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STRUCTURE AND MOLECULAR REGULATION OF THE LIGNIN PEROXIDASE GENES FROM THE WHITE-ROT FUNGUS TRAMETES VERSICOLOR

By

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ABSTRACT

STRUCTURE AND MOLECULAR REGULATION OF
THE LIGNIN PEROXIDASE GENES FROM
THE WHITE-ROT FUNGUS TRAMETES VERSICOLOR

By

Andrew James Kavanaugh Black

White-rot fungi are among the few organisms able to degrade all three constituents of wood: cellulose, hemicellulose and lignin. Lignin peroxidases (LIPs), produced by white-rot fungi are involved in the key reactions leading to depolymerization of the lignin polymer. However the *LIP* gene structure for lignin peroxidases and their molecular regulation has only been characterized for one of the white-rot fungi, *Phanerochaete chrysosporium.* Little is known about the gene structure or molecular regulation of another well studied white-rot fungus, *Trametes versicolor.*

Six lignin peroxidase genes (*VLG1-VLG6*) were isolated from a genomic lambda library of *T. versicolor* strain ATCC 12679. DNA sequence analysis of the genomic sequence for *VLG1* and *VLG2*, as well as cDNA sequences for *VLG2*, revealed substantial stretches of conserved coding sequence between the LIPs of *T. versicolor*, *P. chrysosporium*, and *Phlebia radiata.*

Study of the molecular regulation of the cloned *VLG* genes induced by growth in nitrogen limiting media revealed that the major *LIP* transcript, *VLG2*, was induced coincidentally with LIP enzyme activity. Also LIP activity was completely repressed by low levels of Mn(II) (2.6 ppm). These results stimulated a study on the molecular regulation of the *VLG* genes induced during growth on poplar and pine wood. No such

study has ever been done for any of the white-rot fungi. Study of the molecular regulation of lignin peroxidase genes in wood revealed that three genes, *VLG2*, *VLG5*, and *VLG3* were induced under various conditions in wood cultures. Differential gene regulation was found for *VLG2* and *VLG5* transcripts induced by growth on pine wood. Transcripts for *VLG3* were only induced under high nitrogen conditions in poplar wood grown cultures.

To my mother and father;

and my wife and children.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

CHAPTER II

LIST OF FIGURES

INTRODUCTION

CHAPTER I

CHAPTER II

CHAPTER III

APPENDIX I

INTRODUCTION

I Wood Structure

Wood is composed of three major constituents: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose make up 50% and 20%, respectively, of wood biomass while lignin comprises 20-30% of biomass weight (22, 76). Although cellulose and hemicellulose are readily metabolized by many soil and forest microbes, lignin is recalcitrant to degradation. Lignin provides the plant cell wall with a water impermeable barrier and physical rigidity. And the close association of lignin with the cellulose and hemicellulose fibrils protects these wood polysaccharides from microbial attack.

The basic structure of a wood cell wall is presented in Figure 1. Lignin is concentrated most heavily in the middle lamellar region between vessels (28). The lignin in the middle lamellar region provides a cement between vessel elements and prevents water permeation between vessels. While heavily concentrated in the middle lamellar region, lignin is most abundant in the secondary cell walls. In birch wood the fiber, vessel, and ray cell secondary walls contained over 81% of the total cellular lignin (28). This lignin surrounds the cellulose fibrils, and is intermixed with the hemicellulose, such that for efficient conversion of the cellulose to glucose the lignin must be depolymerized.

Lignin is synthesized in the plant cell by diversion of tyrosine and phenylalanine to produce the three lignin precursors p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These lignin phenylpropanoid subunits are dehydrogenated by a plant peroxidase (E.C1.11.1.7, donor; hydrogen peroxide oxidoreductase) to produce

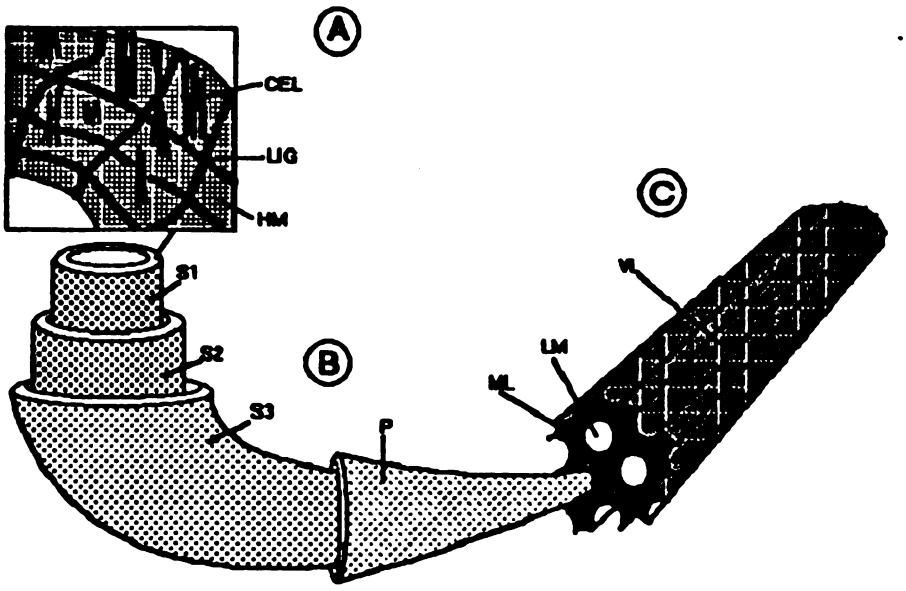

Figure 1 Expanded section of the secondary cell wall. A. The cellulose fibers (CEL), hemicellulose (HM), and lignin (LIG) within the secondary cell wall. B. Organization of plant cell walls. Vessel elements (VL) with their secondary cell walls (S1, S2, and S3) and primary cell walls (P) are surrounded by the middle lamellar region (ML). C. Tracheids (from 22).

phenoxyradicals. Because the unpaired electron is stabilized by π shell electrons in the phenylpropanoid precursor, multiple resonance forms are produced (10). Polymerization of the precursor resonance forms results in an extended, high molecular weight polymer which has a variety of intermonomer linkages (10). Since the C-α and C-ß carbons in the phenylpropanoid sidechains can be either in the R or S form, the lignin polymer is also non-stereospecific (76). The actual lignin molecule is large, 1 x 10^5 daltons or greater, and contains a great variety of the linkage types seen in Figure 2.

Angiosperms (hardwoods) and Gymnosperms (softwoods) share the same basic ultrastructural characteristics described above for the distribution of their lignin within the cell wall; however, they differ in the types of plant tissues they have and in their lignin content and type. Softwood tissues have a simple organization, as pictured in Figure 1. The wood is basically composed of tracheid cells within the xylem which form the bulk of the conductive tissue within the wood. Hardwoods are composed of three basic tissue types within the xylem; rays, vessels, and fibers. These tissue types are organized within a complex network which differs depending upon the tree genus. In both hardwoods and softwoods these xylem elements are not only the conductive tissues, but provide physical rigidity to the tree as well. Gymnosperms generally have a higher lignin content than angiosperms. For example aspen wood (*Populos tremuloides*), an angiosperm, has a lignin content of 22% while Pine wood (*Pinus banksiana*), a gymnosperm, has a lignin content of 29.9% (22). Also the phenylpropanoid subunits are different between softwoods and hardwoods. Gymnosperms contain mostly guaiacyl lignin, while angiosperms contain widely varying ratios of syringyl and guaiacyl lignin. Guaiacyl lignin is composed mostly of coniferyl alcohol subunits with smaller amounts of coumaryl and sinapyl alcohol (ratio for spruce lignin is 80:14:6, respectively) (76). Syringyl lignin contains relatively high levels of sinapyl and coniferyl alcohol, with smaller amounts of coumaryl alcohol. The various tissue types of the hardwoods also contain various concentrations and types of lignin. Fiber cells are the predominant tissue

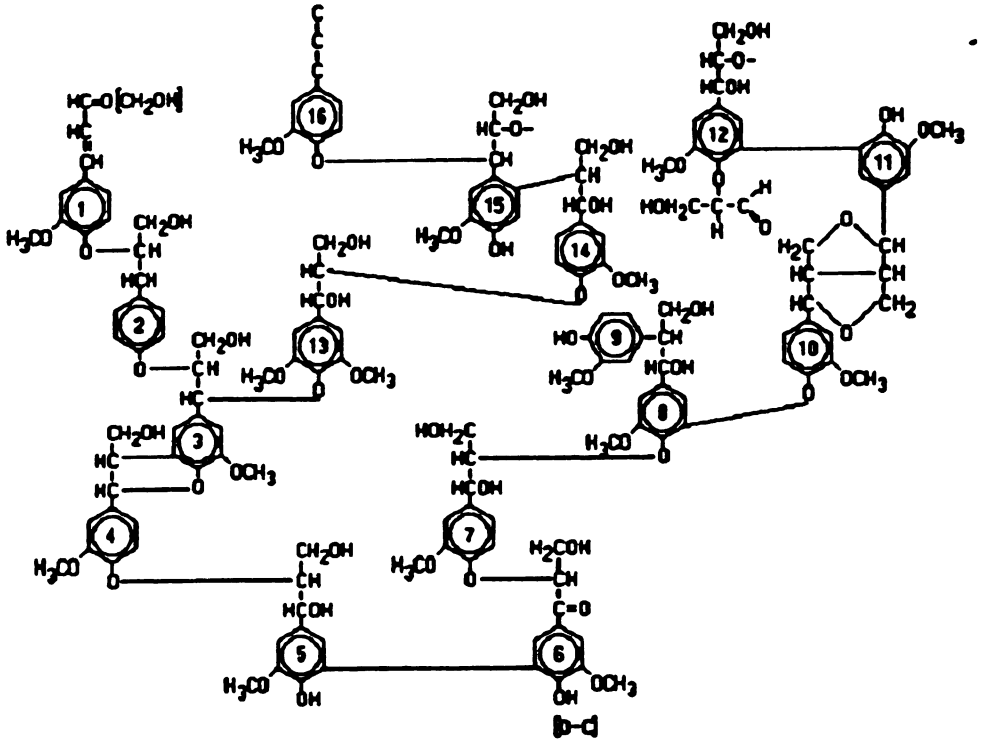

Figure 2 Chemical linkages for the lignin polymer of spruce wood (from 1).

type in birch xylem. They contain 77% of the xylem's lignin and are 19-22% lignin. They are also almost 90% syringyl lignin and only 10% guaiacyl lignin. The vessel cells are more like softwoods in having a higher lignin content, 24-28% lignin, and contain predominantly guaiacyl lignin. Because of the different lignin types, softwood is harder to delignify and takes longer to pulp than hardwoods (28).

Possibly related to the relative difficulty of chemically degrading guaiacyl lignin versus syringyl/guaiacyl lignin, white-rot fungi also generally degrade gymnosperm softwoods less effectively than angiosperm hardwoods (40). *Trametes versicolor* degrades angiosperm wood (from *Eryrithrina cristagalli*), which contains guaiacyl lignin levels similar to gymnosperms, at the same rate as it would a true gymnosperm wood (40). The faster rate of degradation of syringyl/guaiacyl lignin is due to the faster depolymerization of syringyl/guaiacyl lignin than guaiacyl lignin by white-rot fungi. *Phanerochaete chrysosporium* was able to depolymerize both synthetic and natural syringyl/guaiacyl lignin into smaller dioxane:water-DMF soluble material faster than it was able to fragment guaiacyl lignin. However once fragmented, the conversion rate of the guaiacyl and syringyl/guaiacyl lignin monomer and dimer molecules to CO_2 occurred at the same rate. The difference in the ability of white-rot fungi to depolymerize guaiacyl versus syringyl/guaiacyl lignin is most likely due to the differences between the linkages between the monomers, with guaiacyl lignin having a more condensed structure with more C-C bonds than C-O-C bonds at C5 (26).

II Microbial Wood Decomposition

Bacteria

Bacteria within the wood decomposition community can be found within several ecological niches. Bacteria have been categorized into four basic areas. 1) Bacteria that utilize the cell contents of ray vessel and parenchyma cells without affecting the structural integrity of the wood fibers and cell walls. 2) Bacteria that degrade the cell

walls of wood directly. 3) Bacteria that are associated with other wood degrading organisms and have a mutualistic relationship with them. 4) Bacteria that are antagonistic to resident wood degrading organisms and inhibit their growth (36).

The identification and isolation of bacteria which can attack the wood cell wall has not been as successful as the identification and isolation of fungal wood degraders. At this time no single bacterial isolate has been found which when grown in pure culture can completely mineralize polymeric lignin. Yet bacterial consortia have been described from wood and soil samples which can partially degrade lignin.

Forney and Reddy (29) identified mixed bacterial consortium, isolated from tropical soil samples, which could degrade kraft lignin. This bacterial consortium appeared to mineralize 2-^{14}C-synthetic lignin albeit to a limited extent. Schmidt et. al. (80) in a study of 57 bacteria isolated from wood, soil, and lake samples found that only a few strains could colonize the non-lignified wood cell membranes of bordered pits, parenchyma cells, and epithelial cells. Under laboratory conditions these *Actinomycetes, Bacilli,* and *Cellulomonas* produced only a minimal loss of weight from the lignified woody substrates.

Tunneling bacteria are perhaps the best examples of bacteria which may be able to degrade lignin completely. Tunneling bacteria are most likely gliding bacteria of the *Myxobacteriales* or *Cytophagales* groups (17). The bacteria gain access to the cell wall by localized dissolution of the substrate, and then form tunnels throughout the S3 and S2 layers of the tracheids and vessels. It has not been possible yet to purify these interesting tunneling bacteria, but in mixed cultures they have been shown to mineralize lignin, labelled with ^{14}C at either the ring or side chains, to ^{14}CO2. A total maximal reduction of 11% was found for ring labelled lignin and 10.4% for side chain labelled lignin after 16 days cultivation of the bacterium. While it has not been possible to conclusively prove that bacteria in pure culture mineralize ^{14}C-lignin, bacteria undoubtedly play a large, if as yet undefined, role in the wood degradation community.

Even if bacteria do not directly mineralize lignin, mutualistic associations between bacteria and fungi maybe important for lignin degradation. For example pretreatment of wood with cellulase-negative mutants of the white-rot fungus *Phlebia gigantea* was effective in allowing the cellulolytic bacteria *Cellvibrio vulgaris*, *Sporocytophaga myxococcoides*, and *Streptomyces olivaceus* access to the carbohydrates within the cell walls. Without pretreatment bacterial degradation was much reduced (41).

In natural wood consortia, bacteria, yeasts, and basidiomycetes can be found together in mutualistic associations. The role which each of these organisms plays in the decay community has not yet been completely elucidated. The bacteria may supply vitamins or other nutrients, and some of the bacterial consortia appear to fix atmospheric nitrogen (6). This is especially interesting considering the fact that wood contains very low levels of available nitrogen in comparison to the carbon sources available in the form of cellulose and hemicellulose. In experiments combining two species of nitrogen-fixing *Enterobacteria*, two species of yeasts (*Pichia pinus*, and *Saccharomyces bailii*), and the white-rot fungus *Trametes versicolor* on mixed western white pine, fir, spruce, and western red cedar wood chips, the fungal growth rate was increased over 200% as compared to the growth of *T. versicolor* alone on the wood chips. Furthermore the extent of weight loss for the wood inocula increased 1-10%, indicating greater efficiency of wood degradation by the consortia than by any one species alone. The bacteria and yeasts were strongly dependent on fungal enzymatic activity since in scanning electron micrographs the bacteria and yeasts were only found in wood tracheids where fungi were actively degrading the cell wall. The bacteria and fungi therefore appeared to be utilizing the carbohydrates released from the wood by the action of the fungal extracellular enzymes (6).

Soft-Rot and Brown-Rot Fungi

The important fungi which cause soft-rot of water-logged wood belong predominantly to the class *Deuteromycotina* (fungi imperfecti) or *Ascomycotina*. Soft-rot fungi colonize the cell wall and depolymerize the cellulose and hemicellulose with concurrent lignin depolymerization (22). Brown-rot fungi belong predominantly to the class Basidiomycotina and are closely related to the white-rot fungi. Brown-rot wood degradation causes a substantial loss in the cellulose and hemicellulose content of the wood with little loss in the lignin content. They appear to have little ability to mineralize the lignin polymer itself. The main modifications to the lignin polymer are demethoxylation and hydroxylation of aromatic rings. The brown crumbly residue left after their colonization is mostly lignin. However, the enzymology of brown-rot or soft-rot lignin degradation has not been studied extensively and very little is known about the mechanisms or enzymes they employ for lignin depolymerization (55).

White-Rot Fungi

White-rot fungi, like brown-rot fungi, are predominantly basidiomycetes. They begin colonizing wood by invading the wood cell lumen, utilizing the cell contents for growth. The cell wall components are degraded sequentially from the cell lumen starting with the secondary cell wall. The middle lamellar region is generally the last to be attacked. A common feature of fungal wood degradation is the presence of an exo-polysaccharide sheath surrounding the hyphal cell and contacting the plant cell wall. The sheath has several possible functions. It is a contact point between the fungal cell membranes and the plant cell wall and therefore provides a microenvironment where fungal enzymes and plant degradation products can traverse the space between the fungus and plant cell wall. The sheath may contain the depolymerization enzymes for cellulose, hemicellulose, and lignin degradation within a microenvironment optimal for their activity (70). *Phanerochaete chrysosporium* and *Trametes versicolor* were the only fungi out of five white-rot fungi, (the others being *Phellinus pini*, *Xylobolus frustulatus*, and

Pycnoporus sanguineus), for which the hyphal polysaccharide sheaths contained cellular products from autolysis of fungal cells (71). Therefore, at least for these two fungi, lysis of cells may be an important mechanism of releasing lignin, cellulose, and hemicellulose degrading enzymes to the plant cell wall.

There are two different strategies which white-rot fungi employ for lignin removal: 1) simultaneous degradation of all three wood components; 2) preferential attack on the lignin and hemicellulose either before, or to the exclusion of cellulose degradation. White-rot fungi which simultaneously decay all three major polymeric components of wood exhibit several distinctive characteristics. Holes and erosion troughs in the cell walls of the wood are evident near the fungal hyphae indicating degradation of all wood components at once in one localized area. Degradation of the wood proceeds progressively from the cell lumen outward to the secondary cell wall, and the middle lamellar region is attacked last. The tracheids in gymnosperms, and parenchyma, vessel, and ray cells in angiosperms stay intact until the final stages of decay. In general, as shown in Table 1, the percentage of loss for lignin, cellulose (as represented by glucose), and hemicellulose (as represented by losses in both xylose and mannose) is approximately equal. Both *T. versicolor* and *P. chrysosporium* are simultaneous lignin degraders (15, 22), although *P. chrysosporium* utilizes xylose residues preferentially over glucose and mannose residues.

For fungi which exhibit preferential decay of lignin, no holes or erosion troughs can be found within cells which have hyphae present in the lumen. Lignin is still removed progressively from the lumen outward towards the secondary cell wall, and finally towards the middle lamellar region; but the cell wall stays intact. Scanning electron micrographs of wood degraded by preferential lignin degraders shows the lignin has been removed from the wood, while the cell structure is unaffected. The progressive delignification of the middle lamellae causes softwood tracheids, and hardwood vessels and rays to separate from each other (4). A distinctive feature of this type of decay is

Table 1 Percentage of Lignin, Cellulose, and Hemicellulose lost from paper birch (*Betula papyrifera*) and Eastern white pine (*Pinus strobus*) after 12 weeks of degradation by white-rot basidiomycete fungi.

Fungus	Wood of	Percentage Loss[a]			
A. Simultaneous Degraders		Lignin	Glucose	Xylose	Mannose
Trametes versicolor	Birch	64.6	65.4	68.8	71.7
	Pine	35.4	22.1	46.7	11.6
P. chrysosporium	Birch	72.9	15.1	55.1	0.0
(BKM-F-1767)	Pine	30.5	3.9	44.1	0.0
B. Preferential Degraders					
Dichomitus squalens	Birch	71.2	43.8	43.5	40.4
	Pine	53.0	48.3	56.9	64.9
Heterobasidion	Birch	54.6	4.1	5.6	2.2
annosum	Pine	24.0	12.6	14.7	19.6
Phellinus pini	Birch	53.9	5.2	12.9	11.4
	Pine	43.5	10.7	34.5	42.5
Phlebia tremellosa	Birch	75.2	4.1	39.4	28.5
	Pine	38.6	20.2	61.2	41.2
Poria medulla-panis	Birch	73.1	0.1	31.9	11.5
	Pine	38.5	28.6	62.9	35.3

[a] Glucose and Xylose/Mannose loss, respectively, represents degradation of the wood polysaccharides cellulose, and hemicellulose (from 22).

that the resulting delignified wood has been depleted of almost all the lignin and hemicellulose but has not lost a corresponding level of cellulose (5). This is represented by the high percentage of glucose left in the wood decayed (degraded) by *Heterobasidion annosum*, *Phlebia tremullosa*, and *Phellinus pini* (Table 1). The concomitant loss of lignin and hemicellulose during preferential lignin degradation is not too surprising considering the close physical and chemical association of the two polymers within the cell wall, and the fact that lignin degradation has been shown to require a readily hydrolyzable carbon source.

III Basidiomycete Life Cycle

The basidiomycete life cycle is an important factor affecting the interactions among fungi within the decay community. Basidiomycetes have an unusual haplodikaryotic vegetative state where the fungal cell contains two (or more) independent haploid nuclei. These haploid nuclei are derived from two monokaryotic sexually compatible parental fungi. After plasmogamy (when the fungal hyphae fuse), the parental nuclei remain separate. Basidiomycetes, unlike Ascomycetes, can and do exist in this heterokaryotic vegetative state for extended periods of time. Only when conditions for sporulation are right do the nuclei fuse (karyogamy) within the sexual cells of the basidiocarp. These diploid basidial cells go through meiosis and daughter haploid nuclei are deposited within basidiospores which are released to form new monokaryotic individuals (75).

Two parental hyphae can only complete plasmogamy if they are of the correct mating type. For heterothallic fungi the parental hyphae must be of different mating types. Homothallic fungi can self-mate and produce genetically identical basidiospores. Mating type in the heterothallic fungi is determined by either one (bipolar) or two (tetrapolar) genes. Thus for a heterothallic, tetrapolar fungus like *Trametes versicolor*, the monokaryotic parental hyphae must have different alleles for the two mating type genes (referred to as AB x ab). The dikaryotic hyphae resulting will be genetically and

physiologically identical to a diploid cell with the mating type alleles AaBb. For *T. versicolor* the number of alleles for each of the two mating type genes is quite large so that the chance encounter of two identical mating types is rare (91). Dikaryotic heterokaryons are antagonistic to every other genetically non-identical mono or dikaryon of the same species. If two such non-identical hyphae should meet, plasmogamy will not result, and a characteristic incompatibility reaction occurs.

IV Lignin Peroxidases

Discovery

The search for peroxidases involved in lignin degradation began with the finding that hydrogen peroxide was required in the extracellular environment for lignin degradation to occur (31). The production of H_2O_2 by the white-rot fungus *P. chrysosporium* was shown to coincide with the cessation of primary growth and the concomitant degradation of synthetic ^{14}C labelled coniferyl alcohol lignin. It was further shown that the hydroxyl radical scavenging agents mannitol, benzoate, and BHT markedly inhibited lignin degradation. This report was substantiated by Kutsuki and Gold (59) who showed that catalase inhibited lignin degradation as well as the hydroxyl radical scavengers, thereby strengthening the relationship between hydrogen peroxide production and lignin degradation.

These results led to the discovery of an enzyme, ligninase, later called lignin peroxidase (abbreviated as LIP), that could degrade synthetic and natural lignins and required for activity (34, 85). Isolation of this lignin peroxidase showed it to be a 42,000 dalton heme protein with one protoheme IX per molecule and an isoelectric point of 3.5. The enzyme was found to catalyze the oxidation of veratryl alcohol (3,4 dimethoxybenzyl alcohol) to veratraldehyde at a Vmax of 84 µmol veratryl alcohol oxidized per min per mg of protein at a pH of 3.5. Maximal enzyme activity was found with 0.15 mM H_2O_2 and the K_m for H_2O_2 was 30 µM. The LIP enzyme was also found to catalyze the cleavage of ß-1 and ß-O-4 lignin model compounds (see Figure 3)

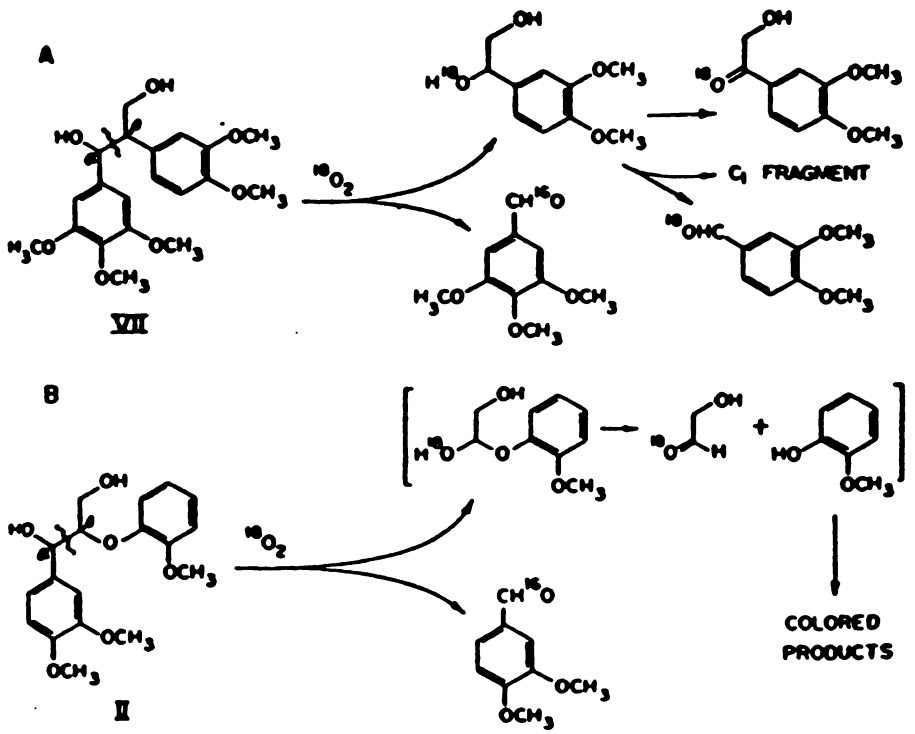

Figure 3 Scheme showing lignin peroxidase action on A. β-1 and B. β-O-4 lignin models (from 86).

with the oxygen molecule coming from molecular oxygen and not hydrogen peroxide (60, 86). Most of the spectral and kinetic studies have been performed on this particular isozyme (designated H8) which was the first lignin peroxidase to be isolated, and the catalytic mechanism for this enzyme has been elucidated. These extensive studies are summarized below from several excellent reviews which have already been published (10, 55, 84).

Activity

Assays for LIP activity have utilized the oxidation of veratryl alcohol to veratraldehyde since the lignin peroxidase enzyme cannot oxidize veratraldehyde further. The stoichiometry is one mole of H_2O_2 consumed per mole of veratraldehyde produced. Saturation kinetic studies using both veratryl alcohol and H_2O_2 indicate that the reaction mechanism is ping-pong. Spectral changes of lignin peroxidase observed upon reaction with H_2O_2, but not with veratryl alcohol, suggest that lignin peroxidase first reacts with H_2O_2 to produce an oxidized enzyme intermediate compound I (Figure 4). Compound I with H_2O_2, but not with veratryl alcohol, suggest that lignin peroxidase first reacts with then performs a single electron oxidation of aromatic structures such as those found in lignin, producing a cation radical in the carbon center of the lignin polymer and the oxidized compound II.

LIP has been found to catalyze the depolymerization of dimeric lignin model compounds by one electron oxidations of the aromatic carbon centers. In studies on lignin model compounds, single electron oxidations of the aromatic carbon centers produces unstable cation radicals which depolymerize into various benzylic compounds. (39, 52, 81). The cation radicals formed in the methoxylated benzene rings lead to cleavage of the propyl carbon bridge linking the two aromatic rings at the Cα-Cß position (Figure 5). The presence of alkoxylated groups on the aromatic ring is necessary for the oxidation to occur since benzene rings with fewer than two methoxyl groups are not oxidized by LIP.

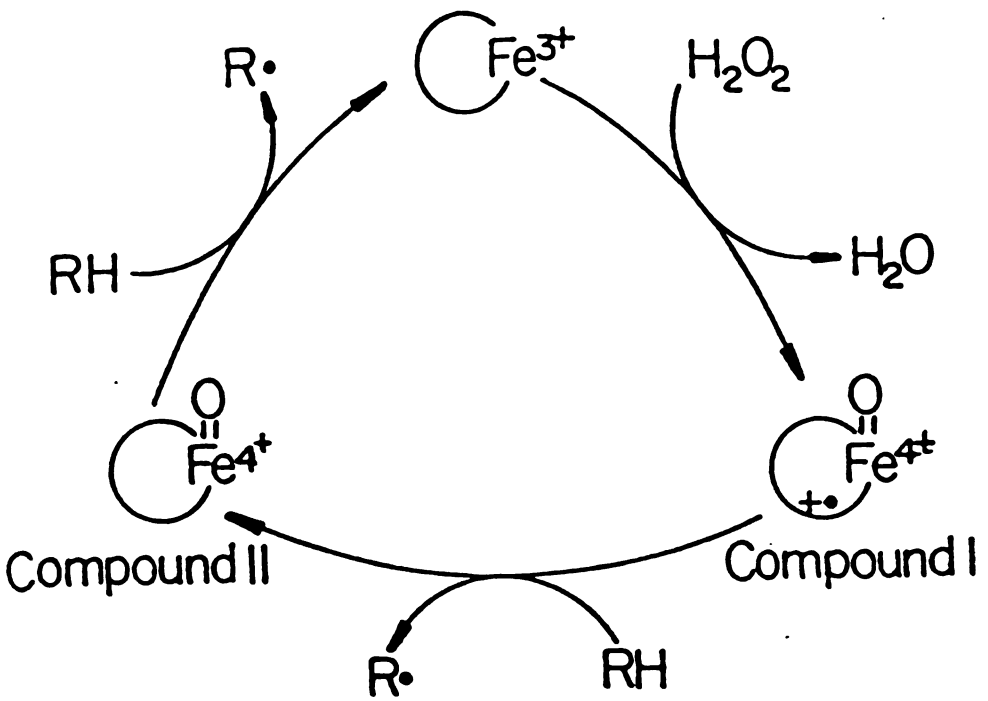

Figure 4 Catalytic cycle of lignin peroxidase showing varied reduction states of the heme (from 84).

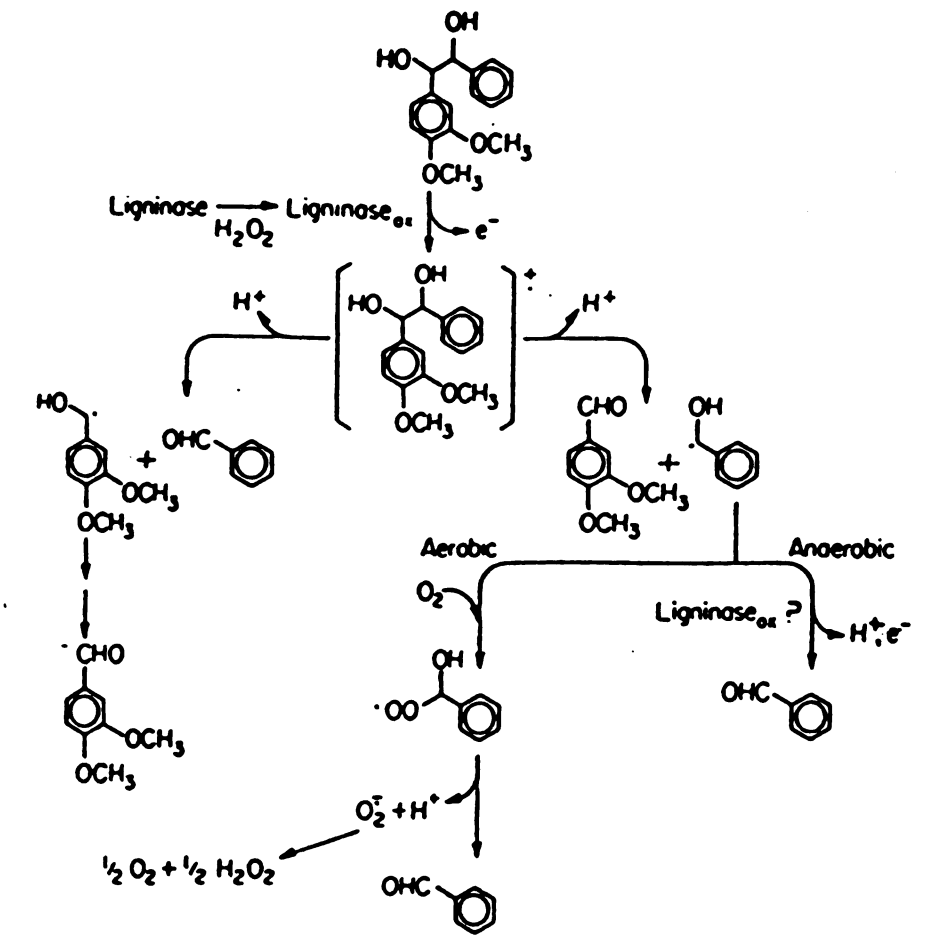

Figure 5 Proposed scheme for aerobic and anaerobic cleavage of DMHB by *P. chrysosporium* lignin peroxidase (from 39).

The alkoxyl groups (or hydroxyl groups) stabilize the positive charge on the aromatic ring and therefore promote formation of aromatic cation radicals (39). The aryl-aldehydes formed result from whichever carbon (either Cα or Cß) accepts the free electron. Since the aromatic cation radicals can also act as electron transfer agents, the action of LIP on one end of the molecule may spread to aromatic centers at distal ends of the lignin polymer. The result would be depolymerization of lignin in regions of the molecule which are inaccessible to the 42 kd LIP. Ultimately the hydroxylated radicals must be reduced further by one electron to prevent their repolymerization.

Although a mechanism for lignin depolymerization by LIPs has been elucidated it has also become clear that for lignin depolymerization to occur other enzymes must be active. Haemmerli et al., (37) showed that alkali straw lignin, HCl-dioxane extracted straw lignin, and milled wood lignin were oxidized by LIPs of *P. chrysosporium* and added H2O2. The result was not depolymerization, but further polymerization. Sarkanen et al., (78) extended this result by showing LIP, in the presence of H_2O_2, was able to polymerize the monomeric lignin precursor coniferyl alcohol into an insoluble high molecular weight synthetic lignin. LIP produced aromatic cation radicals from the coniferyl alcohol, and these were able to form a dehydropolymerizate. These observations are not surprising given that there was a high concentration of substrate (coniferyl alcohol) and the product (dehydropolymerizate) was insoluble and therefore removed from solution by precipitation. LIPs are able to cause net depolymerization of synthetic lignin if three conditions are met: (1) The synthetic lignin is in a dilute suspension in H_2O/dimethylformamide in order to increase its solubility, (2) the H_2O_2 concentration is maintained at a low level to assure that aromatic free radical concentrations are kept low, and (3) veratryl alcohol is added to assist in stabilizing the LIP and extending its active life (38).

The importance of LIP to lignin degradation has been further substantiated by studies on a LIP negative mutant (8, 9). This mutant (*lip5*) has no detectable LIP

activity, yet retains the important ligninolytic enzymes glucose oxidase and manganese peroxidase (see section VI and VII below). The loss of LIP activity was correlated with a substantial decrease in mineralization of ^{14}C-labeled synthetic lignin (6%), as compared to 31% degradation observed with the wild type.

The equilibrium between the forward and reverse reactions catalyzed by LIP may be made to favor the forward direction by the removal of aromatic lignin degradation intermediates. The rapid removal of substituted quinones by quinone reductases, could possibly fulfill this role. Both extracellular and intracellular quinone reductases have been identified, and an intracellular NADH:quinone oxidoreductase has now been purified from *P. chrysosporium* (14). Further studies on these enzymes and the role they play in lignin depolymerization are being investigated.

V Manganese Peroxidases

A second major class of peroxidases involved in lignin degradation were discovered soon after the initial isolation of LIP. They contain one molecule of heme as iron protoporphyrin IX per enzyme molecule. The activity of the enzyme on lignin model compounds was found to be dependent on both H_2O_2 and Mn(II), and its principal enzyme activity appeared to be the oxidation of Mn(II) to Mn(III) (41). At a pH optimum of 5.0 the enzyme oxidized Mn(II) to Mn(III) at a higher rate than any other available substrate, and the Mn(III) complexed with lactate, or other α-hydroxy acids, was an obligatory intermediate in the oxidation of polymeric dyes or lignin model compounds by the enzyme. The enzyme has been called manganese peroxidase (MNP) or manganese-dependent LIP (32) and have a slightly higher M_r of 46,000 daltons than LIP. The catalytic cycle of MNP is similar to LIP in that the intermediate compound MNP I is formed by a two electron oxidation of the resting state enzyme by H_2O_2. The subsequent individual one electron oxidations of two Mn(II) molecules produces two Mn(III) molecules and the native state MNP enzyme (88, 90).

Considering that both LIP and MNP utilize H_2O_2 to bring about one electron oxidations and depolymerization of the lignin polymer, the discovery that Mn(II) levels regulate both LIP and MNP enzyme levels in *P. chrysosporium* is highly significant (127). In Mn(II) free cultures LIP activity appeared earlier, and was 2.5 times higher than that in basal media containing 11.15 ppm Mn(II), while MNP activity was very low or undetectable in media containing no Mn(II) and was 24 times higher in media with 11.15 ppm Mn(II). While concentrations below 1.6 ppm stimulated LIP activity, concentrations above 8.0 ppm suppressed LIP activity. At 40 ppm Mn(II), LIP activity was completely suppressed. MNP activity was completely suppressed in the absence of Mn(II) and increased steadily to a peak level at 40 ppm Mn(II). Similar results were obtained with the white-rot fungi *Phanerochaete flavido alba*, *Phanerochaete magnolia*, *Phlebia radiata*, *Phlebia tremellosa*, *Phlebia subserialis*, *Lentinus edodes*, and *Phellinus pini*. Yet, even though MNP's and LIP's are theoretically able to depolymerize lignin to the same extent, ligninolytic activity (as measured by mineralization of synthetic lignin) was more than seven fold higher at low Mn(II) levels (0.32 ppm) than at high Mn(II) levels (39.88 ppm). Therefore, low Mn(II) levels correlated with low MNP activity, high LIP activity, and high levels of lignin degradation. High Mn(II) levels correlated with high MNP activity, low LIP activity, and low levels of lignin degradation. Natural concentrations of manganese in wood are reported to range from 5 to 200 ppm, (4, 7, Blanchette; personal communication), however the concentrations of Mn(II) available to the fungus are not known since Mn(II) can be bound by the wood in an insoluble form. Still, Mn(II) may play a role in regulating LIP activity in wood. MNP's would act by oxidizing the available Mn(II) to Mn(III). Mn(III) could then diffuse into the wood and cause changes in the lignin polymer which would make the lignin more susceptible to LIP activity. After some time Mn(II) may become depleted around the fungal hyphae, and the LIP's would be derepressed (7). It is known that white-rot fungi can mobilize the

manganese in wood and concentrate manganese in white-rot degraded wood into deposits with 100 fold greater manganese concentrations than in sound wood (4).

Two different *MNP* cDNA clones have been isolated from *P. chrysosporium*; *MP-1*, coding for MNP2 (72), and *MNP-1* coding for a different as yet unidentified MNP peak (73). The homology of *MP-1* to the first LIP enzyme isolated (H8) is 58% at the nucleotide level, and 65% at the protein level. Sequencing of the genomic clone for *MNP-1* indicates that while splice junction sequences are the same, the position of the introns between *LIP*'s and *MNP*'s have not been conserved (35). It has been found that Mn(II) levels regulate the transcription of the *MNP-1* gene (12). Very low levels of *MNP-1* mRNA were present at 0 ppm Mn levels, but as Mn(II) concentrations were increased, the level of *MNP-1* transcript also increased substantially to a peak at 180 µM MnSO4, (10 ppm Mn(II)), with transcripts appearing within 40 mins of the Mn(II) addition (12).

VI Localization of Lignin Degrading Enzymes.

Decomposition of ^{14}C-lignin to $^{14}CO_2$ by *P. chrysosporium* occurs simultaneously with the depolymerization of lignin to smaller molecular weight substances (13). The progressive changes in the molecular size of lignin indicated that the depolymerization was not a separate temporal event during lignin degradation, and that smaller molecular weight lignin degradation products are catabolized concomitantly with lignin depolymerization.

Part of this system requires the production of hydrogen peroxide for its activity. The search for the enzymatic source of the H_2O_2 production has been an important goal in elucidating the complete lignin degradation system. Production of H_2O_2 by *P. chrysosporium* has been shown to occur within the periplasmic space of the ligninolytic cells (30). The H_2O_2 produced there is thought to diffuse out to the extracellular environment for lignin degradation, thus protecting the intracellular cytoplasm from harmful oxidations by the reactive H_2O_2. Glucose oxidase (GOX), an intracellular

H_2O_2 producing enzyme was discovered by Kelley and Reddy (50). GOX produces H_2O_2 from D-glucose, or less efficiently, from L-sorbose, D-cellobiose, D-maltose, D-mannose, and D-xylose. All of the GOX activity was associated with the cellular fractions from ligninolytic cultures of *P. chrysosporium* and, therefore, no glucose-1-oxidase activity could be detected in the extracellular culture fluid. Since H_2O_2 production does occur in the periplasmic space of cells, the cellular location of GOX activity does not preclude its activity in lignin degradation. And its use of glucose, the main metabolite from cellulose degradation, further supports its role in lignin degradation.

Still, the search for an extracellular H_2O_2 producing enzyme continued. One was found by Kersten and Kirk (51) which utilized glyoxal or methylglyoxal to produce H_2O_2 in ligninolytic cultures of *P chrysosporium.* Significantly the substrates glyoxal and methylglyoxal are also present in the extracellular environment of ligninolytic cultures. However, the enzyme glyoxal oxidase (GLOX) is a minor protein component of the extracellular fluid of ligninolytic cultures, and, therefore, whether it can supply all of the required H_2O_2 for ligninolytic activity is debatable.

Many studies have attempted to determine the location of LIP activity during lignin degradation. Various groups have identified LIP as being located predominantly within the cell, in the periplasmic space, or within the wood. The first studies using immunogold-labelled antibodies to LIP found it located predominantly in either the intracellular vesicles, or bound to the plasma membrane in the periplasmic space (83). These results corroborated earlier studies which reported a relationship between the strong binding of lignin to the fungal mycelium and lignin degradation (13). Improvements in staining and fixing techniques, however, showed that while depolymerized lignin may attach to the mycelial membrane, contact between fungal hyphae and degradation of the plant cell wall are not necessary (15). No differences are noted between degradation patterns of hyphae adpressed to the cell wall, and those

located in the center of the lumen. Degradation produces characteristic erosion troughs and decay zones where the attack on wood polymers is localized within a small region and progresses outward. Subsequent studies have found that LIP is located not only within the fungal periplasmic space, fungal slime layer, and membranous materials bound to wood cell walls, but also is found to have penetrated into decay zones within the wood.

The decay zones are thought to be caused by fungal depolymerization of the S2 layer. LIP enzyme was found to consistently penetrate the S2 layer within the decay zone and concentrate at the interface between degraded and undegraded wood. Also LIP was found to accumulate within erosion troughs. The enzyme was never found to have penetrated undecayed wood. Therefore the partial depolymerization of wood in the S3, S2, or S1 cell wall layers allows the LIP (Mr 42,000) to penetrate the wood and concentrate in regions where active lignin depolymerization is taking place (15). Parallel studies using immunogold labelled antibodies to MNP showed a similar distribution to MNP localization. The polyclonal antibodies made against MNPs did not cross-react with LIP. Double labelling experiments using 5 nm gold probes for MNP and 15 nm gold probes for LIP showed that both peroxidases were localized within decay zones and concentrated at interfaces between degraded and undegraded wood (16). These results support a role for MNP in lignin degradation as has been claimed by other investigators (32).

VII Multiple LIP Genes

Within three years of the initial discovery of LIP, it was found that *P. chrysosporium* produces multiple LIPs. Under carbon sufficiency, nitrogen limitation conditions (56 mM glucose, 2.2 mM NH4), and dimethyl succinate buffer *P. chrysosporium* strain BKM-F 1767 produces ten extracellular hemeproteins, six of which show LIP activity (54). The hemeproteins were separated by high pressure liquid chromatography (HPLC) and were arbitrarily named H1 to H10 according to their

elution from the column. LIP isozymes H2, H8, and H10 were predominant under these conditions. LIP H8, induced to the highest level under these conditions, corresponds to the LIP isozyme first isolated and was the enzyme used for all the preceding kinetic and mechanistic studies. The other two hemeproteins, H2 and H10, were shown to be homologous to H8 by their cross reactivity on western blots to polyclonal antibody prepared against H8. Under similar nitrogen limitation conditions utilizing acetate buffer instead of dimethyl succinate buffer LIP isozyme H6 predominates. Other workers have reported up to 15 separate LIPs and six MNPs isolated from nitrogen and carbon starved cultures of *P. chrysosporium* BKM-F 1767 (62).

Rapidly following the discovery of the first LIP enzyme, H8 (85), the first LIP cDNA clones were isolated (93). It was originally assumed that the multiple LIP isozymes arose by glycosylation and other post-translational modifications of a single LIP gene product. But sequencing of the LIP clones (*LIP*'s) showed that each of the hemeprotein LIPs may have a distinct gene coding for it. In order to clarify the relationships between the various LIP enzymes, and their corresponding genes, Boominathan and Reddy (10) have proposed the following nomenclature. LIP enzymes should be designated LIP1-LIP6 instead of the arbitrary heme peak assignments H1-H10. The corresponding enzymes would then be LIP1 (H1), LIP2 (H2), LIP3 (H6), LIP4 (H7), LIP5 (H8), and LIP6 (H10). The genes encoding these enzymes are given the same name but always in italics (*LIP1-LIP6*). Sequence analysis of the cDNA clones of *LIP2* and *LIP6*, previously designated, respectively, *CLG4* and *CLG5* (19, 20), and *LIP5* (87), showed the genes encoded homologous proteins of mature M_r 37,000 which upon glycosylation increased to 38,000-42,000. The mature proteins are preceded by signal sequences of 27 (for *LIP2*) to 28 (for *LIP5* and *LIP6*) amino acids. All the *LIP* genes sequenced so far have two conserved regions which correspond to putative proximal and distal histidine peroxidase active sites.

The isolation of genomic *LIP* clones has defined the relationships between the *LIP* genes of *P. chrysosporium*. Most of the genomic clones isolated are identical to, or differ only by a few nucleotides from, the *LIP5* cDNA clone (3, 11, 43, 79, 82, 89). Studies of the gene structure for the other two major *LIP* genes, *LIP2* and *LIP6*, have revealed a subfamily organization between the genes. *LIP2* is less closely related to all the other *LIP* genes studied so far, with 73% and 72% amino acid homology to *LIP5* and *LIP6*, respectively, as compared to 80% amino acid homology between *LIP5* and *LIP6* (68, 92). Furthermore the position of the introns is more similar between *LIP5* and *LIP6* than either is to *LIP2*. The close relationship between *LIP5* and *LIP6* is further substantiated by similar peptidase digestion patterns and close immunological cross reactivity (27, 62).

The discovery of structurally related *LIP* genes within a multigene family spurred the search for *LIP* gene linkage patterns within the chromosome of *P. chrysosporium*. Genetic studies on *P. chrysosporium* strain ME446 found that this strain contains two sets of *LIP* genes clustered within its genome (74). The clusters were defined by an RFLP-based genetic map which showed that at least some of the *LIP* genes occur on the same chromosome. To date, only two *LIP* genes of *P. chrysosporium* strain ME446 have been shown to be closely linked (separated by 1.3 kb of intergenic DNA) (43). The genes are orientated for convergent transcription and are highly homologous with amino acid similarities of 91.7%.

VIII Regulation of the LIP Enzymes and Genes

The ligninolytic enzyme system of *P. chrysosporium* has long been recognized to be induced by nitrogen starvation. The cessation of growth by the fungus caused by nitrogen starvation coincides with the appearance of ligninolytic enzymes which can degrade ^{14}C labelled synthetic lignins (see Figure 6) (53). Within 48 hours of growth, *P. chrysosporium* had depleted 90% of the available $NH4^+$ initially present at 2.2 mM, while glucose had decreased only 60% from an initial level of 56 mM. The nitrogen

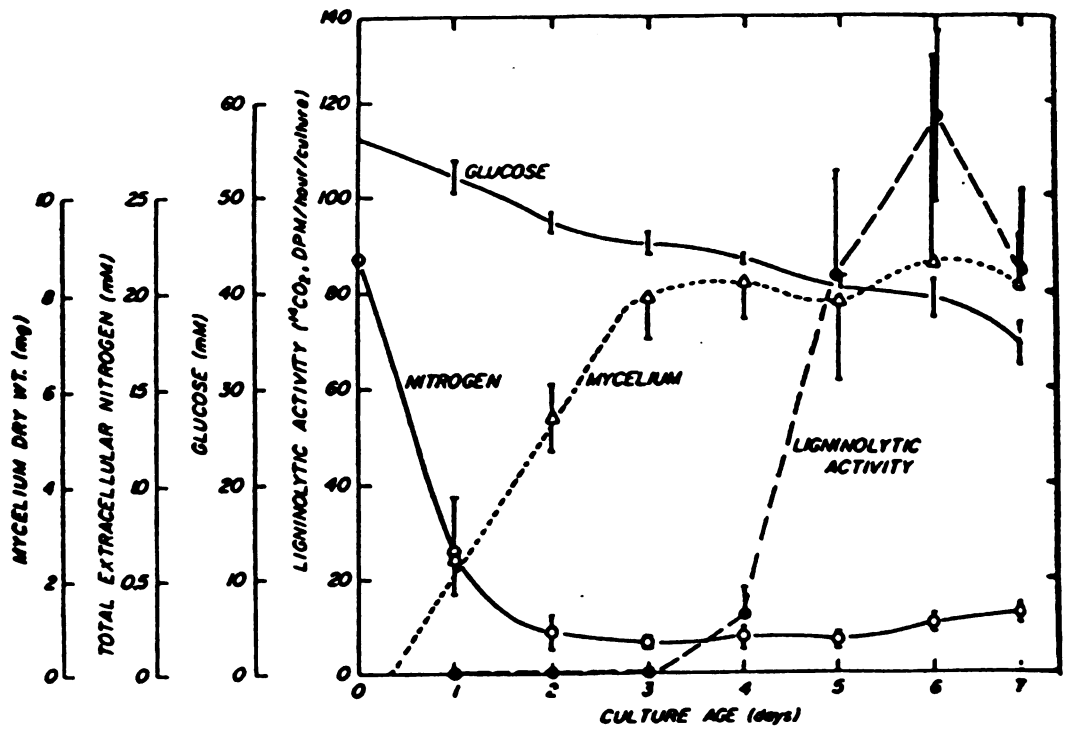

Figure 6 Relationship between culture parameters and ligninolytic activity during 7 days of growth by *P. chrysosporium* (from 53).

limitation signal was sufficient to induce ligninolytic activity, and did not require lignin to be present. Similar results were also obtained with *T. versicolor* indicating the broad relevance of this induction signal to white-rot fungi. Based upon the ubiquitous nature of the induction of ligninolysis by nitrogen starvation, and the fact that wood is a nitrogen-poor growth substrate, it is generally assumed that nitrogen starvation is an environmental signal for the presence of wood as a growth substrate and the need to express the fungal ligninolytic enzymes (53).

It has also been found that high levels of initial $NH4^+$ causes a substantial decrease in overall lignin decomposition. Raising the initial $NH4^+$ concentration from 2.4 mM to 24 mM causes a 65-75% decrease in the ability to mineralize ^{14}C-labelled synthetic lignin. Incubation of the fungus under these high nitrogen conditions causes glucose starvation before the available nitrogen has become depleted (56). However carbon limitation is also a signal for induction of ligninolytic activity. With initial levels of 4.4 mM cellobiose and 7.7 mM nitrogen, carbohydrate becomes limiting first. Under these conditions ligninolytic activity appears even earlier on day 2 than the appearance of ligninolytic activity on day 3 when limiting nitrogen (2.6 mM) is used (44).

While *P. chrysosporium* shows almost complete repression of the lignin degradation system by nitrogen and carbon sufficiency (34 mM glutamate, 139 mM glucose), *T. versicolor* is only moderately repressed in its ligninolytic activity by the same nitrogen and carbon sufficient medium which had synthetic lignin added to it at the time of inoculation. And *Lentinus edodes* and *Pleurotus ostreatus* are actually stimulated in their ligninolytic activity by these conditions (61). Therefore, while nitrogen and/or carbon limitation may be a common induction signal for expression of ligninolytic activity by white-rot fungi, fungi may differ in their sensitivity to high nitrogen or carbon levels.

The induction conditions for the LIPs of *P. chrysosporium* are identical to those for the entire lignin degradation system. LIPs appear in the extracellular culture fluid

coincidentally with the degradation of ^{14}C-labelled lignin; within 2-3 days in nitrogen limited cultures (2.2 mM nitrogen, 1% glucose), and in 3 days for carbon limited cultures (6.6 mM nitrogen, 0.15% glucose). In the nitrogen limited cultures, LIP activity could be further increased two- to five-fold by the addition of lignin or lignin model compounds, such as the dimeric lignin model compound arylglycerol-ß-aryl (ß-O-4). Birch lignin also stimulated the production of H_2O_2 which is required for LIP activity (24). A lignin related compound, veratryl alcohol, was found to be the best inducer of LIP activity in nutrient-limited cultures. Interestingly veratryl alcohol is synthesized by white-rot fungi themselves after they shift from primary to secondary metabolism. Veratryl alcohol is an actual inducer of LIP enzyme expression, and does not simply protect the LIP enzyme already produced from inactivation. Veratryl alcohol moreover induces the protein expression of some of the LIP enzymes better than others, since LIP2 was induced 1.8-fold while LIP5 was only induced 1.1-fold. However, the overall induction of LIP activity was still under the control of nutrient limitation since veratryl alcohol, or lignin, added without nutrient limitation caused no lignin degradation or LIP induction, and LIP induction could be repressed by the addition of macronutrients even in the presence of veratryl alcohol (25).

The genetic regulation of LIP enzyme has begun to be studied with both mutants of *P. chrysosporium* and by using molecular techniques. Nitrogen-deregulated (*der*) mutants of *P. chrysosporium* which produce high levels of LIP enzymes and degrade ^{14}C synthetic lignin to $^{14}CO_2$ under conditions of nitrogen sufficiency (24 mM nitrogen, 1% glucose) have been produced by gamma irradiation (9). Removal of nitrogen regulation not only allows expression of LIP, MNP, and GOX activity in high nitrogen media, but the levels of LIP activity in low nitrogen media, (2.4 mM nitrogen, 1% glucose), are increased 4-fold over levels in the wild type parental strain ME446 under identical limiting nitrogen conditions. Since LIP, MNP, and GOX enzymes were all deregulated in this mutant by gamma irradiation, it suggests a single common

regulatory element which controls nitrogen regulation of ligninolytic activity for these separate families of secondary metabolic enzymes. However the onset of LIP, MNP, and GOX activity was still delayed in the *der* mutants until growth had stopped and secondary metabolism begun (8). Hence, there must be at least two sets of signals for LIP and MNP gene induction, one for nitrogen repression, and another for the shift to secondary metabolism. A mutant of *P. chrysosporium*, PSBL-1, has been described recently (69) which produces 4- to 10 fold higher levels of LIP, MNP, and GLOX enzymes under not only nitrogen sufficiency, but during primary metabolism (while the fungus is still growing). Therefore, the genetic signals controlling the shift to secondary metabolism can be overcome as well.

Differential Gene Expression

Once the existence of multiple LIP genes in *P. chrysosporium* was realized, the question arose as to why there were multiple genes for enzymes which seemed to catalyze the same activity. Studies were begun to determine if there were any regulatory or enzymatic differences between the many genes. The first indication of differential gene regulation came from a report by Holzbaur and Tien (42) that while *LIP2* specific transcripts were present in the poly(A)-RNA isolated from both nitrogen- and carbon-limited cultures, *LIP5* transcripts could only be found in poly(A)-RNA from nitrogen-limited cultures. The enzyme expression pattern corroborated this result in that nitrogen-limited cultures had major LIP peaks for LIP2, LIP5, and LIP6. But in carbon-limited cultures only a single LIP peak, that for isozyme LIP2, could be seen. There are different hypotheses to explain these results. One hypothesis is that the multiple genes exist because each gene has a promoter which is differentially activated by a separate set of environmental signals. This appears to be important because of the diverse environmental signals the fungus must respond to, and because each environmental signal apparently activates a separate regulatory system each of which induces a different subset of *LIP* genes. Under this theory, while different *LIP* genes may be induced

transcriptionally by different environmental signals, the isozymes coded for by these genes would all perform the same basic catalytic function.

The other hypothesis to explain the observation of differential gene regulation is that the different LIP isozymes have some catalytic or substrate binding differences, and they are differentially regulated so that the enzyme is only made when its specific catalytic or substrate binding function is required. Evidence in support of this has come from two studies. The catalytic properties of the LIP enzymes induced under nitrogen limitation were studied by Farrell et al., (27). Their results are summarized in Table 2. Surprisingly some of the isozymes have different ranking orders with respect to K_m when tested against veratryl alcohol and the lignin model dimer. For example LIP4 has a high K_m for veratryl alcohol, but only a moderate K_m for Model 1. However LIP6 has a moderate K_m for veratryl alcohol but a high K_m for Model 1. These differences suggest that different isozymes have different substrate affinities or different substrate accessibilities to the active sites. These results were expanded by Dass and Reddy (18) and correlated with differential expression of LIP enzymes induced when the buffer dimethylsuccinate (DMS) in nitrogen limited cultures is switched to sodium acetate. Switching the buffer from DMS to sodium acetate in shaken cultures causes major changes in the isozyme expression pattern. In shaken DMS cultures, as stated earlier,LIP2, LIP5, and LIP6 are the major LIP isozymes expressed. In shaken sodium acetate cultures LIP2 and LIP3 are the major LIP isozymes expressed, and the MNP isozymes MNP2 and MNP3 become the major hemeproteins expressed in the culture.

Also a new LIP protein, HA, appears as a minor peak when the buffers are changed. What the buffers DMS and sodium acetate may be signaling to *P. chrysosporium* about the environment is still a mystery, yet the large change in isozyme expression indicates that a major change has occurred in gene regulation. The LIP isozymes were also shown to exhibit a great deal of difference in their enzyme activity towards different dyes. These polymeric dyes are considered colorimetric indicators of

Table 2 Catalytic Properties of Different Lignin Peroxidase Isozymes of *Phanerochaete chrysosporium*

Isozyme	H_2O_2	Veratryl alcohol		1,4-dimethoxy Model 1		Benzene[c]	
	K_m[a]	K_m	TN[b]	K_m	TN	K_m	TN
LIP1	13	86	3.20	99	1.43	42	1.50
LIP2	42	250	8.33	78	3.07	132	3.01
LIP3	34	122	6.58	27	1.06	78	4.17
LIP4	77	**483**	5.46	**88**	0.76	20	--
LIP5	17	89	1.32	111	0.50	73	8.55
LIP6	24	**190**	2.78	**444**	0.43	192	0.84

Table from Farrell et al., (27)

a K_m units are micromolar

b TN = Turnover number (per second)

c Model 1 is a diaryl propane lignin model dimer, [1-(3.4-dimethoxyphenyl)-2-(o-methoxyphenoxy)-propane-1 ,3-diol

lignin degradation activity (33) and have been used to isolate *lip* mutants of *P. chrysosporium* (8, 9). Dass and Reddy (18) used these results, along with those of Farrell et al., (27), to postulate that the different LIP isozymes play different roles in lignin degradation.

IX Ligninolytic Enzymes of *Trametes versicolor*

Trametes versicolor has long been studied for its ability to degrade lignin, and the volume of research done on its ligninolytic enzyme system is second only to that of *P. chrysosporium*. *T. versicolor* (*Coriolus versicolor*) is known to degrade the lignin component of wood completely to CO_2 (77). The induction conditions for ligninolytic activity were similar, but not identical to, conditions reported for *P. chrysosporium*. In experiments on mineralization of synthetic ^{14}C-lignin overlaid on cellulose plates, *T. versicolor* converted over 75% of the lignin to CO_2 by day 14 when low nitrogen conditions were employed (2.2 mM NH_4). When nitrogen levels were raised to 5.5 mM NH_4, the ligninolytic activity was decreased but not repressed completely as seen in *P. chrysosporium*. *T. versicolor* converted 45% of the original lignin to CO_2 by day 14. A lag period of four days was observed before ligninolytic activity occurred and the presence of cellulose was absolutely required for ligninolysis. In the absence of cellulose no lignin was mineralized (23).

Similar results were also obtained with ^{14}C labelled wheat lignin. The onset of ^{14}C evolution was delayed for 3 days while primary growth occurred, and CO2 evolution was greater in low nitrogen medium (^{14}C labelled wheat seedlings in 56 mM glucose, 2.4 mM NH4) than in high nitrogen medium (56 mM glucose, 24 mM NH4). Still *T. versicolor* mineralized more ^{14}C lignin in high nitrogen medium than either *P. chrysosporium* or *Sporotrichum pulverulentum* (an asexual anamorph of *P. chrysosporium*). Though the conversion of lignin to CO2 was largely suppressed by these high nitrogen conditions, the lignin was substantially solubilized by the white-rot

fungi in high nitrogen medium, up to 20% by *P. chrysosporium* (65). This indicates that while lignin mineralization may have been suppressed, lignin depolymerization under these high ammonia, high glucose conditions may still have occurred. Low nitrogen ligninolytic conditions as described above were also shown to induce *T. versicolor* to produce enzymes capable of catalyzing the cleavage of ß-O-4 lignin model dimer linkages (48).

These findings led to the discovery of extracellular peroxidases in *T. versicolor* which had similar enzymatic properties as those found for the LIPs and MNPs of *P. chrysosporium* (21). The peroxidase activity could be induced in low nitrogen media (2.2 mM NH4, 11 mM glucose) after seven days growth, or in carbon limited cultures (10.2 mM NH4, 1.1 mM glucose) on day seven, but only if veratryl alcohol was added as an inducer. The peroxidase isolated was a glycosylated 50 kd enzyme which could catalyze the oxidation of veratryl alcohol, free phenolics, and the lignin model dimer 1,2-di(3,4 methoxyphenyl)-1,3-propanediol which resulted in Cα-Cß cleavage to yield veratraldehyde. Surprisingly, oxygenation of cultures was found to be inhibitory to enzyme activity. This is contrary to previous reports of increases in ligninolytic activity upon oxygenation of *T. versicolor* cultures (77). The LIP (46) and the MNP (45) of *T. versicolor* were isolated and characterized from day 11 carbon limited, stationary, non-oxygenated cultures. Three LIP isozymes were isolated by anion-exchange chromatography and chromatofocusing which ranged in size from 43,000 to 45,000 daltons and with pI values between 3.2 to 3.4. Several smaller peaks were also observed, but not isolated. An MNP was also isolated and found to be around M_r 49,000. Both sets of enzymes had enzymatic activities definitive for LIPs and MNPs. The LIP isozymes were found to oxidize non-phenolic ß-O-4 and ß-1 lignin model compounds in the presence of H_2O_2 and were, therefore, confirmed as LIP enzymes. Sequencing of the amino termini of the three isozymes revealed minor differences between them which suggested the existence of several structural genes for LIPs in the genome of *T.*

versicolor. The amino termini of all three isozymes were more similar in sequence to the *P. chrysosporium* amino terminus of LIP2 than to the amino termini of the other LIPs *P. chrysosporium* (47).

T. versicolor is also known to have a glucose oxidase activity as seen in *P. chrysosporium*, and a laccase activity which *P. chrysosporium* does not have (64). Laccase is a copper containing oxidase which catalyzes one-electron oxidations of phenols to yield phenoxy radicals. Ultimately four electrons are used to reduce one O_2 molecule to H_2O during one catalytic cycle (55). Laccase has been implicated in lignin degradation by *T. versicolor*, and has been shown to depolymerize lignin model dimers (49, 66). The ability of *T. versicolor* to degrade the lignin component of wood efficiently has spurred research on using this fungus as an alternative to *P. chrysosporium* in the important industrial applications of biological bleaching of kraft hardwood pulp (2, 57, 58, 63), and the degradation of xenobiotics (67). In both applications *T. versicolor* has proven to perform as well, and in some cases better than, *P. chrysosporium*.

The goal of the research presented in this dissertation is to characterize the structure of the LIP genes of *T. versicolor* and to determine the environmental signals which regulate the *LIP* gene expression in this important white-rot fungus. A comparison of the number of *LIP* genes present in *T. versicolor* to those in *P. chrysosporium* will indicate if the presence of multiple *LIP* genes is a common feature among the lignin degrading white-rot fungi. The homology relationships between the *LIP* gene(s) of *T. versicolor* and *P. chrysosporium* will provide valuable information on the evolution of *LIP* multigene families, and may indicate the location of important conserved regions within the amino acid sequence such as LIP active sites or substrate binding sites.

Considering that both fungi utilize the same resources found in the lignocellulosic material of wood, it is of special interest to find out how the *LIP* genes of *T. versicolor* are regulated in comparison with those of *P. chrysosporium*. Nitrogen and carbon

limitation have been shown to be general signals for induction of the ligninolytic system in both *P. chrysosporium* and *T. versicolor*. Are the LIP genes of *T. versicolor* also induced by nitrogen limitation in defined medium, and is there differential regulation such that some of the gene transcripts are more abundant in the total RNA than others? Most importantly which of the *LIP* genes produce transcripts when the fungus is grown in wood and how does this compare with the major gene transcript(s) produced in nitrogen limiting medium? The answers to these questions will indicate how well the general signal of nitrogen limitation simulates the gene induction signals the fungus responds to in wood, and if there are other as yet unknown environmental signals which activate *LIP* gene expression.

Also both *P. chrysosporium* and *T. versicolor* have been shown to degrade hardwoods (angiosperm) faster than softwoods (gymnosperm). This has been related to the type of lignin present in hardwoods (syringyl/guaiacyl) versus that found in softwoods (guaiacyl). Furthermore, it has been shown that the rate limiting step in guaiacyl lignin degradation is the depolymerization step, and that mineralization of the depolymerized subunits of guaiacyl and syringyl/guaiacyl lignin occurs at equal rates (26, 40). The differences between hardwood and softwood degradation rates may be related to LIP gene expression induced by growth in either gymnosperm or angiosperm wood. If that is so, then that may indicate that the general phenomena of differential *LIP* gene expression is related to differences in the catalytic activity or substrate binding ability of the various LIP enzymes coded for by the *VLG* genes of *T. versicolor* to the widely varying types of lignin polymers present in nature.

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CHAPTER I

CLONING AND CHARACTERIZATION OF A LIGNIN PEROXIDASE GENE FROM THE WHITE-ROT FUNGUS *Trametes versicolor*

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CLONING AND CHARACTERIZATION OF A LIGNIN PEROXIDASE GENE FROM THE WHITE-ROT FUNGUS *Trametes versicolor*

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Six putative lignin peroxidase (LIP) genes were isolated from a λEMBL3 phage library of the white-rot fungus, *Trametes versicolor*, using the *Phanerochaete chrysosporium LIP* cDNA *CLG5* as the probe. Sequence analysis of one of the genes, *VLG1*, showed that its coding region is interrupted by six small introns (49-64 bp) and that it encodes a mature LIP protein (341 aa; M_r: 36,714) that is preceded by a 25 aa signal sequence. This protein has a relatively high degree of aa homology to the *N*-termini of the LIP proteins purified from *T. versicolor* and has an aa homology of 55-60% to the LIP proteins of *P. chrysosporium*, which is comparable to that found between *P. chrysosporium* and *Phlebia radiata* LIP proteins.

Lignin peroxidases (LIPs), a family of extracellular, glycosylated, heme proteins demonstrated in white-rot fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Phlebia radiata*, are believed to be important in lignin degradation, as well as in degrading certain xenobiotics (1). Several *LIP* cDNAs (2,3) and genes (reviewed in ref. 4) of *P. chrysosporium* and one cDNA from *Phlebia radiata* (5), have been isolated and characterized. However, there has been no report to date on the *LIP* genes of the over 1,600 species of other wood-rotting fungi.

Trametes versicolor, the next best studied white-rot fungus after *P. chrysosporium*, produces several lignin peroxidases that, similar to the *P. chrysosporium* LIPs, are produced only during secondary metabolism in response to nutrient starvation (6,7). Furthermore, antibodies against lignin peroxidases of *P. chrysosporium* were shown to cross react with *T. versicolor*

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LIPs indicating the structural relatedness of LIPs from these two organisms (8). The objective of this study was to isolate and characterize *LIP* genomic clones of *T. versicolor* and compare the sequence and other structural features of one of the *LIP* genes with those of *P. chrysosporium* and the LIP encoding cDNA of *Ph. radiata*.

Materials and Methods

Lignin peroxidase gene isolation and sequencing. Genomic DNA of *T. versicolor* (*Coriolus versicolor*) strain ATCC 12679 was extracted as described by Rao and Reddy (9) and a λEMBL3 genomic library was constructed (10) which was screened using ^{32}P-labeled *P. chrysosporium LIP* cDNA *CLG5* as the probe (2,11). The genomic fragments from the positive lambda clones were subcloned into pUC18 and pUC19 vectors and one of the *LIP* genes designated *VLG1* (see Fig. 1) was sequenced as described previously (12), in both directions, using the dideoxy chain termination procedure.

Results and Discussion

Isolation of LIP-encoding genomic clones. Three λEMBL3 clones containing six putative LIP-encoding regions were isolated. Probing of various restriction digests of these three clones with ^{32}P-labeled *CLG5* cDNA showed that *LIP* gene *VLG1* occurs alone on clone 1; genes *VLG2*, *VLG3*, and *VLG4* are located on clone 2; and genes *VLG5* and *VLG6* are located on clone 3 (Fig. 1). The linked *LIP* genes on clones 2 and 3 are separated by 1.5-2.0 kb DNA. Similar linkage has been reported for the *LIP* genes of *P. chrysosporium* (13,14). The results, however, showed that the transcriptional orientation of the linked genes in *T. versicolor* is unidirectional (Fig. 1) whereas in *P. chrysosporium* the

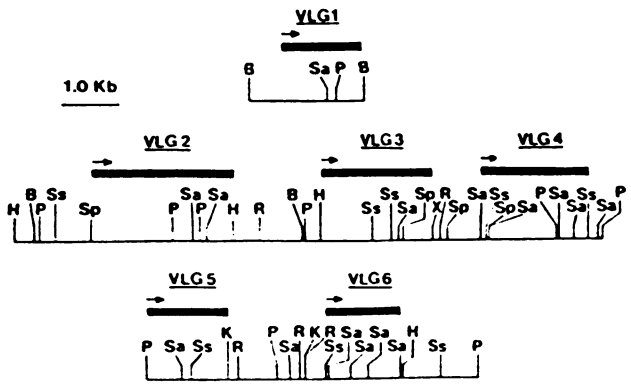

Fig. 1. Restriction maps of the LIP-encoding genomic clones of *T. versicolor*. The abbreviations used for restriction enzymes are B, *Bam*HI; R, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; and X, *Xba*I;. The boundary and the transcriptional direction of each gene are represented by a dark box and an arrow, respectively, above each *LIP* gene.

transcription of linked genes, *lpoA* and *lpoB*, was reported to be in opposite directions (13).

Sequence analysis of *VLG1*. Complete sequencing of *VLG1* and aligning its sequences with the *LIP* genes of *P. chrysosporium* (4) revealed an open reading frame (ORF) of 1,098 bp that encodes 366 aa (Fig. 2). The *VLG1* ORF was identified by using the intron splice site consensus sequences compiled for *P. chrysosporium* (see ref. 4) and the homology to the ORF of *CLG5* cDNA as guides. The mature LIP protein encoded by *VLG1* is 341 aa (M_r 36,714) and is preceded by a 25 aa signal peptide which ends in the consensus proteolytic cleavage site Arg-Arg. The *N*-terminal sequence of the VLG1 protein is very similar to the experimentally determined *N*-terminal aa sequences of the lignin peroxidase isozymes of *T. versicolor* (15), and the lignin peroxidase isozyme H2 of *P. chrysosporium* (2) shown below. This suggests that *VLG1* encodes a protein that shares a high degree of homology to the known LIP isozymes of *T. versicolor* and *P. chrysosporium*.

LIGNIN PEROXIDASE	AMINO-TERMINAL SEQUENCE
P. chrysosporium H2	VAL-ALA-CYS-PRO-ASP-GLY-VAL-HIS-THR-ALA-SER-ASN-ALA
T. versicolor A	VAL-THR- ? -PRO-ASP-GLY-LYS-ASN-THR-ALA-THR-ASN-ALA
T. versicolor B	VAL-THR- ? -PRO-ASP-GLY-VAL-ASN-THR-ALA-THR-ASN-ALA
T. versicolor C	VAL-THR- ? -PRO-ASP-GLY-VAL-ASN-THR-ALA-THR-ASN-ALA
T. versicolor VLG1	VAL-ALA-CYS-PRO-ASP-GLY-ARG-HIS-THR-ALA-THR-ASN-ALA

Consistent with the fact that LIP proteins are glycosylated, the VLG1 protein contains one *N*-glycosylation site with the general sequence Asn-Xaa-Thr/Ser (see Fig. 2), similar to that seen in *P. chrysosporium* LIP proteins. In comparison to the *VLG1*-encoded LIP protein, the mature proteins encoded by the three major *LIP* genes of *P. chrysosporium* each contains 344 aa and are preceded by a 27-28 aa signal peptide that ends in the consensus Lys-Arg cleavage site (see ref. 4). The LIP-encoding cDNA of *Ph. radiata* on the other hand encodes a mature protein of 337 aa and a 24 aa signal peptide which,

```
GGCGCGACACACCGACGTGTCGGACGCTCCCGCGGGCACGCTGCACATGGTCGGCCCCGCTCGTGTTCCTCGGGG 75
TGGACAAAATACTTGCGTTGCGATTGGTCCGAGGCGCGGTTGCAACAGAGGACGGCTCACAATCCATTGTGGCGG 150
TCGTCCTCGGTTCCTTGAGGAGGGAGCCGGGATGAGCCCCGACATCGAGAGGGCCAGGAAATATAAAAGGTGGAC 225
GAAACGGCGCAGAACCTTCAGAGCACTCCAGCGTACTCCTCTCCCCTCTCTTCCACCTCCCTCGGCACCGGCAAC 300
ATG GTT TCC AAG TTC TTC ACC TCC CTC GTC TCC CTC GCT GCT GTC CTG GGT GCT AAC 357
Met Val Ser Lys Phe Phe Thr Ser Leu Val Ser Leu Ala Ala Val Leu Gly Ala Asn
G gtacgtggacgttgtttaaatcgtagtcgcctagctgatcctgttatag  CT TCC CTG ACG CGC CGT 424
A                        IVS 1                        la Ser Leu Thr Arg Arg
GTT GCG TGC CCC GAC GGC AGG CAC ACC GCT ACC AAC GCG GCT TGC TGC GCT CTC TTC 481
Val Ala Cys Pro Asp Gly Arg His Thr Ala Thr Asn Ala Ala Cys Cys Ala Leu Phe
CCT CTC CGG GAC GAT CTC CAG GCC AAC CTC TTC GAC GGC GGC AAG TGC AAC GCT GAG 538
Pro Leu Arg Asp Asp Leu Gln Ala Asn Leu Phe Asp Gly Gly Lys Cys Asn Ala Glu
GCG CAC GAG TCT CTC CGC TTG ACG TTC CAC GAC GCC ATC GCC ATC TCG CCG GCC CTG 595
Ala His Glu Ser Leu Arg Leu Thr Phe His Asp Ala Ile Ala Ile Ser Pro Ala Leu
GAG GCG CAG GGC AA  gttcgggttagtggatacgcaatgcattgcagatcatcatcactcactagactac 664
Glu Ala Gln Gly As                          IVS 2
gcattacag C GGT GGA GGT GCC GAC GGC TCC ATC ACG ATT TTC TCG CAC ATC GAG ACG 722
          n Gly Gly Gly Ala Asp Gly Ser Ile Thr Ile Phe Ser His Ile Glu Thr
GGC TTC CAC CCC AAC ATC GGT CTC GAC GAG GTT GTC GAG AAG CAG CGG CCT TTC CTC 779
Gly Phe His Pro Asn Ile Gly Leu Asp Glu Val Val Glu Lys Gln Arg Pro Phe Leu
CAG CGC CAC AAC ATC GGT GTT GCT GAC TT gtgagttgcacagcacgccccagggttttcacgggc 844
Gln Arg His Asn Ile Gly Val Ala Asp Ph                      IVS 3
tcccgctcatgcttcgttcacag   C ATT CAA TTC GCC GGT GCC CTC GGT GCG TCC AAC TGC 904
                          e Ile Gln Phe Ala Gly Ala Leu Gly Ala Ser Asn Cys
GCA GGT GCT CCC CAG CTC AGC GCC TTC GTC GGC CGC AAG GAG CCG ACG CGC CCC GCC 961
Ala Gly Ala Pro Gln Leu Ser Ala Phe Val Gly Arg Lys Glu Pro Thr Arg Pro Ala
CCC GAC GGC CTC GTC CCG GAG CCG TTC CAC ACG CCC GAC CAG ATC TTC GCC CGC ATC 1018
Pro Asp Gly Leu Val Pro Glu Pro Phe His Thr Pro Asp Gln Ile Phe Ala Arg Ile
GCC GAC GCG TCC TCG GGC GAG TTC GAC GAG ATC CTG ACC GTC TGG CTG CTC ACC GCG 1075
Ala Asp Ala Ser Ser Gly Glu Phe Asp Glu Ile Leu Thr Val Trp Leu Leu Thr Ala
CAC ACG ATC GCC GCC GCC AAC GAC GTC GAC CCG ACC GTG CCC GGC TCG CCG TTC GAC 1132
His Thr Ile Ala Ala Ala Asn Asp Val Asp Pro Thr Val Pro Gly Ser Pro Phe Asp
TCC ACC CCC GAG ATC TTC GAC TCG CAG TTC TTC CTC GAG ACG CAG CTC AAG GGC ACC 1189
Ser Thr Pro Glu Ile Phe Asp Ser Gln Phe Phe Leu Glu Thr Gln Leu Lys Gly Thr
GCC TTC ACC GGG CGC GGC CCC GTG CAG GGC GAG GTC ACG TGC CCG TGC GCG GGC GAG 1246
Ala Phe Thr Gly Arg Gly Pro Val Gln Gly Glu Val Thr Cys Pro Cys Ala Gly Glu
TTC CGC CTG CAG TCC GAC TTC GCG ATC GCG CGC GAC CAG GCC ACC GCG TGC GAG TGG 1303
Phe Arg Leu Gln Ser Asp Phe Ala Ile Ala Arg Asp Gln Ala Thr Ala Cys Glu Trp
CAG TCG TTC GTC AAC AAC CAG ACC AAG GTC CAG CAG ATG TTC CAG TTC GTC TTC CAC 1360
Gln Ser Phe Val Asn Asn Gln Thr Lys Val Gln Gln Met Phe Gln Phe Val Phe His
GAC CTC TCC ATC CTC GGC CAG AAC ATC GAC GAC CTC GTT GAC TGC ACG GAA GTG gta 1417
Asp Leu Ser Ile Leu Gly Gln Asn Ile Asp Asp Leu Val Asp Cys Thr Glu Val
ctatacatttctcgtcagaggatgctcaacgatctgacttgttcttgtcgtag   ATC CCG ATC CCC AGG 1483
                              IVS 4                      Ile Pro Ile Pro Arg
CCC CTC ACC ACC AGG ACC CAC TTC CCC GCC GGC ATG ACC CAC CGC GAC ATC GAG CAG 1540
Pro Leu Thr Thr Arg Thr His Phe Pro Ala Gly Met Thr His Arg Asp Ile Glu Gln
GCT gtgagtcattcagttccattagacacttgccgtgctcacacatcctatcag TGC TTG GAG ACC CCC 1611
Ala                         IVS 5                       Cys Leu Glu Thr Pro
TTC CCC ACC CTC CCC ACC GAC CCC GGA CCC CGC ACC GGT GTC GCC CCC GT gtaagtct 1669
Phe Pro Thr Leu Pro Thr Asp Pro Gly Pro Arg Thr Gly Val Ala Pro Va
cttcttcaactcacgaccgaccacaatctgaccgctcctccag   C ATC CCC AAG CGG GTC TAG     1731
                  IVS 6                           l Ile Pro Lys Arg Val Stop
GTAAACGGAGCAGCAACGCTCTCCCCGGCACACGGCTATCGGCGGTTCAGGATCC                    1786
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Fig. 2. Complete nucleotide sequence of *T. versicolor LIP* gene *VLG1* (EMBL accession number: M55294) and the deduced amino acid sequence. The nucleotide sequence of the introns (*IVS1* to *IVS6*) are given in lower case letters. Putative TATA box and CAAT box, signal peptide cleavage site, and putative *N*-glycosylation sites are given in bold face letters and underlined.

similar to that of *T. versicolor* LIP protein VLG1, ends in the putative proteolytic cleavage site, Arg-Arg (5).

The codon usage for the LIP protein encoded by *VLG1* is extremely biased in favor of codons ending in C (67%) or G (30%). Thus, 97% of the total codons used for the VLG1 LIP protein end in a C or G. In LIP proteins of *P. chrysosporium* also, the codon usage is heavily biased in favor of the codons ending in a C or G (2).

The ATG initiation site (Fig. 2) is located within the consensus eukaryotic initiation sequence A/GNNATGG (16) and is very similar to that found in the genes encoding LIP isozymes H2, H8, and H10 in *P. chrysosporium* (see ref. 4). Putative TATAA box and CAAT box, the consensus eukaryotic promoter elements, are, respectively, located 80 bp and 165 bp upstream of the ATG initiation site.

Amino acid homology to the LIP proteins of other white-rot fungi. A comparison of the aa sequence of the LIP protein *VLG1* to that of *P. chrysosporium* LIP isozymes H2, H8, and H10 (Fig. 3A) showed 57-61% homology, whereas the aa homologies are much higher among the latter three LIP isozymes of *P. chrysosporium* (70-80%). The LIP protein VLG1 shares 60% homology to the LIP protein LIII of *Ph. radiata*.

It has been well established that in a variety of peroxidases, including the turnip peroxidase, cytochrome c peroxidase, horseradish peroxidase, LIP isozymes of *P. chrysosporium*, and *Ph. radiata* LIP protein, a proximal histidine (which serves as an axial ligand of heme) and a distal histidine and arginine (which are involved in charge stabilization during reaction of the heme with H_2O_2) residues are well conserved. These critical aa residues and the aa sequences surrounding these residues are also highly conserved in *VLG1* encoded LIP proteins (Fig. 3B).

Introns. The coding region of *VLG1* is interrupted by six relatively small introns (size range 49 to 64 bp), including one intron *(IVS1)* which interrupts the signal peptide coding region (Fig. 2 and Fig. 4A). In comparison, the *LIP* genes of *P. chrysosporium* described to date contain eight to nine introns and the positions of these introns are different from those in *VLG1* (Fig. 4A).

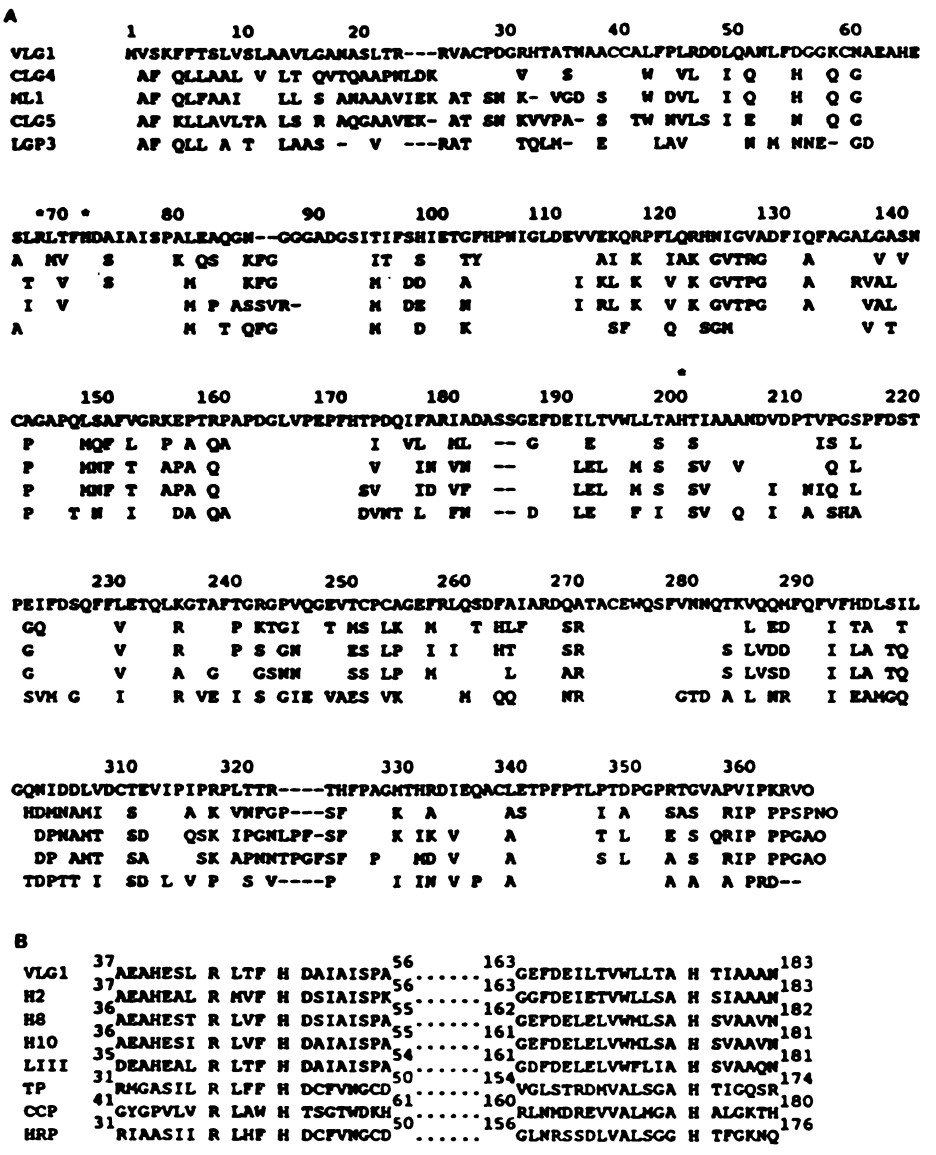
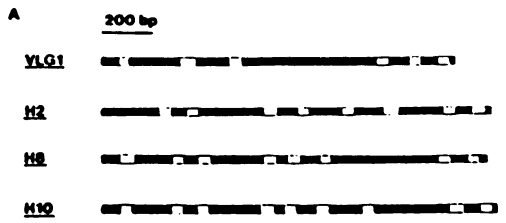


Fig. 3. Conservation of amino acid sequences between the LIP proteins of *P. chrysosporium*, *Ph. radiata*, and VLG1 of *T. versicolor*. A. Amino acid homolgy between the LIP protein encoded by *T. versicolor* gene *VLG1* and those encoded by the *LIP* cDNAs *CLG4*, *CLG5*, and *ML1* of *P. chrysosporium* and *LGP3* of *Ph. radiata*. *CLG4*, *ML1*, and *CLG5* are *LIP* cDNAs of *P. chrysosporium* that, respectively, encode LIP proteins, H2, H8, and H10 (2,3). *LGP3* is a *LIP* cDNA of *Ph. radiata* that encodes the LIP protein LIII of this organism (5). Proximal and distal histidine and arginine residues known to be required for catalytic activity of peroxidase proteins are marked with an asterisk. B. Comparison of the active site regions of the LIP protein VLG1 of *T. versicolor*, LIP proteins H2, H8, and H10 of *P. chrysosporium* (2,3), LIII of *Ph. radiata* (5), turnip peroxidase, cytochrome c peroxidase, and horse radish peroxidase.

However, the consensus exon/intron splice junction sequences and the conserved internal sequences of *VLG1* N/GTRNGT.....CTSAY.....YAG/Y, (Fig. 4B) are similar to those seen in the *LIP* genes of *P. chrysosporium* and other eukaryotic



B

IVS1	ACG/GTACGTGCACGTTTGTTTAAATCGTAGTCGCCTAG		CTGAT		CCTGTTATAG/CT
IVS2	CAA/GTTCGCGTTAGTCGATACGCAATCCATTCCAGATCATCATCA		CTCAC		TAGACTACGCATTACAG/CG
IVS3	CTT/GTGAGTTGCACAGCACGCCCCCAGCGTTTTCACGGGCTCCCG		CTCAT		GCTTCGTTCACAG/CA
IVS4	GTG/GTAGTATACATTTCTCGTCAGAGCATCCTCAACGAT		CTCAC		TTGTTCTTGTCGTAG/AT
IVS5	GCT/GTGAGTCATTCAGTTCCATTAGACACTTCCCGTG		CTCAC		ACATCCTATCAG/TG
IVS6	CGT/GTAAGTCTCTTCTTCAACTCACGACCGACCACAAT		CTCAC		CCCTCCTCCAG/CA
T. versicolor	NK/GTRNGT	34-41	CTSAY	10-17	YAG/Y
P. chrysosporium	SK/GTRNGY	32-37	YTGAY	9-19	YAG/N
Higher Eukaryotes	CAG/GTAAGT		CTAAT		NCAG/G
Yeast	N/GTATGT		TACTAAC		NYAG/N
Filamentous fungi	g/GTAYGTT		TGCTAAC		ACAG/g

Fig. 4. Comparison of the intron positions and the splice junction sequences in the *LIP* genes of *T. versicolor* and *P. chrysosporium*. A. A comparison of the position of introns in the *T. versicolor LIP* gene *VLG1* and *P. chrysosporium* genes which encode LIP proteins H2, H8, and H10 (from ref. 17). Closed boxes represent exons and open boxes represent introns. B. Conserved exon/intron junction sequences and internal conserved sequences of introns of the *VLG1* gene. The boundaries of exons and introns are marked by slashes. Abbreviations for single letter codons are: N, A or C or G or T; R, A or G; Y, C or T; S, C or G. The conserved intron/exon junction sequences and internal conserved sequences of *P. chrysosporium LIP* genes (4), and of genes of higher eukaryotes, yeasts, and filamentous fungi (16) are also presented.

genes suggesting that these genes share similar splicing mechanisms (4,16). The sizes of the introns in *VLG1* and *LIP* genes of *P. chrysosporium* are also very similar.

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CHAPTER II

Structure and Molecular Regulation of *VLG2*, a Lignin Peroxidase Gene From the White-Rot fungus *Trametes versicolor*.

ABSTRACT

Transcripts (1.25 kb) of the lignin peroxidase gene *VLG2* from the white-rot fungus *T. versicolor* were detected in a nitrogen limited medium (56 mM glucose, 2.2 mM NH_4) containing low levels of Mn(II) (0.13 ppb). The presence of transcripts of the other 5 *LIP* genes cloned from this fungus could not be confirmed under the above conditions. At moderate Mn(II) levels (2.6 ppm), LIP activity of *T. versicolor* was completely suppressed. A cDNA library was prepared, screened, and *VLG2*-specific cDNA (designated *VLC2*) was isolated. Sequencing of both *VLG2* and *VLC2* revealed that *VLC2* is 79% homologous at the nt level and 76% homologous at the aa level to the coding region of *VLG1*.

INTRODUCTION

Woody plant tissues are made up of three major structural polymers: cellulose, hemicellulose, and lignin. The lignin component makes up 20-30% of the dry mass of woody plants, and is the most abundant renewable aromatic polymer on the planet (32). Because it is a random polymer of phenylpropanoid subunits that is chemically associated with cellulose and hemicellulose in the cell wall, lignin provides physical rigidity to plants as well as a barrier to fungal and bacterial plant pathogens. White-rot fungi are among the few organisms capable of mineralizing all the three components of wood (4).

Interest in these organisms has increased since the discovery of lignin peroxidases (LIP) and manganese peroxidases (MNP), which are the key enzymes responsible for the initial depolymerization of the lignin polymer (12, 26, 37, 38). LIPs catalyze H_2O_2-dependent one electron oxidations of both the phenolic and non-phenolic groups in the lignin polymer (13, 21, 35, 36). MNPs catalyze one electron oxidations of Mn(II) to Mn(III) which then oxidizes the aromatic carbons in the lignin (11, 41). These enzymes can be induced by either carbon or nitrogen starvation during secondary metabolism (9).

The lignin peroxidases of the white-rot fungus *P. chrysosporium* have been the most well studied. Multiple LIP isozymes have been isolated and cDNA as well as the genomic clones encoding these isozymes have been characterized (6, 23, 28-30, 39, 40, 43). These studies have shown that each LIP isozyme is encoded by a distinct gene of the *LIP* multi-gene family (5). The *LIP* genes are reported to be clustered within the genome of *P. chrysosporium* (31), but only two such clusters have been identified to date.

The LIP isozymes are differentially regulated such that under nitrogen limitation at least six readily identifiable LIPs (designated LIP1-LIP6) are induced, but three

predominate (23): LIP2, LIP5 and LIP6. A study of the molecular regulation of *LIP* genes has shown that the levels of expression of LIP isozymes is correlated with the levels of the corresponding *LIP* RNA transcripts (4, 15).

T. versicolor has also been shown to produce multiple LIP isozymes (18, 19) and a single MNP isozyme (17) under both nitrogen and carbon limitation conditions (7). We have previously described the cloning of a family of *LIP* genes from the white-rot fungus *T. versicolor* (1). We present here the sequence analysis of the second *LIP* gene, (*VLG2*) of *T. versicolor*. Furthermore, since *T. versicolor* has been shown to mineralize lignin and express LIP activity under conditions of nitrogen or carbon starvation, we studied the molecular regulation of the six *LIP* genes cloned from *T. versicolor*. We present evidence that under nitrogen limited conditions, *T. versicolor* produces an abundance of *VLG2* transcripts, and that LIP activity of *T. versicolor* is regulated by Mn(II) concentration in the medium.

MATERIALS AND METHODS

Culture Conditions. *T. versicolor* (ATCC 12679) was used for all experiments. The strain was maintained at 4° C on modified malt extract plates (2% malt extract, 2% glucose, 0.1% peptone agar, pH 4.5). Modified low nitrogen medium (LN medium) of Kirk et al (23) was used. This medium contained the following: Vitamins per liter: 2 µg biotin, 2 µg folic acid, 5 µg thiamine, 5 µg riboflavin, 10 µg pyridoxine, 100 pg cyanocobalamine, 5 µg nicotinic acid, 5 µg DL-calcium pantothenate, 5 µg p-aminobenzoic acid, and 5 µg thiotic acid. Except where mentioned otherwise, $MnSO_4.H_2O$ was added to a final concentration of 400 pg per liter (0.13 parts per billion) and Tween 80 was added to a final concentration of 0.001%. Four plugs of *T. versicolor* were added from malt extract agar plates to 50 ml of LN medium without Tween 80 in a 2.8 L Fernbach flask and grown at 25° C for 7 days. The culture was homogenized and the homogenized inoculum was added to 10% of the final culture volume (10 ml of low N medium in 125 ml of sterile foam-plugged Erlenmeyer flasks) and then incubated at 25° C under static conditions.

Enzyme Assays and Analytical Assays. LIP and MNP assays were performed as described previously (36). Glucose was measured as the reducing sugar by the dinitrosalicylic acid method, using D-glucose as the standard (42). Mycelial dry weight was determined by vacuum filtration of the mycelial mass onto tared Whatman #1 filter disks, dried to a constant weight at 80° C, and weighed. Ammonium ion concentration was determined by using an ammonia electrode (Fisher model 13-299-96, Fisher Scientific Company, Pittsburg, PA) per manufacturers specifications.

mRNA Extraction and Northern Blots. Total RNA was extracted as described by Haylock and Broda (14), and the poly(A) RNA fraction was purified by oligo-dT

cellulose (Bethesda Research Laboratories, Gaithersburg, MD) chromatography. 2 µg of this RNA was electrophoresed on a 1.2% agarose gels with 2.2 M formaldehyde as previously described (33). RNA was transferred to nylon membranes (Micron Separations Incorporated, Westborough, MA) by capillary transfer and immobilized by UV crosslinking (Stratalinker 1800, Stratagene, La Jolla, CA) at 400 J/m^2. The blot was hybridized with a ^{32}P-dCTP labelled DNA probe containing the complete coding region of *VLG2*at 42^{o} C in 50% formamide, 1.0 M NaCl hybridization buffer.

cDNA probe preparation. cDNA probes were prepared as described by Sambrook et al. (34). In brief, a poly-dT 17 bp primer was added to poly(A) purified RNA and annealed at room temperature. The primer was elongated by addition of MMLV-reverse transcriptase (BRL), and labelled with ^{32}P-dCTP added along with the other nucleotides. The resulting single stranded cDNA probe, representative of the mRNA species present in the RNA sample, was hybridized to a Southern blot containing the *VLG1, VLG2, VLG3, VLG4, VLG5,* and *VLG6 LIP* genomic clones of *T. versicolor.* The genes occured in linked sets on lambda clones as previously described (1). To separate the genes, the lambda clones were digested with different enzymes. Clone 1 containing *VLG2*, *VLG3*, and *VLG4* was digested with *EcoR*I and *Hin*dIII; clone 2 containing *VLG5* and *VLG6* was digested with *Pst*I; and clone 3 containing a single gene, *VLG1*, was digested with *Bam*HI. The digested clones were electrophoresed on a 0.7% agarose gel, blotted to nylon and immobilized as above. The Southern blot was hybridized to the cDNA probe at 48^{o} C in 50% formamide and 400 mM NaCl hybridization buffer; washed extensively at 68^{o} C in 0.1 X SSC, 1% sodium dodecyl sulphate; and an autoradiogram was prepared.

cDNA Library Construction. 5 µg of poly(A) purified RNA extracted from day 9 cultures grown in LN medium was used to construct a lambda phage cDNA library in the vector gt22A using the Lambda Superscript system (BRL) as described by the manufacturer. The library consisted of 1.4 x 10^6 phage clones; 50,000 of the phage

clones were screened on a 150 x 15 mm petri plate by immobilization of the plaques onto nitrocellulose filters and hybridization to a ^{32}P-dCTP labelled *VLG2* probe. A lambda cDNA clone, which hybridized to the *VLG2* probe, was purified and the cDNA insert was subcloned out of the *Eco*RI, *Not*I site on the purified lambda clone into the *Eco*RI, *Sma*I site of pUC19. The insert was verified as *VLC2* by sequencing 200 bp from its 5' end and comparing it with the corresponding *VLG2* DNA sequence.

Sequencing of *VLG2* and *VLC2*. A 3.8 kb *Hin*dIII fragment from the lambda genomic clone 1 containing the complete coding sequence of *VLG2* was subcloned into M13mp19 in both orientations. The complete 3.8 kb fragment was sequenced by generating ordered deletions with nucleases ExoIII and S1 (34). *VLC2* was sequenced similarly in both directions by using appropriate restriction fragments.

RESULTS

Nitrogen limitation. Growth of *T. versicolor* in low nitrogen medium (initially 2.2 mM NH4) leads to nitrogen depletion after 5 days, whereas glucose levels are non-limiting through day 13 (Fig. 1). Biomass continues to increase in the cultures, even after nitrogen depletion, until day 7 when biomass increase levels off and LIP activity appears.

Lignin peroxidase enzyme induction and appearance of transcripts. Low levels (0.13 ppb) of Mn (II) allowed relatively high levels of activity while higher levels were inhibitory and at 2.6 ppm LIP enzyme production was completely suppressed (Table 1). Measurable LIP enzyme activity appeared on day 7 (Fig. 2) under nitrogen limiting conditions. Manganese peroxidase (MNP) activity appeared earlier than LIP activity with peak values of 101.5 units/liter occurring on or before day 5 and decreasing to 19.23 units/liter by day 11. To determine the temporal production of *LIP* transcripts vs LIP enzyme activity, RNA was extracted from the samples on days 5, 7, 9, and 11. The poly(A) RNA fraction was purified and a northern blot was prepared, (Fig. 2). Hybridizing the blot to the *VLG2* probe indicated that a low level of *VLG2* transcript (1.25 kb) was present on day 5 which correlated well with the onset of nitrogen limitation on day 5. Transcript levels rose dramatically to peak levels on days 7 and 9, and then fell somewhat by day 11. A positive correlation was observed between LIP enzyme production and the level of *VLG2* transcripts although there was a time lag (about 24 hours) between the transcript levels and the translation of these into LIP activity levels. The corresponding MNP transcript levels in the above cultures were not determined since *MNP* genes have not yet been cloned from *T. versicolor.* Carbon limited and nitrogen-sufficient medium, previously used to induce LIP enzymes in *T.*

versicolor strains PRL 28A (6) and PRL 572 (17), was tried with strain ATCC 12679 at 0.13 ppb Mn(II); however, no measurable LIP enzyme activity was seen under these conditions.

Major *LIP* gene transcripts under nitrogen limitation. Six *LIP* genes have been cloned previously from the *T. versicolor* genome (1). Some of the genes are tightly linked within the genome (*VLG2*, *VLG3*, *VLG4*, and *VLG5*, *VLG6*) with 1.5-2.0 kb separating them (1). To determine which of the poly A RNA transcripts from these genes was most abundant under nitrogen-limited conditions, a cDNA probe (see materials and methods) was prepared from day 9 mRNA, and hybridized to the cloned *LIP* genes immobilized on a southern blot. High stringency hybridization and washing conditions were employed to minimize cross-hybridization between related *LIP* cDNA probes. The resulting autoradiogram (Fig. 3) clearly showed that *VLG2* is actively transcribed under nitrogen limiting conditions. Similar results were obtained using day ll mRNA. The second and third most intensely hybridizing genes were *VLG1* and *VLG3*, respectively. However, at least for *VLG1*, this hybridization must be due to cross-hybridization since *VLG1* specific transcripts could not be found when northern blots were probed with a 5' *VLG1* specific gene probe, nor could any cDNA clones specific for *VLG1* could be found in the cDNA library constructed from day 9 mRNA (data not shown).

Isolation of *VLC2* and sequence analysis of *VLG2*. A cDNA library was constructed from the day 9 mRNA and a cDNA clone, which corresponds to the *LIP* genomic clone *VLG2*, was isolated. Both clones were sequenced and the intron/exon junctions located. Fig. 4 contains the sequence of *VLG2* with the intron sequences in lower case letters. The amino acid sequence of VLG2 was deduced from the open reading frame provided by *VLC2* and placed below the corresponding DNA coding sequence. A consensus TATAA box precedes the ATG translation initiation codon by 83 base pairs. Translation of *VLG2* from this ATG results in a 368 residue protein. A

consensus signal sequence of 26 residues ending at the proteolytic cleavage site of **Arg-Arg** (underlined and in boldface in Fig. 4) is located at the N terminus of the protein, and is very similar to the signal sequence found in *VLG1* (1). Cleavage of the signal sequence results in a 342 amino acid mature protein of M_r 36,075. Two potential N-glycosylation sites of the general sequence *Asn-X-Thr/Ser* are located within the mature protein at residues 103 and 214.

DISCUSSION

Nutrient limitations such as nitrogen or carbon starvation have been shown to be necessary for induction of lignin degradation and lignin peroxidase enzyme production in the white-rot fungi *P. chrysosporium* and *T. versicolor* (7-9, 20, 22, 25, 27). Lignin model compounds, or the white-rot fungal secondary metabolite veratryl alcohol, are also known to induce LIP enzyme levels (9, 10). Moreover LIP isozymes of *P. chrysosporium* have been shown to be repressed in nitrogen- or carbon-limited media in the presence of $\geq$ 8 ppm Mn(II) (3).

The results of this study showed that nitrogen-limiting culture conditions are a necessary pre-requisite for the appearance of LIP enzyme activity in *T. versicolor*, but only in the presence of very low levels of Mn(II) in the medium. The level at which the LIP enzymes of *T. versicolor* were repressed (i.e. 2.6 ppm) is lower than the reported range of 5-200 ppm Mn(II) present in wood (2, 3). However, the actual percentage of this manganese available to the fungus is not known since the Mn(II) may be bound to wood in insoluble complexes.

The results showed good correlation between LIP enzyme levels and the level of the *VLG2* transcripts. *VLG2* transcripts only appear when nitrogen has been depleted from the culture. The observed correlation between the appearance of *VLG2* transcripts and the appearance of LIP enzyme activity is similar to that observed for *LIP2* and *LIP5* transcripts and the corresponding LIP isozymes H2 and H8 in *P. chrysosporium* (15). It is possible that other *LIP* genes exist in this strain of *T. versicolor* that have not yet been described, and that these genes are also induced under these conditions. It is also possible that *VLG1* transcripts are present, but at levels too low to be detected by present molecular techniques.

As the DNA hybridization results predicted, *VLG2* has strong nucleotide homology to *VLG1* and the protein sequences deduced from *VLG1* and *VLG2* sequences have a high degree of amino acid homology (1).

Amino Acid Homology Amongst The LIP Enzymes

	VLG2	VLG1	LIP2	LIP5	LIP6	LGP3
VLG2		76%	63%	61%	58%	62%
LIP2				72%	66%	58%
LIP5					82%	61%
LIP6						58%

In fact when comparing the homology of the LIP enzymes of *T. versicolor* to that of the other white rot fungi it is obvious that VLG1 and VLG2 are more closely related to each other than either is to the LIP enzymes from *P. chrysosporium*, or the LGP3 enzyme from the white-rot fungus *Phlebia radiata* (33). VLG2, next to VLG1, is most homologous to the LIP enzyme LIP2 of *P. chrysosporium*, and LGP3 of *P. radiata.*

The molecular regulation of the LIP genes of *T. versicolor* and *P. chrysosporium* is quite similar. Nitrogen and Mn (II) limitations are common environmental signals for the appearance of the LIP transcripts of *T. versicolor.* Also, nitrogen limitation leads to increases in transcript levels for some of the *LIP* genes more so than for the others, as shown here for the increase in transcript levels of the *VLG2* gene.

The presence of *LIP* multi-gene families in both *T. versicolor* and *P. chrysosporium* with differential expression of the *LIP* genes under nutrient limiting conditions raises the question of what the function of the multiple *LIP* genes is. Defined low nitrogen media simulates some of the natural conditions the fungus encounters in wood. Wood has a low nitrogen content and therefore white-rot fungi probably exist in a constant state of nitrogen limitation when subsisting on wood. Therefore, it would be of interest to determine if the same *LIP* genes are induced in natural substrates such as wood as are induced in defined nitrogen limiting media.

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TABLE 1. Effect of Mn(II) concentration on lignin peroxidase activity of *T. versicolor*[a]

Mn(II) concentration[b] (parts per billion)	Lignin Peroxidase[c] activity (Units/Liter)
0.00	7.40
0.13	13.10
13.00	8.36
2600.00	0.00

a) *T. versicolor* was grown in low nitrogen media as described in Materials and Methods. LIP and MNP activities were measured in 11 day old cultures.

b) Mn(II) was added as MnSO4.

c) LIP activity was measured as described (35). One unit of LIP activity represents 1 µmol of veratraldehyde produced from veratryl alcohol per minute.

FIG. 1. Nitrogen depletion versus lignin peroxidase production during growth of *T. versicolor* in nitrogen limiting medium. Filled diamonds (♦) represent glucose concentration (mM); open circles (O) represent ammonium ion concentration (mM); filled circles (•) represent biomass (dry weight in mg/flask); and crosses (X) represent LIP activity (Units/Liter) of *T. versicolor*.

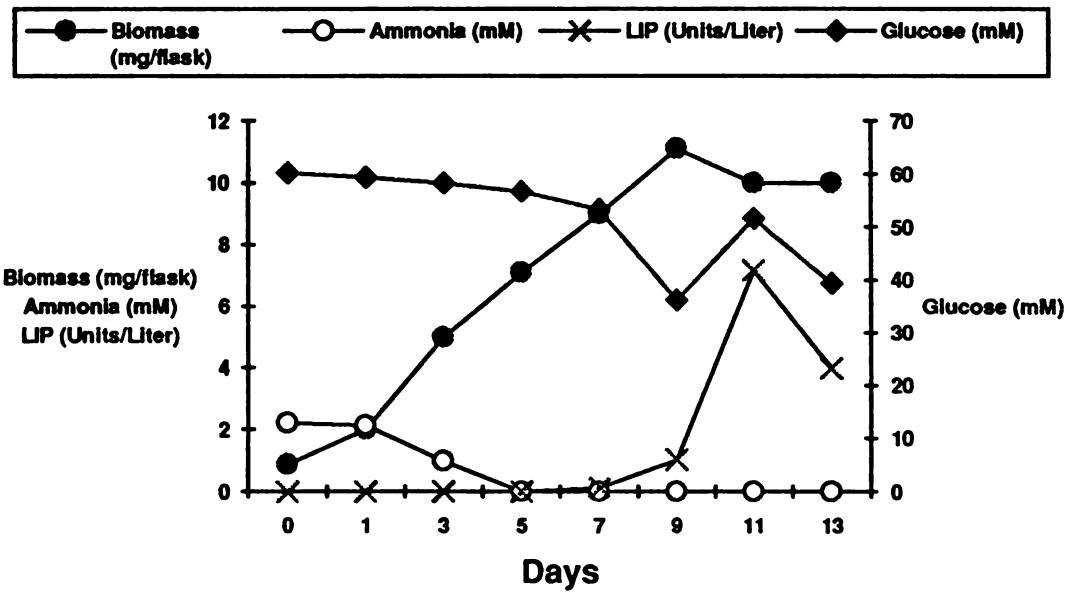
Biomass (mg/flask)
Ammonia (mM)
LIP (Units/Liter)
Glucose (mM)
Biomass (mg/flask)
Ammonia (mM)
LIP (Units/Liter)
Glucose (mM)
12
10
8
6
4
2
0
70
60
50
40
30
20
10
0
0
1
3
5
7
9
11
13
Days

FIG. 2. *VLG2* transcript levels on different days of incubation. *T. versicolor* was grown in low nitrogen media as described in Materials and Methods. LIP activity (Units/Liter) was determined as described in the footnote of Table 1. Lanes 1 through 4 in the Northern blot correspond, respectively, to poly(A) RNA from cultures on day 5, 7, 9, and 11. The blot was hybridized with a ^{32}P-dCTP labelled DNA probe containing the complete coding region of *VLG2* at 42^{o} C in 50% formamide, 1.0 M NaCl hybridization buffer.

FIG. 3 Concentration of transcripts of *VLG1*, *VLG2*, *VLG3*, *VLG4*, *VLG5* and *VLG6* in poly(A) RNA from day 9 cultures grown on low nitrogen medium. The Southern blot was hybridized with a cDNA probe prepared from a day 9 poly(A) fraction of RNA (as described in Materials and Methods), and washed under high stringency conditions. Lane 1 contained *Eco*RI and *Hin*dIII digested DNA from lambda clone 1 which contained the LIP genes (*VLG4*, *VLG2*, *VLG3*); lane 2 contained DNA from lambda clone 2 (*VLG6*, *VLG5*) digested with *Pst*I; lane 3 contained DNA from lambda clone 3 (*VLG1*) digested with *Bam*HI.

1 2 3

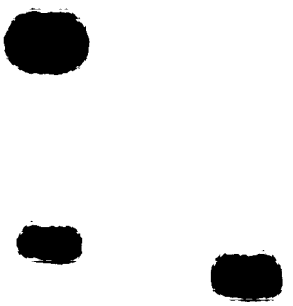

FIG. 4. Nucleotide sequence of *VLG2* and the deduced amino acid sequence of the protein encoded by this gene. 5' upstream, 3' downstream, and exon sequences are in upper case letters intron sequences are in lower case letters. The first 6 nucleotides from the 5' end of *VLC2* are in boldface. The sequence of *VLG2* ends at the polyadenylation site for *VLC2*.

```
CCTACGTTCACAGTAGGTTACGGCAGCGATACAGCTTGGGCACACGAACTAGGATGGCCCTGGCGGGGAA   70
GGATCAGGGCGATGCTACAGCTCGCCGCCATTGGTCGCCTTGCACAACGTCACCGCCACACCAGAGTTGT  140
GGACCTCTCGCTAGTGCGCTTCATGGCGGCAAGGTTGTGAACGGCACCGAGAATGGTGGAATGGCAGAGC  210
TGCCATTGGGTCCTTGTGTCGATCTGTGCATCACAGCTCGGCTACCTATCCTTGGTCGCGGAGAGCCGGT  280
GAGGACCTTGCAGAGACACCGCCCAGTTTATTCACTATTTTCGAGGTTCACGACAGATATAAAAGAGGTG  350
ATGCGGCAGCCCAAAGACCTCAGAGCACTCGAGTTCTCCTCTCTTCGCCTCCCAACAGCTCGGCACCGAC  420
ATG GCC TTC AAA ACC TTA CTC TCC ATC GTC TCC CTT TTG GCG GCG TTC CAG GGC  474
Met Ala Phe Lys Thr Leu Leu Ser Ile Val Ser Leu Leu Ala Ala Phe Gln Gly
GCG ACC G gtacgtggctagcgcggccgacctcgacatcatttgtcacgacaatctgacgtcttttcat  542
Ala Thr A
ccgctag  CC GCA TTG ACC CGC CGC GTC GCT TGC CCC GAC GGA GTG AAC ACC GCG  596
         la Ala Leu Thr Arg Arg Val Ala Cys Pro Asp Gly Val Asn Thr Ala
ACT AAC GCG GCG TGC TGC CAG CTC TTC GCT GTG CGC GAC GAC CTC CAG GAG AAC  650
Thr Asn Ala Ala Cys Cys Gln Leu Phe Ala Val Arg Asp Asp Leu Gln Glu Asn
CTG TTC CAC GGC GGT CTC TGC ACG GCC GAG GCG CAC GAG TCT CTT CGC TTG ACG  704
Leu Phe His Gly Gly Leu Cys Thr Ala Glu Ala His Glu Ser Leu Arg Leu Thr
TTC CAC GAC GCT ATC GCT ATC TCC CCT GCC CTT GAG CAG CAG GGT ATC TTT GG   757
Phe His Asp Ala Ile Ala Ile Ser Pro Ala Leu Glu Gln Gln Gly Ile Phe Gl
gtatgtcaagcacgtcttacacattccgacatcgttgttgatgacgctctactgcacag   T GGC GGA  823
                                                              y Gly Gly
GGT GCC GAT GGT TCC ATC GCG ATC TTC TCA GAC ATC GAG ACC GCC TTC CAC CCC  877
Gly Ala Asp Gly Ser Ile Ala Ile Phe Ser Asp Ile Glu Thr Ala Phe His Pro
AAC ATC GGT CTT GAC GAG ATC GTC GAG CTG CAG AAG CCG TTC ATC GCG CGC CAC  931
Asn Ile Gly Leu Asp Glu Ile Val Glu Leu Gln Lys Pro Phe Ile Ala Arg His
AAC CTC TCC GTC GCT GAC TT  gtgagcgttactgctattggtgtcaccataacaatatgctaac  994
Asn Leu Ser Val Ala Asp Ph
tcgtggccacag  C ATT CAA TTC GCC GGA GCC ATC GGT GCC TCG AAC TGT GCG GGT 1049
              e Ile Gln Phe Ala Gly Ala Ile Gly Ala Ser Asn Cys Ala Gly
GCT CCC CAG CTC GCC GCC TTC GTC GGC CGC GTG GAT GCT ACT CAG CCC GCC CCC 1103
Ala Pro Gln Leu Ala Ala Phe Val Gly Arg Val Asp Ala Thr Gln Pro Ala Pro
GAC GGT CTT GTC CCC GAG CCG TTC CAC ACG CCC GAC CAG ATC TTC GCC CGC CTC 1157
Asp Gly Leu Val Pro Glu Pro Phe His Thr Pro Asp Gln Ile Phe Ala Arg Leu
GCC GAC GCA TCC CAG GGC GAG TTC GAC GAA ATC TTG ACC GTG TGG CTT CTC GTC 1211
Ala Asp Ala Ser Gln Gly Glu Phe Asp Glu Ile Leu Thr Vla Trp Leu Leu Val
GCG CAC ACC GTC GCT GCG GCC AAT GAC GTC GAC CCG ACT GTC CCC GGT TCG CCC 1265
Ala His Thr Val Ala Ala Ala Asn Asp Val Asp Pro Thr Val Pro Gly Ser Pro
TTC GAC TCG ACT CCA GAG GTC TGG GAC ACG CAG TTC TTC GTC GAG GTC CTC CTG 1319
Phe Asp Ser Thr Pro Glu Val Trp Asp Thr Gln Phe Phe Val Glu Val Leu Leu
AAC GGC ACG ACC TTC CCC GGC ACC GGC GAC AAC CAG GGT GAG GTC GCG TCC CCG 1373
Asn Gly Thr Thr Phe Pro Gly Thr Gly Asp Asn Gln Gly Glu Val Ala Ser Pro
ATC GCC GGC GAG TTC CGC CTG CAG TCC GAC TTC GCG ATC GCG CGC GAC AGC CGC 1427
Ile Ala Gly Leu Phe Arg Leu Gln Ser Asp Phe Ala Ile Ala Arg Asp Ser Arg
TCC GCG TGC GAG TGG CAG TCG TTC GTC GAC AAC CAG CCC AAG GCG CAG GCC ATG 1481
Ser Ala Cys Glu Trp Gln Ser Phe Val Asp Asn Gln Pro Lys Ala Gln Ala Met
TTC CAG TTC GTG TTC CAC GAC CTT TCC ATC TTC GGC CAG GAC ATC AAC TCG CTC 1535
Phe Gln Phe Val Phe His Asp Leu Ser Ile Phe Gly Gln Asp Ile Asn Ser Leu
GTC GAC TGC ACG GAA GTT  gtgcgtgtttctgctcctatagaacgttttagacaccaactaacca 1599
Val Asp Cys Thr Glu Val
gcaagcatag  GTT CCG ATT CCG GCA CCG CTC CAG GGC GTG ACC CAC TTC CCC GCT 1654
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GGT TTG ACC GTC AAC GAC ATC GAC CAG CCT gtacgtatttctgatgcagccctagcctgtt 1715
Gly Leu Thr Val Asn Asp Ile Asp Gln Pro
gcagtactgatcaccggctatctcggacag  TGC GTC GAG ACC CCC TTC CCC ACT CTC CCG 1775
                                Cys Val Glu Thr Pro Phe Pro Thr Leu Pro
ACT GAC CCC GGT CCC GCG ACC TCT GTC GCC CCC GT  gtaagttttcagctatgatcgat 1833
Thr Asp Pro Gly Pro Ala Thr Ser Val Ala Pro Va
atgactagtcacttactacagccttttgtttcatag  C CCC CTT CCG TGA GAGAATATATTAGAG 1897
                                      l Pro Leu Pro Stop
GCGGTAACGCAAAGCTCATTTGAGCGTTGAAGGCGCGTGATGTGGATTAAAGCTTATGACTTCGATCTGG 1967
TGGTTGTGTTTTGTTGTATGGGTACTACCGCAGCTGCGGGACGTTGTATAACGGGTATACATGATGTTGA 2037
TGATC                                                                    2042
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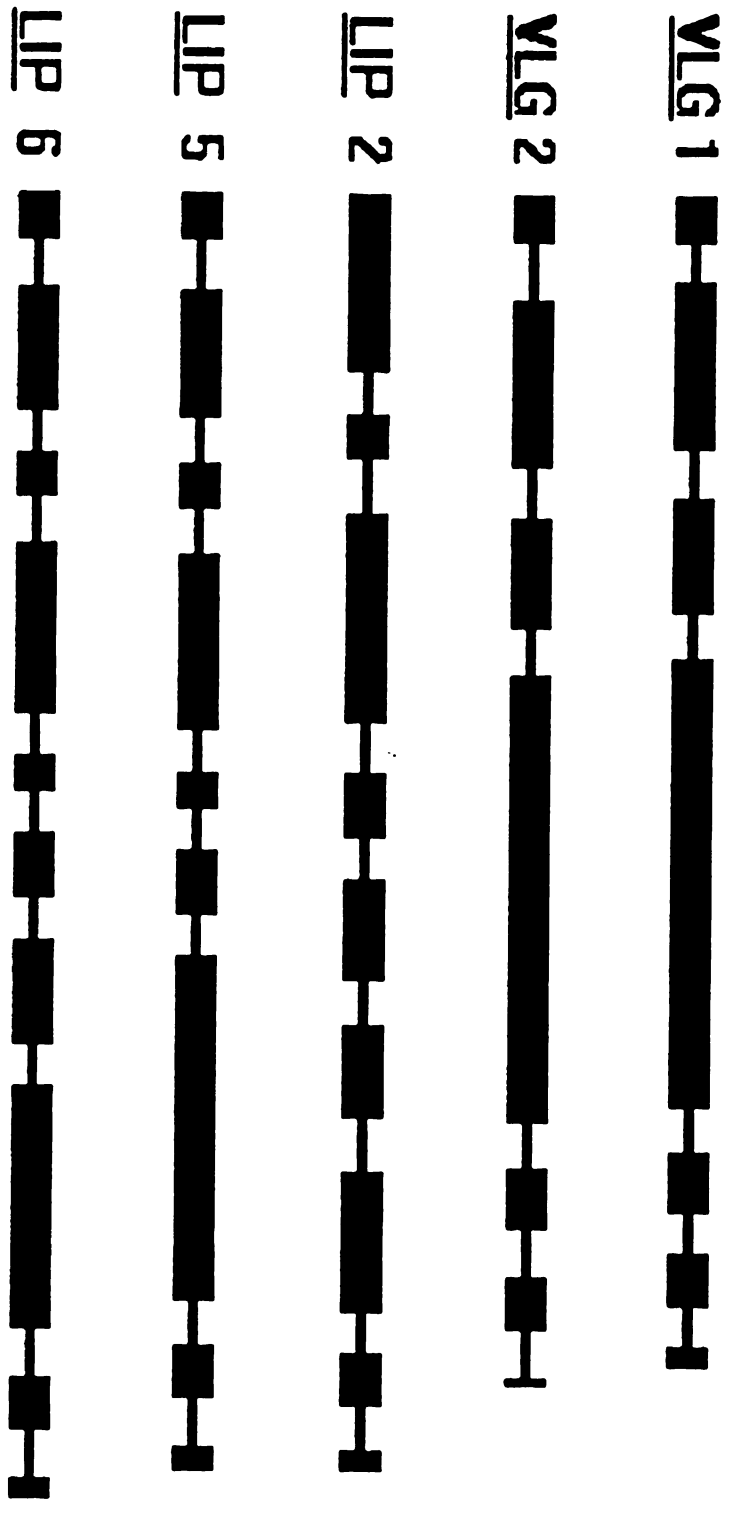
FIG. 5. Comparison of intron locations between the *T. versicolor* genes *VLG1* and *VLG2*, and the *P. chrysosporium* genes, *LIP2*, *LIP5* (6), and *LIP6* (39). Solid bars represent exon locations, solid lines represent intron locations.


LIP 6
LIP 5
LIP 2
VLG 2
VLG 1

CHAPTER III

Regulation of the Lignin Peroxidase Gene Transcripts in the White Rot Fungus *Trametes versicolor* Grown in Wood

ABSTRACT

Temporal expression of the mRNA transcript levels for the lignin peroxidase genes *VLG1-VLG6* of the white-rot fungus *Trametes versicolor*, grown in a defined basal medium with either pine (gymnosperm) or poplar (angiosperm) wood powder as the growth substrate, was studied. Transcripts of the lignin peroxidase genes *VLG2* and *VLG5* were detected in both poplar and pine wood grown cultures, though only *VLG5* transcripts were abundant during the early days of culturing on pine wood. Exogenous NH_4, glucose, and Mn(II) were added to the poplar grown cultures to study the effect of these nutrients on *lip* gene expression. Transcripts of *VLG3* could only be detected in poplar wood grown cultures when excess nitrogen was added, and not under any other experimental conditions. Also transcripts of *VLG2* and *VLG5* were found to be differentially regulated by the addition of excess glucose (56 mM) or nitrogen (22 mM) to poplar wood cultures such that *VLG2* was repressed by the addition of excess nitrogen while *VLG5* was repressed by excess glucose levels. In contrast to previous observations in defined medium, Mn(II) (at 15.4 ppm) failed to repress *VLG2* or *VLG5* transcripts in wood cultures. The differential expression of *VLG2* and *VLG5* transcripts by growth in poplar vs. pine wood, indicates that these genes are regulated by different environmental signals present in angiosperm and gymnosperm wood.

INTRODUCTION

Wood is composed of three major biopolymers: cellulose, hemicellulose, and lignin. Lignin forms a matrix with hemicellulose surrounding the cellulose fibrils (22). The lignin in wood protects the plant polysaccharides cellulose and hemicellulose from attack by pathogens (7). The complex and random nature of the linkages between the phenyl propanoid subunits of lignin make it recalcitrant to degradation by many of the soil and plant microbes (23). However, white-rot fungi, such as *Trametes versicolor* and *Phanerochaete chrysosporium*, are known to degrade all three wood biopolymers to CO_2 (7). White-rot fungi degrade softwoods (gymnosperms) that contain guiacyl lignin slower than hardwoods (angiosperms), which contain guiacyl/syringyl lignin (12, 16).

The white-rot fungi produce two sets of enzymes, lignin peroxidases (LIP) and manganese peroxidases (MNP) which are believed to be important in the degradation of the lignin polymer (14, 26). LIPs and MNPs utilize H_2O_2 to perform one electron oxidations of the aromatic carbon centers of the lignin polymer(15, 20). LIPs directly oxidize the aromatic carbon rings, while MNPs oxidize Mn(II) to Mn(III), which then performs the oxidation of lignin (29). LIPs and MNPs are only expressed in nitrogen or carbon limited defined media (10, 11). LIP activity in *P. chrysosporium* can be repressed and MNP activity enhanced by high levels (100 ppm) of Mn(II) in nitrogen limiting media (4). The cloning of *LIP* genes from *P. chrysosporium* (8, 27, 28, 30) has shown that a multigene family exists for LIPs and that LIP mRNA transcripts appear concurrently with lignin degradation (27). Furthermore, *LIP* genes are differentially regulated such that under nitrogen limitation the *LIP* genes *LIP2*, *LIP5* and *LIP6* are actively transcribed, while under carbon limitation only *LIP2* is actively transcribed (17).

An *LIP* multigene family has now been cloned from the important lignin degrading fungus *T. versicolor* (*Coriolus versicolor*). Six *LIP* genes (*VLG1* through *VLG6*) have been identified (2). This white-rot fungus also degrades lignin, and expresses LIP and MNP activity, in either nitrogen or carbon limiting media (9, 18, 19). We have studied the regulation of transcript levels for the *VLG* genes in nitrogen limiting media, and have found that the major transcript detected is that for *VLG2*. Also, in this strain of *T. versicolor*, Mn(II) levels regulate LIP activity such that greater than 13 parts per billion of Mn(II) represses LIP activity, and at 2.6 parts per million LIP activity is completely absent (3).

Wood generally contains low levels of fixed nitrogen. Since lignin surrounds the wood polysaccharides, it is possible that, carbon availability to the fungus is also limited by the presence of lignin (22). Therefore, nitrogen or carbon limitation in defined media has been assumed to realistically simulate the environment the fungi encounter when growing on wood. However, it is not known if the most highly expressed *LIP* genes in defined low nitrogen or carbon media are expressed at all in wood grown cultures. Also, the finding of LIP gene repression in both *T. versicolor* (3) and *P. chrysosporium* (4) by levels of Mn(II) normally found in wood (200 ppm), raises questions about the Mn(II) regulation of LIP genes during growth in wood. Furthermore, considering the differences in lignin content and degradation rates of angiosperm vs. gymnosperm wood, we investigated the differences in the transcription patterns of the *VLG* genes when *T. versicolor* was grown on white pine (a gymnosperm) vs poplar (an angiosperm) wood as the substrate. Also, we report on the effect of addition of excess nitrogen, carbon, or Mn(II) to *LIP* gene expression in wood-grown cultures by *T. versicolor*.

MATERIALS AND METHODS

Growth of *T. versicolor* in wood. *T. versicolor* strain (ATCC 12679) was used for all experiments, and was maintained at 4° C. on malt extract slants. Milled poplar wood (*Poplus eugini*) and milled eastern white pine wood (*Pinus strobus*) were obtained from Dr. Hans Grethlein (Michigan Biotechnology Institute, East Lansing, MI). 5.0 grams dry weight of a given milled wood was weighed out, soaked for 16 hours in 1.0 liter of double distilled H_2O at 4° C, filtered to remove the water, and stored at -20° C. The soaked wood was added to the basal medium described previously (3, 21) at 5 g (dry weight) wood per liter of medium, and sterilized by autoclaving. Except where stated otherwise, exogenous NH_4, glucose, and $MnSO_4$ were excluded completely. *T. versicolor* inoculum was grown in defined low nitrogen (2.2 mM NH_4, 56 mM glucose) medium for seven days and homogenized as previously described (3), and was added to the wood media at 10% of the final volume. The inoculated medium was mixed and 10 ml aliquots were transferred aseptically to sterile 125 ml Erlenmeyer flasks. For experiments where additional nitrogen, carbon, or Mn(II) were added, nitrogen was added as diammonium tartrate to 11 mM; carbon was added as glucose to 56 mM; and Mn(II) was added as $MnSO_4$ to 15.4 ppm.

RNA extraction, Northern blots, and enzyme assays. Ten flasks were harvested on days 4, 7, 10, 13, 16, 19, 22, and 25 after inoculation of the wood media. The contents of the ten flasks were pooled, the extracellular fluid was separated from mycelium by vacuum filtration, and the mycelium was frozen at -70° C until RNA extraction. LIP and MNP assays on the extracellular fluid were performed as described previously (25). Total RNA was extracted as described by Baker et al., (1), and 10 µg per lane was loaded (as measured by UV260 absorbance) on a 1.2% formaldehyde agarose gel for the experiments presented in Fig. 1-4, and 6. While 5 µg of total RNA was loaded per lane

for experiments presented in Fig. 5. Northern blots were prepared on nylon (Micron Separations Incorporated, Westborough, MA), UV-crosslinked at 600 J/m^2 in a Stratalinker 1800 (Stratagene, La Jolla, CA), and hybridized to random primer ^{32}P-dCTP labeled probes. Blots were hybridized at 42° C in a 50% formamide and 200 mM NaCl hybridization buffer (24). Blots were washed at high stringency (twice for 15 min each at room temperature with 5X SSC, 0.5% sodium dodecyl sulfate; twice for 15 minutes each at 37° C with 1X SSC, 1% SDS; three times for 15 minutes each at 65° C with 0.1X SSC, 1% SDS). Blots were stripped of probe as described previously (24).

RESULTS

No LIP or MNP activity has been measurable in the extracellular fluid from wood-grown cultures, possibly due to the low fungal biomass in these cultures, or because of strong adsorption of LIPs and MNPs to the wood substrates. Northern blotting analyses showed that the *VLG2* transcript (≈1.3 kb) reaches maximum abundance on days 10 and 22 (Fig. 1); however, the variation in transcript abundance seen on different days may be in part due to variations in the loading of total RNA as well. Therefore, the same blot was stripped of the *VLG2* probe and was hybridized with a ribosomal gene probe from *Neurospora crassa* (13) as a control for differences in loading of total RNA between lanes (Fig 4A). This is a better measure of the concentration of total RNA in each sample than UV260 absorbance because ribosomal RNA content within the cell is relatively constant, and therefore variations between samples would indicate differences in loading of total RNA. The results showed variation between samples, especially under loading in lane 5.

The above blot was stripped and re-hybridized, along with a northern blot prepared with identical levels of total RNA from cultures grown on pine wood, with *VLG2* in order to observe the transcription patterns in pine wood as compared to those in poplar. The results showed that the transcripts of *VLG2* are also most abundant on day 22 when the fungus is grown on pine wood instead of poplar (Fig. 2). Fig. 4B indicates that there are once again variations in loading of the total RNA from different samples of pine grown cultures. However, it should be noted that transcript levels for *VLG2* and *VLG5* peak on different days for the samples from pine wood grown cultures which suggests that the observed patterns of transcript abundance are not due to the variations in amount of RNA loaded between samples.

The pattern of appearance for *VLG5* transcripts is similar to that for *VLG2* when

poplar wood is used as the growth substrate (Fig. 3A) with peak transcript levels observed on days 10 and 22. On the other hand, the pattern of transcription of *VLG5* is different from *VLG2* when pine wood is used as the growth substrate. In pine wood, *VLG5* transcript levels are higher during early days of culturing with peak transcript levels on days 10 and 19, with the transcript levels on day 10 being higher than those on day 19. Therefore, in pine wood grown cultures, *VLG5* transcript levels are higher during the early days of culturing than during later days, while *VLG2* transcript levels are higher during the later days of growth (around 22 days) in pine wood cultures. Also, transcripts for *VLG5* are apparent in pine wood grown cultures during the early days of growth at a time when transcripts from *VLG2* cannot be detected.

In order to compare the transcript levels of different *VLG* genes, we loaded RNA samples from pine and poplar grown cultures into side by side lanes and prepared a northern blot which was then probed with all the *VLG* genes. The results indicate that the transcripts for *VLG5* are expressed early (day 10) in pine grown cultures while transcripts for *VLG2* are more abundant on day 19 (Fig. 5A and 5B). In poplar grown cultures, transcript levels for both *VLG2* and *VLG5* were higher on day 22 than on day 10. Northern blots of the pine and poplar cultures were then hybridized with the other *VLG* genes cloned from this strain of *T. versicolor*. No transcripts could be found for *VLG1*, *VLG3*, *VLG4*, or *VLG6*.

Differential regulation of several lignin peroxidase genes has been shown to occur in *P. chrysosporium* when nitrogen vs. carbon limiting defined media were used, and Mn(II) levels of 2.6 ppm or greater were shown to repress the lignin peroxidase activity of *T. versicolor* in defined low nitrogen media. Therefore, we investigated if the molecular regulation of the *LIP* genes of *T. versicolor* would be altered by addition of exogenous nitrogen, carbon, nitrogen and carbon together, or Mn(II) to cultures grown on poplar. The results showed that the addition of 22 mM NH_4 results in lower *VLG2* transcript levels as compared to the addition of 56 mM glucose (Fig. 6A, lanes 1-6) with

few differences apparent between early and late days of culturing. However, no *VLG2* transcripts were detected when high levels of both nitrogen and carbon (Fig. 6A, lanes 7-9) were added. Furthermore, in contrast to previous results obtained using defined low nitrogen media, addition of 5 ppm Mn(II) (Fig. 6A, lanes 10-12) did not repress *VLG2* transcripts.

The concentration of *VLG5* transcripts is somewhat different from *VLG2* transcripts under nitrogen and carbon repression conditions. Addition of 56 mM glucose completely represses the appearance of *VLG5* transcripts on the later days of culturing (Fig. 6B lanes 4-6), while addition of nitrogen (Fig. 6B, lanes 1-3) did not repress transcript levels as effectively. As seen for *VLG2* transcript levels, addition of 5 ppm Mn(II) did not repress *VLG5* transcripts levels (Fig. 6B, lanes 10-12). Moreover *VLG5* transcript levels were relatively constant throughout the culturing period when compared to the levels of *VLG2* transcripts.

The results show that the addition of high levels of nitrogen (22 mM) to poplar wood grown cultures of *T. versicolor* results in strong expression of *VLG3* transcripts (Fig. 6C lanes 1-3), while transcripts of *VLG3* were not observed in poplar or pine grown cultures not supplemented with nitrogen. However, supplementation of the poplar medium with glucose, glucose plus 22 mM NH_4, or Mn (II) did not result in expression of *VLG3* transcripts.

DISCUSSION

This study of the transcriptional abundance for the *lip* genes (*VLG1* to *VLG6* of *T. versicolor* grown on wood has revealed a complex pattern of expression of various *VLG* genes. Both *VLG2* and *VLG5* transcripts are seen in cultures grown in pine and poplar wood, no other *VLG* transcripts could be detected in these cultures.

Transcript levels for *VLG2* and *VLG5* appear to be differentially regulated by addition of excess nitrogen and carbon to poplar wood grown cultures. Addition of excess glucose to poplar grown cultures causes suppression of *VLG5* transcripts after day 7 (Fig. 6B lanes 4-6). Yet, addition of excess nitrogen to poplar grown cultures did not appear to suppress *VLG5* transcripts as severely as glucose addition did (Fig. 6B lanes 1-3). In contrast to this, addition of excess nitrogen to poplar wood grown cultures appeared to suppress *VLG2* transcripts more than that seen upon addition of excess glucose (Fig. 6A lanes 1-6). This result is similar to the differential gene regulation found for *P. chrysosporium* in defined medium. Under nitrogen limiting conditions the *LIP* genes *LIP2*, *LIP5*, and *LIP6* are actively transcribed, while under carbon limitation only *LIP2* is actively transcribed (17). Also, it was found that the major transcript in nitrogen limiting defined medium for *T. versicolor* was *VLG2*, while transcripts for *VLG5* were less abundant, if not absent all together (3). It would appear, therefore, that the most highly expressed *T. versicolor LIP* genes in defined low nitrogen media are also expressed in wood grown cultures, and that the genes are differentially regulated under nitrogen or carbon limiting conditions.

Transcripts of *VLG5* are present in pine wood grown cultures during the first weeks of growth, (Fig. 3B and Fig. 5B) when transcripts of *VLG2* are much less abundant, if not absent altogether. Transcripts of *VLG2* appear to become much more abundant in pine wood grown cultures between 19-25 days. In poplar wood grown

cultures both *VLG2* and *VLG5* transcripts are present during the early and the later days of culturing. Since lignin depolymerization/degradation has been shown to be the rate limiting step in the degradation of gymnosperm vs. angiosperm wood (12, 16), and lignin peroxidases have been implicated in catalyzing the initial depolymerization of the lignin polymer (26), it is interesting to find differences in the *VLG* transcript levels when the two types of wood are used as growth substrates.

The appearance of *VLG3* transcripts with high levels of added nitrogen only in wood grown cultures indicates that this gene is differentially regulated by nitrogen and carbon limitation in poplar wood grown cultures. This is somewhat analogous to previous observations with the nitrogen deregulated mutant of *P. chrysosporium*, *der8-5* (5, 6). This mutant is deregulated for nitrogen limitation such that the lignin peroxidases are induced in both low and high nitrogen media. In low nitrogen media, *der8-5* produces the normal wild-type set of lignin peroxidases but in high nitrogen media a new lignin peroxidase (LN) appeared. LN was not produced by the wild type strain of *P. chrysosporium* under low or high nitrogen conditions (5, 6).

The repression of LIP activity in both *P. chrysosporium* and *T. versicolor* by 40 ppm (4) and 2.6 ppm (3) Mn(II) concentrations, respectively, in defined media has been firmly established. The results reported here indicate that either soluble Mn(II) becomes bound by the wood in a form not available to the fungus, or that in wood grown cultures the lignin peroxidase genes are not as susceptible to repression by moderate levels of Mn(II).

The differential regulation of transcripts of *VLG2*, *VLG3*, and *VLG5* suggests that there are environmental signals inducing these genes which are separate from the global signal of nutrient starvation. That nutrient starvation is a pre-requisite to lignin peroxidase gene expression is supported by the complete suppression of *LIP* gene expression in cultures containing excess nitrogen and carbon. The appearance of *VLG3* transcripts only in wood grown cultures containing excess nitrogen indicates that specific

VLG genes respond differentially to different nutritional signals. The expression of different lignin peroxidase genes under different nutritional and environmental conditions may explain, at least in part, *LIP* gene multiplicity in *T. versicolor* (2) and *P. chrysosporium* (8, 27, 28, 30). This is exemplified by the presence of *VLG3* transcripts only under high nitrogen conditions in poplar wood. Since *VLG2* is partially repressed by nitrogen sufficiency, the discovery of a lignin peroxidase gene (*VLG3*) which is expressed in the presence of high nitrogen, and silent in low nitrogen is significant. More significantly, some of the signals may be specific to the type of wood (gymnosperm vs. angiosperm) as illustrated by the differential expression of various *VLG* genes of *T. versicolor* grown on poplar vs pine wood.

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FIG. 1. Temporal expression of the *LIP* gene, *VLG2*, of *T. versicolor* in poplar wood grown cultures. Lanes 1-8 contain 10 µg total RNA extracted, respectively, from cultures on days 4, 7, 10, 13, 16, 19, 22 and 25 after inoculation.

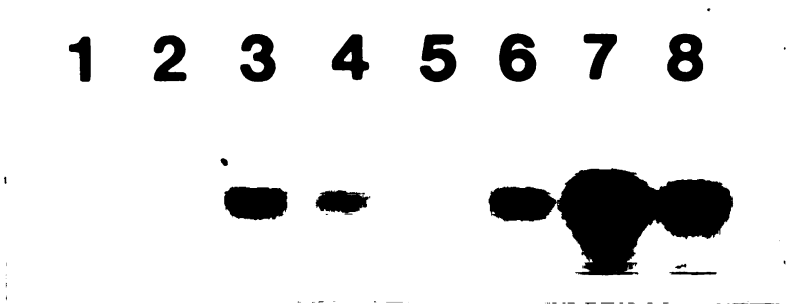

1 2 3 4 5 6 7 8

FIG. 2. Regulation of expression of the transcripts of the *LIP* gene *VLG2* of *T. versicolor* in **A.** Poplar wood medium versus **B.** Pine wood medium. In both **A.** and **B.** lanes 1-8 contained, respectively, 10 µg of total RNA extracted from cultures on days 4, 7, 10, 13, 16, 19, 22 and 25 after inoculation was loaded on a 1.2% formaldehyde agarose gel. Northern blots were prepared and hybridized to ^{32}P-CTP labeled *VLG2* (3), and subsequently washed at high stringency as described in materials and methods.

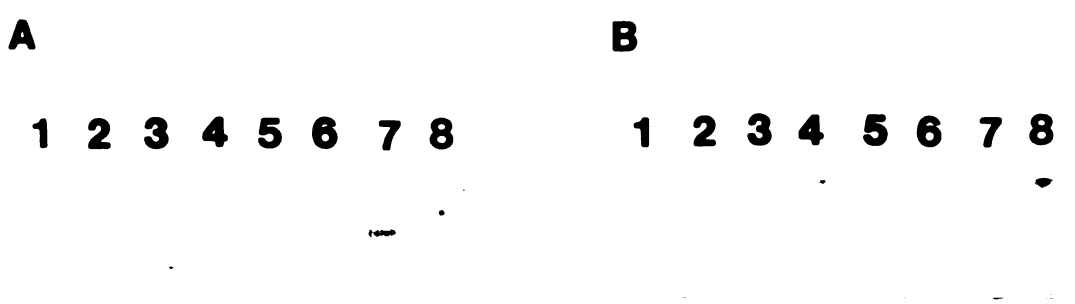

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

FIG. 3. Temporal expression of the transcripts of the *LIP* gene, *VLG5*, of *T. versicolor* in **A.** Poplar wood medium, and **B.** Pine wood medium. Blots identical to those in Fig. 2 were hybridized to labeled *VLG5* gene (2) as described in Fig. 2.

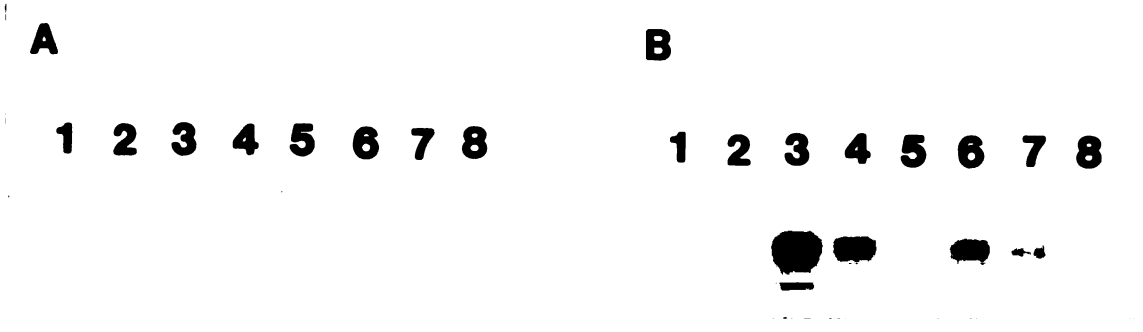

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

FIG. 4. Differences in total RNA loaded between samples of **A.** poplar wood grown cultures, and **B.** pine wood grown cultures. These blots are identical to those in Figs. 2-3 except that these were hybridized with the ribosomal gene of *Neurospora crassa* (13). Hybridization and washing conditions were identical to those in Figs. 2-3.

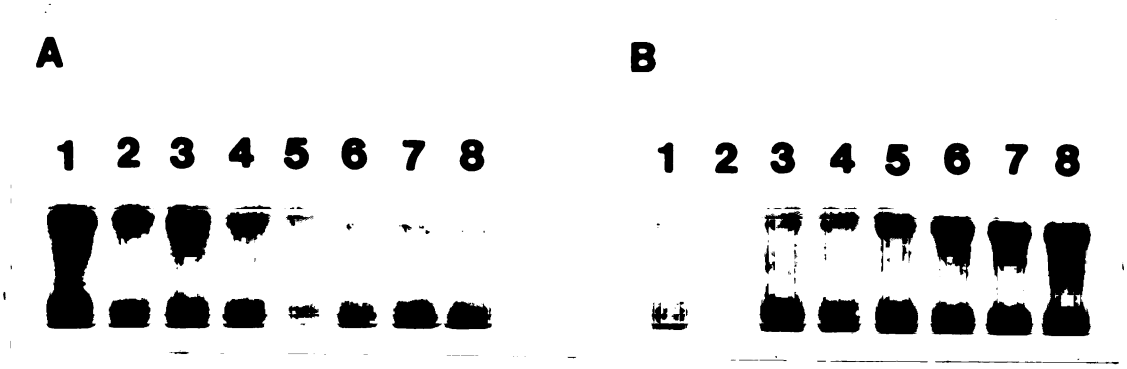

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

FIG. 5. Comparison of lignin peroxidase gene transcript levels in pine vs. poplar wood. Panels **A.** and **B.** represent identical northern blots probed, respectively, with labeled *VLG2* and *VLG5*. In each panel, lanes 1 and 2 contained 5 µg of total RNA extracted, respectively, from pine wood grown cultures of *T. versicolor* on days 13 and 19 after inoculation. Lanes 3 and 4 contain 5 µg of total RNA extracted, respectively, from poplar induced cultures on days 10 and 22 after inoculation.

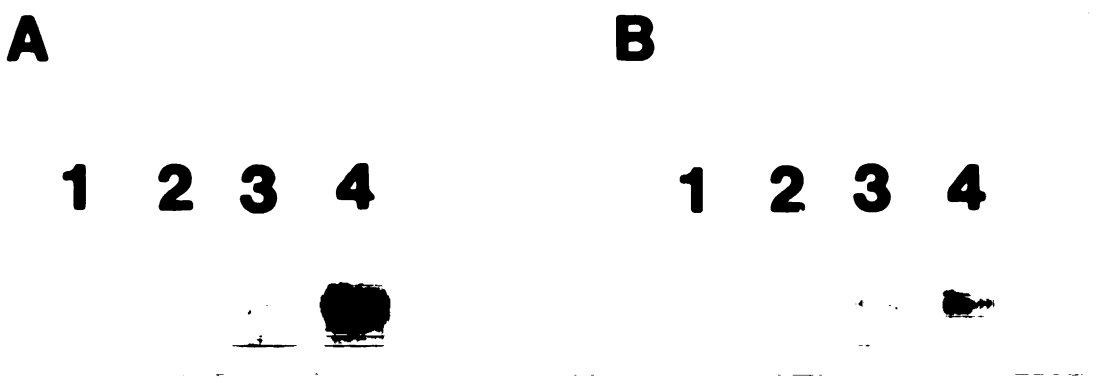
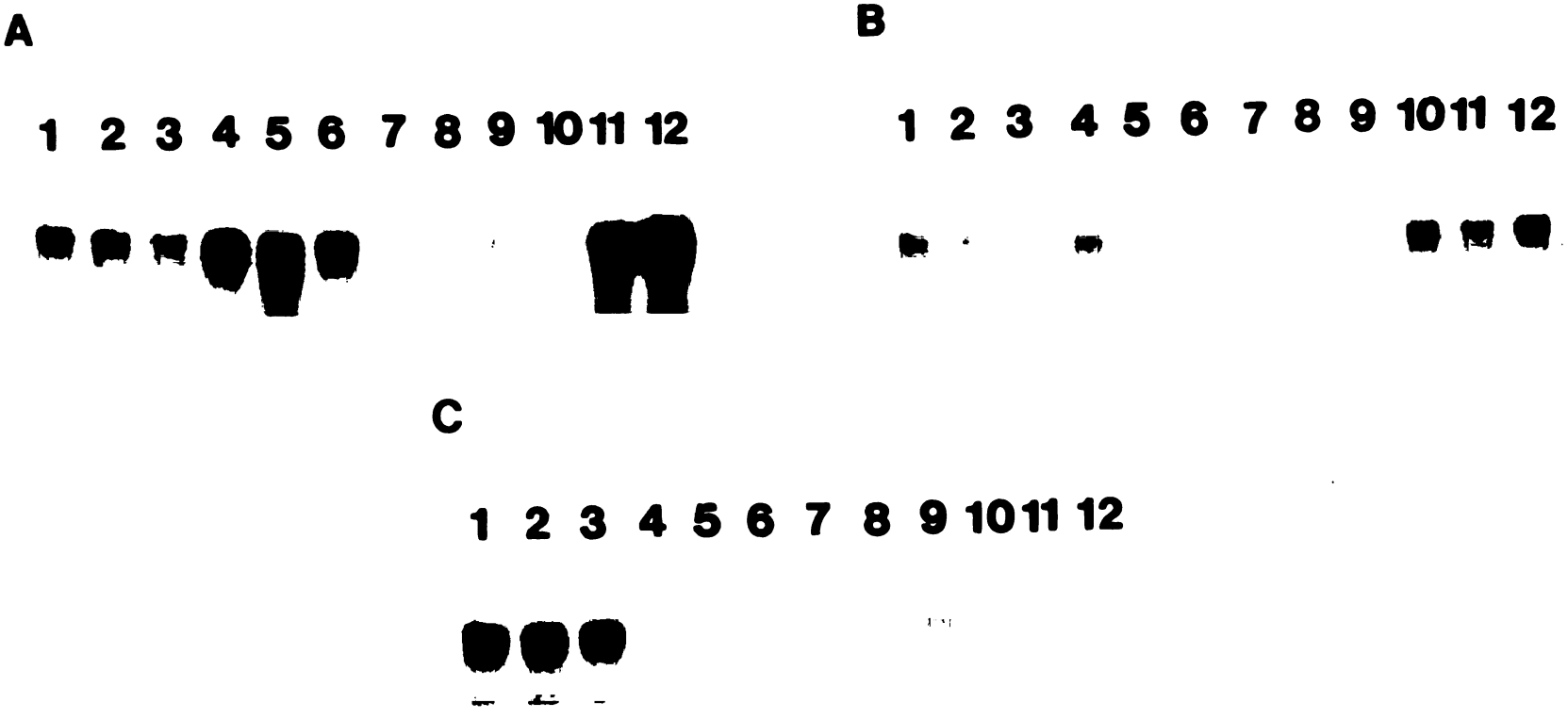

A
1 2 3 4
B
1 2 3 4

FIG. 6. Regulation of expression of lignin peroxidase genes as determined by northern blot analyses. Identical blots were probed with *VLG2*,(A.) *VLG5*, (B.) and *VLG3* (C.) Different lanes were loaded with total RNA from poplar grown cultures containing 22 mM NH_4 (lanes 1-3); 56 mM glucose (lanes 4-6); 22 mM NH_4 and 56 mM glucose (lanes 7-9); or 5 ppm Mn(II) (lanes 10-12). Cultures were harvested on day 7 (lanes 1, 4, 7 and 10); day 14 (lanes 2, 5, 8 and 11); and day 21 (lanes 3, 6, 9 and 12) after inoculation. Each lane contained 10 μg of total RNA.


A
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11 12
C
1 2 3 4 5 6 7 8 9 10 11 12

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Six lignin peroxidase genes were isolated from the white-rot fungus *Trametes versicolor* by screening a genomic EMBL3 lambda phage library with a lignin peroxidase cDNA clone, *CLG5* (1, 2) from the related white-rot fungus *Phanerochaete chrysosporium*. The genes of *T. versicolor* occurred in linked sets within the lambda clones: *VLG1* on clone 1; *VLG2*, *VLG3*, and *VLG4* on clone 2;and *VLG5* and *VLG6* on clone 3. The transcription of the genes was unidirectional within each linked set.

Sequence analysis of two of the genomic clones *VLG1* and *VLG2*, and the cDNA clone corresponding to *VLG2*, revealed substantial similarities to the structural organization of the lignin peroxidase genes of *P. chrysosporium*. The coding region of *VLG2* is interrupted by six small introns ranging in size from 51 to 68 bp. The putative intron locations for *VLG1* and *VLG2* are substantially similar. The mature proteins for both VLG1 and VLG2 are preceded by 25 and 26 amino acid signal sequences, respectively, which end with the putative proteolytic cleavage site Arg-Arg. The mature proteins coded for by both *VLG1* and *VLG2* are substantially similar, but not identical, to the LIP proteins isolated from a different strain of *T. versicolor* induced by carbon limitation conditions (3, 4). The mature protein encoded by both VLG1 and VLG2 contains 341 amino acids (M_r = 36,714). 90.0% of the codons for *VLG1*, and 83.0% of the codons for *VLG2* end in G or C. Glycosylation at the single N-glycosylation site in VLG1 and at either, or both, of the N-glycosylation sites in VLG2 would increase the molecular mass of these proteins to approximately 43-45 kd as previously reported for the lignin peroxidase enzymes isolated from *T. versicolor*.

In comparison to the *T. versicolor* genes, the coding regions of the *LIP* genes of

P. chrysosporium are interrupted by eight to nine introns ranging in size from 50-62 bp. The mature proteins are preceded by 27 to 28 amino acid signal sequences which end with the amino acids Lys-Arg. The mature proteins are 344 amino acids long (Mr of approximately 36,500 - 36,600). The codon usage for the *LIP* genes is also strongly biased towards codons that end in G or C. Glycosylation at the N-glycosylation site(s) would increase the size of the proteins to approximately 42 kd as has been measured for the lignin peroxidase enzymes isolated from *P. chrysosporium.*

Substantial stretches of conserved amino acid sequences were found in the protein encoded by *VLG1* versus those encoded by the lignin peroxidase genes of *P. chrysosporium* and *Phlebia radiata* (1, 2, 5, 6). The regions between amino acids 62-81 and 188-208 correspond to the respective distal and proximal active histidine residues already identified for peroxidases in general. However, other long stretches of homology at: 88-115, 162-174, 218-241, 273-286, and 335-354 have not had a function assigned to them. These may correspond to substrate binding sites, or sequences conserved for correct tertiary structure.

The coding sequence of *VLG1* is 79% homologous at the DNA level and 76% homologous at the protein level to *VLG2*. The VLG2 protein is only 58-63% homologous at the amino acid level to the lignin peroxidases of *P. chrysosporium* and 62% homologous to the lignin peroxidase LGP3 of *Phlebia radiata.* VLG1 is slightly less homologous than VLG2 to the LIP's of *P. chrysosporium*, with 57-61% homology at the amino acid level. But both are more homologous to *P. chrysosporium* gene LIP2 compared to the other LIP proteins of this organism.

The homology relationships between the lignin peroxidases of *T. versicolor* and *P. chrysosporium* suggest the two multigene families arose independently by gene duplication events from a common ancestral *LIP* gene. However the *lip* genes of *P. chrysosporium* are more homologous to each other (70-80% homology at the amino acid level) than they are to either *VLG1* or *VLG2* (61% and 63% respectively). This is further

substantiated by DNA-DNA hybridization studies between *LIP2* and *LIP6*, and *VLG1-VLG6* (see Appendix) which indicates that there are no separate homologs to either *LIP2* or *LIP6* amongst the *VLG* genes.

Two schools of thought currently exist as to the need for a multigene family in the white-rot fungus *P. chrysosporium*. As already described, the first theory is that each gene has a promoter controlled by a separate regulatory signal, such as nitrogen, carbon, or Mn(II), and that each regulatory system responds to different environmental signals. The second theory holds that each lignin peroxidase gene performs a different catalytic activity or has a different substrate specificity, and that differential gene expression allows only those *LIP* genes to be expressed as are needed for a particular lignin substrate. According to this a different set of *LIP* genes are expressed when *T. versicolor* (or *P. chrysosporium* or some other white-rot fungus) if grown on pine vs. poplar vs. maple vs. some other wood species. Alternatively, it is possible that both the theories proposed above may be true with regard to *LIP* gene regulation in white-rot fungi.

The discovery of two independently derived lignin peroxidase multigene families in the white-rot fungi stimulated the question as to why so many lignin peroxidase genes are necessary, and why this is a common feature in two genera of white-rot fungi? A study of the differential gene regulation of the *VLG* genes was initiated so as to find out which *VLG* genes were transcriptionally induced, and what the environmental signals for that induction were. It was found that nitrogen limiting defined media triggered the production of lignin peroxidase activity in this strain of *T. versicolor*. The appearance and levels of lignin peroxidase transcripts coincided with the appearance and levels of lignin peroxidase in the culture supernatant and the transcripts appeared immediately upon or following nitrogen limitation. VLG2 transcript was the major one present under these conditions. Also Mn(II) concentrations above 13 parts per billion were found to repress lignin peroxidase activity. Since wood is reported to have manganese levels of 5-200 parts per million, this result called into question the relevance of either; a) lignin

peroxidase activity to lignin degradation, or b) these induction conditions to those encountered during wood degradation in nature.

Study of the lignin peroxidase genes expressed by *T. versicolor* cultures grown in defined media with poplar or pine wood added as the sole source of nitrogen and carbon provided further information on the environmental signals regulating transcript levels in this fungus. Transcript levels appeared to be higher (i.e. they were easier to detect and were produced more reliably) when poplar or pine wood was used as the growth substrate than when nitrogen limiting media were used.

VLG2 transcripts appear to be produced in an undulent temporal pattern during growth on poplar wood; however, the possibility that this is due to variations in loading of total RNA cannot be totally excluded. The reason for the observed temporal variations is unknown, but could be related to a continuum of fungal transitions between primary and secondary metabolism when growing on woody substrates. *VLG2* transcript levels appear to be greater on the later peak days as compared to transcript levels on earlier days. The second major transcript in poplar wood grown cultures was *VLG5*, which appeared to follow the same pattern of undulating transcript levels. The observed inhibition of *VLG2* and *VLG5* expression in defined media even by low levels of Mn(II) was relieved by growth on poplar wood. Since the poplar wood already contained bound manganese, and the soluble exogenous Mn(II) was added to replace any Mn(II) that was lost by leaching during preparation of the wood samples, it can be concluded that the lignin peroxidases of *T. versicolor* are expressed in wood which contains Mn(II).

VLG2 and *VLG5* were differentially regulated by growth in pine wood as opposed to poplar wood. *VLG2* transcripts could not be detected during the early days of growth when a substantial increase in biomass was visually apparent. *VLG5* was the only transcript detectable up until day 19 and appeared to have a undulent induction pattern similar to both *VLG2* and *VLG5* induced by poplar wood. However peak days were separated by about nine days and *VLG5* transcript levels appeared to be decreasing as the

culture age increased.

Addition of excess nitrogen to poplar grown cultures resulted in lower levels of *VLG2* transcripts, but also allowed the expression of *VLG3* transcripts; however, this did not affect *VLG5* transcript levels. Furthermore, addition of excess glucose to poplar wood grown cultures led to a moderate decrease in *VLG5* transcript levels while *VLG2* transcript levels were not lowered. Transcripts from *VLG3* could not be detected in poplar wood grown cultures with excess glucose added. These results suggest a possible explanation for the existence of a lignin peroxidase multigene family in *T. versicolor*. It has been observed that both *VLG2* and *VLG5* transcripts are abundant in poplar wood grown cultures. However, only transcripts from *VLG5* are abundant in early age pine wood grown cultures. This indicates that poplar wood contains different induction signals than pine wood. Since pine contains a different type of lignin than poplar, the intriguing possibility is that *VLG5* has a catalytic activity, or substrate specificity that is more suited to gymnosperm lignin than is *VLG2*. This may be verified by purification of the VLG2 and VLG5 proteins and a comparative study made of their respective km and Vmax values on pine and poplar lignin or model lignin compounds. If the above hypothesis is correct, then VLG5 would have different km or Vmax values from VLG2 towards lignin model compounds which more closely resemble gymnosperm lignin than angiosperm lignin.

The need to induce lignin peroxidase activity under a wide variety of environmental conditions may also be one of the causes for *VLG* gene multiplicity. This is suggested by the presence of *VLG3* transcripts only under high nitrogen conditions in poplar wood. Since *VLG2* expression is partially inhibited in the presence of excess nitrogen, the discovery of a lignin peroxidase gene which is induced by high nitrogen, and silent in low nitrogen is significant. The fact that *VLG2* and *VLG3* are closely related, while *VLG2* and *VLG5* are more distantly related tends to support the above hypothesis.

In summary it can be concluded that transcript levels for *VLG2*, *VLG3*, and *VLG5* are differentially regulated by glucose and nitrogen levels present in poplar wood grown cultures, and by unknown factors present in pine and poplar wood. It may be possible to extract these unknown factors from pine and poplar wood, and add them to defined media. This may lead to high level, reliable induction of transcripts from *VLG2*, *VLG3*, and *VLG5*, and possibly to higher level induction of these VLG enzymes. The purification of the VLG2 and VLG5 lignin peroxidases would allow researchers to determine if there are catalytic or substrate affinity differences between these two very interesting enzymes. Since there are no homologs for either *VLG2*, or *VLG5* amongst the *LIP* genes of *P. chrysosporium* these results cannot be extended to the unanswered questions surrounding the differential gene regulation observed in *P. chrysosporium.* It would be interesting to determine if similar results could be obtained with the well studied *LIP* genes of *P. chrysosporium.*

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APPENDIX

APPENDIX I

Determination of the Homology Relationships Within the Lignin Peroxidase Multigene Family of *Trametes versicolor*, and Between the Lignin Peroxidase Subfamilies of *Trametes versicolor* and *Phanerochaete chrysosporium*

The lignin peroxidase genes of *Trametes versicolor VLG1-VLG6* can be grouped into two subfamilies based upon DNA-DNA hybridization studies (Fig. 1. A-C). Lignin peroxidase genes *VLG1*, *VLG2*, *VLG3*, and *VLG4* constitute one subfamily, while *VLG5* and *VLG6* make up a second subfamily. Interestingly *VLG2*, *VLG3* and *VLG4* are linked together within the genome, separated by 1.0-2.0 kb of non-LIP DNA. *VLG5* and *VLG6* are likewise closely linked together within the genome.

P. chrysosporium is also known to have two subfamilies of lignin peroxidase genes, represented by the *LIP* genes *LIP2*, *LIP5*, and *LIP6*. The existence of lignin peroxidase multigene families in both *P. chrysosporium* and *T. versicolor* raises the question if some of the genes between the two species might be homologs or share some other common evolutionary history. The existence of homologs between the subfamilies would be indicated by one set of *VLG* genes being more closely related to one of the *P. chrysosporium* subfamilies than to the others. Since *LIP2* and *LIP6* represent separate *P. chrysosporium* subfamilies, they were used to screen the *VLG1-VLG6* genes of *T. versicolor* for any clones which hybridized more intensely to either *LIP2* or *LIP6*. The results in Fig. 2. indicate that while *VLG1* through *VLG4* hybridize more intensely to *LIP2* than either *VLG5* or *VLG6*, *VLG1* through *VLG4* also hybridize more intensely to *LIP6*. This indicates that *VLG1*, *VLG2*, *VLG3*, and *VLG4* are more closely related to the *LIP* genes of *P. chrysosporium*. In particular *VLG3* is most closely related to the *LIP* genes *LIP2*, and *LIP6*. However, no *LIP2* or *LIP6* homologs in the *T. versicolor VLG* multigene family could be found. Furthermore the DNA-DNA hybridization studies and stringency conditions necessary to promote hybridization between the *LIP* genes and the

VLG genes indicates that all the *VLG* genes are more homologous to each other than any is to a particular *LIP* gene in *P. chrysosporium*. The collective evidence suggests that the multiple lignin peroxidase genes presently known for *P. chrysosporium* and *T. versicolor* arose by independent gene duplication events. A common ancestral gene would appear to be most homologous to the *P. chrysosporium* gene *LIP2* and the *T. versicolor* gene *VLG3*.

FIG. 1. Homology Relationships amongst the Lignin Peroxidase genomic clones of *T. versicolor*. Genomic lambda clones (lane 1, clone 2; lane 2, clone 3; and lane 3, clone 1) were cut with various restriction enzymes; lane 1, *EcoR*I and *Hind*III; lane 2, *Pst*I; and lane 3, *BamH*I; and electrophoresed in a 0.7% agarose gel in order to separate the restriction fragments containing *VLG* genes. The *VLG* genes contained in each lane, listed from the top of the lane to the bottom are: lane 1, *VLG4*, *VLG2* and *VLG3*; lane 2, *VLG6* and *VLG5*; and lane 3, *VLG1*. The resulting southern blot was hybridized to ^{32}P-dCTP labelled probes of **A.** the 2.1 kb *BamH*I fragment from lambda clone 2 containing *VLG2*, **B.** the 2.5 kb *Pst*I fragment from lambda clone 3 containing *VLG5* and, **C.** the 2.3 kb *Hind*III-*EcoR*I fragment from lambda clone 2 containing *VLG3*.

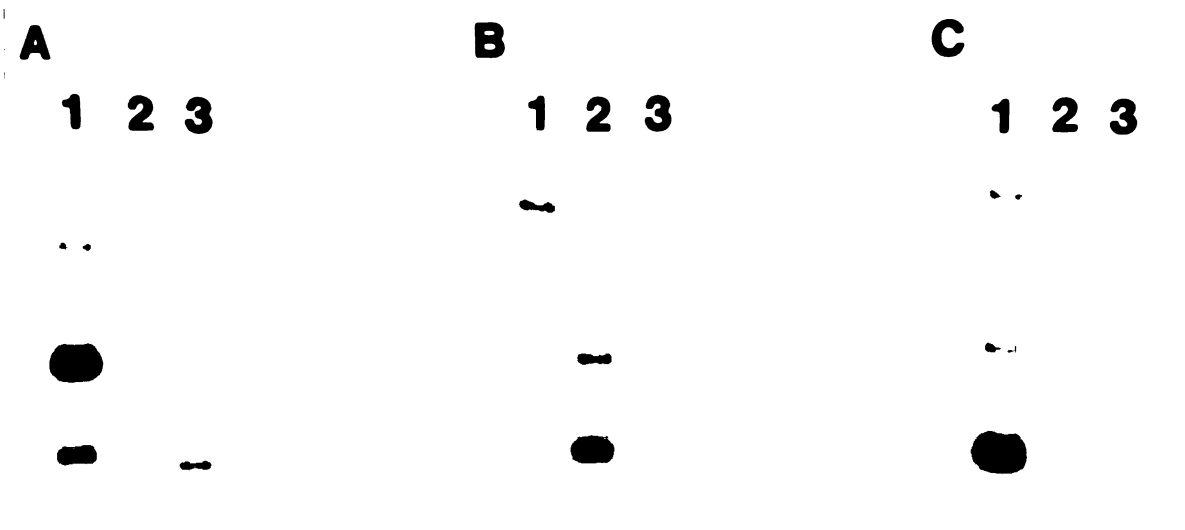
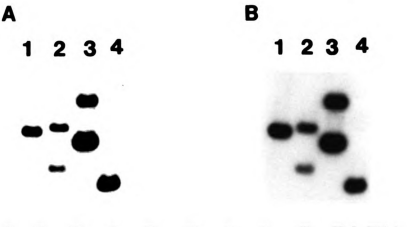

A
1 2 3
B
1 2 3
C
1 2 3

FIG. 2. Homology relationships between the *LIP* subfamilies of *P. chrysosporium* and *T. versicolor*. Genomic lambda clones (lane 1, clone 2; lane 2, clone 3; lane 3, clone 4; and lane 4, clone 1) were cut with various restriction enzymes; lane 1, *Hind*III; lane 2, *Pst*I; lane 3, *EcoR*I; and lane 4, *BamH*I; and electrophoresed in a 0.7% agarose gel in order to separate the restriction fragments containing *VLG* genes. The *VLG* genes contained in each lane, listed from the top of the lane to the bottom are: lane 1, *VLG2*; lane 2, *VLG6* and *VLG5*; and lane 3, *VLG4* and *VLG3*; and lane 4, *VLG1*. The resulting southern blot was hybridized to ^{32}P-dCTP labelled cDNA probes of *CLG4* (A.) and *CLG5* (B.) which encode, respectively, lignin peroxidases H2 and H10 isozymes of *P. chrysosporium*.


A
1 2 3 4
B
1 2 3 4