Molecular cloning of aryl-alcohol oxidase from the fungus *Pleurotus eryngii*, an enzyme involved in lignin degradation

Elisa VARELA, Angel T. MARTÍNEZ and María Jesús MARTÍNEZ[1]

Department of Molecular Microbiology, Centro de Investigaciones Biológicas, Censejo Superior de Investigaciones Científicas, Velázquez 144, E-28006 Madrid, Spain

Aryl-alcohol oxidase (AAO), an extracellular enzyme characteristic of fungi from the genus *Pleurotus*, constitutes a source for H_2O_2 required in lignin biodegradation. The gene *aao* has been cloned, sequenced and characterized for the first time in *Pleurotus eryngii*. Both cDNA and genomic libraries were screened with probes obtained by PCR using as primers oligonucleotides corresponding to the N-terminus and internal sequences of AAO. DNA sequences from positive clones showed a unique open reading frame of 1779 nucleotides interrupted by 12 introns. The conceptual translation of the protein agrees with the partial amino acid sequences obtained from protein sequencing. A search for proteins with related amino-acid sequences revealed that glucose oxidase from *Aspergillus niger* has 33 % identity and 51 % similarity. A comparison with other oxidoreductases showed common motifs in both N- and C-terminal regions corresponding, respectively, to the FAD-binding region and the enzyme active site. However, AAO probably has structural differences with other oxidases, as deduced from its unique ability to generate H_2O_2 from the oxidation of aromatic alcohols.

Key words: aryl-alcohol oxidase, biodegradation, gene cloning, hydrogen peroxide.

INTRODUCTION

Lignin biodegradation is an oxidative process carried out principally by white-rot basidiomycetes. These fungi have developed an unspecified extracellular system which includes ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase, and enzymes responsible for the production of hydrogen peroxide (H_2O_2), such as glyoxal oxidase and aryl-alcohol oxidase (AAO) [1–3]. *Pleurotus eryngii* and related species degrade lignin preferentially [4] (i.e. with a limited degradation of cellulose), a relevant characteristic for environmentally friendly biotechnological applications in the pulp and paper industry [5]. This white-rot fungus excretes, both in liquid and solid-state fermentation cultures, AAO, laccases and peroxidases [6–8]. Amongst the latter group of enzymes has been reported a new type of peroxidase that can oxidize Mn^{2+} as well as phenolic and non-phenolic aromatic compounds [7]. The laccase isoenzymes oxidize different substituted phenols and aromatic amines [8], and non-phenolic compounds in the presence of mediators [9]. The AAO presents wide substrate specificity on benzyl, cinnamyl, naphthyl and aliphatic unsaturated primary alcohols [6]. AAO activity was first reported in cultures of *Polystictus versicolor* (a synonym of *Trametes versicolor*) [10] and has been purified from other basidiomycetes [6,11–14]. *P. eryngii* AAO is involved in the continuous production of H_2O_2 a co-substrate of ligninolytic peroxidases [3], via aromatic aldehydes redox cycling [15,16]. On the other hand, H_2O_2 reduction by Fe^{2+} (Fenton's reaction) produces the hydroxyl free radical which is considered to be involved in the initial attack on lignin when the ligninolytic enzymes are not able to penetrate the lignocellulose matrix [17,18]. Recently, gene cloning and molecular modelling of Mn^{2+}-oxidizing peroxidases involved in degradation of lignin by *P. eryngii* have been reported [19,20]. However, AAO had not been cloned in this or other fungi, despite the importance of extracellular production of H_2O_2 in lignin biodegradation. The aim of the present work was to provide the first molecular characterization of the gene encoding AAO.

MATERIALS AND METHODS

Organisms, media and growth conditions

P. eryngii A.T.C.C. 90787 (IJFM A 169) was grown in glucose–ammonium medium [6]. Homogenized 6-day-old pellets were used as inoculum (1 g/l) and the cultures were grown at 28 °C and 180 rev./min in an orbital shaker. The *Escherichia coli* strains used were DH5α (Life Technologies Inc.) for cloning and plasmid propagation, XL1-Blue MRA and MRA-P_2 strains (Stratagene) for titering and screening of the genomic library (prepared in the replacement λEMBL3 vector), XL1-Blue MRF′, for cDNA and *Sol*R, for *in vivo* excision. They were grown in Luria-Bertani (LB) or amine-yeast extract medium (NYZ) [21]. All fragments were subcloned in pBluescript II SK (+/−) (Stratagene).

AAO activity and enzyme purification, characterization and hydrolysis

AAO activity was assayed as the oxidation of veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde (ϵ_{310} 9300 $M^{-1} \cdot cm^{-1}$) in 0.1 M sodium phosphate, pH 6.0 [6]. One unit of enzymic activity was defined as the amount of enzyme transforming 1 μmol of substrate/min.

AAO was purified from *P. eryngii* cultures grown in glucose–ammonium medium as described by Guillén et al. [6]. After the last chromatographic step on a Mono-Q column, AAO was *N*-deglycosylated with 125 m-units/ml of endo-β-*N*-acetylglucosaminidase (Boehringer) and SDS/PAGE of native and deglycosylated proteins was performed in 7.5 % (w/v) polyacrylamide gels which were stained with $AgNO_3$. Samples of deglycosylated protein were hydrolysed with trypsin (5 % w/w) in 0.4 M NH_4HCO_3 (37 °C for 24 h), and peptides were separated in a C_{18} column using 0–70 % (v/v) acetonitrile gradient in 0.1 % (w/v) trifluoroacetic acid (1 ml/min flow). N-terminal sequences

Abbreviations used: AAO, aryl-alcohol oxidase.

[1] To whom correspondence should be addressed (e-mail mjmartinez@cib.csic.es).

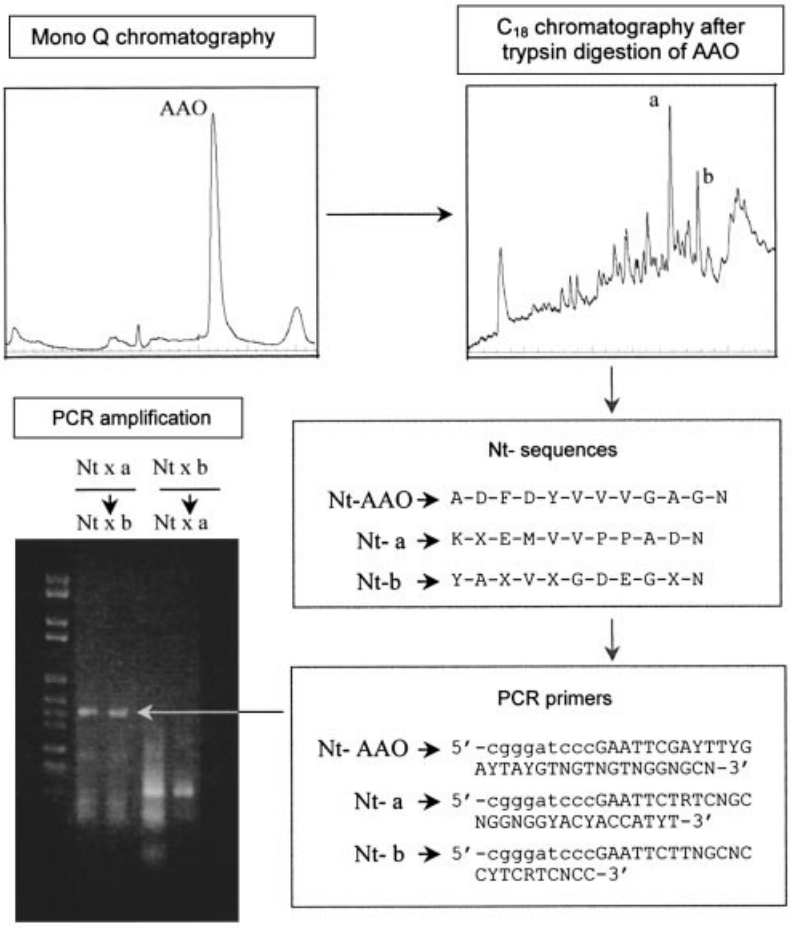

Figure 1 Strategy used for cloning *P. eryngii* aryl-alcohol oxidase

A unique protein peak was obtained after the last purification step with Mono-Q chromatography (top-left-hand panel). The protein was hydrolysed with trypsin and peptides were purified by C_{18} chromatography (top-right-hand panel). The N-terminal sequence of AAO and tryptic peptides a and b were obtained and degenerate oligonucleotides were synthesized which included *Eco*R1 cleavage sites (bottom-right-hand panel). Two successive PCRs were performed (bottom-left-hand panel). In the first, genomic DNA was used as template, and oligo Nt-AAO together with oligos Nt-a or Nt-b were used as primers. The two amplified products were used as templates for the second PCR with oligo Nt-AAO as first primer and oligos Nt-b or Nt-a, as second primers. Finally, a 0.5 kb fragment was amplified, sequenced and cloned in pBluescript. This fragment was used a probe for screening the genomic DNA library.

were obtained by automated Edman degradation of 7 μg of protein in a Applied Biosystems protein sequencer.

Preparation of DNA probes, cloning and sequencing

DNA was extracted from 6-day-old mycelium with phenol/chloroform/3-methylbutan-1-ol [22]. mRNA was obtained from *P. eryngii* 6-day-old cultures using the Ultraspec RNA isolation system (Biotech).

Three DNA primers were synthesized, using an oligonucleotide synthesizer (Beckman-oligo 1000 M), corresponding to: the sequence encoding part of the N-terminus of the mature protein (oligo Nt) and reverse and complementary sequences encoding part of the N-terminal sequences of two tryptic peptides. *Eco*RI cleavage sites were included in the 5′ terminal regions of the primers for subsequent cloning. PCR was used to prepare the DNA probe for screening a genomic DNA library. Reactions were carried out in 100 μl volumes with 0.1 μg of DNA, 400 pmol of each primer and 2.5 units of Amplitaq (Perkin-Elmer Cetus). The reaction program consisted of: (i) one cycle of 2 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C; (ii) 35 cycles of 35 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C; and (iii) one cycle of 10 min at 72 °C. PCR products from genomic DNA were separated in 0.8 % agarose gels in 1 × Tris/acetate buffer [21], excised and purified using the Geneclean BIO101 kit (BIO 101, Vista, CA, U.S.A.) and the fragments cloned.

mRNA was purified from total RNA using oligo(dT)-cellulose (mRNA purification kit from Pharmacia-Biotech). For preparation of the first and second cDNA strand the ZAP-cDNA Synthesis Kit (Stratagene) was used. The cDNA was ligated into the Uni-ZAP XR Vector (GigapackII Gold Cloning Kit, Stratagene) and products were packaged using Gigapack II Gold extracts (Stratagene).

Both DNA chains were sequenced using an automatic sequencer ABI model 377 version 2.2.1. Synthetic oligonucleotides were used as primers in automatic sequencing. GCC software package and BLAST and EXPASY programs were used for analysis, alignment and comparison sequences.

RESULTS AND DISCUSSION

The time-course of AAO activity showed a pattern similar to that described by Guillén et al. [23], although the maximal activity

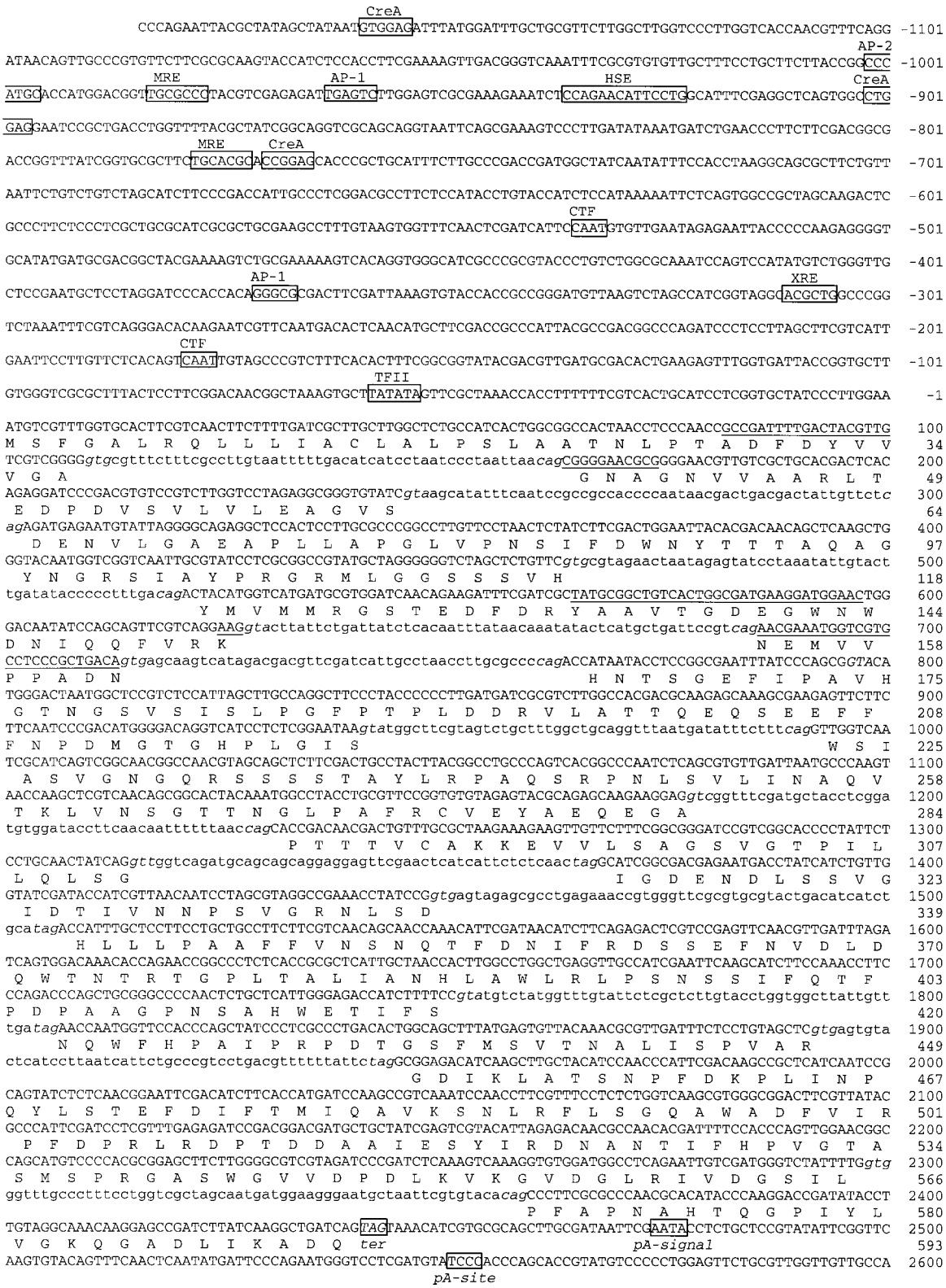

Figure 2 Nucleotide and predicted amino acid sequences of gene *aao* from *P. eryngii*

Putative general transcription (one TATATA and two CAAT boxes) and response elements, to metals (MRE), xenobiotics (XRE), heat-shock (HSE), and signals for regulatory proteins implicated in oxidative stress (AP-1), regulation by nitrogen (AP-2) and carbon sources (CreA), are indicated in the promoter region, while the polyadenylation signal and site are indicated in the 3′-flanking region (boxes). The gene includes 12 introns, which show typical initiation and end sequences (italics). The amino acid sequences confirmed by N-terminal sequencing of the mature AAO protein and tryptic peptides are underlined. The accession number for these sequences is AFO64069.

Table 1 Multiple alignment of N-terminal region of AAO and other oxidoreductases

AAO, aryl-alcohol oxidase from *P. eryngii*; GOX, glucose oxidase from *Aspergillus niger*; AOD1, alcohol oxidase from *Candida boidinii*; AOD2, alcohol oxidase from *Hansenula polymorpha*; AOD3, alcohol oxidase from *Pichia pastoris*; GRED, glutathione reductase from *Escherichia coli*; LPDH, lipoamide dehydrogenase from *Saccharomyces cerevisiae* (conserved residues are in bold) [32,33].

Protein	Position	FAD-binding site
		---β---- -----α----- ----β---
AAO	26-60	NLPTADFDYVVV **GAGNAG** NVVAARLTEDPD --VSVLVL**E**
GOX	20-50	VDYIIA **GGGLTG** LTTAARLTENPN ISVL--VI**E**
AOD1	1-38	MAIPEEFDVIVC **GGGSTG** CVIAGRLANVDE -NLKVLLI**E**
AOD2	1-39	MAIPDEFDIIVV **GGGSTG** CCIAGRLANLDD QNLTVALI**E**
AOD3	1-38	MAIPEEFDILVL **GGGSSG** SCIAGRLANLDH SLK-VGLI**E**
GRED	1-34	MTKHYDYIAI **GGGSGG** IASINRAAMYGQ KCA---LI**E**
LPDH	21-56	LTINKSHDVVII **GGGPAG** YVAAIKAAQLGF -NTA--CV**E**

Table 2 Multiple alignment of C-terminal region of AAO and other oxidoreductases

For abbreviations, see legend to Table 1 (conserved residues are in bold).

Protein	Position	Active site
AAO	526-543	IFHPV **G**TASM---S**P**-R GASWGVV
GOX	514-533	NYHGV **G**TCSM---M**P**-K EMGGVVD
AOD1	565-584	TWHCL **G**TNSM---A**P**-R EGNKNAP
AOD2	566-585	TWHCL **G**TCSM---A**P**-R EGSKIAP
AOD3	565-584	TWHCL **G**TCSI---G**P**-R EGSKIVK
GRED	35-58	AKELG **G**TCVNVGCV**P**KK VMWHAAQ
LPDH	58-81	RGKLG **G**TCLNVGCI**P**SK ALLNNSH

levels were lower. The AAO was isolated from 15-day-old cultures showing the maximal activities (15 units/l). An homogeneous peak with absorbance at 280 and 460 nm, the latter characteristic of flavoproteins, was obtained after Sephacryl S-200 and Mono-Q chromatography (Figure 1, top-left-hand panel). Homogeneity was confirmed by SDS/PAGE.

The strategy to obtain the probe for the gene *aao* of *P. eryngii* is shown in Figure 1. The N-terminal sequences of AAO and the tryptic peptides corresponding to peaks a and b were obtained and degenerate oligonucleotides encoding part of these sequences were synthesized. Using genomic DNA as a template a 0.5 kb fragment was amplified (Figure 1), which was cloned into the pBluescript SK(+/−) vector, and used as a probe for plaque-hybridization screening of the genomic DNA library. Two overlapping positive clones were purified and their DNA was digested with restriction enzymes. A *Sal*I fragment (around 9 kb) that hybridized with the probe was digested with *Pst*I, yielding fragments of 3.9 and 5 kb. Although only the 3.9 kb fragment hybridized with the *aao* probe, both were subcloned into the pBluescript SK (+/−) vector and sequenced. The 3.9 kb fragment contained 2.1 kb of the *aao* gene, including the N-terminal region of the protein, and the 5 kb fragment contained 2.2 kb including the C-terminus. The 3.9 kb fragment was used as a probe for screening of the cDNA library. Plaques selected with cDNA inserts were *in vivo* excised and sequenced.

The main characteristics of the *aao* gene from *P. eryngii* and its 5′ and 3′-flanking sequences are indicated in Figure 2. A total of 4375 bp was sequenced, 747 nucleotides corresponding to the 3′-flanking region (the accession number for the full sequence is AFO64069). The *aao* gene has an open reading frame of 1779 bp, which starts with the ATG codon in position +1 and ends with the TAG codon in position 2443. It is interrupted by 12 introns, of average length 55 bp (51–62), showing 5′-splice (GTDHSY), 3′-splice (YAG) and internal lariat (NNYTNAY) sequences typical of fungal genes [24]. The *aao* gene encodes a 593 amino acid protein, including a 27 amino acid signal peptide which was deduced after identifying the sequence corresponding to the N-terminus of mature protein. The molecular mass from the protein sequence was estimated to be 71.2 kDa, 2% lower than calculated for the *N*-deglycosylated protein after SDS/PAGE [6]. The mature protein contains six potential *N*-glycosylation sites, at Asn-89, -165, -178, -249, -352 and -396, according to the sequence (Asn-X-Ser/Thr) described for other fungal proteins [25]. The preference in codon usage is for those ending in C and A.

The 1185 bp upstream sequence of the *aao* gene includes, in addition to the general transcription sequences CAAT and TATA, putative binding sites for regulatory proteins mediating the response to metals (MRE) [26], heat shock (HSE) [27] and xenobiotic compounds (XRE) [28]. The influence of Mn^{2+} in the regulation of ligninolytic enzymes of *P. chrysosporium* has been reported [29]. Therefore, the positive effect of Mn^{2+} on AAO levels in cultures of *P. eryngii* [7] could indicate a regulation by Mn^{2+} of *aao* expression. The XRE sequence suggests possible regulation by aromatic compounds, as described for the cytochrome P450 gene [30,31]. Studies on regulation of *P. eryngii* AAO by H_2O_2, Mn^{2+} and aromatic compound are in progress.

The *P. eryngii* AAO has 33% and 18% identity (51% and 41% similarity) with glucose oxidase from the ascomycetous fungus *Aspergillus niger* and with aryl-alcohol dehydrogenase from the ligninolytic basidiomycete *P. chrysosporium*, respectively. A comparison of the mature AAO sequence with other oxidoreductases indicates motifs common with other flavoenzymes. The N-terminal region (Table 1) includes a β-α-β motif involved in binding of the AMP moiety of the FAD cofactor [32]. The second homologous region shared by AAO and other oxidoreductases is near the C-terminus (Table 2), corresponding to the active site [32]. AAO and other alcohol oxidases have a deletion of three amino acids (Val-Gly-Cys) compared with disulphide oxidoreductases [32]. Most of the alcohol oxidases oxidize methanol, propan-1-ol and butan-1-ol, and have no activity on aromatic alcohols [33]. However, the AAO found in *Pleurotus* species [6,13,14], *Bjerkandera adusta* [11] and *P. chrysosporium* [12] is able to oxidize aromatic alcohols. This enzyme has no activity on aliphatic saturated alcohols, although the AAO from *P. eryngii* has high activity on some aliphatic polyunsaturated alcohols [6]. Differences in substrate specificity of alcohol oxidases are probably due to differences in the active site of these enzymes. Crystallization and molecular modelling of AAO are currently in progress to provide a structural basis for the unique catalytic properties of this alcohol oxidase, which acts synergistically with Mn-oxidizing peroxidase to provide the H_2O_2 necessary for lignin degradation.

We thank Teresa Suárez for her valuable suggestions and critical reading of the manuscript, and Teresa Raposo and Angeles Guijarro for skilful technical assistance. This work was supported by the project BIO96-393 'Evaluation of enzyme and radical-mediated mechanisms for lignin degradation by *Pleurotus* and *Phanerochaete*' of the Spanish Biotechnology Programme.

REFERENCES

1 Buswell, J. A. and Odier, E. (1987) CRC Crit. Rev. Biotechnol. **6**, 1–60
2 Higuchi, T. (1990) Wood Sci. Technol. **24**, 23–63
3 Kirk, T. K. and Farrell, R. L. (1987) Annu. Rev. Microbiol. **41**, 465–505
4 Martínez, A. T., Camarero, S., Guillén, F., Gutiérrez, A., Muñoz, C., Varela, E., Martínez, M. J., Barrasa, J. M., Ruel, K. and Pelayo, M. (1994) FEMS Microbiol. Rev. **13**, 265–274
5 Eriksson, K.-E. L. (1990) Wood Sci. Technol. **29**, 79–101
6 Guillén, F., Martínez, A. T. and Martínez, M. J. (1992) Eur. J. Biochem. **209**, 603–611
7 Martínez, M. J., Ruiz-Dueñas, F. J., Guillén, F. and Martínez, A. T. (1996) Eur. J. Biochem. **237**, 424–432
8 Muñoz, C., Guillén, F., Martínez, A. T. and Martínez, M. J. (1997) Appl. Environ. Microbiol. **63**, 2166–2174
9 Martínez, M. J., Muñoz, C., Guillén, F. and Martínez, A. T. (1994) Appl. Microbiol. Biotechnol. **41**, 500–504
10 Farmer, V. C., Henderson, M. E. K. and Russell, J. D. (1960) Biochem. J. **74**, 257–262
11 Muheim, A., Waldner, R., Leisola, M. S. A. and Fiechter, A. (1990) Enzyme Microb. Technol. **12**, 204–209
12 Asada, Y., Watanabe, A., Ohtsu, Y. and Kuwahara, M. (1995) Biosci. Biotechnol. Biochem. **59**, 1339–1341
13 Bourbonnais, R. and Paice, M. G. (1988) Biochem. J. **255**, 445–450
14 Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G. and Giardina, P. (1991) Biochim. Biophys. Acta **1073**, 114–119
15 Guillén, F., Martínez, A. T., Martínez, M. J. and Evans, C. S. (1994) Appl. Microbiol. Biotechnol. **41**, 465–470
16 Guillén, F. and Evans, C. S. (1994) Appl. Environ. Microbiol. **60**, 2811–2817
17 Backa, S., Gierer, J., Reitberger, T. and Nilsson, T. (1993) Holzforschung **47**, 181–187
18 Evans, C. S., Dutton, M. V., Guillén, F. and Veness, R. G. (1994) FEMS Microbiol. Rev. **13**, 235–240
19 Ruiz-Dueñas, F. J., Martínez, M. J. and Martínez, A. T. (1999) Mol. Microbiol. **31**, 223–236
20 Camarero, S., Sarkar, S., Ruiz-Dueñas, F. J., Martínez, M. J. and Martínez, A. T. (1999) J. Biol. Chem. **274**, 10324–10330
21 Sambrook, J., Fritsch, E. F. and Martínez, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York
22 González, R., Ramón, D. and Pérez González, J. A. (1992) Appl. Microbiol. Biotechnol. **38**, 370–378
23 Guillén, F., Martínez, A. T. and Martínez, M. J. (1990) Appl. Microbiol. Biotechnol. **32**, 465–469
24 Johansson, T. and Nyman, P. O. (1995) Gene **170**, 31–38
25 Kukuuruzinska, M. A., Bergh, M. L. E. and Jackson, B. J. (1987) Annu. Rev. Biochem. **56**, 915–944
26 Labbé, S., Prévost, J., Remondelli, P., Leone, A. and Séguin, C. (1991) Nucleic Acids Res. **19**, 4225–4231
27 Dynan, W. S. and Tjian, R. (1985) Nature (London) **316**, 774–778
28 Hapgood, J., Cuthill, S., Denis, M., Poellinger, L. and Gustafsson, J. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 60–64
29 Gettemy, J. M., Ma, B., Alic, M. and Gold, M. H. (1998) Appl. Environ. Microbiol. **64**, 569–574
30 Kullman, S. W. and Matsumura, F. (1997) Appl. Environ. Microbiol. **63**, 2741–2746
31 Fujisawa-Sehara, A., Yamane, M. and Fujii-Kuriyama, Y. (1998) Proc. Natl. Acad. Sci. U.S.A. **85**, 5859–5863
32 Frederick, K. R., Tung, J., Emerick, R. S., Masiarz, F., Chamberlain, S. H., Vasavada, A., Rosenberg, S., Chakraborty, S., Schopter, L. M. and Massey, V. (1990) J. Biol. Chem. **265**, 3793–3802
33 Reid, M. F. and Fewson, C. A. (1994) CRC Crit. Rev. Microbiol. **20**, 13–56

Received 1 March 1999/25 March 1999; accepted 22 April 1999