAA GGT AGA TTC AAT GGA GGA CCC	1336
o Val Gln Asp Ser Gly Leu Ile Asn Gly Lys Gly Arg Phe Asn Gly Gly Pro	
CTC AgtaagtagatcaaaacagaaaaggtgatgatgaaaatttgactttacttgcttagCA CCA TTC GCC GTC GTC AAT GTT	1418
Leu T INTRON VI hr Pro Phe Ala Val Val Asn Val	
CGC CGA GGC AAA CGT TAC AGG CTC CGT GTC ATA GCT ATT TCT TGC AGG CCG TTC TTC ACC TTC TCC GTC	1487
Arg Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile Ala Ile Ser Cys Arg Pro Phe Phe Thr Phe Ser Val	
GAT AAC CAT AGC TTG GTC TTC ATG GAA GCC GAT GGT GTT GAA CAT GAC CCT GTC GAA GTC CAA AAC GTC	1556
Asp Asn His Ser Leu Val Phe Met Glu Ala Asp Gly Val Glu His Asp Pro Val Glu Val Gln Asn Val	
GAC ATC TAC GCG GCC CAG CGT GTC TCG GTT ATT CTG CAT GCC AAT CAA CCC ATC GAT AAC TAC TGG ATC	1625
Asp Ile Tyr Ala Ala Gln Arg Val Ser Val Ile Leu His Ala Asn Gln Pro Ile Asp Asn Tyr Trp Ile	
AGA GCT CCT ATG ACT GGC GGC AAC CCA GAC AGG AAC CCC AAT Cgtaagcgtcattttgccacctgtagcagctgta	1702
Arg Ala Pro Met Thr Gly Gly Asn Pro Asp Arg Asn Pro Asn L INTRON VII	

Fig. 1. For legend see p. 1212.

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atgaaacgtgtttccaaagTA AAC ATC AGC CTC ACT CTC GCC ATC CTG CGC TAC CAT GGT GCA CGT CAC GTC GAA 1777
    eu Asn Ile Ser Leu Thr Leu Ala Ile Leu Arg Tyr His Gly Ala Arg His Val Glu
        * 300                                310
CCG ACT ACG GTC AAC GTT CCC GGA CAC AAA CTC CTT GAT CAA GAA ATG CATgtaagaataacttgcgtaaagg 1850
Pro Thr Thr Val Asn Val Pro Gly His Lys Leu Leu Asp Gln Glu Met His
    320                                330
cattccgaaataacttactaacgcgcaccaatctagCCG ATC CGC CAG GAA GGA CCA GGC AAAGtgtgtatatatttaca 1929
    INTRON VIII                                Pro Ile Arg Gln Glu Gly Pro Gly Lys
                                                340
tgaacataatcatcaactcaacgttctctagCTC GGT GAC GGT CCC CCC GAT AAG CAC ATC ACC CTC AAC ATT GCT 2005
    INTRON IX                                Leu Gly Asp Gly Pro Pro Asp Lys His Ile Thr Leu Asn Ile Ala
                                                350
CAAGtaagcgtctcattattcaaatcaaaactcgacttctgacatgagcacagCCC AAC GCT CCC TTC TTC GAT ATC AAC 2084
Gln                                INTRON X                                Pro Asn Ala Pro Phe Phe Asp Ile Asn
                                                360
GGA ATC TCT TAC ATC TCC CCG ACT GTC CCT GTT CTT CTC CAA ATC CTC AGC GGA GCC AAG AGG CCC GAA 2154
Gly Ile Ser Tyr Ile Ser Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Lys Arg Pro Glu
    370                                380                                390
GAT GTT CTT CCA TCC GAA CAA ATC TTC TTT GTC CCC AAG AAT TCA TTG ATT GAA GTC AAC ATT CCA GGA 2222
Asp Val Leu Pro Ser Glu Gln Ile Phe Phe Val Pro Lys Asn Ser Leu Ile Glu Val Asn Ile Pro Gly
    400                                410
GAA GGA GCT CAT CCA TTC CAT TgtatggtaacctcattttctcctcaattcctctacttacgtccctgtttcagTG CAC GGT 2304
Glu Gly Ala His Pro Phe His L                                INTRON XI                                eu His Gly
    420
CAT AAC TTT GAT GTC GTT CTG GCT TCT AAC GAC GAC ACT TTC AAC TTC AAG AAgtagttaattgaaaaagga 2377
His Asn Phe Asp Val Val Leu Ala Ser Asn Asp Asp Thr Phe Asn Phe Lys As
    430                                440
agttacctgctgaacactaatctcaaatgaaagC CCG CCT CGT CGT GAC GTA TAC CCC ATC AAC GGT GGC AAC ACC 2453
    INTRON XII                                n Pro Pro Arg Arg Asp Val Tyr Pro Ile Asn Gly Gly Asn Thr
                                                450
ACT TTC CGC TTC TgtaagattccacgtttctttctgaagaataactctttctgaacgacccctcagTC ACC GAT AAC CCA 2533
Thr Phe Arg Phe P                                INTRON XIII                                he Thr Asp Asn Pro
    460
GGC GCA TGG TTC TTG CAT TGgtgaggaacatttctatccatttctctcgaggcgaataactgattgaatcttgtgacatagC 2614
Gly Ala Trp Phe Leu His Cy                                INTRON XIV                                s
    470
CAT ATC GAT TGG CAT CTT GAA GCT GGT CTC GCT ATT GTG TTT GCT GAG GCT CCG GAG GAT AAC GTT TCA 2683
His Ile Asp Trp His Leu Glu Ala Gly Leu Ala Ile Val Phe Ala Glu Ala Pro Glu Asp Asn Val Ser
    480                                490
GGT CCC CAG TCT CAG ATC ACT CCT CAA GAT TGG TTG GAT CTC TGT CCA GAA TAC AAC GCA ATC GAG CCT 2752
Gly Pro Gln Ser Gln Ile Thr Pro Gln Asp Trp Leu Asp Leu Cys Pro Glu Tyr Asn Ala Ile Glu Pro
    500                                510
GAA TTC CAG TAG TTTATTCAAATATTATATTCCTCTCACAAGGAAATCTATCTCTGTGTACAAGTAGGAATTTTAAACAATGTTTA 2839
Glu Phe Gln STOP
    520
CCctaaaacaaaatgatcgtaagtattaagtggcggcattaacgaggcaggtagtaatgataacaaacCCAGGCACTTGATGCGCTTTGAA 2930
TTGAAGTCTTAAGGGCCCATGTGTAACAAGAATGCATTCACTGACATGACCATGAGACTCCGAACGGTCTATATGCAC 3008

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Fig. 1. The nucleotide sequence of the laccase gene *lcc1* 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 M1 of *Ag. bisporus* (overline marked 'a', residues 117–129, cf. Harmsen *et al.*, 1991); a 10 bp sequence homologous to residues –59 to –50 of the *N. crassa* laccase gene 5' region (overline marked '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 (overline marked 'c', residues 297–306, cf. Kojima *et al.*, 1990). Possible N-glycosylation sites in the deduced amino acid sequence are shown with an asterisk. Lys₂₀ 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 *Δlcc611* 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

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 λ ccg1 was obtained. From subclones obtained as *Kpn*I, *Eco*RI and *Sal*I fragments, the sequence shown in Fig. 1

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 ^{32}P -labelled plcc5a DNA (lane 2). The laccase probe also hybridized to some bands of marker *Hind*III-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 (λlcc511 , λlcc411 and λlcc451) differed from the cDNA clone λl611 and the sequence found as exons in the genomic sequence at 36 out of 520 residues. The genomic sequence is named *lcc1* and is shown in Fig. 1. The sequence that we have described only in cDNA is named as *lcc2* and its deduced coding sequence is shown in Fig. 2, together with indication of those residues that differ in the coding sequence of *lcc1* (at the nucleic acid level, *lcc1* and *lcc2* 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 *lcc1* and *lcc2* 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 *lcc1* gene

The coding sequence of *lcc1* 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 *cel1* gene and glyceraldehyde-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 *cel1* gene or the glyceraldehyde-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 *lcc1* and *lcc2*

The coding sequence of *lcc1* 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 λ Ab22 (b). Lanes 1–4 contain DNA from strains B237, B238, B239 and B240, respectively. In (a) DNA was restricted with *Eco*RI (E), *Sal*I (S) and *Hind*III (H). In (b), digests were with *Eco*RI only. The laccase probe gives identical patterns for all four strains, whereas the control probe λ Ab22 gives two distinct patterns (1 and 3 are different from 2 and 4 in b).

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.

	<i>N. crassa</i>	<i>Asp. nidulans</i>	<i>C. hirsutus</i>	<i>P. radiata</i>	<i>Ag. bisporus</i>	Asc. oxidase (squash)	Asc. oxidase (cucumber)
Deduced protein sequence alignment							
<i>N. crassa</i>		46.9	55.1	53.2	52.8	49.9	52.0
<i>Asp. nidulans</i>	38.7		48.8	48.1	52.4	51.7	45.1
<i>C. hirsutus</i>	41.6	38.3		77.2	66.4	51.6	53.7
<i>P. radiata</i>	38.2	39.2	57.6		64.7	53.4	51.5
<i>Ag. bisporus</i>	38.7	38.7	45.3	44.8		53.9	51.5
Asc. oxidase (squash)	39.1	37.0	40.6	42.6	38.6		88.5
Asc. oxidase (cucumber)	38.5	38.4	36.7	41.8	38.7	73.1	
Nucleic acid sequence alignment							

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_r 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 more 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 Ser₂₃₉ and Val₂₄₁ in the *lcc1* product compared with the *lcc2* product, corrupt the potential glycosylation site at Asn₂₃₉ and create an alternative site at Asn₂₄₁.

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 *cell1* 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 (Asn₄₅₄) 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 (Cys₁₀₃–Cys₅₀₉ and Cys₁₃₅–Cys₂₂₉ 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

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 ascorbate 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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