

Lignin-degrading Peroxidases from Genome of Selective Ligninolytic Fungus *Ceriporiopsis subvermispora*^{*[5]}

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Background: The first genome of a selective lignin degrader is available.

Results: Its screening shows 26 peroxidase genes, and 5 genes were heterologously expressed and the catalytic properties investigated.

Conclusion: Two new peroxidases oxidize simple and dimeric lignin models and efficiently depolymerize lignin.

Significance: Although lignin peroxidase and versatile peroxidase had not been reported in *C. subvermispora*, the genomic screening suggests the presence of related lignin-degrading enzymes.

The white-rot fungus *Ceriporiopsis subvermispora* delignifies lignocellulose with high selectivity, but until now it has appeared to lack the specialized peroxidases, termed lignin peroxidases (LiPs) and versatile peroxidases (VPs), that are generally thought important for ligninolysis. We screened the recently sequenced *C. subvermispora* genome for genes that encode peroxidases with a potential ligninolytic role. A total of 26 peroxidase genes was apparent after a structural-functional classification based on homology modeling and a search for diagnostic catalytic amino acid residues. In addition to revealing the presence of nine heme-thiolate peroxidase superfamily members and the unexpected absence of the dye-decolorizing peroxidase superfamily, the search showed that the *C. subvermispora* genome encodes 16 class II enzymes in the plant-fungal-bacterial peroxidase superfamily, where LiPs and VPs are classified. The 16 encoded enzymes include 13 putative manganese peroxidases and one generic peroxidase but most notably two peroxidases containing the catalytic tryptophan characteristic of LiPs and VPs. We expressed these two enzymes in *Escherichia coli* and determined their substrate specificities on typical LiP/VP substrates, including nonphenolic lignin model monomers and dimers, as well as synthetic lignin. The results show that the two newly discovered *C. subvermispora* peroxidases are functionally competent LiPs and also suggest that they are phylogenetically and catalytically intermediate between clas-

sical LiPs and VPs. These results offer new insight into selective lignin degradation by *C. subvermispora*.

Most of the carbon fixed by land photosynthesis is stored in the plant polymers cellulose, hemicellulose, and lignin. Lignin is an aromatic macromolecule (1) that protects the other two polysaccharidic polymers against biodegradation. After plant death, only certain specialized filamentous fungi, especially the highly active basidiomycetes termed white-rot fungi (2), are able to degrade lignin via oxidative mechanisms. Lignin biodegradation is a key step in terrestrial carbon recycling, enabling subsequent degradation of plant polysaccharides by complex microbial communities. Its removal is also a central issue for the current industrial use of lignocellulosic biomass (e.g. in cellulose production for paper) as well as in lignocellulose biorefineries for the sustainable production of fuels, chemicals, and materials from plant feedstocks (3). Industrial removal of lignin often involves harsh chemical treatments, but lignin-degrading fungi and their enzymes may present environmentally friendly alternatives (4).

Two decay patterns are produced by white-rot fungi, being characterized by degradation of both lignin and polysaccharides (simultaneous decay) and by a preferential removal of lignin leaving most of the polysaccharides unaffected (selective decay) (5). Among selective decay basidiomycetes, *Ceriporiopsis* (*Gelatoporia*) *subvermispora* has been selected for delignification of different types of wood (6–8) in paper pulp manufacture (9). More recently, it has also been assayed for delignification in bioethanol production (10). The usual model fungus in lignin biodegradation studies, *Phanerochaete chrysosporium* (11), is of limited interest for such applications because it generally causes a simultaneous decay pattern (2). From the first pilot scale trials on wood biopulping with *C. subvermispora*, a high interest in its lignocellulolytic enzymatic machinery has been sustained, with the double purpose of enhancing enzyme production and discovering new or more efficient enzymes of interest as industrial biocatalysts.

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White-rot fungi secrete multiple isoenzymes of three heme peroxidases with proposed roles in oxidative ligninolysis as follows: lignin peroxidases (LiPs),⁵ manganese peroxidases (MnPs), and hybrid enzymes known as versatile peroxidases (VPs) that combine the structural-functional properties of LiPs and MnPs. In addition, these fungi secrete various oxidases to produce the H₂O₂ required by peroxidases, and most of them also secrete laccases (12). LiPs and VPs are able to oxidize and cleave the recalcitrant nonphenolic structures that compose the bulk of lignin, as shown by studies with dimeric lignin model compounds (13, 14), and also by depolymerization experiments with synthetic lignins in the case of LiPs (15). MnPs and VPs oxidize Mn²⁺ to Mn³⁺, which can oxidize only the minor phenolic units in lignin (16). Likewise, laccases are directly able to oxidize only the phenolic units in lignin (17).

Because the cleavage of nonphenolic lignin structures is central to efficient ligninolysis, it might be expected that LiPs and/or VPs are essential participants in the process. However, among the above oxidoreductases, only laccase and MnP isoenzymes have been found in *C. subvermispora* cultures (18, 19), although DNA hybridization/amplification with LiP sequences was reported in initial studies (20, 21), and some manganese-independent peroxidase activity has been detected (22). The unexpected lack of LiPs or VPs in this selective delignifier has led to various alternative hypotheses. One is that lipid peroxidation reactions catalyzed by MnP might generate fatty acid-derived oxyradicals that cleave lignin (23). There is some experimental support for this mechanism, as it can depolymerize nonphenolic synthetic lignin *in vitro*, but the reaction is slow and inefficient (23–25). Alternatively, the ability of laccases to oxidize nonphenolic lignin structures in the presence of various low molecular weight naturally occurring redox mediators has been demonstrated (26). This might lead to ligninolysis if the fungus produces such mediators as extracellular metabolites or lignin degradation products, but so far this has not been demonstrated.

The recent sequencing of the *C. subvermispora* genome at the Joint Genome Institute, Department of Energy, allows a new look at this longstanding problem. This project supplements the first white-rot (27) and brown-rot (28) fungal genome sequences to be obtained and reflects strong current interest in lignocellulose decay mechanisms that may inspire new biotechnological applications. In this study, an inventory of all the peroxidase genes, with special emphasis on class II heme peroxidases (29), was obtained with manual curation. Then a structural-functional annotation of the predicted proteins was produced, as recently reported for the *Pleurotus ostreatus* genome (30), and their evolutionary history was established by comparison with all the basidiomycete peroxidase sequences currently available. Surprisingly, this annotation revealed the presence in *C. subvermispora* of two genes that encode putative LiP/VP enzymes. To assess the significance of this finding, we have expressed these two peroxidases

heterologously (together with other *C. subvermispora* and reference class II peroxidases) and have estimated their kinetic constants using high and low redox potential substrates. Furthermore, we have assessed the ligninolytic capability of these two peroxidases in experiments with a nonphenolic lignin model dimer and with synthetic lignin.

MATERIALS AND METHODS

Fungal Strain and Genome Sequencing—The genome of monokaryotic *C. subvermispora* strain B (31) was sequenced at the Joint Genome Institute using a shotgun approach (39.0 Mb total) in a project coordinated by Daniel Cullen (United States Department of Agriculture Forest Products Laboratory, Madison, WI) and Rafael Vicuña (Pontificia Universidad Católica de Chile, Santiago, Chile). The results from gene prediction and annotation are available for searching at the Joint Genome Institute genome portal.

Genome Screening and Analysis of Peroxidase Models—The process to obtain the complete inventory of *C. subvermispora* heme peroxidase genes consisted of the following steps: (i) screening the automatically annotated genome at the Joint Genome Institute genome portal; (ii) revising and manually curating (when necessary) the positions of introns, and the N and C termini of the selected models; (iii) comparing the amino acid sequence identities with related proteins; and (iv) confirming the presence of characteristic residues at the heme pocket and substrate oxidation sites, after homology modeling using crystal structures of related proteins from the RCSB Protein Data Bank as templates.

Three MnP genes previously cloned from *C. subvermispora* (GenBankTM accession numbers AAB03480, AAD45725, and AAO61784 and described as MnP1, MnP3, and MnP4, respectively) (32, 33) were identified (as models 116608, 139965, and 94398, respectively, showing 98–100% identities with the corresponding GenBankTM sequences). *C. subvermispora* MnP2 (GenBankTM entries AAB92247 and AAD43581 with 99% sequence identity) was not localized in the genome but could be an allelic variant of gene 50297 (94% identity). The deduced amino acid sequences of the *C. subvermispora* heme peroxidase gene models were compared with all the basidiomycete heme peroxidase sequences (from GenBankTM and genomes) available to date (a total of 376 sequences, prokaryotic class I peroxidases excluded), and phylogenetic trees were constructed using Poisson-corrected distances and unweighted pair group method with arithmetic mean clustering (bootstrap consensus trees were inferred from 1000 replicates).

The programs used in the above analyses include the following: (i) BLAST (Basic Local Alignment Tool) at the National Center for Biotechnology Information for searching nucleotide and protein databases; (ii) MEGA5 (34) for conducting automatic and manual sequence alignment during the heme peroxidase gene search and curation, and for inferring evolutionary trees; and (iii) SignalP 3.0 (35) for predicting the presence and location of signal peptides and cleavage sites. The latter predictions were validated by multiple alignment (from MEGA5) of the 196 class II sequences available, including proteins whose N termini have been sequenced. Theoretical molecular models

⁵ The abbreviations used are: LiP, lignin peroxidase; DHP, dehydrogenation (lignin) polymer; GPC, gel permeation chromatography; HTP, heme-thiolate peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

were generated at the Swiss-Model automated protein homology-modeling server (36).

Gene Synthesis and Directed Mutagenesis—The mature protein-coding sequences of five selected *C. subvermispora* peroxidase genes (49863, 99382, 112162, 118677, and 117436 models) after the above manual curation, two VP genes from *Pleurotus eryngii* (GenBankTM AF007244) and *Pleurotus ostreatus* (genome 137757), and a *P. chrysosporium* LiP gene (GenBankTM Y00262) were synthesized by ATG:biosynthetics (Merzhausen, Germany) after verifying that all the codons had been previously used for expressing other genes in the same *Escherichia coli* strains (and substituting them when required).

The E183K mutation was introduced in *C. subvermispora* gene 99382 by PCR using the expression plasmid pFLAG1-99382 (see below) as template, and the QuikChange kit from Stratagene. The 5'-CGCGGCTGCCGACAAGGTCGATCCTACCATCCCG-3' direct (mutated triplet underlined) and reverse primers were synthesized. The PCR (50- μ l volume) was carried out in a PerkinElmer Life Sciences GeneAmp PCR System 240 using 20 ng of template DNA, 500 μ M each dNTP, 125 ng of each primer, 2.5 units of *Pfu*Turbo DNA polymerase (Stratagene), and the manufacturer's buffer. Reaction conditions were as follows: (i) a "hot start" at 95 °C for 1 min; (ii) 18 cycles at 95 °C for 50 s, 55 °C for 50 s, and 68 °C for 10 min; and (iii) a final cycle at 68 °C for 10 min.

***E. coli* Expression of Heme Peroxidase Genes**—The five *C. subvermispora* peroxidases, the *P. eryngii* and *P. ostreatus* VPs, and the *P. chrysosporium* LiP mature protein-coding sequences were cloned in the expression vectors pFLAG1 (International Biotechnologies Inc.) or pET23a(+) (Novagen). The resulting plasmids pET23a-49863, pFLAG1-99382, pET23a-112162, pFLAG1-118677, pET23a-117436, pFLAG1-AF007244, pFLAG1-137757, and pFLAG1-Y00262 were directly used for expression, and for the above-described directed mutagenesis (pFLAG1-99382). *E. coli* DH5 α was used for plasmid propagation.

The different peroxidases (and the 99382 E183K variant) were produced in *E. coli* W3110 (pFLAG1-derived plasmids) and BL21(DE3)pLysS (pET23a-derived plasmids). Cells were grown for 3 h in Terrific Broth (37), induced with 1 mM isopropyl β -D-thiogalactopyranoside, and grown further for 4 h. The apoenzyme accumulated in inclusion bodies, as observed by SDS-PAGE, and was solubilized using 8 M urea. Subsequent *in vitro* refolding of *C. subvermispora* peroxidases and *Pleurotus* VPs was performed using 0.16 M urea, 5 mM Ca²⁺, 20 μ M hemin, 0.5 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.1 mg/ml protein, at pH 9.5 (38). *C. subvermispora* peroxidase 112162 was refolded using 1 M urea, 5 mM Ca²⁺, 10 μ M hemin, 0.5 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.1 mg/ml protein, at pH 9. Refolding of *P. chrysosporium* LiP was performed using 2.1 M urea, 5 mM Ca²⁺, 10 μ M hemin, 0.7 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.2 mg/ml protein, at pH 8 (39). Active enzyme was purified by Resource-Q chromatography using a 0–300 mM NaCl gradient (2 ml·min⁻¹, 20 min) in 10 mM sodium tartrate (pH 5.5) containing 1 mM CaCl₂.

Kinetic Constants on Selected Substrates—Absorbance changes during substrate oxidation in 0.1 M tartrate (at various

pH values) by the above *E. coli*-expressed peroxidases were recorded in a Thermo Scientific Biomate5 spectrophotometer (using $\sim$ 0.01 μ M enzyme). Oxidation of Mn²⁺ was followed at pH 5 for the formation of the Mn³⁺-tartrate complex (ϵ_{238} 6.5 mM⁻¹·cm⁻¹). Veratryl alcohol oxidation was followed at pH 3 for veratraldehyde (ϵ_{310} 9.3 mM⁻¹·cm⁻¹) formation. Reactive Black 5, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and 2,6-dimethoxyphenol oxidation were all assayed at pH 3.5. These reactions were monitored for Reactive Black 5 disappearance (ϵ_{598} 30 mM⁻¹·cm⁻¹), for ABTS cation radical formation (ϵ_{436} 29.3 mM⁻¹·cm⁻¹), and for dimeric coeruleinone formation (ϵ_{469} 5.5 mM⁻¹·cm⁻¹), respectively. All reactions were at 25 °C and were initiated by addition of 0.1 mM H₂O₂. ABTS and 2,6-dimethoxyphenol oxidation by some *C. subvermispora* peroxidases shows double kinetics with sigmoidal activity curves (at increasing substrate concentrations) that enable calculation of two sets of kinetic constants (for high and low efficiency oxidation sites), as reported previously for VP (40, 41).

Means and standard errors for affinity constant (K_m) and enzyme turnover (k_{cat}) values were obtained by nonlinear least squares fitting of the experimental measurements to the Michaelis-Menten model. Fitting of these constants to the normalized equation $v = (k_{cat}/K_m)[S]/(1 + [S]/K_m)$ yielded the catalytic efficiency values (k_{cat}/K_m) with their corresponding standard errors.

***p*-Dimethoxybenzene and Ferrocycytochrome *c* Oxidation**—Spectral changes during oxidation of *p*-dimethoxybenzene (0.2 mM) and ferrocycytochrome *c* (15 μ M) by the above peroxidases ($\sim$ 0.1 μ M) at 25 °C in the presence of 0.1 mM H₂O₂ were recorded with an Agilent 8453 diode-array spectrophotometer, showing the formation of *p*-benzoquinone and ferricytochrome *c*, respectively. Ferrocycytochrome *c* was prepared before use by reducing ferricytochrome *c* with sodium dithionite, followed by removal of excess dithionite on a Sephadex G-25 column. *p*-Benzoquinone formation was followed at 245 nm in 20 mM sodium succinate (pH 3.5). Decrease of ferrocycytochrome *c* concentration was followed at 550 nm in 20 mM sodium succinate (pH 4). Controls without enzyme and without H₂O₂ were included.

Oxidative Degradation of a Lignin Model Dimer—Unlabeled and ring-¹⁴C-labeled (1.0 mCi mmol⁻¹) 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether were prepared, and their *erythro* and *threo* isomers were chromatographically separated, as described earlier (42).

For reactions involving product analysis, the radiolabeled *erythro* or *threo* dimer was treated either with one of the two *C. subvermispora* LiP-like peroxidases (from genes 118677 and 99382) or with *P. chrysosporium* LiPH8 in 10 mM sodium acetate (pH 3.0) at 25 °C for 1 h. The products formed were analyzed by reversed phase high performance liquid chromatography (HPLC) using a Gilson system equipped with a C-18 column (Phenomenex Luna C18(2); 150 by 4.6 mm, 5- μ m particle size), and methanol/water as mobile phase (35:65 for 15 min, followed by 50:50) at a flow rate of 1 ml·min⁻¹. Elution was monitored at 255 nm, and the ¹⁴C content in collected fractions (0.5 ml) was measured in a liquid scintillation counter. HPLC in conjunction with gas chromatography/mass spectrometry was

used in parallel analyses with unlabeled dimers to confirm the identity of the products obtained.

For experiments to determine reaction kinetics, unlabeled *erythro* or *threo* dimer was treated with *C. subvermispora* peroxidase at 25 °C in the same buffer. Approximate turnover numbers (s^{-1}) at various dimer concentrations were obtained from the increase in absorbance at 310 nm due to benzylic carbonyl formation, *i.e.* production of 4-ethoxy-3-methoxybenzaldehyde, 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)-propan-1-one, and 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropan-1-one (ϵ_{310} 9 $mm^{-1} \cdot cm^{-1}$). Kinetic constants were then calculated as described above.

Enzymatic Depolymerization of Synthetic Lignin—A radiolabeled syringyl/guaiacyl dehydrogenation polymer (DHP) with a syringyl/guaiacyl ratio of ~4:1 was prepared by enzymatic copolymerization of β -[^{14}C]sinapyl alcohol (0.01 mCi $mmol^{-1}$) and unlabeled coniferyl alcohol using horseradish peroxidase as described previously (43). The synthetic lignin was then fractionated on a column of Sephadex LH-20 as described (43), and the high molecular mass fractions (approximately ≥ 21 kDa) were pooled for use in depolymerization experiments.

Enzymatic depolymerization of the DHP by the same peroxidases used in the dimer degradation assays was investigated in 10 mM sodium acetate (pH 4.5) containing 0.25% Tween 20, 1.5×10^4 dpm (188 μg) DHP, and 0.01 μM enzyme in a final volume of 40 ml, in the presence or absence of 10 mM veratryl alcohol. Reactions were conducted at 25 °C by adding H_2O_2 (7.5 mM final concentration in experiments with veratryl alcohol and 0.3 mM in experiments without veratryl alcohol) continuously over 24 h with a syringe pump (15). Control reactions without enzyme were run for comparison. The reaction mixtures were then concentrated by rotary vacuum evaporation, redissolved in *N,N'*-dimethylformamide containing 0.1 M LiCl, and centrifuged as described earlier (15). Molecular mass distributions of the supernatant fractions were assessed by gel permeation chromatography (GPC) on a 1.8×30 -cm column of Sephadex LH20, using *N,N'*-dimethylformamide containing 0.1 M LiCl as the mobile phase. Fractions (2 ml) were collected and assayed for ^{14}C in a liquid scintillation counter (15).

Chemicals—*p*-Dimethoxybenzene, *N,N'*-dimethylformamide, dithiothreitol, 2,6-dimethoxyphenol, ferricytochrome *c*, hemin, isopropyl β -D-thiogalactopyranoside, lithium chloride, manganese sulfate, methanol, oxidized glutathione, Reactive Black 5, sodium dithionite, sodium tartrate, Tween 20, veratryl alcohol, and other unlabeled chemicals were purchased from Sigma, with the exception of H_2O_2 that was from Merck, and ABTS that was from Roche Applied Science.

RESULTS

Inventory of Heme Peroxidase Genes in the *C. subvermispora* Genome—Among the 12,125 gene models identified in the *C. subvermispora* genome distributed in 740 main scaffolds, 26 models were found to encode heme peroxidases after manual revision of the possible candidates from automatic annotation (supplemental Table S1 provides the reference, scaffold, position, best hit, amino acid sequence identity, and peroxidase types encoded by these genes).

For the class II peroxidases we found (numbers 1–16 supplemental Table S1), the classification was based on the presence/absence in the molecular models (obtained using crystal structures of related enzymes as templates) of the following: (i) an exposed tryptophan (two models) putatively involved in oxidation of veratryl alcohol and other high redox potential aromatics (Wc, homologous to Trp-171 in *P. chrysosporium* LiP isoenzyme-H8 and to Trp-164 in *P. eryngii* VP isoenzyme-VPL) (Fig. 1, A and B); and/or (ii) a putative Mn^{2+} oxidation site (14 models) formed by three acidic residues near the most internal heme propionate (Ea, Eb, and Ec, homologous to *P. chrysosporium* MnP1 Glu-35, Glu-39, and Asp-179, and to *P. eryngii* VPL Glu-36, Glu-40 and Asp-175, respectively) (Fig. 1, B and D).

The only *C. subvermispora* peroxidase showing both of the above two catalytic sites (gene 99382) was initially classified as a putative VP (Fig. 1B). The 13 models showing only the Mn^{2+} oxidation site (genes 1–13 in supplemental Table S1) were classified as likely MnPs (Fig. 1D). The single peroxidase containing only the catalytic tryptophan (gene 118677) was classified as a putative LiP (Fig. 1A). Finally, the model lacking both oxidation sites (gene 112162) was classified as a generic peroxidase (Fig. 1C), related to the *Coprinopsis cinerea* peroxidase (44).

Among the MnPs, two types were identified (see supplemental Table S1). (i) MNP-long, represented by 12 gene models in the *C. subvermispora* genome, resembles typical MnPs described in *P. chrysosporium*, whose amino acid sequence is longer than those of LiPs and VPs and includes an additional disulfide bridge. (ii) MNP-short, represented by only one gene model (gene 124076), resembles some *Phlebia radiata* MnPs. MNP-long includes five “extra long” MnPs, whose differential catalytic properties are still to be reported.

An additional single model (supplemental Table S1, gene 83438) has predicted protein folding and heme pocket architecture similar to that of the above class II peroxidases. However, it is a class I (of prokaryotic origin but in the same superfamily) cytochrome *c* peroxidase and is therefore not included in the subsequent comparisons.

The other nine models (numbers 18–26 in supplemental Table S1) correspond to putative peroxidases from the HTP superfamily and are most probably related to the *Agrocybe aegerita* peroxygenase (GenBankTM entries CAV28568 and CAV28569) (45). This superfamily is characterized by the presence in the molecular structures (that of gene 80799 is shown in Fig. 2A) of a cysteine residue acting as the fifth heme ligand, instead of the histidine residue present in the above peroxidases, among other structural details (Fig. 2B).

Catalytic Properties of Peroxidases Expressed in *E. coli*—Among the 15 class II genes in the *C. subvermispora* genome, including genes putatively encoding ligninolytic peroxidases, we selected 118677, 99382, 117436, 49863, and 112162 for heterologous expression, as representative for the main families identified. Our first goal was to investigate their catalytic properties on selected peroxidase substrates and in this way to confirm or modify their putative structural/functional classifications. Additionally, we aimed to evaluate their ability to degrade lignin using a simple dimeric model compound and a polymeric synthetic lignin (DHP).

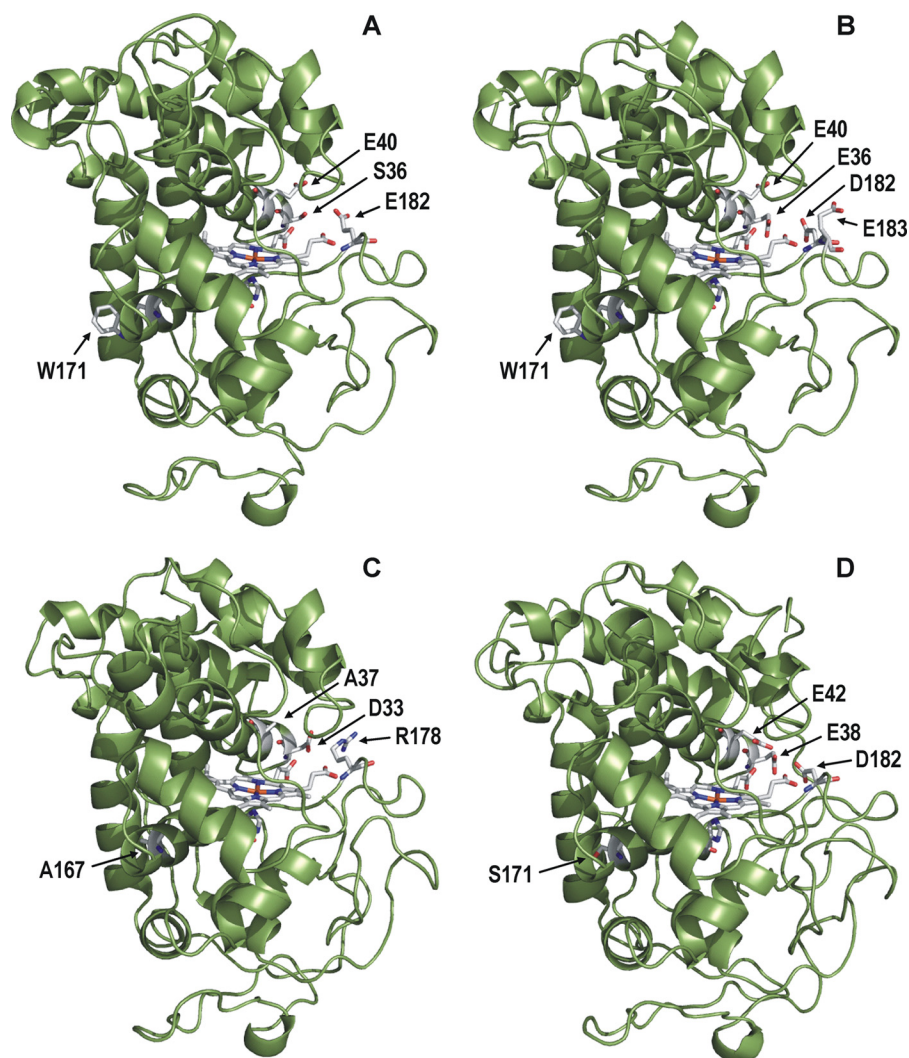


FIGURE 1. **Homology models for the molecular structures of four hypothetical members of class II (fungal peroxidases) from the *C. subvermispora* genome.** A, 118677 peroxidase; B, 99382 peroxidase; C, 112162 peroxidase; and D, 117436 peroxidase. The general folding is shown, together with details of the heme cofactor and residues forming the substrate oxidation sites of MnP/VP (two glutamates and one aspartate, B and D) and LiP/VP (exposed tryptophan, A and B)-type enzymes, as well as the homologous residues in the other proteins (residues and cofactor as Corey-Pauling-Koltun (CPK), colored sticks). The crystal structures of *P. eryngii* VP (Protein Data Bank code 2BOQ) and *P. chrysosporium* MnP (Protein Data Bank code 3M5Q) were used as templates. The amino acid numbering refers to putative mature sequences, after manual curation of their signal peptide sequences.

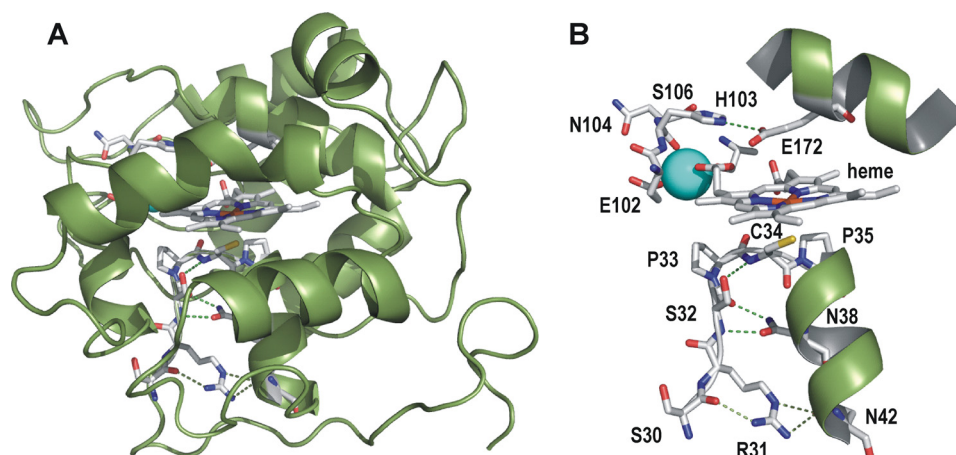


FIGURE 2. **Homology model for the molecular structure of a member of the HTP superfamily from the *C. subvermispora* genome.** The general folding of a putative *C. subvermispora* HTP (model 80799) is shown (A) together with details of its heme region (B, residues and cofactor as CPK-colored sticks, and metal ion as a blue van der Waals sphere). The crystal structure of *L. fumago* chloroperoxidase (Protein Data Bank code 1CPO) was used as a template. The amino acid numbering refers to putative mature sequence after manual curation of signal peptide sequences.

After manual curation, which entailed revision of predicted introns and N and C termini, we used the codifying DNA sequences to express the mature proteins in *E. coli* (see supplemental Fig. S1, A–D for sequences). We used this expression system because, although it requires heme insertion and protein refolding *in vitro*, the yields are high (~200 mg of protein per 5 liters of culture). Moreover, the lack of host-dependent protein glycosylation in this prokaryotic expression system facilitated comparisons of catalytic properties in our subsequent investigations. We purified the resulting active peroxidases to electrophoretic homogeneity in one chromatographic step using a Resource-Q column (Fig. 3). The average yield of the refolding process was ~6 mg of pure active protein per each 200 mg of protein recovered from the inclusion bodies. The molecular masses estimated by SDS-PAGE (Fig. 3, inset) coincided with the values obtained from the mature protein sequences, and electronic absorption spectra revealed the presence of Soret (406 nm) and other typical bands of resting state peroxidases, thus confirming correct incorporation of the heme cofactor (see the electronic absorption spectra in supplemental Fig. S2). Moreover, the A_{410}/A_{280} ratio was similar for all the refolded enzymes, with an average value of 4.

Next, we characterized the substrate specificity of our heterologously expressed peroxidases, including for comparison several well characterized enzymes as follows: *P. eryngii* VP, a closely homologous *P. ostreatus* VP, and *P. chrysosporium* LiPH8. For this experiment we used the following: (i) two simple high redox potential substrates (veratryl alcohol and Reactive Black 5), which can be oxidized only at the LiP or VP catalytic tryptophan; (ii) two low redox-potential substrates (ABTS and 2,6-dimethoxyphenol), which are oxidized at the above catalytic tryptophan with high efficiency but are also oxidized at a second site by VPs and generic peroxidases; and (iii) Mn^{2+} , which is oxidized only at the MnP or VP Mn^{2+} oxidation site (Table 1). In this way, we were able to check each enzyme's putative peroxidase classification as an LiP, VP, MnP, or generic peroxidase. The abovementioned existence of two oxidation sites for some phenols and dyes has been reported for VP (40, 41), although the second one is still to be fully characterized.

As shown by the kinetic constants obtained (Table 1), the putative MnP classification for model 117436 was confirmed by its very high catalytic efficiency for Mn^{2+} oxidation ($>5000\text{ s}^{-1}\cdot\text{mM}^{-1}$), its negligible efficiency (nonsaturation of the enzyme prevented estimation of other kinetic constants) for 2,6-dimethoxyphenol oxidation at a low efficiency site ($<0.05\text{ s}^{-1}\cdot\text{mM}^{-1}$), and its complete lack of activity in all the other reactions investigated (and very similar results were obtained for the 49863 peroxidase). The classification of peroxidase 112161 as a generic (nonligninolytic) peroxidase was also confirmed by its inability to oxidize veratryl alcohol and Mn^{2+} , as well as Reactive Black 5, although it oxidizes ABTS and 2,6-dimethoxyphenol (Table 1).

Likewise, the putative LiP classification for model 118677 appears appropriate, as it exhibited no activity toward Mn^{2+} but was able to oxidize the standard LiP substrate veratryl alcohol (Table 1). Although the catalytic efficiency of peroxidase 118677 is considerably lower than that of *P. chrysosporium*

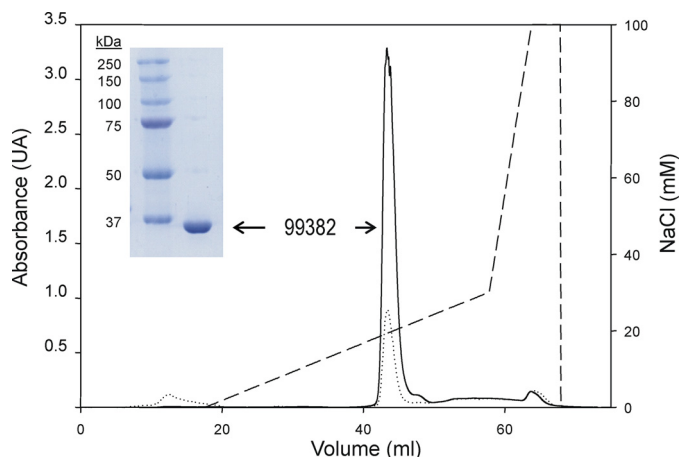


FIGURE 3. Purification of the enzyme product of the putative *C. subvermispora* VP gene (99382) after *E. coli* expression and *in vitro* activation. The gene (optimized manually curated codifying sequence) was synthesized, cloned in pFLAG1, expressed in *E. coli* W3110, refolded *in vitro*, and purified (38). The VP peak obtained using a Resource-Q column and a 0–300 mM NaCl gradient is shown. The elution profiles at 280 nm (total protein) and 410 nm (heme proteins) are shown by dotted and continuous lines, respectively (the actual NaCl gradient, from conductivity measurement, is shown by the dashed line). The electrophoretic homogeneity of the enzyme was confirmed by SDS-PAGE, as shown in the inset (right lane), and a molecular mass of ~35 kDa was estimated.

LiPH8 ($\sim 5\text{ s}^{-1}\cdot\text{mM}^{-1}$ versus $\sim 90\text{ s}^{-1}\cdot\text{mM}^{-1}$), it is clearly a LiP-like enzyme.

The putative VP classification for model 99382 was not confirmed, as it was unable to oxidize Mn^{2+} (Table 1) despite having the three acidic residues that form the typical Mn^{2+} oxidation site in MnPs and VPs (Fig. 1B). This finding, together with our observation that peroxidase 99382 oxidized veratryl alcohol (Table 1), suggested that it, like peroxidase 118677, is an LiP-like enzyme with a low catalytic efficiency on veratryl alcohol ($\sim 3\text{ s}^{-1}\cdot\text{mM}^{-1}$). In agreement, the catalytic efficiencies of enzyme 99382 for ABTS and 2,6-dimethoxyphenol oxidation at high and low efficiency sites (Table 1) were similar to those we found for the LiP-like peroxidase 118677, and its substrate specificity resembles that of *P. chrysosporium* LiPs (although typical LiPs lack the low efficiency site).

One peculiarity of the two *C. subvermispora* LiP-like enzymes, as compared with *P. chrysosporium* LiPs, is their ability to directly oxidize Reactive Black 5 (Table 1), a typical VP substrate that LiP cannot oxidize in the absence of veratryl alcohol as a mediator. Moreover, as noted above, the catalytic efficiencies of the two *C. subvermispora* LiP-like enzymes on veratryl alcohol are low relative to values for typical LiPs. Instead, they are similar to values found for typical VPs ($\sim 2\text{ s}^{-1}\cdot\text{mM}^{-1}$). Accordingly, we surmise that *C. subvermispora* peroxidases 99382 and 118677 are VP-type enzymes that have lost the ability to oxidize Mn^{2+} and therefore must be formally classified as LiPs. They appear as such in Tables 1 and supplemental Table S1 and in Fig. 7.

The lack of Mn^{2+} oxidation activity in the 118677 peroxidase is explained by its having a serine in place of one of the three acidic (glutamate or aspartate) residues required for Mn^{2+} oxidation (Fig. 1, A and D). However, the lack of Mn^{2+} oxidation activity by the 99382 peroxidase is more difficult to explain because it has the three necessary acidic residues (Fig. 1B) that

TABLE 1

Kinetic constants (K_m (μM), k_{cat} (s^{-1}), and k_{cat}/K_m ($\text{s}^{-1}\cdot\text{mM}^{-1}$)) for *C. subvermispota* class II peroxidases (and the 99382 E183K variant) on the simple substrates veratryl alcohol (VA), Reactive Black 5 (RB5), ABTS, 2,6-dimethoxyphenol (DMP), and Mn^{2+} , compared with typical LiP and VP. Kinetic constants were estimated at 25 °C in 0.1 M tartrate (pH 3) for veratryl alcohol, pH 3.5 for Reactive Black 5, 2,6-dimethoxyphenol and ABTS, and pH 5 for Mn^{2+} . GP is generic peroxidase. Means and 95% confidence limits are shown. Dashes correspond to efficiency values (i.e. k_{cat}/K_m ratios) where both k_{cat} and K_m are 0.

		<i>C. subvermispota</i> genome						
		99382 (LiP1 ^a)	118677 (LiP2 ^a)	99382 (E183K)	117436 (MnP10)	112162 (GP)	LiP ^b	VP ^c
VA	K_m	3120 $\pm$ 526	1620 $\pm$ 290	2860 $\pm$ 179	0	0	190 $\pm$ 17	4130 $\pm$ 320
	k_{cat}	8.6 $\pm$ 0.7	8.7 $\pm$ 0.6	8.40 $\pm$ 0.37	0	0	17.5 $\pm$ 0.5	9.5 $\pm$ 0.2
	k_{cat}/K_m	2.8 $\pm$ 0.3	5.4 $\pm$ 0.7	2.9 $\pm$ 0.4	-	-	92.0 $\pm$ 6.0	2.3 $\pm$ 0.1
RB5	K_m	4.0 $\pm$ 0.7	4.5 $\pm$ 0.6	4.6 $\pm$ 0.6	0	0	0	3.4 $\pm$ 0.3
	k_{cat}	9.8 $\pm$ 0.9	7.3 $\pm$ 0.5	7.2 $\pm$ 0.4	0	0	0	5.5 $\pm$ 0.3
	k_{cat}/K_m	2460 $\pm$ 185	1620 $\pm$ 138	1560 $\pm$ 120	-	-	-	1310 $\pm$ 90
ABTS ^d	K_m	4.0 $\pm$ 0.4 (4250 $\pm$ 508)	7.2 $\pm$ 1.0 (NS) ^e	4.2 $\pm$ 0.3 (4110 $\pm$ 468)	0	40.5 $\pm$ 5	23.0 $\pm$ 1.6	3.0 $\pm$ 0.2 (1090 $\pm$ 42)
	k_{cat}	9.3 $\pm$ 0.2 (56 $\pm$ 2)	11.3 $\pm$ 0.5 (NS)	9.5 $\pm$ 0.4 (61 $\pm$ 3)	0	39.6 $\pm$ 2.6	13.0 $\pm$ 0.2	8.1 $\pm$ 0.2 (186 $\pm$ 3)
	k_{cat}/K_m	2300 $\pm$ 200 (13 $\pm$ 1)	1600 $\pm$ 200 (3 $\pm$ 0.1)	2260 $\pm$ 236 (15 $\pm$ 1)	-	976 $\pm$ 16	563 $\pm$ 36	2700 $\pm$ 140 (200 $\pm$ 3)
DMP ^d	K_m	29 $\pm$ 2 (NS)	37 $\pm$ 4 (NS)	30 $\pm$ 2 (NS)	NS	185 $\pm$ 28	6 $\pm$ 1	78 $\pm$ 8 (10500 $\pm$ 400)
	k_{cat}	6.4 $\pm$ 0.1 (NS)	6.1 $\pm$ 0.1 (NS)	6.9 $\pm$ 0.1 (NS)	NS	33.7 $\pm$ 2	10.0 $\pm$ 0.1	56 $\pm$ 1 (30 $\pm$ 0)
	k_{cat}/K_m	222 $\pm$ 13 (0.1 $\pm$ 0)	167 $\pm$ 17 (0.1 $\pm$ 0)	235 $\pm$ 15 (0.1 $\pm$ 0)	<0.1	181 $\pm$ 13	1720 $\pm$ 153	71 $\pm$ 6 (2.8 $\pm$ 0.1)
Mn^{2+}	K_m	0	0	9720 $\pm$ 1530	59 $\pm$ 9	0	0	181 $\pm$ 10
	k_{cat}	0	0	135 $\pm$ 8	331 $\pm$ 20	0	0	275 $\pm$ 4
	k_{cat}/K_m	-	-	14 $\pm$ 0	5600 $\pm$ 500	-	-	1520 $\pm$ 70

^a LiP-VP transition enzymes.

^b Data are from GenBankTM accession number Y00262 LiPH8.

^c Data are from GenBankTM accession number AF007244 VPL.

^d ABTS and DMP showed biphasic kinetics when oxidized by some VP-type and LiP-type enzymes enabling determination of two sets of constants (the second one, characterized by a low catalytic efficiency, is shown in parentheses).

^e NS, K_m and k_{cat} values were not determined because of nonsaturation of the enzyme, but catalytic efficiencies were estimated from slope of observed activity versus substrate concentration.

are conserved in the Mn-oxidizing enzymes (Fig. 1D). The explanation may lie in the presence of a fourth acidic residue (Glu-183 contiguous to conserved Asp-182) (Fig. 1B), which constitutes a difference with respect to typical MnPs and VPs. In support, the E183K variant of the 99382 peroxidase is able to oxidize Mn^{2+} , as shown in Table 1, albeit with lower catalytic efficiency than typical MnPs and VPs, whereas its other catalytic properties are not significantly modified.

Oxidation of Recalcitrant Substrates by *C. subvermispota* Peroxidases—LiPs and VPs, unlike other peroxidases, are able to oxidize aromatics that lack strongly electron-donating $-\text{OH}$ or $-\text{NH}_2$ substituents. This ability stems from their high redox potential and is central to their degradative activity on lignin. In addition, the ligninolytic activity of LiPs and VPs is likely promoted by their ability to oxidize bulky molecules due to the presence on their surface of an exposed tryptophan, which serves to receive electrons from the solvent region and transfer them to the heme cofactor.

To assess the reactivity of our heterologously expressed enzymes on recalcitrant substrates, we began by performing oxidations of two electron donors classically used to show the above properties, *p*-dimethoxybenzene and ferrocyanochrome *c*. As shown in Fig. 4, where the same concentration of each enzyme (0.1 μM) was used, the *C. subvermispota* MnP (model 117436) was unable to oxidize either substrate, in agreement with the lack of a catalytic tryptophan. *P. chrysosporium* LiP catalyzed the fastest oxidation of *p*-dimethoxybenzene to *p*-benzoquinone, followed by the two *C. subvermispota* peroxidases (models 99382 and 118677) and the *Pleurotus* VP, which all exhibited similar oxidation rates (Fig. 4A). However, the *P. chrysosporium* LiP did not catalyze the fastest oxidation of ferrocyanochrome *c*, as slightly higher rates were observed for one

of the *C. subvermispota* peroxidases (model 118677) and the *Pleurotus* VP (Fig. 4B).

Next, we assayed the ability of the heterologously expressed *C. subvermispota* LiPs to cleave two nonphenolic lignin model dimers, the *erythro* and *threo* isomers of 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether, which represent the principal β -O-4 interunit linkage in lignin (1). The substrates were ¹⁴C-labeled to facilitate detection and quantification of oxidized products by HPLC. The results (Fig. 5A) revealed that *C. subvermispota* LiP 118677 oxidized the dimer (peak 1) to yield 4-ethoxy-3-methoxybenzaldehyde (peak 2), the uncleaved ketone with an oxo group at C α (peak 3), and 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropan-1-one (peak 4), together with small amounts of 1-(4-ethoxy-3-methoxyphenyl)glycerol (peak 5). Similar reaction products were produced by the 99382 peroxidase (data not shown). The *threo* isomer was oxidized with a higher k_{cat} than the *erythro* isomer but was not cleaved more rapidly, as virtually all of the additional oxidized product consisted of the uncleaved ketone 3 (Fig. 5, A and B).

Finally, we assessed the ability of the heterologously expressed *C. subvermispota* LiPs to depolymerize a radiolabeled synthetic lignin (DHP). Fig. 6, A and B, shows the results of GPC analyses of lignin treated with *C. subvermispota* peroxidase 118677 in the presence and absence of veratryl alcohol, respectively, together with controls without enzyme. Similar GPC profiles were obtained for the 99382 peroxidase (data not shown). These results show that the two *C. subvermispota* LiPs partially depolymerized polymeric lignin, provided veratryl alcohol was present. For comparison, we show here the result obtained in this work using *P. chrysosporium* LiPH8 in the presence of veratryl alcohol (Fig. 6C). We conclude that *C. subvermispota* peroxidases 99382 and 118677 are fully competent

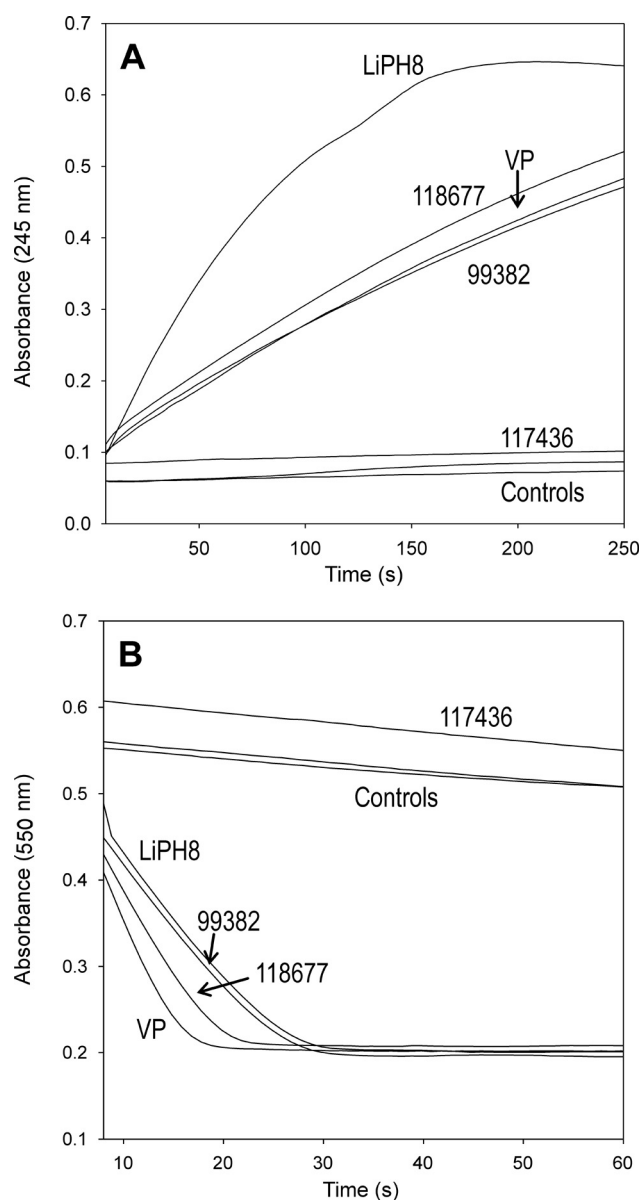


FIGURE 4. Oxidation of *p*-dimethoxybenzene (A) and ferrocytochrome *c* (B) by three class II peroxidases from the *C. subvermisporea* genome compared with oxidation by VP and LiP. *p*-Dimethoxybenzene oxidation to *p*-benzoquinone by *E. coli*-expressed *C. subvermisporea* 99382 (LiP), 118677 (LiP), and 117436 (MnP), *P. ostreatus* VP (genome 137757) and *P. chrysosporium* LiPH8 (GenBank™ Y00262) was followed at 245 nm in the presence of H_2O_2 (A). Ferrocytochrome *c* oxidation to ferricytochrome *c* was followed at 550 nm (B). Controls without enzyme and without H_2O_2 were included in both cases.

LiPs, despite their relatively low catalytic efficiency with the standard LiP substrate veratryl alcohol.

Evolutionary Relationships of *C. subvermisporea* and Other Basidiomycete Peroxidases—Over 400 sequences of basidiomycete heme peroxidases are available to date (from GenBank™ and genomes). To establish the evolutionary relationships of *C. subvermisporea* peroxidases, a comparison of their deduced protein sequences with the other peroxidases from basidiomycetes was performed with MEGA5 (44 class I sequences were excluded). In the dendrogram obtained (Fig. 7), all branches that do not include at least one *C. subvermisporea* sequence are collapsed for simplicity. This is the case for the branch, includ-

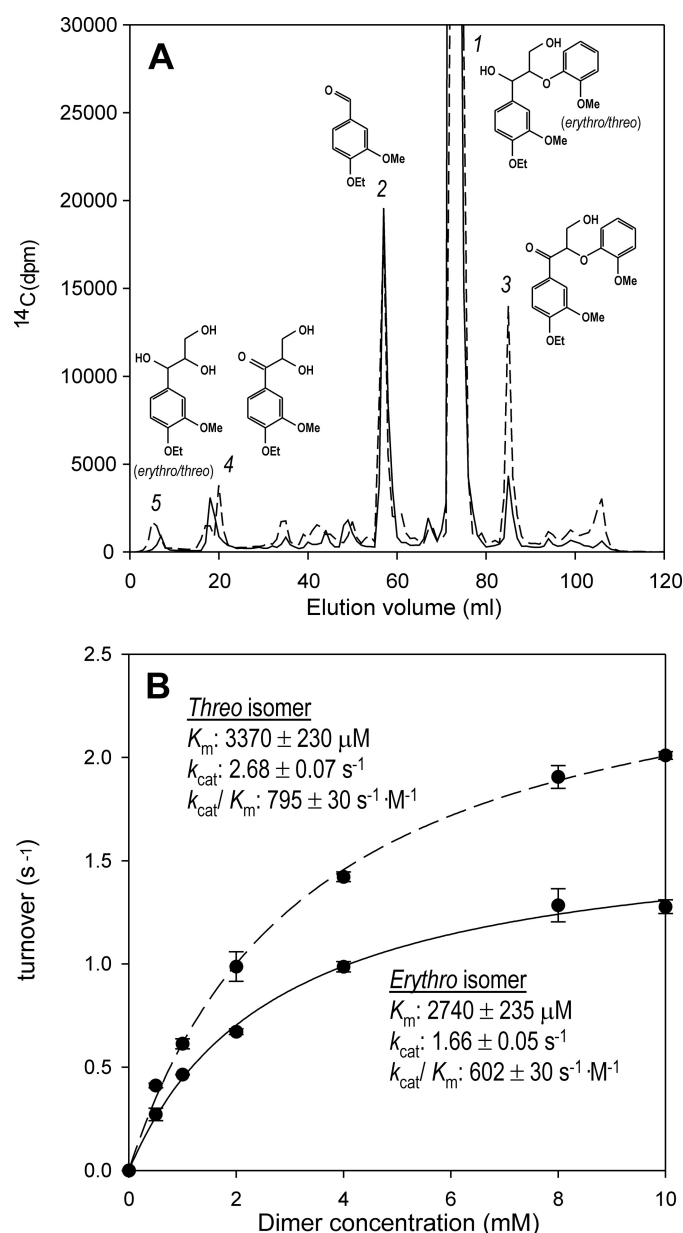


FIGURE 5. Oxidative degradation of a nonphenolic lignin model dimer and estimation of the corresponding kinetic constants. A, erythro (continuous line) and threo (dashed line) isomers of ^{14}C -labeled 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether (peak 1) were treated with *E. coli*-expressed *C. subvermisporea* LiP (gene 118677), and the completed reactions were analyzed by HPLC. The main products were 4-ethoxy-3-methoxybenzaldehyde (peak 2), 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propan-1-one (peak 3), and 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropan-1-one (peak 4). The minor peak 5 corresponds to small amounts of erythro and threo 1-(4-ethoxy-3-methoxyphenyl)glycerol formed from the erythro and threo dimers, respectively. B, different concentrations of the erythro (continuous line) and threo (dashed line) isomers of the lignin model dimer were treated with *C. subvermisporea* LiP 118677. Turnover numbers (s⁻¹) were obtained at different dimer concentrations by spectrophotometric estimation of the main products formed, and the kinetic constants were then calculated.

ing all members (47 sequences), of the dye-decolorizing peroxidase superfamily (group D). As for the HTPs (a total of 133 sequences), the nine *C. subvermisporea* sequences are distributed in three of the four main clusters defined in this superfamily (Fig. 7, E, G, and H) with sequences 122198, 81391, and 114787 forming a homogeneous subcluster in cluster E.

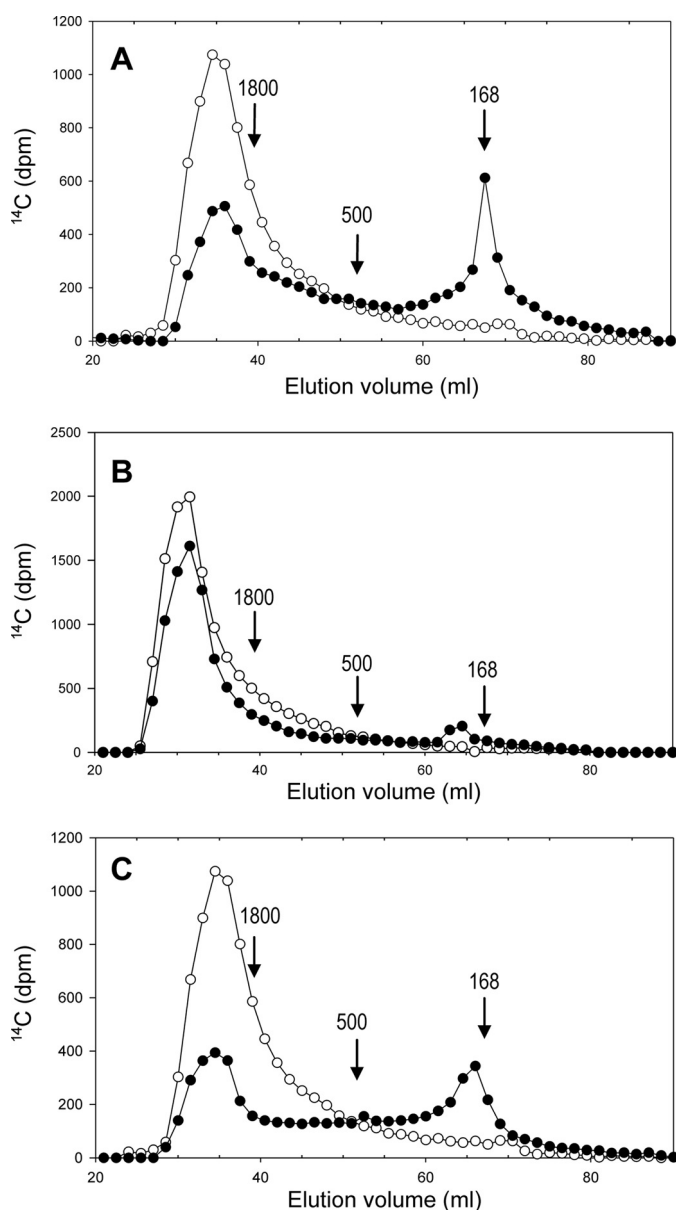


FIGURE 6. Lignin depolymerization by *C. subvermispora* LiP as compared with depolymerization by *P. chrysosporium* LiP. ^{14}C -Labeled synthetic lignin was treated with *E. coli*-expressed *C. subvermispora* LiP (gene 118677) in the presence (A) and absence (B) of veratryl alcohol and with *P. chrysosporium* LiPH8 (GenBankTM Y00262) in the presence of veratryl alcohol (C). Black symbols indicate reactions with enzymes, and open symbols indicate controls without enzymes. Total recoveries of initially added ^{14}C from complete reactions before GPC analysis were 72% for reaction A, 81% for reaction B, and 68% for reaction C. Recoveries from control reactions were somewhat higher as reported earlier (15). The arrows indicate elution volumes of two polystyrene molecular mass standards (1800 and 500) and of veratryl alcohol (168).

The most interesting information for our purposes was obtained for the class II peroxidases (a total of 196 sequences) in the plant-fungal-bacterial peroxidase superfamily. Most of the *C. subvermispora* sequences in this group correspond to long and extra long MnPs (12 sequences) in cluster B, together with all the related enzymes from *P. chrysosporium* and *Dichomitus squalens*, among other basidiomycetes. However, the unique MNP-short sequence and the two LiP-type sequences (from genes 118677 and 99382) are included in cluster A, where intermixed MNP-short and VP subclusters, as well as LiP/VP sub-

clusters (including the 10 *P. chrysosporium* LiP genes) are included, together with additional VP and LiP sequences from other fungi. The position of these two *C. subvermispora* peroxidases (118677 and 99382), grouping together in cluster A, agrees with their LiP-like catalytic and lignin-degrading properties as described above, and thus with their description as new lignin-degrading peroxidases occupying an intermediate position between typical LiPs and VPs.

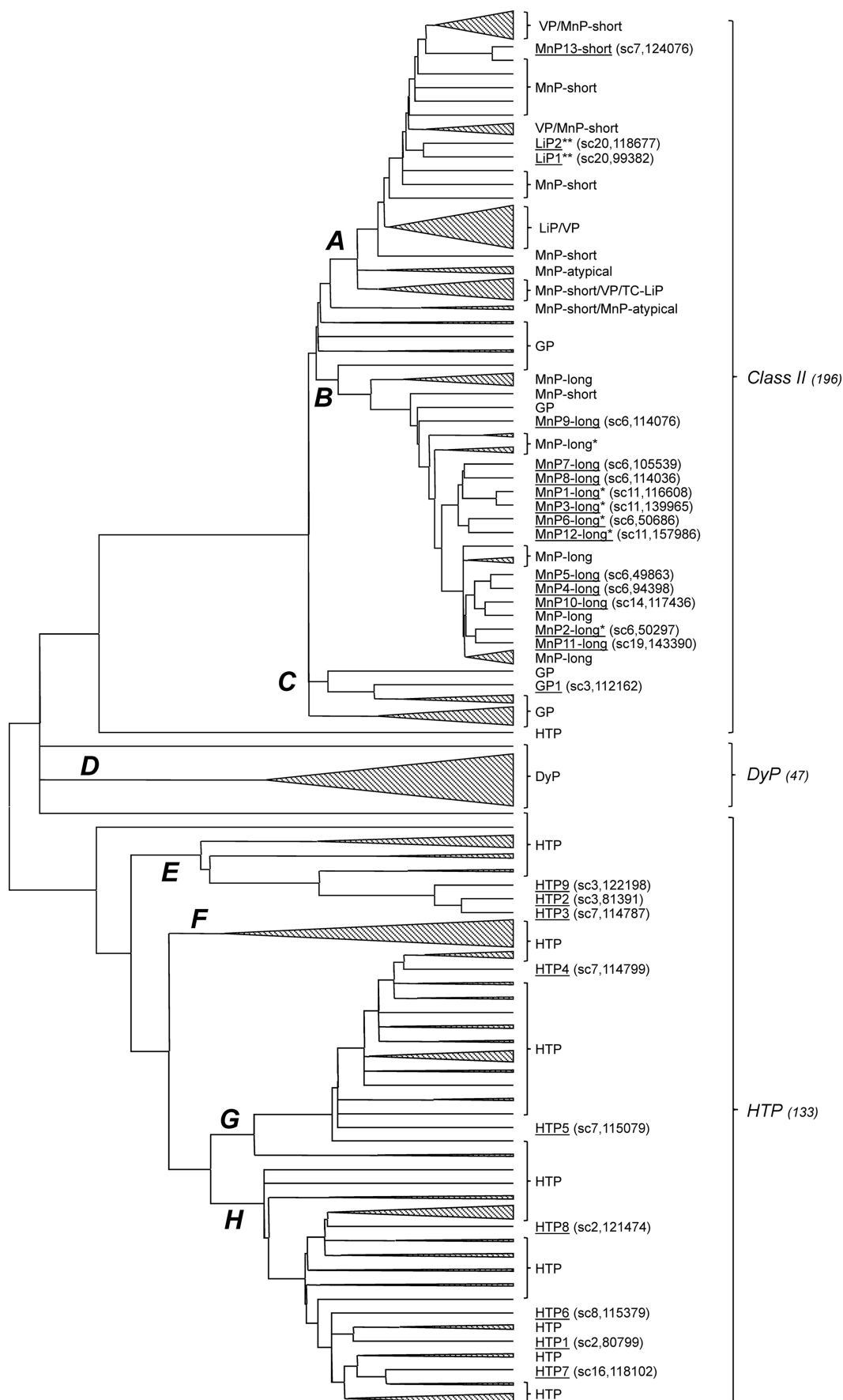
DISCUSSION

Overview—One of the most important pieces of evidence on the central role of ligninolytic peroxidases in lignin degradation comes from the comparison of the first white-rot (*P. chrysosporium*) (27) and brown-rot (*Postia placenta*) (28) genomes to be sequenced. Up to 15 ligninolytic peroxidase genes (10 *lips* and 5 *mnps*) were identified in the *P. chrysosporium* genome. By contrast, no such peroxidases are encoded in the genome of *P. placenta*, a related species that degrades polysaccharides while removing little of the lignin (4). These findings confirm earlier genetic and biochemical studies with the two fungi and point to a central role for LiPs and MnPs in efficient biological ligninolysis (47, 48). VP genes substitute for LiP genes in some genomes, such as that of *P. ostreatus* (30).

In light of these results, the apparent paucity of ligninolytic peroxidases in *C. subvermispora*, a white rot fungus closely related to *P. chrysosporium*, has been a perplexing research problem. When the *C. subvermispora* genome project was initiated, four MNP isoforms were the only peroxidases previously isolated (and/or cloned) from this organism (19, 32, 33, 49–51). The genomic screening results we report here now expand this number to include 26 heme peroxidase genes belonging to the plant-fungal-bacterial peroxidase superfamily (17 genes) and the HTP superfamily (nine genes) (29, 52). Surprisingly, no members of the growing dye-decolorizing peroxidase superfamily were identified, although they seem widespread among white-rot fungi (47 basidiomycete sequences available to date) and can oxidize nonphenolic lignin model compounds (53). The absence of related genes in *C. subvermispora* rules out their involvement in lignin degradation by this fungus.

HTPs—The chloroperoxidase from the ascomycete *Leptoxylum fumago* was the only known fungal HTP for years, but the number of similar genes now apparent in basidiomycetes has increased greatly after the sequencing of the *A. aegerita* peroxygenase gene (45). Basidiomycete HTPs are among the most versatile peroxidase types, able to catalyze a wide variety of oxygenation and oxidation reactions on aromatic and aliphatic compounds (52, 54). However, the *A. aegerita* peroxygenase fails to cleave synthetic lignin, although it can oxidize nonphenolic lignin model dimers (55). It is also unable to oxidize chloride efficiently, although it has bromoperoxidase activity (56), and for this reason it is unlikely to cleave lignin through the agency of hypochlorous acid as the *L. fumago* chloroperoxidase has been shown to do (57). Accordingly, a role for *C. subvermispora* HTPs in ligninolysis appears unlikely.

Plant-Fungal-Bacterial Peroxidases—The *C. subvermispora* genome encodes a single class I cytochrome *c* peroxidase, a mitochondrial enzyme of prokaryotic origin. It encodes numer-



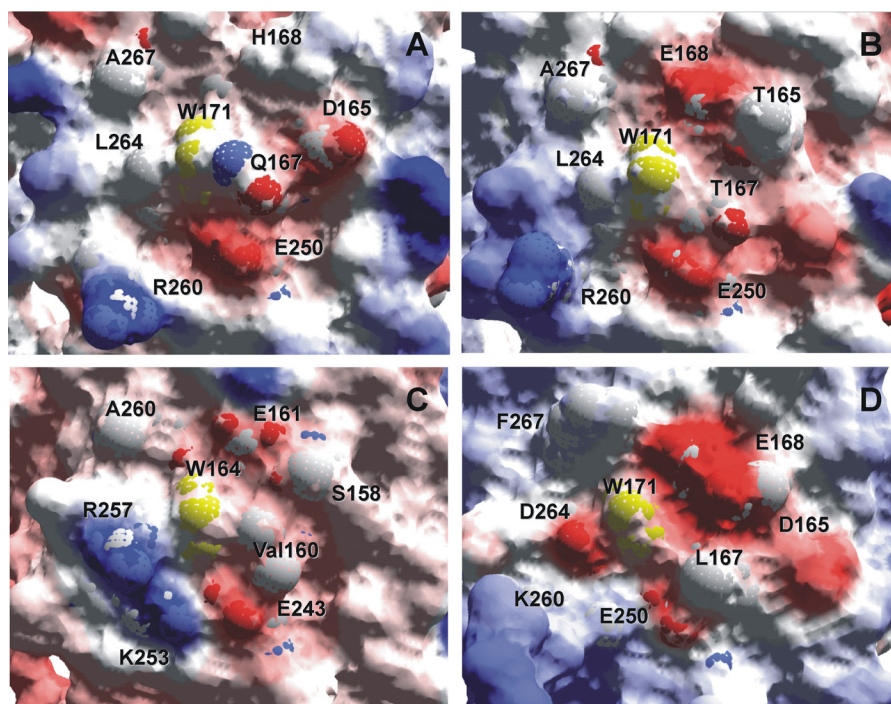


FIGURE 8. Detail of the catalytic tryptophan surface environment (7 residues) in two *C. subvermispura* peroxidases (top) compared with typical VP and LiP (bottom). A, *C. subvermispura* 118677 peroxidase (homology model) solvent access surface (showing the Asp-165, Gln-167, His-168, Trp-171, Glu-250, Arg-260, Leu-264, and Ala-267). B, *C. subvermispura* 99382 peroxidase (homology model) solvent access surface (showing Thr-165, Thr-167, Glu-168, Trp-171, Glu-250, Arg-260, Leu-264, and Ala-267). C, *P. eryngii* VPL crystal structure (Protein Data Bank code 2BOQ) solvent access surface (showing Ser-158, Val-160, Glu-161, Trp-164, Glu-243, Lys-253, Arg-257, and Ala-260). D, *P. chrysosporium* LiPH8 crystal structure (Protein Data Bank code 1LGA) solvent access surface (showing Asp-165, Leu-167, Glu-168, Trp-171, Lys-260, Asp-264, and Phe-267). Semitransparent solvent access surfaces colored by electrostatic potential (red, acidic residues; blue, basic residues) are shown. Amino acids are depicted as white or yellow (catalytic tryptophan) van der Waals spheres. The *C. subvermispura* peroxidase homology models correspond to those shown in Fig. 1, A and B. The amino acid numbering refers to putative mature sequences after manual curation of their signal peptide sequences.

ous class II peroxidases, compared with the other basidiomycete genomes available. Interestingly, all the class II peroxidase genes in the *C. subvermispura* genome appear to be functional, because transcripts for all have been detected in fungal cultures (transcriptomic data recently available under GEO accession GSE34636). This class II expansion is particularly true for the MnP models, which include a total of 13 genes, a greater number than in any sequenced basidiomycete genome except that of *Fomitiporia mediterranea* (58).

These *C. subvermispura* MnPs can be classified as short or long MnPs based on structural and catalytic properties. The latter corresponds to typical MnPs, first described in *P. chrysosporium*, and the former appears similar to some *P. radiata* MnPs that are able to oxidize certain phenols and dyes directly without the involvement of Mn^{2+} (59). The long *C. subvermispura* MnPs include five extra long MnPs similar to a thermostable MnP found in *D. squalens* (60), but their catalytic properties remain to be defined.

In addition and most interesting is the presence in the *C. subvermispura* genome of two LiP/VP-type genes that had never been detected in this fungus at the gene or protein level despite intensive efforts in past work. Our initial structural-functional classification of these peroxidases was based on whether they contain a conserved exposed tryptophan residue that could participate in electron transfer reactions or contain formal oxidation sites for Mn^{2+} (61). This analysis suggested that one gene encoded a VP (gene 99382) and the other encoded an LiP (gene 118677). However, heterologous expression showed that neither oxidized Mn^{2+} , although both oxidized veratryl alcohol, and therefore we classify both as LiPs.

Nevertheless, the relatively low catalytic efficiencies of the *C. subvermispura* LiPs on veratryl alcohol and 1,4-dimethoxybenzene, as compared with *P. chrysosporium* LiP, are reminiscent of a typical *Pleurotus* VP. This property could be related to the environment near the catalytic tryptophan (Fig. 8), which in the two *C. subvermispura* peroxidases (118677 and 99382) is closer

FIGURE 7. Dendrogram of 376 sequences of basidiomycete heme peroxidases (from GenBank™ and different genomes) showing the position of 25 sequences from the *C. subvermispura* genome (underlined). The evolutionary analysis was performed with MEGA5 (34) using Poisson distances and unweighted pair group method with arithmetic mean clustering. Those clusters where *C. subvermispura* sequences are not included were collapsed, and prokaryotic origin class I heme peroxidases, including the putative cytochrome c peroxidase from *C. subvermispura* genome (model 83438), are not included for simplicity. The three main peroxidase groups (class II in the plant, fungal, and prokaryotic peroxidase superfamily; dye-decolorizing peroxidase (DyP) superfamily; and HTP superfamily) and the number of sequences in each of them (parentheses) are indicated (right), together with their eight subclusters (A–H), and the scaffold and model numbers of the different *C. subvermispura* gene models (parentheses). Peroxidase (see supplemental Table S1 for details) include five MnP-long enzymes (*) that could be classified as extra long MnPs (60) and two LiP-type enzymes (**) representing LiP/VP transition stages. Additionally, MnP-atypical corresponds to a MnP type with only two acidic residues at the oxidation site (as found in the *Stereum hirsutum* genome), TC-LiP corresponds to the unique LiP from *Trametes cervina* (46), and GP corresponds to generic peroxidases. See Ruiz-Dueñas and Martínez (29) for references of class II basidiomycete peroxidases (from GenBank™ and genomes).

to that of *P. eryngii* VPL than to *P. chrysosporium* LiPH8 in terms of electrostatic charge distribution. A typical LiP, with four acidic residues, provides a significantly more electronegative environment than do the three other peroxidases with only two acidic residues.

Likewise, the ability of the *C. subvermispota* LiPs to directly oxidize the dye Reactive Black 5 is a typical property of VPs but not of LiPs. This property may be due to the absence in the *C. subvermispota* peroxidases of bulky residues near the catalytic tryptophan that could interfere with the binding of Reactive Black 5; LiPH8 has Phe-267 near Trp-171, whereas the two *C. subvermispota* LiPs and *P. eryngii* VPL have much smaller alanines at analogous positions. The differences in catalytic tryptophan environments of typical VPs and LiPs, as well as their possible implications in catalysis, have already been discussed (62).

The above catalytic properties, together with sequence-based phylogenetic analyses that group the *C. subvermispota* LiPs with VPs and short MnPs from other fungi, suggest that the two new *C. subvermispota* peroxidases represent VP-LiP transition stages. The discrepancy between the initial structural-functional classification of the 99382 peroxidase (as a VP-type enzyme) and the lack of Mn²⁺ oxidation activity after heterologous expression can be explained by conservation, near its hypothetical Mn²⁺ oxidation site, of a glutamate residue present in LiPs, which could disturb productive Mn²⁺ oxidation due to an excess of negative charge in this region of the protein. The Mn²⁺ oxidation activity of our 99382 E183K variant confirms this hypothesis.

Relevance of *C. subvermispota* LiPs to Ligninolysis—One diagnostic property of LiPs and VPs is their ability to cleave nonphenolic lignin model dimers by the one-electron abstraction mechanism reported earlier (2, 63). Our results show that the two *C. subvermispota* LiPs cleaved a dimer based on the principal nonphenolic β -O-4-linked lignin structure, giving product distributions very similar to those produced by *P. chrysosporium* LiPs. Even the well documented higher rate of LiPs on the *threo* isomer of this lignin structure is reproduced by the *C. subvermispota* enzymes. However, as with the monomeric substrate veratryl alcohol, the catalytic efficiency of LiPs 99382 and 118677 on dimeric lignin models is lower than that reported for *P. chrysosporium* LiPH8 (64–67).

The most stringent test available for whether a peroxidase should be classified as a LiP is to determine whether it partially depolymerizes a dispersion of synthetic lignin *in vitro* (15). Our results show that the two *C. subvermispota* LiPs were as effective as *P. chrysosporium* LiPH8 in this reaction, despite their lower catalytic efficiencies on monomeric and dimeric lignin models. As found also with other LiPs, veratryl alcohol was required for depolymerization.

The reason for the veratryl alcohol requirement remains unclear despite extensive research (68). One possibility is that veratryl alcohol is oxidized to its aryl cation radical by the LiP, and this radical then acts as a mediator to abstract an electron from insoluble lignin that cannot make sufficiently close contact with the enzyme's catalytic tryptophan. If this hypothesis turns out to be correct, it will be necessary to address the ques-

tion of why veratryl alcohol, a natural metabolite of *P. chrysosporium*, has not so far been found in *C. subvermispota*.

Alternatively, because veratryl alcohol has been shown to stabilize LiPs against oxidative inactivation by H₂O₂ (68), and assays using synthetic lignins employ large quantities of this oxidant, the veratryl alcohol requirement may be an artifact of *in vitro* reaction conditions. If this second possibility, rather than the mediator hypothesis, proves to be correct, LiPs probably cleave lignin by making direct contact with it. Previous work showing that LiP can bind to lignin favors this second view (69). Moreover, our observation that the *C. subvermispota* LiPs are as effective as *P. chrysosporium* LiPH8 at oxidizing the bulky substrate ferrocyclochrome *c* (46, 70) is consistent with our finding that these enzymes all exhibit similar activities on synthetic lignin.

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Note added in proof—While this article was being published, a description of the whole *C. subvermispota* genome was published online (71).

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