Mycoscience (2003) 44:11–17
DOI 10.1007/s10267-002-0075-y

FULL PAPER

Kenji Okamoto · Yasuhiro Ito · Ichirou Shigematsu
Sonoe Ochiai Yanagi · Hideshi Yanase

Cloning and characterization of a laccase gene from the white-rot basidiomycete *Pleurotus ostreatus*

Received: August 22, 2002 / Accepted: October 9, 2002

Abstract The gene *lccK* encoding a laccase of the white-rot basidiomycete *Pleurotus ostreatus* wild-type strain collected in Japan has been cloned, sequenced, and characterized. The isolated gene consists of 2929 bp with the coding region interrupted by 19 introns and flanked by an upstream region in which putative CAAT and TATA elements were identified. Two putative N-glycosylation sites and four putative copper-binding sites found in other fungal laccase are conserved in *lccK*. The cDNA contains an open reading frame of 1599 bp and the gene encodes 533 amino acids preceded by a signal peptide of 23 amino acids. The nucleotide sequence of the *lccK* cDNA showed high homology with those of laccases of other basidiomycetes.

Key words Copper-binding sites · Intron · Laccase gene · N-Glycosylation sites · *Pleurotus ostreatus* · Promoter

Introduction

Laccase (EC 1.10.3.2) is one of the important enzymes that contribute to lignin degradation in conjunction with lignin peroxidase and manganese peroxidase in white-rot basidiomycetes (Youn et al. 1995). The enzyme contains sugar and copper atoms, which are extracellularly excreted, as its major characteristic. Several studies have been carried out on the applications of laccase in the pulp and paper industry, such as biopulping and biobleaching processes (Reid and Paice 1994; Decarvalho et al. 1999). Moreover, much attention has been paid to the application of laccase to the detoxification of environmental pollutants (Martirani et al. 1996; Kadhim et al. 1999; Ullah et al. 2000; Abadulla et al. 2000; Pointing 2001; Leontievsky et al. 2001). Laccase genes from various basidiomycetes, such as *Coriorus hirsutus* (Wulf.: Fr.) Pilát (Kojima et al. 1990), *Phlebia* (*Ph.*) *radiata* Fr. (Saloheimo et al. 1991), basidiomycete PM1 (Coll et al. 1993), *Agaricus* (*Ag.*) *bisporus* (J. Lange) Imbach (Perry et al. 1993), *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (type, Florida) (Giardina et al. 1995, 1996, 1999), *Trametes villosa* (Fr.) Kreisel (Yaver et al. 1996), *Trametes versicolor* (L.: Fr.) Lloyd (Ong et al. 1997), *Pycnoporus* (*Py.*) *cinnabarinus* (Jacq.: Fr.) P. Karst. (Eggert et al. 1998), *Schizophyllum commune* Fr.: Fr. (Hatamoto et al. 1999), and *Lentinula edodes* (Berk.) Pegler. (Zhao and Kwan 1999) have been cloned and characterized.

Pleurotus ostreatus shows strong laccase activity among edible mushrooms, and it is relatively easy to culture in a medium. We have been studying the heterologous expression of laccase from *P. ostreatus* (Okamoto et al. 1995), and we have previously reported the purification and characterization of laccase from *P. ostreatus* as part of our study (Okamoto et al. 2000). Although there have been various reports on laccase cDNA already described, the comparison of the promoter region and intron of the laccase gene from various fungi has not been described in detail. Here we report the sequences of the laccase gene including those of the promoter region and introns from *P. ostreatus* wild-type strain collected in Japan, comparing it with sequences of other fungal laccases.

Materials and methods

Organism and culture conditions

The basidiomycete *Pleurotus ostreatus* K16-2 is a monokaryon screened from the protoplasts of a dikaryon collected in Kanagawa prefecture (Okamoto et al. 2000).

K. Okamoto (✉) · I. Shigematsu · H. Yanase
Department of Biotechnology, Faculty of Engineering, Tottori University, 4-101 Koyamacho-Minami, Tottori 680-8552, Japan
Tel. +81-857-31-5276; Fax +81-857-31-0881
e-mail: okamoto@bio.tottori-u.ac.jp

Y. Ito · S.O. Yanagi[1]
National Food Research Institute, Tsukuba, Japan

Present address:
[1]Faculty of Bioresource Sciences, Akita Prefectural University, Shimoshinjo-nakano, Akita 010-0195, Japan

The strain was maintained on 1.5% agar slants containing 1% (w/v) malt extract (Difco, Detroit, MI, USA), 0.4% (w/v) yeast extract (Difco), and 0.4% (w/v) glucose (MYG medium, pH 6.3). For the preparation of genomic DNA and total RNA encoding laccase, the mycelia grown on an agar slants were suspended in 8 ml MYG medium, transferred to a 500-ml Erlenmeyer flask containing 80 ml MYG medium, and incubated at 30°C for 7 days in a static culture.

mRNA isolation

Total RNA was isolated from 7-day-old mycelia of *P. ostreatus* K16-2 that were snap-frozen and ground in liquid N_2, with a RNeasy plant minikit (Qiagen, Hilden, Germany). Poly $(A)^+$ mRNA was purified using an Oligotex™-dT30 ⟨Super⟩ kit (Roche Diagnostics, Basel, Switzerland).

cDNA synthesis

Purified poly $(A)^+$ mRNA was used to synthesize the cDNA using a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Primers for cDNA amplification were designed based on the sequence of the N-terminus and the internal peptides of purified *P. ostreatus* laccase. The forward primer NF (5′-CTGGCAACATGTACATCG TCAACG-3′) was designed on the basis of the N-terminal sequence, and the reverse primer IR (5′-TGCTCGTCGA CGAGGTTGTGGTG-3′) was based on the sequence of the internal peptides. With these primers, an approximately 900-bp cDNA fragment was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequenced. Then, primers for rapid amplification of cDNA ends (RACE) were designed from the cDNA sequence: RT5R (5′-GAAACTATCCCCAGAGGCGAC-3′) for 5′ and RT3F (5′-TTGCTCGTCATTGAAGCAGATGC-3′) for 3′ RACE. RACE PCR was carried out using the two primer pairs, RT5R or RT3F and an adapter primer (AP1, 5′-CCATCCTAATACGACCACTATAGGGC-3′), and the PCR products were sequenced.

Genomic DNA preparation

Genomic DNA as a template for PCR amplification was isolated from frozen mycelia of *P. ostreatus* by cetyltrimethylammonium bromide treatment, chloroform–octanol extraction, and isopropanol precipitation (Ausubel et al. 1994).

PCR primers

The primers designed from the nucleotide sequences of the cDNA from *P. ostreatus* laccase and used for PCR amplifications were as follows: N1, 5′-ATGTTTCCAGGC GCACGGATTCTT-3′; M361, 5′-GTCGCCTCTGGGG ATAGTTTC-3′; M490R, 5′-CGTATAAACTCAAGT GCGGATCCGA-3′; M532, 5′-ACGGTCATTACGC TTGAGGATTGG-3′; M661, 5′-ATCAACGTCGAAA GCAACAAGCG-3′; M753R, 5′-CATCTGCTTCAA TGACGAGC-3′; M966, 5′-CACCACAACCTCGTCGA CGAGC-3′; M1004R, 5′-AGGATTCTCAAGCGGAA CAAGGTTGG-3′; M1395R, 5′-ACGAAGCGAATAG TGACGTTGTCG-3′; and M1578R, 5′-TTAGGACGG AACGATGCCTCCTTTG-3′. The full-length laccase genomic DNA from *P. ostreatus* K16-2 was amplified by PCR using the primer pairs N1 and M1578R. A 2.6-kb fragment was isolated and analyzed partially by PCR using five primer pairs: N1 and M490R, M361 and M753R, M532 and M1004R, M661 and M1395R, and M966 and M1578R. The upstream region including the promoter of the gene was isolated by inverse PCR using *Aat*II-cleaved genomic DNA.

PCR amplification

The reaction mixture for the DNA amplification contained 2.5 U ProofStart DNA polymerase (Qiagen), 1X ProofStart PCR buffer (Qiagen), 2.5 mM $MgSO_4$, 300 μM each dNTP, 100 ng template DNA, and 1.0 μM each of the primers in a 50-μl reaction volume. A mineral oil overlay that completely covers the solution was added to prevent condensation. PCR amplification was conducted with a DNA thermal cycler model 480 (Perkin-Elmer, Norwalk, CT, USA), using an initial denaturation step of 94°C for 2 min followed by 30 amplification cycles of denaturation at 94°C for 1 min, annealing at 57°C for 2 min, and primer extension at 72°C for 2 min, with a final extension at 72°C for 5 min. Aliquots (10 μl) of the amplification products were separated by gel electrophoresis through a 0.8% horizontal agarose gel at 100 V in Tris-acetate-ethylenediaminetetraacetic acid buffer for 2 h. Gels were stained with ethidium bromide and visualized under UV light. Molecular sizes were determined by comparison with a λ DNA-*Eco*T14I digest marker. A target amplification product was recovered from the agarose gel using the QIAquick gel extraction kit (Qiagen).

DNA sequencing

Sequencing reaction was performed using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequence data were analyzed using DNASIS-Mac v. 3.7 software (Hitachi Software, Yokohama, Japan). DNA sequences and deduced protein sequences obtained in this study were compared using the BLAST program.

Nucleotide sequence accession number

The nucleotide sequence data reported in this article has been submitted to DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB089612.

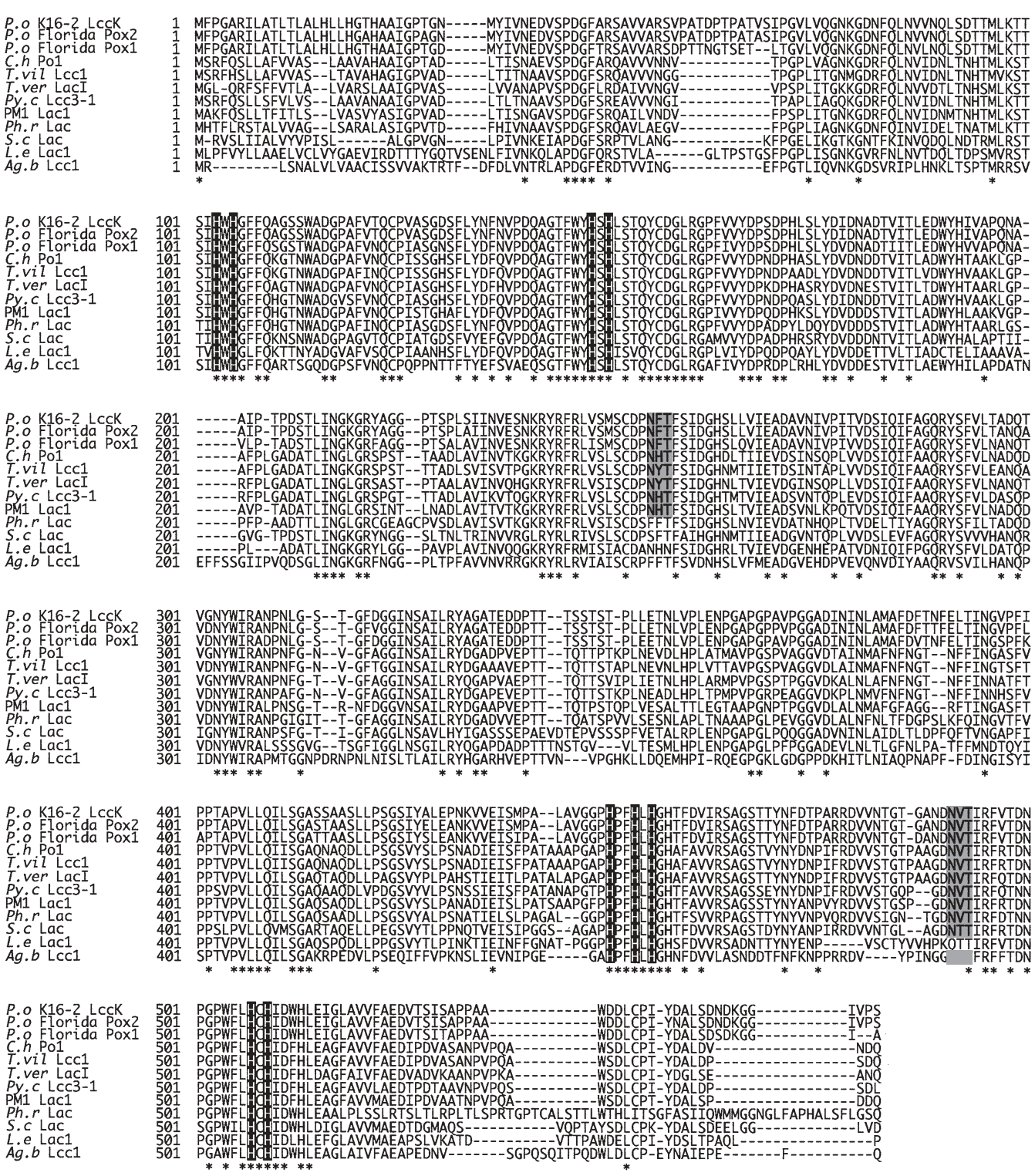

Fig. 1. Amino acid sequence alignment of LccK from *Pleurotus ostreatus* K16-2 and laccases from different basidiomycetes. Possible copper-containing domains are shown in *white with a black background*. Possible N-glycosylation sites (N-X-S/T) are highlighted in *gray boxes*. Identical residues are shown with *asterisks*. *P.o* K16-2 LccK, *Pleurotus ostreatus* K16-2 LccK (GenBank accession number, AB089612); *P.o* Florida Pox2, *P. ostreatus* (strain Florida) Pox2 (Q12739); *P.o* Florida Pox1, *P. ostreatus* (strain Florida) Pox1 (Q12729); *C.h* Lac, *Coriorus hirsutus* Lac (Q02497); *T.vil* Lcc1, *Trametes villosa* Lcc1 (Q99044); *T.ver* LacI, *T. versicolor* LacI (U44430); *Py.c* Lcc3-1, *Pycnoporus cinnabarinus* Lcc3-1 (AF025481); PM1 Lac1, basidiomycete PM1 Lac1 (Z12156); *Ph.r* Lac, *Phlebia radiata* Lac (Q01679); *S.c* Lac, *Schizophyllum commune* Lac (AB015758); *L.e* Lac1, *Lentinula edodes* Lac1 (AF153610); *Ag.b* Lcc1, *Agaricus bisporus* Lcc1 (Q12541)

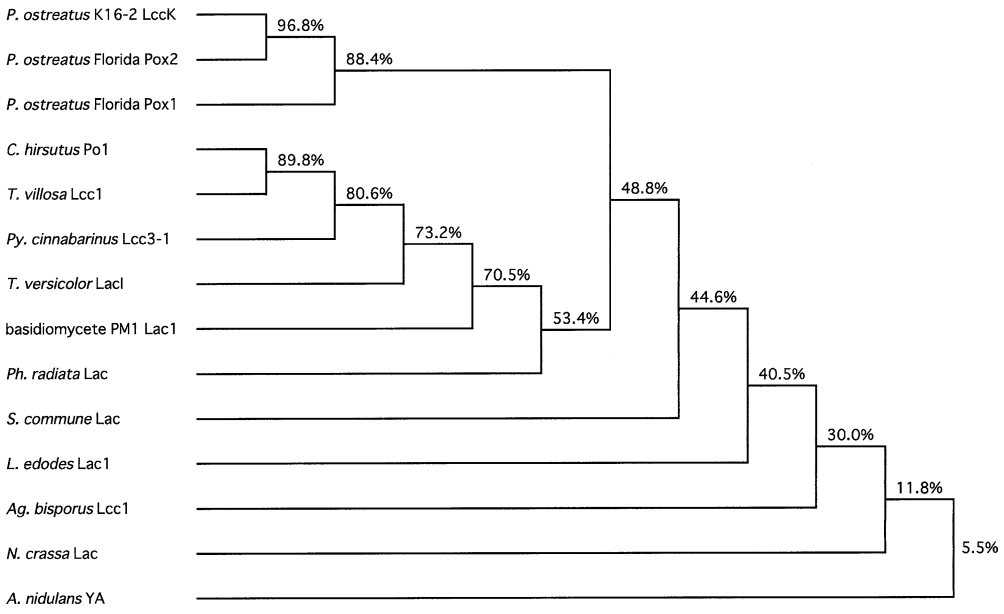

Fig. 2. Phylogenetic tree constructed based on the deduced amino acid sequences of fungal laccases. Calculated matching percentages are indicated at each branch point of the dendrogram and were generated using the DNASIS-Mac program

Results and discussion

Nucleotide sequence of *lccK* cDNA

The full-length *lccK* cDNA from *P. ostreatus* was amplified by RT-PCR and RACE. Sequence analysis revealed one open reading frame of 1599 bp and 533 amino acids including a signal sequence of the first 23 amino acids, and its mature laccase with a molecular mass of 56798 Da, which is almost identical to that of the purified enzyme (Okamoto et al. 2000). The open reading frame showed a G+C content of 53%.

The deduced amino acid sequence of laccase from *P. ostreatus* K16-2 was compared with those of other laccases (Fig. 1). The laccase amino acid sequence shows similarity to those of Pox2 (97% identity) and Pox1 (89% identity) from strain Florida, which is a variety of *P. ostreatus*, *C. hirsutus* Po1 (64% identity), *T. villosa* Lcc1 (62% identity), *T. versicolor* LacI (62% identity), *Py. cinnabarinus* Lcc3-1 (61% identity), basidiomycete PM1 Lac1 (61% identity), *S. commune* Lac (60% identity), *Ph. radiata* Lac (58% identity), *L. edodes* Lac1 (54% identity), and *Ag. bisporus* Lcc1 (47% identity). On the other hand, the primary sequence of LccK exhibits a lower similarity to ascomycetes, such as *Neurospora crassa* Lac (34% identity) (German et al. 1988) and *Aspergillus nidulans* YA (30% identity) (Aramayo and Timberlake 1990). These identity values are the same as those obtained when compared with N-terminal amino acid sequences among them (Okamoto et al. 2000). The percentage amino acid identity was calculated for pairwise combinations of fungal laccases and presented as a phylogenetic tree (Fig. 2), which shows that the *P. ostreatus* laccase is slightly different from laccases of other white-rot fungi. The signal amino acid sequence, MFPGARILATLTLALHLL HGTHA, is perfectly conserved among *P. ostreatus* laccases, but shows low identity (below 26%) to those of other laccases from different fungi. Purified laccase from *P. ostreatus* K16-2 was a copper-containing glycoprotein (Okamoto et al. 2000). It is confirmed that the deduced amino acid sequence of the laccase from *P. ostreatus* K16-2 contains two potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) and four potential copper-binding histidine-rich sites as the other fungal laccases mentioned earlier.

Nucleotide sequence of *lccK* genomic DNA

To determine the upstream N-terminal sequences and introns, the *lccK* genomic DNA from *P. ostreatus* K16-2 was amplified by PCR using some primers designed based on the nucleotide sequence of the *lccK* cDNA as described in Materials and methods. A fragment of 2929 bp was amplified by PCR and sequenced. The structure of the laccase gene was characterized by comparison of the sequences of the genomic DNA and cDNA (Fig. 3). Nineteen introns that ranged in size from 48 to 61 bp were found to be localized in the gene, and the sequences at the splicing junctions in their introns adhered to the GT-AG rule (Gurr et al. 1987). The number and localization of introns in the laccase gene were compared with those of other fungal laccases in Fig. 4. There are various numbers of introns among laccases from fungi, such as *P. ostreatus* (strain Florida) *pox1* (19 introns) and *pox2* (19 introns), *C. hirsutus po1* (10 introns), *T. villosa lcc1* (8 introns), basidiomycete PM1 *lac1* (10 introns), *Py. cinnabarinus lcc3-1* (10 introns), *Ag. bisporus lcc1* (14 introns), and *A. nidulans yA* (5 introns). The variation of intron number and localization in the genes indicate a mutual vestige of divergence. *P. ostreatus* laccase have a large number of introns as compared with other fungal laccases, which confirms that *P. ostreatus* laccase is diverged a little differently from laccases of other basidiomycetes, as can be seen in the phylogenetic tree (see Fig. 2).

Analysis of the upstream N-terminal region revealed typical promoter sequences that were the same as those of

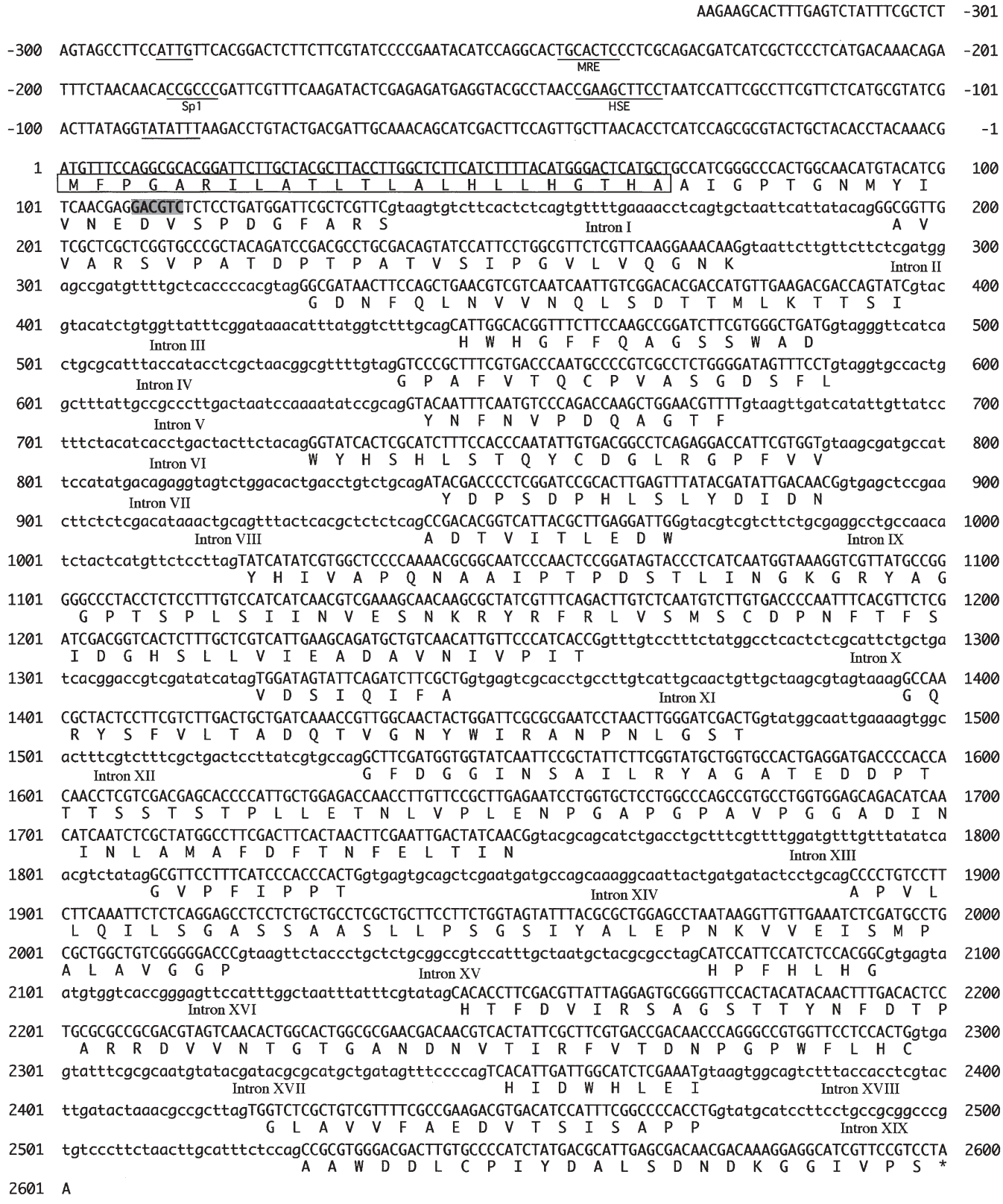

Fig. 3. Nucleotide and deduced amino acid sequences of the *lccK* genomic DNA from *P. ostreatus* K16-2. The putative CAAT element, TATA element, Sp1, heat shock element (HSE), and metal response element (MRE) are *underlined*. The predicted signal peptide is *boxed*. The 19 putative introns are indicated in *lowercase type* and *numbered* (*I–XIX*). Recognition site for restriction endonuclease *Aat*II is highlighted in the *gray box*

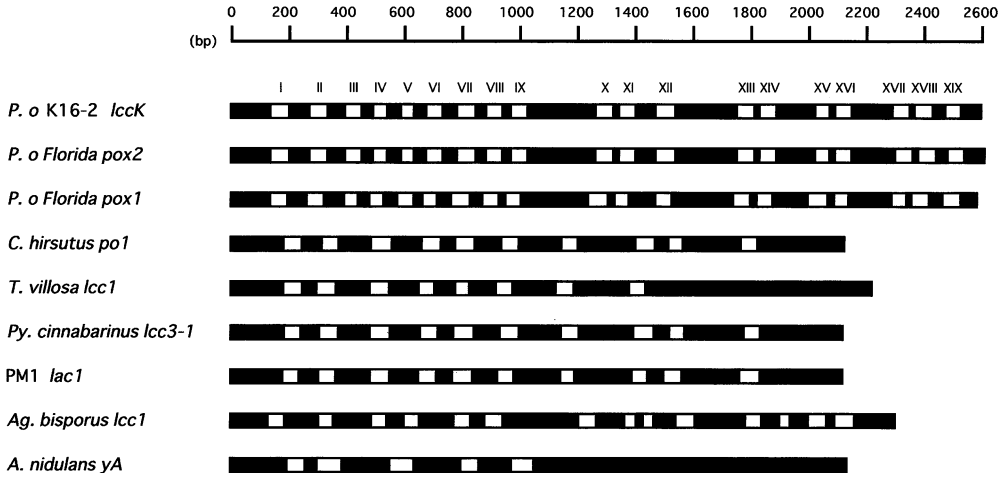

Fig. 4. Comparison of intron/exon structure of *lccK* from *P. ostreatus* strain K16-2, *pox1* and *pox2* from *P. ostreatus* (strain Florida), *po1* from *C. hirsutus*, *lcc1* from *T. villosa*, *lcc3-1* from *Py. cinnabarinus*, *lac1* from basidiomycete PM1, *lcc1* from *Ag. bisporus*, and *yA* from *A. nidulans*. The exons are in *black boxes*; *white boxes* correspond to the introns

```
P.o K16-2 lccK          -328  --------AAGAAGCACTTTGAGTCTATTTCGCTCTAGTAGCCTTCCATTGTTCACGGACTCTTCTTCGTATCCCCGAATACAT
P.o Florida pox2        -334  GCCATGAAAAGAAGCACTTTGAGTCTATTTCGCTCTAGTAGCCTTCCATTGTTCACGGAGTCTTCTTCGTATCCCCGAATACAT
P.o Florida pox1        -327  GCTATGAATAGAAGCACTTTGAGTCTATTTCGCTCTAATAGCCTTCCATTCCTCACGGGCTCTTCTTCGCATCTCCGAATATAC
C.hirsutus po1          -336  CCGACACTGTTCGGGACGCGCGTCTTACCGCCGTGAGACGCAGGGCGTGTCGCGACCTCTGCAAGCTCACACGCTTACCAGGGG
T.villosa lcc1                ----------------------------------------------------------------------------------
Py.cinnabarinus lcc3-1  -307  ----------------------------AAGCGGGTCTCTCCCGCCTCTGTTGGCTCTCACGCATTCCGCATGGTTCGCGCGA
PM1 lac1                -300  ----------------------------------CGTGAGAGGCATTCCTACTCCCTCTCTCTCTCCCAGCTCTCACGCACC
Ag.bisporus lcc1        -336  AAACTGAACCACAGTACAACGCCTGCAAGACACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGA
A.nidulans yA                 ----------------------------------------------------------------------------------

                                        MRE                                                     Sp1
P.o K16-2 lccK          -252  CCAGGCACTGCACTCCCTCGCAGACGATCATCGCTCCCTCATGACAAACAGATTTCTAACAACACCGCCCGATTCGTTTCAAGA
P.o Florida pox2        -250  CCAGGCACTGCACTCCCTCGCAGACGATCATCGCTCCCTCATGACAAACAGATTTCTAACAACACCGCCCGATTCGTTTCAAGA
P.o Florida pox1        -243  CCAGGTTATGCACTGACTCGCGGACGATCATCGCTCCCTTACGACAAACGGATCTTTAACAACACCGCCCCAATTCGGTGCAAGA
C.hirsutus po1          -252  ACTCGCGCGATGGCCGCGTTCCAGGGCCGGCTTGACAGATGCTGACACCGGTGCAATCTTGACACTGTGCCAACCGGGTAAGGC
T.villosa lcc1          -229  ----------------------AGATACTGACACCGGTGCAATCTTGACACTGTACCAACCGGGCAAGTCTCGTCCTTGGTTC
Py.cinnabarinus lcc3-1  -252  CGGCCGCTCCTCAGGGCCAGACCAGTGTCAATGTCTGGTGCCGGTGCAGTGGTGACAGTGCGACAACTGAGTAAGGCTCGTCGG
PM1 lac1                -252  CTCTAGGCTCGCGCGATGCCCGCCTCAGGGCAGATGTCATTCAATACTTCCCTGCGCCACGTCGTCCCGACATGGCGACAATCC
Ag.bisporus lcc1        -252  GAGAAACCTCGTGAAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATAAATACCCAGCAG
A.nidulans yA                 ----------------------------------------------------------------------------------

                                                HSE
P.o K16-2 lccK          -168  TACTCGAGAGATGAGGTACGCCTAACCGAAGCTTCCTAATCCATTCGCCTTCGTTCTCATGCGTATCGACTTATAGGTATATTT
P.o Florida pox2        -166  TACTCGAGA--TGAGGTACGCCTAACCGAAGCTTCCTAATCCATTCGGCTTCGTTCTCATGCGTATCGACTTATAGATATATTT
P.o Florida pox1        -159  TCCTTGAGA--TGAGGTACGCCTAACCGAGGCTTCCTAATCCGTTCATTTTCGTTCTCATGCATATCGTTCTGTAGGTATATTT
C.hirsutus po1          -168  TCGTCCTTGGTTTGCTGGAGGCGCCCACCGTTGAGCCTTGGCCATACAGAGCGCTGTTCTTCGACGGGGTATAAAGGATGCCGC
T.villosa lcc1          -168  TCGGGGACTGGCGCCGGTCGCTACCCCTTGGTCATTCACTCTACCAGAGCGCTGGCTTCGCCGAGGTATAAAGGATGTTGCGCG
Py.cinnabarinus lcc3-1  -168  TTTCCTGCCCCGCCGCTTATGCTGACCACGGGCGTGTTGCTGTACAGACTTCAAGGGCGTCTGTACTTGCGTAGTATAAAGGAT
PM1 lac1                -168  GACGATACCCTCTACGGCAAGGCGTCCATCGTTACACAGCACAATGACTGCTCAGAGCAGAATGACGCTAGCCTTCGTCTACGA
Ag.bisporus lcc1        -168  AGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTTTAGAAAATTTCCGTGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCT
A.nidulans yA           -143  -------------------------CAGGTACATTCCAATCAACAATGCAAGAATGTGAAACGCGAAGTATATATCAGCGAACA

P.o K16-2 lccK          - 84  AAGACCTGTACTGACGATTGCAAACAGCATCGACTTCCAGTTGCTTAACACCTCATCCAGCGCGTACTGCTACACCTACAAACG  ATG•••
P.o Florida pox2        - 84  AAGACCTGTACTGACGATTGCAAACAGCATCGACTTCCAGTTGCTTAACACCTCATCCAGCGCGTACTGCTACACCTACCAACG  ATG•••
P.o Florida pox1        - 77  AAGACGTGCATGGACGACCGAAAATATCATCATCAAC-------TTAACACCTCATCCAGCGCGTACTGCTACACCTACGAACG  ATG•••
C.hirsutus po1          - 84  AGCGAACTCCCAACAGCACAACTCGAGCCCCGCTTGAGTTTCTACGAGGTCCTGCAAACCACTGCCCCTCCTCCCGTCACAGCC  ATG•••
T.villosa lcc1          - 84  ACACCCTCAACACCCCAACTCAAGCCCCACTTGAGCTTTTGCGAGATCCTCCACATACCACTCACTACTTTCAAGTTCTTCAAC  ATG•••
Py.cinnabarinus lcc3-1  - 84  GTCGCTCGAGAGCCGTTCGAAGACAACTCGAGTCCCACTTGAGTTTCGACGAGGACATCCCTTTCAGCGACCCTTCTTTCAGTC  ATG•••
PM1 lac1                - 84  GTATAAAGGCCGCGAGGTATTGTCCGTTCAGACCAACCCCAGGCCCTCTCGAGTCTTCGGCAGTCCTCCCCAATCACTCCAGAC  ATG•••
Ag. bisporus lcc1       - 84  CCTTGTTGATAAAAATATGTATCTTCAGGCTATAAAGACGGGGGCAGAGGACCGTGGATTGACTCTCCAGCGGTAGGATCAACA  ATG•••
A.nidulans yA           - 84  ACATCAAGCCGACATAATCAAACGGTTTCAGTCTCTATCGCGTTTTGCACTTGAGCGTCATTTTGATTCCTCCGGGATCCAGCC  ATG•••
```

Fig. 5. Comparison of nucleotide sequence of the region upstream of the *lccK* gene of *P. ostreatus* with those of other basidiomycetes. The CAAT and TATA elements are highlighted in *gray boxes*. The putative Sp1, heat shock element (HSE), and metal response element (MRE) are *boxed*

previously characterized laccase genes (Fig. 5). The promoter region of this gene contains a TATA element that is an important sequence for initiating transcription (Ballance 1986) and an inverted CAAT element. The putative TATA and CAAT elements were localized at nucleotide positions −91 and −289, respectively, of the 5′-flanking region. A putative inverted Sp1 transcription factor recognition site (GGGCGG) (Dynan and Tjian 1985) was found at −188. A putative heat shock element (NGAANNTTCN) (Bonner et al. 1994) was found at −142, whereas a putative metal re-

sponse element (TGC(A/G)CNC) (Thiele 1992) was found at −244. The positions of CAAT and TATA elements differ among fungal laccase genes. No characteristic common to all except for the TATA and CAAT elements was found in a promoter region. The important point to note, however, is that there are some consensus sequences beyond the genus among the same group, revealing phylogenetic proximity (see Fig. 2). Two consensus sequences, 5′-TATAAAGGATG...CAACTC(G/A)AG(C/T)CCC(G/A)CTTGAG(T/C)TT...CGAG-3′, 5′-(G/C)A(C/G)ACC(G/A)GTG(T)CAAT...TGACA(C/G)TG(T/C)(G/A)(C/A)CAAC(C/T)G(G/A)G(T/C)AAG(G/T)CTCGTC-3′, could be seen at nucleotide positions −102 to −32, and −235 to −164, respectively, among *C. hirsutus po1*, *T. villosa lcc1*, and *Py. cinnabarinus lcc3-1*. These motifs may have some relation to promoter activity for strict conservation within these genes. So far as we know, various reports published previously have made no mention of the matter. This is an interesting finding supplementing dendogram and intron analyses to determine divergence among fungal laccases.

From what has been discussed here, we can conclude that *P. ostreatus* possesses a slightly different type of laccase from others. The sequence reported here should be helpful in the mass production of fungal laccase, which is expected to be an application to various methods. As the next step, we are now investigating the heterologous expression of laccase from *P. ostreatus* K16-2 in yeast cells.

References

Abadulla E, Tzanov T, Costa S, Robra K-H, Cavaco-Paulo A, Gübitz G (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes versicolor*. Appl Environ Microbiol 66:3357–3362

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1994) Current protocols in molecular biology. Wiley, New York

Aramayo R, Timberlake WE (1990) Sequence and molecular structure of the *Aspergillus nidulans ya* (laccase I) gene. Nucleic Acids Res 18:3415

Ballance DJ (1986) Sequences important for gene expression in filamentous fungi. Yeast 2:229–236

Bonner JJ, Ballou C, Fackenthal DL (1994) Interactions between DNA-bound trimers of the yeast heat shock factor. Mol Cell Biol 14:501–508

Coll PM, Tabernero C, Santamaría R, Pérez P (1993) Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). Appl Environ Mcrobiol 59:4129–4135

Decarvalho MEA, Monteiro MC, Sant´Anna GL Jr (1999) Laccase from *Trametes versicolor*. Stability at temperature and alkaline conditions and its effect on biobleaching of hardwood kraft pulp. Appl Biochem Biotechnol 77:723–733

Dynan WS, Tjian R (1985) Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (Lond) 316:774–778

Eggert C, Lafayette PR, Temp U, Eriksson K-EL, Dean JFD (1998) Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. Appl Environ Microbiol 64:1766–1772

German UA, Müller G, Hunziker PE, Lerch K (1988) Characterization of two allelic forms of *Neurospora crassa* laccase. J Biol Chem 263:885–896

Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G, Sannia G (1995) Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. Appl Environ Microbiol 61:2408–2413

Giardina P, Aurilia V, Cannio R, Marzullo L, Amoresano A, Siciliano R, Pucci P, Sannia G (1996) The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*. Eur J Biochem 235:508–515

Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G, Sannia G (1999) Protein and gene structure of a blue laccase gene from *Pleurotus ostreatus*. Biochem J 341:655–663

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. IRL Press, Oxford, pp 93–139

Hatamoto O, Sekine H, Nakano E, Abe K (1999) Cloning and expression of a cDNA encoding the laccase from *Schizophyllum commune*. Biosci Biotechnol Biochem 63:58–64

Kadhim H, Graham C, Barrat P, Evans CS, Rastall RA (1999) Removal of phenolic compounds in water using *Coriolus versicolor* grown on wheat bran. Enzyme Microb Technol 24:303–307

Kojima Y, Tsukuda Y, Kawai Y, Tsukamoto A, Sugiura J, Sakaino M, Kita Y (1990) Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 265:15224–15230

Leontievsky AA, Myasoedova NM, Baskunov BP, Golovleva LA, Bucke C, Evans CS (2001) Transformation of 2,4,6-trichlorophenol by free and immobilized fungal laccase. Appl Microbiol Biotechnol 57:85–91

Martirani L, Giardina P, Marzullo L, Sannia G (1996) Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus*. Water Res 30:1914–1918

Okamoto K, Sakamoto H, Sakai T, Yanagi SO (1995) Phenoloxidase activity in *Coprinus cinereus* due to protoplasting and insertion treatment with *Pleurotus ostreatus* DNA. Biotechnol Lett 17:1127–1130

Okamoto K, Yanagi SO, Sakai T (2000) Purification and characterization of extracellular laccase from *Pleurotus ostreatus*. Mycoscience 41:7–13

Ong E, Pollock WBR, Smith M (1997) Cloning and sequence analysis of two laccase complementary DNAs from the ligninolytic basidiomycete *Trametes versicolor*. Gene (Amst) 196:113–119

Perry CR, Smith M, Britnell CH, Wood DA, Thurston CF (1993) Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. J Gen Microbiol 139:1209–1218

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33

Reid ID, Paice MG (1994) Biological bleaching of kraft pulp by white-rot fungi and their enzymes. FEMS Microbiol Rev 13:369–373

Saloheimo M, Niku-Paavola M-L, Knowles JKC (1991) Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J Gen Microbiol 137:1537–1544

Thiele DJ (1992) Metal-regulated transcription in eukaryotes. Nucleic Acids Res 20:1183–1191

Ullah MA, Bedford CT, Evans CS (2000) Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. Appl Microbiol Biotechnol 53:230–234

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K Dalbøge H (1996) Purification, characterization, molecular cloning, and expression of two laccase genes from the white-rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62:834–841

Youn H-D, Hah YC, Kang S-O (1995) Role of laccase in lignin degradation by white-rot fungi. FEMS Microbiol Lett 132:183–188

Zhao J, Kwan HS (1999) Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*. Appl Environ Microbiol 65:4908–4913