Cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea*

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A novel ligninolytic peroxidase gene (*ACLnP*) was cloned and characterized from a poroid brown-rot fungus, *Antrodia cinnamomea*. The genomic DNA of the fungus harboured two copies of *ACLnP*, with a length of 2111 bp, interlaced with 12 introns, while the full-length cDNA was 1183 bp, with a 66 bp signal peptide and an ORF of 990 bp. The three-dimensional molecular structure model was comparable to that of the versatile peroxidase of *Pleurotus eryngii*. *ACLnP* was cloned into vector pQE31, successfully expressed in *Escherichia coli* strain M15 under the control of the T5 promoter and produced a non-glycosylated protein of about 38 kDa, pI 5.42. The native and recombinant *ACLnP* was capable of oxidizing the redox mediator veratryl alcohol, and also decolorized bromophenol blue and 2,6-dimethoxyphenol dyes, implicating a functional extracellular peroxidase activity. The significance of discovering a functional *ACLnP* gene in *A. cinnamomea* in terms of wood degradation and colonization capacity in its unique niche is discussed.

**INTRODUCTION**

*Antrodia cinnamomea* is an endemic, resupinate to pileate perennial, polyporoid mushroom, inhabiting the empty cavity of *Cinnamomum kanehirae* Hey, causing brown-rot of heart-wood (Chang & Chou, 1995). The host *C. kanehirae* is an endemic evergreen broad-leaved tree of Taiwan, distributed on hillsides in an altitude range from 500 to 1500 m. Interestingly, long before its description as a new taxon, the fruiting body of *A. cinnamomea* was used as a folk medicine for alleviating itching, pain, diarrhoea, inflammation, hangover, hepatic dysfunction and even cancer by local people (Chang & Chou, 1995; Chen et al., 1995; Han et al., 2006; Hsiao et al., 2003; Hsu et al., 2005). The usage of *A. cinnamomea* in medicine has been boosted in vivo and in vitro studies in cell and animal model systems using extracts from the culture filtrate, mycelium or fruiting body. The active ingredients appear to consist mainly of polysaccharides, terpenoids, steroid acid and zhankuic acid (Chen et al., 1995; Cherng et al., 1995; Hsiao et al., 2003; Hsu et al., 2005; Wu et al., 2006).

To help address the molecular and biochemical basis of the fungus and the colonizing capacity of *A. cinnamomea*, a cDNA library has been constructed (S. S. Tzean, unpublished data). Of the 6125 sequenced and annotated ESTs (expressed sequence tags), 3153 putative genes were defined by searching the NCBI GenBank (www.ncbi.nlm.nih.gov), Swiss-Prot (www.expasy.ch/sprot), JGI (www.jgi.doe.gov) or TIGR (www.tigr.org/index.shtml) databases using BLAST. Among them, an EST encoding a putative ligninolytic peroxidase (LnP) homologue with an E-value of 3e-54 was identified. This was surprising since brown-rot basidiomycetes are usually considered to lack the gene responsible for ligninolytic enzyme production, and therefore could not appreciably and efficiently degrade the recalcitrant lignin in wood (Gilbertson, 1980; Hibbett & Donoghue, 2001; Worrall et al., 1997). In contrast, white-rot basidiomycetes are characterized by producing ligninolytic enzymes, i.e. lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), versatile peroxidase (VP), peroxidase or laccase, which co-operate with cellulase or hemicellulase to degrade the pycocarbohydrates and lignin in woods, either simultaneously or independently to retrieve the nutrients needed...
More recently, Morgenstern et al. (2008) have examined the molecular evolution and diversity of lignin-degrading haem peroxidase in the agaricomycetes. Their phylogenetic analysis showed that Class II fungal peroxidases, including a set of haem peroxidases derived from *Phanerochaete chrysosporium* and *Trametes versicolor*, which cause white rot, and *Coprinus cinereus* and *Antrodia cinnamomea*, which either live on litter saprophytically or produce brown-rot in wood, form the basal clade on the phylogram. They suggested that these haem peroxidases would be best categorized as hypothetical or hybrid peroxidases, although a capacity for lignin degradation could not be ruled out (Morgenstern et al., 2008).

The potential for *A. cinnamomea* and allied brown- or soft-rot fungi remains to be clarified. To address these questions, we report the cloning and characterization of the LnP gene from *A. cinnamomea* (ACLnP) and its heterologous expression in *Escherichia coli*. We have also verified its enzymic activity as a native (nACLnP) or recombinant (rACLnP) protein. Additionally, the three-dimensional (3-D) molecular structure of ACLnP was modelled and compared to the structure of peroxidases from some of the white-rot homobasidiomycetes, *Pleurotus eryngii*, *Phanerochaete chrysosporium* and *Arthromyces ramosus* (Martinez, 2002; Piontek et al., 1993, 2001; Ruiz-Duenas et al., 1999; Sundaramoorthy et al., 1994).

**METHODS**

**Organisms.** *Antrodia cinnamomea* used in this study was isolated from the inner cavity of a fallen hollow tree of *Cinnamomum kanehira* in Snow Mountain Valley, Taichung County, Taiwan, ROC, and maintained on potato dextrose agar (PDA).

**Rapid amplification of cDNA ends of ACLnP.** Total RNA was used for biosynthesis of first-strand cDNA, and also for rapid amplification of cDNA ends by the SMART RACE cDNA amplification kit (BD Bioscience-Clontech), according to the procedures provided by the manufacturer. Two gene-specific primers (Supplementary Table S1, available with the online version of this paper), LnP1 for 3'-RACE, and LnP2 for 5'-RACE, were designed based on the EST sequence of a putative LnP gene determined from a previously annotated cDNA library (S. S. Tzean, unpublished data). Amplification of cDNA ends was performed in a thermal cycler (Biometra) under the following conditions: 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min.

**Fosmid colony hybridization, fosmid clone primer walking and gene characterization.** Fosmid primer walking using gene-specific primers forward LnP3/reverse LnP4 (Table S1), and the nested gene-specific primers forward Nested F1, F2 and F3/reverse Nested R1 and R2, was used to define the full-length genomic gene, which in turn was compared with the full-length cDNA to assess the introns in the gene. The regulatory elements of the promoter region were analysed and predicted mainly by MatInspector (www.genomatix.de) and Alibaba 2.1 (www.gene-regulation.com) software, and also by consulting several key references (Camarero et al., 2000b; Gold & Alic, 1993; Hilden et al., 2005; Ruiz-Duenas et al., 1999).

**Southern blot analysis of ACLnP in genomic DNA.** To determine the copy number of ACLnP, genomic DNA of *A. cinnamomea* was digested with EcoRV, PstI or EcoRI, and separated by electrophoresis on a 0.8% agarose gel at 140 V for 110 min using an OWL B2 electrophoresis apparatus. The restricted DNA was vacuum-blotted onto a nylon membrane (GenePure Tech). For Southern blotting, the protocol of Sambrook & Russell (2001) was followed. The membrane was first probed with a DIG-labelled ACLnP gene-specific probe (770 bp, spanning from 901 to 1671 bp in the ORF without EcoRV, PstI or EcoRI restriction sites), followed by immunological staining using anti-DIG antibody conjugated to alkaline phosphatase, and finally incubated with chemiluminescent CDP-Star substrate (Roche). The emitted light was recorded on X-ray film.

**Western blot analysis of ACLnP.** Protein in the gel was transferred onto a nitrocellulose membrane by using a Mini Trans-Blot in accordance with the manufacturer’s instructions (Bio-Rad). For Western blotting, the protocol of Sambrook & Russell (2001) was followed. The His-tag-fused ACLnP on the membrane was first hybridized with the anti-His-tag monoclonal antibody (Santa Cruz), followed by hybridization with 2000-fold-diluted secondary anti-mouse antibody conjugated with horseradish peroxidase (HRP; Jackson Laboratories). The membrane was finally incubated with chemiluminescent CDP-Star substrate (Roche). The emitted light was recorded on X-ray film.

**Real-time PCR (Q-PCR).** Relative quantification and expression of ACLnP, β-glucosidase (BG), exo-1,3-β-glucanase (EBG) and 18S RNA genes in the mycelium or fruiting body of *A. cinnamomea* were optimized and measured using an ABI Prism SDS 7000 sequence detection system using the SYBR Green PCR Master Mix kit (Applied Biosystems). Q-PCR mixtures (25 μl) contained 10 ng first-strand cDNA product, 400 mM gene-specific primers (Table S1) for ACLnP, BG and EBG, and 12.5 μl fluorescent dye SYBR Green mixture. Thermal amplification was conducted under the following conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Double-distilled water instead of first-strand cDNA was used as negative control, and 18S rRNA was used as positive control – this has been shown to be stable and reproducible in Q-PCR.

**Virtual Northern blotting.** cDNA (1 μg) was electrophoresed in a 1% agarose gel, and after denaturation and neutralization was transferred to a Hybond-N nylon membrane (Amersham Biosciences). DIG-labelled (Roche) specific gene fragments for ACLnP, BG, EBG and 18S RNA were used as probes to hybridize the cDNA on the membrane. Virtual Northern blot procedures and signal detection were the same as for the Southern blots described above.

**Homology modelling of ACLnP.** The translated ACLnP protein sequence was modelled using SWISS-MODEL software (http://swissmodel.expasy.org/repository/) using the 3-D molecular structure in the PDB database (www.rcsb.org/pdb/home/home.do) as template to predict the 3-D molecular structure. The preliminary 3-D structures were compared to available LiP, MnP, VP or peroxidase 3-D molecular structures (Kishi et al., 1996, 1997; Martinez, 2002; Perez-Boada et al., 2005; Piontek et al., 1993, 2001; Ruiz-Duenas et al., 1999) for further analysis using PyMOL software (http://pymol.sourceforge.net).

**Phylogeny of ACLnP.** The sequence of the full-length cDNA of ACLnP was translated and aligned by using CLUSTAL_W (CLUSTAL_X 1.83), along with the sequences of 39 LiP, MnP, VP and peroxidase
proteins, representing 22 species of white-rot and one species of brown-rot poroid basidiomycetes, and of the ascomycetous Magnaporthe grisea plant peroxidase superfamily (used as an outgroup) retrieved from the GenBank (www.ncbi.nlm.nih.gov). The data were analysed using the Gonet 250 distance matrix in combination with the neighbour-joining (NJ) and maximum-parsimony (MP) methods provided in the PAUP Version 4.0b10 and Bayesian analysis (MrBayes 3.1) (Hu et al., 2007; Swofford, 2002). The bootstrap support was strengthened by 1000 replicates for both in NJ and MP analysis. The higher-level analysis was run for 1 million generations, using the mixed model for amino acid substitutions, and trees were sampled every 100 generations by using Bayesian analyses. The remaining trees were used to calculate a 60% consensus tree using PAUP.

Construction of ACLnP gene expression vector pQE31-ACLnP. Gene-specific primers LnP5, with an SphI site in the middle, and LnP6, with a HindIII site in the middle, were constructed. The commercially available ampicillin-resistant E. coli expression vector pQE31 with an IPTG-inducible T5 promoter (Qiagen) was modified to express the cloned ACLnP gene. A. cinnamomea ACLnP, initially cloned into the pGEM-T Easy Vector, was digested with SphI and HindIII, and after electrophoresis and purification using the QiAquick gel extraction kit (Qiagen), was ligated to the pQE31 vector which had been subjected to restriction by the same enzyme. The resultant plasmid, pQE31-ACLnP (Supplementary Fig. S1, available with the online version of this paper), was transformed into E. coli strain T10 by electroporation. Positive clones were digested with Avai, sequenced and verified by using an ABI 3730 DNA analyser. After confirmation, plasmid pQE31-ACLnP was subsequently transformed into E. coli strain M15 as described above, processed for colony PCR and sequenced to verify the identity further.

Expression, purification and refolding of rACLnP. E. coli strain M15 harbouring the plasmid pQE31-ACLnP was inoculated in 1 L LB medium containing 100 µg ampicillin ml−1. The cultures were incubated at 37 °C with shaking on a rotary shaker at 200 r.p.m. to an OD₆₀₀ of 0.6, measured using a Hitachi U-2000 spectrophotometer, then IPTG was added to a final concentration 1 mM and incubated for an additional 6 h. The harvested cultures were centrifuged at 3000 g at 4 °C for 10 min. The pellets were washed twice in buffer A (2 mM EDTA, 2 mM DTT, 20 mM Tris-HCl, pH 8, and 1 µl protease inhibitor cocktail (Bioman)) at 4 °C. The cells were resuspended in buffer A with 1% Triton X-100 and 0.1 g DNAse and RNase ml⁻¹, and disintegrated using a French Press (Thermo Scientific IEC) at 16000 p.s.i. with a 3/8” diameter piston. The cell lysates were incubated on ice for 1 h, and subjected to three cycles of freezing-thawing in liquid nitrogen and a water bath at 42 °C. Then the cell lysates were rinsed and centrifuged three times with buffer A, each time at 13200 r.p.m. for 10 min at 4 °C. An equal volume of lysates was added to Ni-NTA agarose (Qiagen) and incubated for 2 h at 4 °C in an end-over-end shaker. After centrifugation, the pellets were resuspended in an equal volume of phosphate buffer with protease inhibitor and boiled on a hot block at 95 °C for 2 min. The released protein was refolded with 8 M urea and 2 mM DTT, standing at 4 °C for 4 h. Then 0.7 mM glutathione and 8 µM haemden were added, incubated at 25 °C for 24 h in darkness, and subsequently subjected to dialysis with sodium succinate buffer (20 mM sodium succinate, pH 3.0) for further assessment of enzyme activity.

nACLnP and rACLnP activity assay. Wild-type A. cinnamomea was grown in malt-extract broth (malt extract, 30 g; peptone, 5 g; distilled water, 1 l) and incubated at 25 °C for 30 days. Approximately 2 l culture filtrates were concentrated to 300 ml by a rotary vacuum evaporator operated at 30 °C (Büchi V800). The protein in the concentrate was saturated by ammonium sulfate on ice overnight and precipitated by centrifugation at 8000 r.p.m. for 20 min, discarding all but the precipitate and 10 ml of the supernatant. The 10 ml suspension mixture was dialysed in 20 mM sodium succinate buffer, pH 4.3, for 24 h. Approximately 10 ml dialysate was subjected to ultrafiltration by using a 10 kDa cut-off YM filter (Amicon) and restored to a final volume of 10 ml with 20 mM sodium acetate buffer, pH 4.3. For qualitative assessment of lignin-modifying enzymes, two methods were used. One is adapted from the test developed by Basvendam (Davidson et al., 1938; Nobles, 1965) with minor modification. In brief, agar discs excised from the colony margin of A. cinnamomea on malt-extract agar (MEA: malt extract, 15 g; agar powder, 20 g; distilled water, 1 l) were transferred to the centre of freshly prepared MEA containing 0.5% 0.22 µm Millipore-filtered gallic acid or tannic acid, incubated at 25 °C and examined for 7–10 days. The appearance of a brown oxidation zone around the colonies indicated positive polyphenoloxidase activity. The second method is a cut plate method (Dingle et al., 1953). Approximately 25 ml 1.5% sterilized agar medium containing 0.001% 0.22 µm Millipore-filtered dyes (Poly R-478, Bromophenol Blue, Remazol Brilliant Blue R, Azure B and 2,6-dimethoxyphenol) was poured into a 9 cm Petri dish. On each plate, three wells at an equal distance from each other or from the centre were cut with a 5 mm cork borer, and the bottom of each well was sealed with agar. To each well, 25 µl culture filtrate concentrate from wild-type A. cinnamomea or 25 µl cell lysates from ACLnP-transformed E. coli M15 containing the reaction mixture with a final concentration of 0.5 mM veratryl alcohol (VA), 0.2 mM H₂O₂ and 50 mM sodium succinate, pH 3.0, was added, respectively. Protein extracted from non-transformed E. coli M15 containing pQE31 only served as a negative control. The reaction was conducted at room temperature in darkness for 12 h.

Spectroscopic and kinetic analysis of rACLnP and nACLnP. For determining ACLnP activity of transformed E. coli M15 or of the culture filtrate of wild-type A. cinnamomea, the method described by Tien & Kirk (1983, 1988) was followed. The oxidation of VA to veratraldehyde was conducted in a UV/VIS spectrophotometer (Bio-Rad SmartSpec 3000) at 310 nm at room temperature with a 1 cm light path cuvette. For steady-state kinetic analysis, VA oxidation was measured at 310 nm in the presence of various concentrations of VA at a constant concentration of 0.2 mM H₂O₂ in 50 mM sodium succinate, pH 3.0 (Solvilegijn Gelpke et al., 1999).

RESULTS

Cloning and characterization of ACLnP

Specific primers based on the specific EST singlet (bda 020260A10.ab1_024) homologous to the predicted putative ACLnP gene were designed to carry out rapid amplification of the cDNA ends. The full-length cDNA of ACLnP consisted of 1183 bp, including a 66 bp signal peptide and an ORF of 990 bp, with homology to the VP gene (spIQUR19.11VPL1_PLEER, VPL1 precursor, E-value = 2e-74) as shown by a BLAST search of the NCBI database (Fig. 1 and Supplementary Fig. S2, available with the online version of this paper). The cloned gene was fused to a 6 × His-tag with a total size of 1026 bp from the start codon to the terminator (Fig. 1). Insertion of the gene into vector pQE31 allowed expression of a 349 aa protein of 38 kDa with a pI of 5.42 (Fig. 2). In total, five fosmid clones showed a positive signal by colony hybridization with a DIG-labelled specific probe, and one of the positive clones (b16) was selected for primer walking to assess the complete gene sequence. The whole gene consists of
2111 bp, interlaced with 12 introns, and has a promoter region with four TATA boxes, three CAAT elements, SP1 (2), HSF (2), AP1 (1), AP2 (1), RFX2 (1) and C/EBP (1) regulatory elements and a GATA binding factor (Supplementary Figs S3 and S4, available with the online version of this paper). The start and termination codons, transmembrane helix, intracellular domain, signal peptide cleavage site and glycosylation sites were predicted by ExPASy software (http://expasy.org/tools) (Figs S3 and S4).

As shown from the comparison and analysis, the ACLnP gene contrasted markedly to genes encoding LiP, MnP and VP from other white-rot basidiomycetes in its promoter-responsive elements, and intron number, size and distribution. However, the gross structure of ACLnP was comparable to members of the class II peroxidase gene family (Fig. S4 and Table S2, available with the online version of this paper). Southern blotting of EcoRI-, EcoRV- and PstI-digested genomic DNA of A. cinnamomea with a DIG-labelled specific probe revealed two copies of the ACLnP gene in the genome (Fig. 3). The four additional fosmid clones (g20, k16, k17, p11) that gave a positive signal in colony hybridization to the probe specific for ACLnP (770 bp from 901 to 1671 bp, without any restriction sites for EcoRV, PstI or EcoRI) were sequenced by fosmid walking, aligned and compared (Supplementary Fig. S5, available with the online version of this paper). With the exception of clone g20, which exhibits 8 nucleotide substitutions at locations 81G(A), 263A(G), 871C(T), 1648C(T), 2146A(C), 2668C(T), 2745C(T) and 3234A(C), the other three ACLnP sequences are identical to the initial ACLnP clone (b16) (Fig. S5). Additionally, the genes encoding BG and EBG were also constitutively expressed both in the mycelium and in the fruiting body, although the former appeared to be expressed at a slightly higher level than the latter, as shown by virtual Northern

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**Fig. 1.** Map of the ACLnP gene with a total length of 2111 bp and interlaced with 12 introns. The full-length cDNA is 1183 bp long, consisting of a 990 bp ORF, and 5' and 3' untranscribed regions (UTR).

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**Fig. 2.** Heterologous expression of ACLnP in *E. coli* M15 as shown by SDS-PAGE. Lanes: M, molecular size marker; 1, supernatant of lysed cells; 2, particulate inclusion of lysed cells; 3, Ni-NTA-purified recombinant protein.

**Fig. 3.** Southern blot analysis indicating the existence of two copies of ACLnP gene (arrows) in the genome after digestion with EcoRV (lane 1), PstI (lane 2), and EcoRI (lane 3). Lane M, molecular size marker.
Expression and enzymic activity of nACLnP and rACLnP

To determine the level of expression of ACLnP in a heterologous bacterial cell, the full-length cDNA of 990 bp was excised by restriction enzymes, and ligated to a 6 × His-tag and the pQE31 expression vector under the control of a strong T5 promoter (Fig. S1). E. coli M15 transformed with pQE31-ACLnP produced an enormous amount of ACLnP protein, with an expected size of about 38 kDa, in the particulate inclusion of the lysed cell as compared to the control, non-transformed cells (Fig. 2, lane 1). The recombinant ACLnP comprised approximately 60% of the total protein in the cell (Fig. 2, lane 2), and after purification almost to homogeneity by Ni-NTA agarose, it produced a band with molecular size of about 38 kDa in an SDS-PAGE gel (Fig. 2, lane 3).

In the Western blotting analysis using a secondary antibody specific for the 6 × His-tag, no signals were detected in the supernatant of transformed and lysed E. coli cells (Fig. 5, lane 1). In contrast, distinctive bands indicating the presence of ACLnP in the particulate inclusion and the recombinant protein purified by the His-tag were detected (Fig. 5, lanes 2 and 3). Purified recombinant MnP from Ganoderma lucidum, expressed from the pET21a(+) vector and used as a positive control, also exhibited an expected band (Fig. 5, lane G). In addition, both the nACLnP and rACLnP from the culture filtrate, or from ACLnP-transformed E. coli M15 were active and functional, as shown by their ability to decolorize Bromophenol Blue (Fig. 6b, c) and 2,6-dimethoxyphenol (data not shown), producing a distinct clear halo in contrast to the blue-purple background; however, they only slightly decolorized Remazol Brilliant Blue R and Azure B (data not shown), and no decoloration reaction occurred at all with Poly R-478. Moreover, no decoloration took place when the dyes were exposed to cell lysate from E. coli M15 transformed with a blank pQE31 plasmid (Fig. 6a). Decoloration of the various phenolic and non-phenolic dyes by ACLnP from the culture filtrate of A. cinnamomea or from rACLnP in

Fig. 4. Virtual Northern analysis of ACLnP, BG, EBG and 18S RNA from the mycelium (M) or fruiting body (F) of A. cinnamomea.

Fig. 5. Western blot of cellular lysates of E. coli M15 transformed with ACLnP. Lanes: G, purified recombinant MnP from Ganoderma lucidum (positive control); 1, supernatant of lysed cells; 2, particulate inclusion of lysed cells; 3, Ni-NTA-purified recombinant protein.

Fig. 6. Bromophenol Blue dye decoloration activity assay by rACLnP or nACLnP by the cut-plate method. (a) Lysate from E. coli transformed with a blank pQE31 plasmid; (b) rACLnP; (c) nACLnP from concentrated culture filtrate of A. cinnamomea. Bar, 1 cm.
transformed *E. coli* M15 is summarized in Table 1. The oxidative activity of ACLnP was further demonstrated by a steady-state kinetic study. rACLnP and nACLnP exhibited a Michaelis–Menten constant \( (K_m) \) of 13.1 (µM) and 20.9 (µM), respectively (Doyle & Smith, 1996; Kishi *et al.*, 1996; Stewart *et al.*, 1996; Tien & Kirk, 1983).

**Modelling the 3-D molecular structure of ACLnP**

The 3-D structure of the ACLnP is shown in Supplementary Fig. S7(a) (available with the online version of this paper), which depicts the predicted 13 α-helices, three β-sheets, the internal haem functional block, and the N- and C-terminal tails. The functional block of the internal haem was associated with several conserved amino acid residues: His-178, Asp-240, Phe-49 and His-50, and the distal Asn-87, Asp-187, Arg-60, Glu-81, Phe-206 and Trp-253. Possible electron transfer between the oxygen and hydrogen bonds of the adjacent amino acid residues, or the presumed long-range electron transfer from the distal superficial tryptophan to the haem is indicated by the dotted line (Fig. S7b). Superimposition of the 3-D structure of ACLnP over LiP, MnP, VP and peroxidase from other white-rot basidiomycetes revealed that ACLnP matched VP better than LiP, MnP or peroxidase (Supplementary Fig. S8, available with the online version of this paper).

**Phylogeny of ACLnP**

The protein sequence translated from the full-length *ACLnP* cDNA was used for phylogenetic analysis with class II fungal peroxidase sequences (LiP, MnP, VP and peroxidase) retrieved from the NCBI database (Fig. S2), representing one brown-rot fungus and 22 white-rot fungi; a protein classified in the plant peroxidase superfamily from the ascomycete *Magnaporthe grisea* was used as the outgroup. The phylogenetic tree displayed five clades, most of the terminal branches receiving high bootstrap and Bayesian inference value support (Fig. 7). The five clades can be summarized as follows: (1) the *Arthromyces* peroxidase clade at the tree base, composed of *Arthromyces ramosus*, *Coprinus cinereus*, *Coprinopsis cinerea*, *Coprinus macrorhizus* and *Coprenellus disseminatus*, bordering *Antrodia cinnamomea*; (2) the *Ganoderma* MnP clade composed of *Ganoderma applanatum*, *Ganoderma australae*, *Ganoderma formosanum* and *Trametes (Coriolus) versicolor*; (3) the *Phanerochaete* MnP clade composed of *Phanerochaete chrysosporium*, *Phanerochaete sordida* and *Dichomitus squalenus*; (4) the *Phanerochaete* LiP clade composed of *Phanerochaete chrysosporium*, *Trametes (Coriolus) versicolor* and *Phlebia radiata*; (5) the *Pleurotus* VP and *Phlebia* MnP composite clade composed of VP of *Pleurotus eryngii*, *Pleurotus sapidus* and *Bjerkandera adusta*, and MnP of *Pleurotus ostreatus*, *Lentinula edodes* and *Ceriporiopsis rivulosa* (Fig. 7).

**DISCUSSION**

In this study, a novel LnP gene (*ACLnP*) was cloned and characterized from a polyoporid brown-rot medicinal mushroom, *Antrodia cinnamomea*, via an EST obtained from an annotated cDNA library. The *ACLnP* gene was successfully expressed in *E. coli*, producing a functional lignonolitic enzyme which could oxidize the redox mediator VA to veratryl aldehyde. Enzymes capable of using VA as substrate can be conclusively defined as LiPs (Tien & Kirk, 1983, 1984, 1988; Martinez *et al.*, 2005). Based on substrate preference, sequence identity and similarity, and the modelled 3-D protein structure, ACLnP appears to be closely related to VP, a more recently established category of polyphenoloxidase found in *Pleurotus eryngii* and *Bjerkandera adusta* (Camarero *et al.*, 2000; Heinfling *et al.*, 1998).

Southern blotting of EcoRI-, EcoRV- and *PstI*-digested genomic DNA of *A. cinnamomea* with a 770 bp gene-specific probe revealed two copies of the *ACLnP* gene in the genome with almost equal intensity. As there are no restriction sites for these enzymes in the specific probe (Supplementary Fig. S9, available with the online version of this paper), the two copies should not come from an internal digestion of the gene. So how similar are the two copies? In order to answer this question, we used degenerate primers derived from the ORF of the initial cloned *ACLnP* gene sequence, to carry out fosmid walking for the four additional fosmid clones (g20, k16, k17, p11) which showed a positive signal with the same probe. The outcome revealed that, with the exception of clone g20 which exhibited 8 nucleotide substitutions at locations 81G(A), 263A(G), 871C(T), 1648C(T), 2146A(C), 2668C(T), 2754C(T) and 3234A(C), the other three clones have a sequence identical to that of the initial *ACLnP* gene clone (b16) (Fig. S5). It would appear that the *ACLnP* gene has duplicated itself by unequal crossing over, and evolved

### Table 1. Decolouration of various phenolic and non-phenolic dyes by nACLnP derived from the culture filtrate of wild-type A. cinnamomea, or rACLnP from transformed E. coli M15

<table>
<thead>
<tr>
<th>Dye*</th>
<th>Decolouration zone (mm)†</th>
<th>rACLnP</th>
<th>nACLnP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly R-478</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>20</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Remazol Brilliant Blue R</td>
<td>7.8</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Azure B</td>
<td>7.8</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>6.2</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

*The decolouration reaction was carried out in the well in a reaction mixture consisting of 25 µl culture filtrate and 0.5 mM VA, 0.2 mM H₂O₂ and 50 mM sodium succinate, pH 3.0, incubated at room temperature in darkness for 12 h.

†Diameter of decolouration zone after subtraction of the diameter of the well.
Fig. 7. Phylogram of class II fungal peroxidases (LiP, MnP, VP, peroxidase) showing the relatedness of 23 polyphenoloxidase sequences of wood-rot fungi, using a protein classified in the Magnaporthe grisea PPC(EAA53513.1) plant oxidase superfamily as the outgroup, inferred by NJ and Bayesian inference algorithms. The bar represents 5% dissimilarity. The number above each branch indicates the bootstrap value of 1000 replicates by parsimonious methods and followed by posterior probabilities from Bayesian inference. LiP represents LnP; P, peroxidase. The taxa analysed included one brown-rot fungus, Antrodia cinnamomea (ACT1), and 22 white-rot fungi: Phanerochaete chrysosporium LiP (Q01775), LiPLG2 (P49012), LiPLG5 (P11543), LiPLG6 (P50622), LiPH2 (P11542), LiPH8 (P06181), LiPA (P31837), LiPH3 (AAA62243.1), Phlebia radiata LiP (AAW71986.1), Trametes versicolor LiP (AA34049.1), Phanerochaete chrysosporium LiPLG3 (P21764), Phanerochaete chrysosporium LiPLG6 (P50622), Phanerochaete chrysosporium LiPH6 (P06181), Phanerochaete chrysosporium LiPH5 (P11543), Phanerochaete chrysosporium LiPLG5 (P11543), Phanerochaete chrysosporium LiPLG5 (P11543), Phanerochaete chrysosporium LiPH3 (AAA62243.1), Dichomitus squalens MnP (A9F31330.1), Ceriporiopsis subvermispora P (AAO61784.1), Phanerochaete chrysosporium MnP2 (AAB30859.1), Phanerochaete chrysosporium MnP (AAA33743.1), Phanerochaete chrysosporium MnP1 (AAA33742.1), Phanerochaete sordida MnP (BAC06187.1), Phanerochaete chrysosporium MnPH3 (AAA62243.1), Antrodia cinnamomea LNPs (ACT1), Magnaporthe grisea PPC(EAA53513.1).

0.05 changes

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concertedly over time (Hurles, 2004; Lynch & Conery, 2000; Moore & Purugganan, 2003; Zhang, 2003). The high level of sequence identity between the two copies of the gene may account for the almost equally strong hybridization signal shown in the Southern analysis. The 8 nucleotide substitutions identified in fosmid clone g20 are located in an exon in the ORF and lead to the following amino acid changes: Asn-184 (AAC) to Lys (AAA), Ser-313 (TCA) to Pro (CCA) and Phe-319 (TTT) to Leu (CTT). However, when the protein sequence of g20 was compared to the NCBI protein database, a similarity was found to the VP precursor VPL1, with an E-value 2e-73 (Supplementary Fig. S10, available with the online version of this paper). None of the amino acid changes described affected conserved amino acids essential for the activity of LnP, so they presumably have no influence on its normal function (Fig. S7b).

In a global sense, ACLnP may belong to the class II fungal peroxidase family. However, it demonstrated a number of differences in ORF length, codon usage and regulatory elements in the promoter region, as well as different phylogenetic relatedness, implying that the regulation or mode of enzymic action of ACLnP might differ from previously documented polyphenol peroxidases of other white-rot fungi (Tien & Kirk, 1983; Alic et al., 1997; Ambert-Balay et al., 2000; Camarero et al., 2000; Godfrey et al., 1990; Piontek et al., 2001; Ruiz-Duenas et al., 1999, 2001). Perhaps the results we have obtained may help to clarify the problem indicated by Morgenstern et al. (2008). They proposed that some haem peroxidase produced by agaricomycetes, i.e. Trametes cervina and A. cinnamomea (an EST sequence retrieved from our database deposited in GenBank), are not seemingly involved in lignin degradation.

By using the partial cDNA sequence of ACLnP to design primers for PCR, specific DNA products were amplified and sequenced from the brown-rot fungi Antrodia salmonea, Antrodia vaillantii, Antrodia xantha, Antrodia oleracea and Laetiporus sulphureus. At least two of the sequences from Antrodia salmonea and Antrodia vaillantii exhibited similarity to a gene encoding a putative MnP. Most of the above-tested brown-rot fungi did not show a positive response in the Barvendamm test, just like A. cinnamomea (Davidson et al., 1938; Nobles, 1965; data not shown). Generally, the brown-rot fungi do not possess lignin degradation capacity, or if they do it is not appreciable. The outcome of our study is unexpected, and suggests the possible existence of extracellular peroxidases in some previously unstudied brown-rot basidiomycetes. The LnPs produced by these brown-rot fungi may allow them to colonize hard- or softwood. Previously, production of MnP by the brown-rot fungus Piptoporus betulinus, lignin-degrading activity in cultures of Oligoporus fragilis, and a laccase gene-specific sequence isolated from Gloeophyllum trabeum have been reported by several research groups (D’Souza et al., 1996; Szklarz et al., 1989; Worrall et al., 1997). Collectively, these studies imply the possibly wide occurrence of ligninolytic enzymes in brown-rot agaricomycetes, with the capacity to degrade lignin.

Based on the 3-D molecular structure of ACLnP, three possible electron passages for the redox reaction may be deduced: (1) Trp-253→Phe-206→Asp-240→His-178→haem; (2) Asp-187→mediator (undefined)→haem; (3) Glu-81→Asn-87→His-50→haem (Fig. S7b) (Hilden et al., 2005; Kishi et al., 1997; Martinez, 2002; Piontek et al., 2001; Ruiz-Duenas et al., 1999). However, it is noteworthy that the current 3-D model of ACLnP is deduced from previous work on LiPs and MnPs from model organisms, i.e. Phanerochaete chrysosporium or Pleurotus eryngii. Therefore, in order to further distinguish its enzymic activity or to elucidate the electron transfer route, site-directed mutagenesis and crystallographic studies of ACLnP are required (Kishi et al., 1996, 1997; Perez-Boada et al., 2005; Piontek et al., 1993, 2001; Martinez et al., 2005; Sundaramoorthy et al., 1994).

A. cinnamomea showed a negative response in the Barvendamm test, a criterion generally used to distinguish white-rot from brown-rot fungi, and to separate morphologically similar genera, i.e. Antrodia from Antrodiella, Neolentinus from Lentinus (Davidson et al., 1938; Hibbett & Donoghue, 2001). This result suggests that A. cinnamomea might be incapable of secretion of extracellular peroxidases to oxidize gallic acid to a quinolic compound. Coincidentally, in nature A. cinnamomea is a typical brown-rot decayer, which colonizes and degrades the heart-wood of the host tree slowly, causing light browning, and indistinct wood block shrinkage and cracking, though the colonized area does increase over time (Chang & Chou, 1995). In contrast, as revealed in the present study, A. cinnamomea is capable of oxidizing the lignin redox mediator VA, and also oxidizes and decolorizes phenolic dyes, i.e. Bromphenol Blue and 2,6-dimethoxyphenol, and non-phenolic dyes, i.e. Remazol Brilliant Blue R and Azure B. Enzymes able to oxidize and modify phenolic and non-phenolic dyes in vitro might be able to oxidize lignin substrates and lead to their eventual disintegration in vivo. If A. cinnamomea recently evolved from a white-rot ancestor in accordance with the proposed hypothesis (Gilbertson, 1980; Hibbett & Donoghue, 2001; Morgenstern et al., 2008), then ACLnP may represent a vestigial or modified hybrid enzyme, with varied oxidative activity toward lignocellulose polymers.

In nature, A. cinnamomea has a quite unique niche, i.e. the inner cavity of C. kanehirae. Extracts from the heart- or sap-wood of C. kanehirae consist of a wide variety of compounds (phenolic or aromatic terpenoids, tropolones, flavonoids, stilbens and tannins) which may act as growth stimulants or inhibitors toward various organisms, i.e. fungi, bacteria or insects. More recently, extracts from C. kanehirae have been demonstrated to have the capability to stimulate the growth of A. cinnamomea while inhibiting the wood-rot basidiomycetes (i.e. Laetiporus sulphureus and Lentinus betulinus) (Wu et al., 2003). Of the extracted
essential oils, eugenol was demonstrated to be most potent, and it can completely inhibit the growth of these fungi (Wu et al., 2003). How can A. cinnamomea grow and colonize the heart-wood of C. kanehirae without suffering from the inhibition or toxicity of these essential terpenoid oils? The production of ACLnP by A. cinnamomea, and some other hydrolases, might contribute to this capability. By using the synergistic activity of ACLnP and lignocellulose hydrolases, i.e. BG or EBG (Fig. 4), A. cinnamomea may be able to degrade lignocellulose, phenolic compounds and aromatic oils, and use the released carbohydrates or nutrients for detoxification, growth, survival and colonization in this unique habitat.

In conclusion, to the best of our knowledge, this is the first LnP gene that has been cloned and characterized from a brown-rot fungus. In addition, we expressed the protein heterologously in bacterial cells, and the generated recombinant protein exhibited normal functions. The enzyme also catalysed the redox reaction of the mediator VA to veratryl aldehyde in a steady-state enzymic kinetic analysis, and also was able to decolorize some phenolic and non-phenolic dyes. Together, the evidence suggests that A. cinnamomea may oxidize lignocellulose substrates in natural habitats by different modes of redox reaction and mechanisms. Based on the current study, as well as the oxidizing capacity shown in our unpublished study of its unique niche more efficiently and successfully.

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