lip-Like Genes in *Phanerochaete sordida* and *Ceriporiopsis subvermispora*, White Rot Fungi with No Detectable Lignin Peroxidase Activity

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Lignin peroxidase-like genes were PCR amplified from *Phanerochaete sordida* and *Ceriporiopsis subvermispora*, fungi lacking lignin peroxidase (LiP) activity. Amplification products were highly similar to previously described LiP genes. Using reverse transcription-coupled PCR a LiP-like cDNA clone was amplified from *P. sordida* RNA. In contrast, no evidence was obtained for transcription of *C. subvermispora* LiP genes.

White rot basidiomycetes are the most efficient lignin-degrading microbes known (8). Extracellular oxidative enzymes involved in lignin depolymerization differ among species, although lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are often produced in culture (16). Of these, the highly oxidizing lignin peroxidases have been regarded as critical because of their ability to cleave the nonphenolic linkages common to lignin (12, 37). Manganese peroxidase, on the other hand, oxidizes Mn(II) to the weak oxidant Mn(III). Phenolic substructures, relatively minor components of lignin, are oxidized by MnP, but nonphenolic lignin is inefficiently depolymerized (11, 24, 27). The peroxidases are ubiquitous among white rot fungi (23), and the most thoroughly studied system is *Phanerochaete chrysosporium*, which secretes both LiP and MnP (2, 25). In *P. chrysosporium*, multiple MnP and LiP isozymes are encoded by large families of structurally related genes (2, 7).

Several studies have identified efficient lignin-degrading fungi apparently lacking LiP (23, 26, 29, 30). *Phanerochaete sordida* is widely distributed and extensively studied for organopollutant degradation (17, 18). Rüttimann-Johnson et al. (30) characterized three *P. sordida* MnP isozymes but failed to detect LiP or laccase activity under a wide range of culture conditions. *Ceriporiopsis subvermispora*, important in biomechanical pulping processes (1), produces large amounts of MnP and laccase but no detectable LiP (29). Amino-terminal sequences of MnP isozymes from liquid and wood chip cultures suggest the presence of multiple, differentially regulated MnP genes in *C. subvermispora* (22).

The mechanism by which lignin is efficiently depolymerized by fungi lacking lignin peroxidases is uncertain. Bao and coworkers recently suggested that MnP-mediated lipid peroxidation systems may play a role in the depolymerization of nonphenolic lignin (4). Another possibility is that LiP is produced but concentrations are relatively low and/or detection is complicated by interfering substances, particularly in woody substrates. Consistent with the second hypothesis, Southern blot hybridizations suggested the presence of LiP-like sequences within the genome of *C. subvermispora* (29). Further supporting this view, we report here the PCR amplification and sequence of LiP-like genes from *C. subvermispora* and *P. sordida*.

PCR amplification and analysis of genomic clones. Sequence alignments of known LiP genes (reference 10 and references therein; 3, 5, 15, 31) identified a conserved region surrounding an essential histidine residue (7, 38) (Fig. 1). Two flanking octapeptides, highly conserved in LiPs but differing in MnPs, were selected for the design of degenerate oligonucleotide primers. Primer 1 [5′-G(C/T)CT(C/T)GT(C/G/T)CC(A/C/G)GAGCC(A/C/G)TTCC-3′] and primer 2 [5′-AGCTG(TCA)GTCTC(G/A)A(T/C)GAAGAACTG-3′] were based on LiP consensus sequences GLVPEPFH and QFFVETQL, respectively (Fig. 1). In contrast, the corresponding MnP consensus sequences are GLIPEPQD and QVFLEVLL and thus unlikely to be amplified by the LiP-specific primers. Genomic DNA was partially purified (20) from *P. sordida* HHB-8922 and *C. subvermispora* L14807 (Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis.). Thirty-five cycle PCRs were used (10). Amplified products were subcloned into pCRII (Invitrogen Inc., San Diego, Calif.), and the double-stranded template was sequenced by the dideoxy termination method (32). Both strands were sequenced.

Three genomic clones were sequenced (Fig. 2). Nucleotide sequences of two *C. subvermispora* clones were remarkably similar. The sequences of clones 1 and 2, including introns, were 96% identical. Their predicted amino acid sequences differed at three positions (Fig. 2), two of which were conservative changes (R↔Q, V↔I). High sequence conservation between *C. subvermispora* clones, particularly within introns, is consistent with allelism, although comparable sequence similarities have been observed for distinct LiP genes of *P. chrysosporium* (33).

All three genomic clones showed substantial sequence similarity to certain *P. chrysosporium* LiP genes (Fig. 2; Table 1). The nucleotide and amino acid sequences of the genomic clones were most similar to *lipA* (92.6 to 95.6%; Table 1), the *P. chrysosporium* gene believed to encode isozyme H8. The relative position and approximate length of introns were conserved among all three clones (Fig. 2) and coincided with known *P. chrysosporium* LiP genes (13).

Sequence and intron structure of the *C. subvermispora* and *P. sordida* LiP-like clones differed substantially from MnP

```
1
       ─────────────────▶
G L V P E P F H T V D Q I I A R V N D A - - G E F D E L E L V W M L S A    P.c.lipA
G L V P E P F H T V D Q I I N R V N D A - - G E F D E L E L V W M L S A    P.c.lipB
G L V P E P F H T I D Q V L A R M L D A - - G G F D E I E T V W L L S A    P.c.lipD
G L V P E P F H T V D Q I I A R V N D A - - G E F D E L E L V W M L S A    P.c.lipE
G L V P E P F H T A D Q I F A R M L D A - - L Q F D E L E T V W G L I A    B.a.lip
G L V P E P F H D V N T I L A R F N D A - - G D F D E L E T V W F L I A    P.r.lip
G L V P E P F H T P D Q I F D R I A D A S Q G E F D P I L T V W L L T A    T.v.lip


H S V A A V N D V D P T V Q G L P F D S T P G I F D S Q F F V E T Q F    P.c.lipA
H S V A A V N D V D P T V Q G L P F D S P P G I F D S Q F F V E T Q L    P.c.lipB
H S I A A A N D V D P T I S G L P F D S T P G Q F D S Q F F V E T Q L    P.c.lipD
H S V A A V N D V D P T V Q G L P F D S T P G I F D S Q F F V E T Q F    P.c.lipE
H T V G A S N D V D P S I P R T P G D S T P S I F D G Q F F I D T Q L    B.a.lip
H S V A A Q N D I D P A V S H A P F D S T P S V M D G Q F F I E T Q L    P.r.lip
H T V A A A N D V D P T K S G L P F D S T P E L W D T Q F F L E T Q L    T.v.lip
                                                              2
                                                          ◣────────
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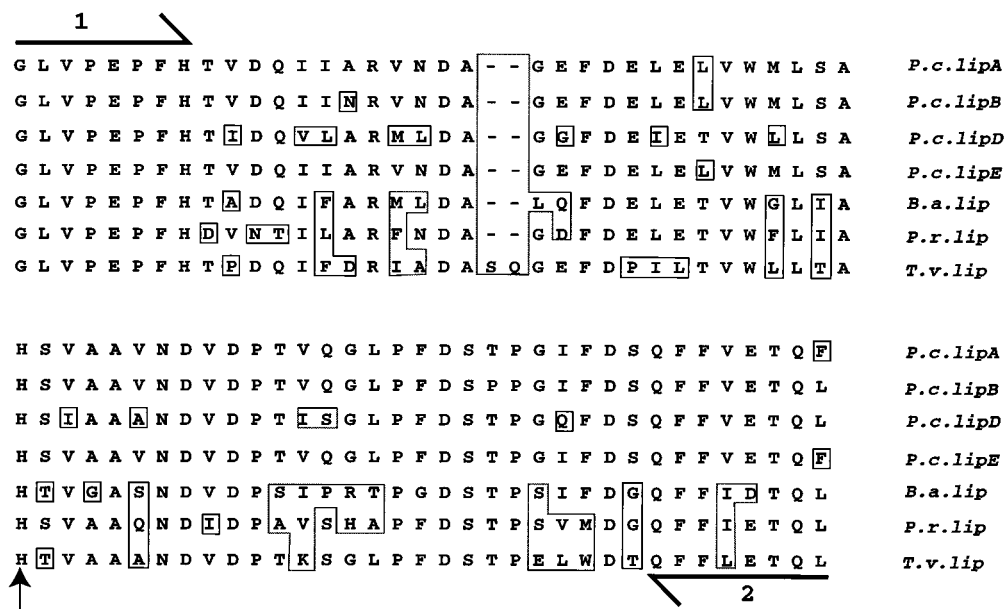
FIG. 1. Multiple alignment of several lignin peroxidase amino acid sequences. *P. chrysosporium* (*P.c.*) gene designations (*lipA, lipB, lipD, lipE*) are based on previously described nomenclature (10). Other sequence abbreviations: *B.a.*, *Bjerkandera adusta* (3); *P.r.*, *Phlebia radiata* (31); *T.v.*, *Trametes versicolor* (15). Sequences were aligned by the Clustal method (14). Residues differing from the consensus are boxed. The proximal histidine, thought to be the axial heme ligand for all peroxidases (38), is indicated by a vertical arrow. Horizontal half arrows show positions of degenerate primers.

genes. All possible pairwise sequence comparisons were made with the five known MnP genes: *P. chrysosporium* clones MP-1 (GenBank accession number J04980), MnP1 (J04624) (28), and MnP2 (U10306); *Trametes versicolor* MPGI (Z30668); and *C. subvermispora* MnP13 (22a). Amino acid sequence similar- ities ranged from 40.6 to 61.8%, with the highest score between the *P. sordida* genomic clone and *T. versicolor* MPG1 (15). In addition, three residues conserved in LiP genes but different in MnP genes were identified within the amplified region (Fig. 2; residues D-23, W-29, and A-33 in *C. subvermispora* genomic 1).

C. subvermispora genomic 1

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CGCTTGTGCCGGAGCCGTTCCgtaagtgcatacttctagacagacgtcaccgtactttcgctcacatctgcacccagACACTGTCGATCAAATCATCAGCCGTGTCAATG    110
  L  V  P  E  P  F                                                          H  T  V  D  Q  I  I  S  R  V  N      17
ATGCC---GGACGGTTCGATGAGCTCGAGCTCGTATGGATGCTTTCGGCgtaag-tctcgagat-gtgtgtcaa-ttcatcctatctgactgcctggcagGCACTCCGTC    214
D  A     G  R  F  D  E  L  E  L  V  W  M  L  S  A                                                       H  S  V    36
GCGGCAGTCAACGACGTCGATCCGACTGTCCAAGGTCTGCCCTTCGACTCGACGCCCGGAAGCTTCGACTCCCAGTTCTTCGTCGAGACTCAGCT    309
A  A  V  N  D  V  D  P  T  V  Q  G  L  P  F  D  S  T  P  G  S  F  D  S  Q  F  F  V  E  T  Q  L        68
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C. subvermispora genomic 2

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GTCTCGTGCCGGAGCCGTTCCgtaagtgcatacttctagacagacgtcaccgtactttcgctcacatctgcacccagACACTGTCGATCAAATCATCAGCCGTGTCAATG    110
  L  V  P  E  P  F                                                          H  T  V  D  Q  I  I  S  R  V  N      17
ATGCC---GGACAGTTCGATGAGCTCGAGCTCGTATGGATGCTTTCGGCgtaagttctcgagattgtgtgtcaggttcatcctatctgactgcctggcagGCACTCCGTC    217
D  A     G  Q  F  D  E  L  E  L  V  W  M  L  S  A                                                       H  S  V    36
GCGGCAGTCAACGACGTCGATCCGACTGTCCAAGGTCTGCCCTTCGACTCGACGCCCGGAATCTTCGACTCCCAGTTCTTCATCGAGACACAGCT    312
A  A  V  N  D  V  D  P  T  V  Q  G  L  P  F  D  S  T  P  G  I  F  D  S  Q  F  F  I  E  T  Q  L        68
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P. sordida genomic

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GTCTCGTGCCGGAGCCCATTCCgtaagtc----aactgcgtgggtctcgcgggacgcagagctgactttacccacagACACTGTCGACCAGATCATCAACCGTGTCAACG    106
  L  V  P  E  P  F                                                          H  T  V  D  Q  I  I  N  R  V  N      17
ACGCC---GGCGAGTTCGATGAGCTTGAGCTCGTCTGGATGCTTTCCGCgtaagtttcacctctgcacccgcgctcccttccctgttgaccgc-atcagCCACTCCGTC    212
D  A     G  E  F  D  E  L  E  L  V  W  M  L  S  A                                                       H  S  V    36
GCCGCCGCAAACGACATTGACCCGACGATCCAAGGCCTGCCGTTCGACTCGACCACCGGGCGTGTTCGACTCGCAGTTCTTCATCGAGACGCAGCT    307
A  A  A  N  D  I  D  P  T  I  Q  G  L  P  F  D  S  T  P  G  V  F  D  S  Q  F  F  I  E  T  Q  L        68
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P. sordida cDNA

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GCCTTGTGCCCGAGCCGTTCC.................................................ACAACCCCGACCAGATCTTCGACCGTCTCGCCG    54
  L  V  P  E  P  F                                                  H  N  P  D  Q  I  F  D  R  L  A          17
ATGGTGAGGGCGAGTTCGACCCCATTCTCACCGTCTGGCTCCTGACCGC..........................................GCACACCGTC    113
D  G  E  G  E  F  D  P  I  L  T  V  W  L  L  T  A                                            H  T  V          37
GCGGCGGCCAACGACGTCGACCCGACGAAGTCCGGGCTGCCGTTCGACTCGACGCCCGAGATCTGGGACACGCAGTTCTTCGTCGAGACGCAGCT    208
A  A  A  N  D  V  D  P  T  K  S  G  L  P  F  D  S  T  P  E  I  W  D  T  Q  F  F  V  E  T  Q  L        69
```

FIG. 2. Nucleotide and deduced amino acid sequences of *C. subvermispora* and *P. sordida* clones. To improve alignments, dashes and dots have been added to genomic and cDNA sequences, respectively. Vertical arrows indicate the conserved proximal histidine. Residues in boldface type are conserved in all known LiP genes but differ from MnP genes.

TABLE 1. Nucleotide and amino acid sequence similarities of lignin peroxidase genes from different sources

Source	C. subvermispora		P. sordida		P. chrysosporium[b]				B. adusta[c]	P. radiata[d]	T. versicolor[e]
	Genomic clone 1	Genomic clone 2	Genomic clone	cDNA	lipA	lipB	lipD	lipE			
Ceriporiopsis subvermispora											
Genomic clone 1		95.5	89.6	62.7	95.5	95.5	77.6	95.5	65.7	68.7	64.2
Genomic clone 2	97.5		91.2	63.2	95.6	94.1	76.5	94.1	70.6	70.6	64.7
Phanerochaete sordida											
Genomic clone	82.3	83.7		63.2	92.6	89.7	67.6	89.7	67.6	72.1	66.2
cDNA	67.0	67.5	69.0		66.2	64.7	67.6	64.7	58.8	55.9	88.4
Phanerochaete chrysosporium[b]											
lipA	88.2	87.7	83.7	69.5		97.1	77.9	97.1	67.6	69.1	66.2
lipB	83.7	84.2	81.3	66.0	88.2		77.9	100	67.6	69.1	64.7
lipD	72.9	72.9	74.4	67.5	74.9	75.4		77.9	69.1	66.2	69.1
lipE	83.7	84.2	82.3	64.5	87.7	94.1	77.3		67.6	69.1	64.7
Bjerkandera adusta[c]	66.0	69.0	67.0	66.0	70.0	71.4	71.9	72.4		67.6	61.8
Phlebia radiata[d]	64.5	65.0	68.5	61.1	69.0	66.0	67.0	67.5	67.5		57.4
Trametes versicolor[e]	63.5	65.0	67.0	85.6	69.5	69.5	68.0	69.0	69.5	62.1	

[a] Nucleotide sequence similarities (lower left) were computed for aligned coding regions (40). Amino acid similarities (upper right) are based on 68 aligned residues (21).
[b] See reference 10.
[c] *B. adusta* LiP clone (3).
[d] *P. radiata lpg3* (31).
[e] *T. versicolor LPGI* (15).

Reverse transcription-PCR (RT-PCR) amplification of cDNA. To determine whether LiP-like genes were transcribed under ligninolytic conditions, RT-PCR was employed. Total RNA was purified (39) from *P. sordida* and *C. subvermispora* grown in nitrogen-limited minimal salts medium (30) and in modified potato dextrose broth (9), respectively. For *P. sordida* RT, a nondegenerate primer based on genomic sequence (Fig. 2) was used: 5'-CGACTCAGAGCTACTTCTTGA-3'. Subsequent PCR amplifications used this 3' primer together with the degenerate 5' primer above. For *C. subvermispora* RT-PCR, degenerate primers were replaced with 5'-GAAGAACTGGG AGTCGAA-3' (downstream) and 5'-TTGTGCCGGAGCCG TTCC-3' (upstream). The latter is identical to *C. subvermispora* genomic clone 1 (Fig. 2), but differs from clone 2 by a single nucleotide. The downstream primer was complementary to both clones.

Initial RT-PCR conditions (35) yielded *P. sordida* cDNA, but no product was obtained with the *C. subvermispora* samples. The LiP-like transcript amplified from *P. sordida* cultures was not identical to the genomic clone (Fig. 2; Table 1). This is not unexpected given the distinct possibility of multiple, differentially expressed LiP genes as observed in *P. chrysosporium*. Presumably, variations in culture conditions would yield additional LiP-like cDNAs. The *P. sordida* cDNA sequence was most closely related to a *T. versicolor* LiP gene LPGI (88.4% similar; Table 1). To formally exclude the possibility that the *P. sordida* cDNA has been amplified from genomic DNA contaminating the total RNA samples, the RT-PCR amplification was successfully repeated with poly(A) RNA as a template. Further, *P. sordida* DNA samples consistently yielded the larger genomic products (~310 bp), never the smaller cDNA (~200 bp).

Numerous experimental modifications were made to enhance *C. subvermispora* cDNA amplifications, including RNA concentrations and alterations of thermocycler conditions. Further, poly(A) RNA was purified (19) from ligninolytic wood chip cultures and used as the RT-PCR template without success. Finally, negative RT-PCR results were also obtained on RNA samples derived from ligninolytic cultures of *C. subvermispora* CZ-3 (6) and FP105752 (29). (Subsequent PCR amplifications of genomic DNA revealed the presence of LiP-like sequences in CZ-3, but not in FP105752 [data not shown].)

In summary, LiP-like sequences were detected within *P. sordida* and *C. subvermispora* genomes, even though both species lack detectable LiP activity. At least one LiP-like gene was transcribed in *P. sordida* cultures. In contrast, no LiP-like transcripts were detected in *C. subvermispora* regardless of substrate or strain.

Clearly, all LiP-like sequences were not identified by our experimental approach. Highly divergent sequences, if present, would not efficiently anneal to the degenerate primers. Subtle differences in primary structure combined with the exponential nature of PCRs might favor the disproportionate amplification of certain sequences. In particular, substantially longer sequences, perhaps due to an unusual intron, would probably be missed.

The role of *P. sordida* and *C. subvermispora* LiP-like genes in ligninolysis, if any, remains to be established. Similar to *P. chrysosporium*, both species may harbor families of structurally related LiP genes which may be differentially expressed. Detection of LiP activity can be obscured by interfering substances (23), and recent evidence suggests that LiPs are more susceptible to certain fungal proteases relative to MnPs (reference 36 and unpublished observations). In short, failure to detect enzyme activity should be viewed with caution. This point is clearly illustrated by the recent identification of laccase activity in *P. chrysosporium*, a species long regarded as lacking laccase (34).

Nucleotide sequence accession numbers. Sequences Csg1, Csg2, Psg, and Psc have been deposited in GenBank and assigned accession numbers L77198, L77199, L77200, and L77201, respectively.

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