13 Enzymology and Molecular Biology of Lignin Degradation

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I. Introduction

Global conversion of organic carbon to CO_2 with concomitant reduction of molecular oxygen involves the combined metabolic activity of numerous microorganisms. The most abundant source of carbon is plant biomass, composed primarily of cellulose, hemicellulose, and lignin. Many microorganisms are capable of degrading and utilizing cellulose and hemicellulose as carbon and energy sources, however, a much smaller group of filamentous fungi has evolved with the ability to breakdown lignin, the most recalcitrant component of plant cell walls. Collectively known as white rot fungi, they possess the unique ability to efficiently degrade lignin to CO_2 in order to gain access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources. These wood-decay fungi are common inhabitants of forest litter and fallen trees. The most widely studied white rot organism, *Phanerochaete chrysosporium*, belongs to the homobasidiomycetes.

The enzymes from white rot fungi that catalyze the initial depolymerization of lignin are extracellular and unusually nonspecific. A constellation of oxidases, peroxidases, and hydrogen peroxide are responsible for generating highly reactive free radicals that undergo a complex series of spontaneous cleavage reactions. The nonspecific nature and extraordinary oxidation potential of these enzymes have attracted considerable interest for industrial applications such as biological pulping of paper, fiber bleaching, and remediation of organopollutants such as pesticides, polyaromatic hydrocarbons, PCBs and various halogenated aromatics (including dioxins), certain textile dyes, TNT, and other environmentally detrimental chemicals including cyanides, azide, carbon tetrachloride, and pentachlorophenol (for review see Cameron et al. 2000; Cullen 2002).

This review provides an overview of the physiology and genetics of lignin degradation by white rot basidiomycetes. Emphasis is on recent advances and the reader is referred to earlier comprehensive reviews for historical perspective and background (Kirk and Farrell 1987; Gold and Alic 1993; Higuchi 1993; Cullen and Kersten 1996; Cullen 1997). Recent completion of a draft genome

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sequence has established *P. chrysosporium* as the premier model system, and our review therefore concentrates on this species. Other wood decay fungi are only occasionally mentioned. Areas of uncertainty are highlighted.

II. Microbiology

Lignin is a formidable substrate (Higuchi 1990; Lewis and Sarkanen 1998). Formed through oxidation and free radical coupling of phenyl alcohol precursors, the insoluble polymer lacks stereoregularity. In contrast to hydrolysable bonds between subunits of other wood polymers (e.g., cellulose and hemicellulose), lignin degradation requires oxidative attack on the carbon—carbon and ether interunit bonds. The lignin polymer encrusts cellulose microfibrils, particularly within the secondary walls. No microbe, including white rot fungi, is known to be capable of utilizing lignin as a sole carbon or energy source, and it is generally believed that lignin depolymerization is necessary to gain access to cellulose and hemicellulose. Extracellular peroxidases and oxidases are thought to play an important role in the initial depolymerization of lignin, and small molecular weight fragments are subsequently metabolized intracellularly ultimately to water and carbon dioxide.

Only white rot basidiomycetes have been convincingly shown to efficiently mineralize lignin, although species differ in their gross morphological patterns of decay (for review see Eriksson et al. 1990; Blanchette 1991; Daniel 1994). Microscope analyses show that *P. chrysosporium* strains simultaneously degrade cellulose, hemicellulose and lignin, whereas others such as *Ceriporiopsis subvermispora* tend to remove lignin in advance of cellulose and hemicellulose. In this connection, an important consideration in understanding the mechanism(s) of degradation is that enzymes are too large to penetrate sound, intact wood (Cowling 1961; Srebotnik et al. 1988; Srebotnik and Messner 1991; Flournoy et al. 1993; Blanchette et al. 1997). Erosion from the exposed lumen surfaces through the cell wall layers should be more efficient when all components are simultaneously degraded by an array of oxidative and hydrolytic enzymes, but it is puzzling how selective delignification can occur deep into the cell wall. Blanchette et al. (1997) have shown that during decay of pine by *C. subvermispora*, the walls gradually become permeable to insulin (5.7 kDa), and then to myoglobin (17.6 kDa), but not to ovalbumin (44.3 kDa), even in relatively advanced stages of decay. As lignin-depolymerizing enzymes and many of the cellulases are in the same size range as ovalbumin, it has been proposed that enzyme-generated lignin-oxidizing species penetrate from the lumens into the walls. Evidence for diffusible oxidative species is described below.

Brown rot fungi, another category among homobasidiomycete wood decay fungi, do not degrade lignin, but merit brief mention. These fungi rapidly depolymerize cellulose, but only slowly modify lignin. Brown rot fungi are a major component of forest soils and litter and they are responsible for most of the destructive decay of wood "in service" (for review Gilbertson 1981; Worral et al. 1997). Depolymerization of crystalline cellulose appears to proceed long before wood porosity would admit cellulases, suggesting the participation of small molecular weight oxidants. Brown rot species tend to show specialization for conifers and recent molecular phylogeny suggests they have been repeatedly derived from white rot fungi (Hibbett and Donoghue 2001).

III. Physiology

Because of the complexity and heterogeneity of lignin polymers, most detailed studies on lignin degrading/modifying enzymes have used lignin model compounds (Fig. 1) to simplify the detection of catalytic activity and the characterization of reaction products. Although lignin is not required to induce the ligninolytic system, I? *chrysosporium* synthesizes veratryl alcohol, which has a lignin substituent pattern (Lundquist and Kirk 1978; Shimada et al. 1981). Ligninolysis, as determined by the mineralization of ^{14}C-lignin, is triggered by nutrient limitation (reviewed Cullen and Kersten 1996). Typically, the fungus is grown with glucose or cellulose as the carbon source and NH_4^+ as the limiting nitrogen source.

For the purposes of this chapter, the enzymology of lignin biodegradation will be limited primarily to those proteins secreted by I? *chrysosporium* under ligninolytic conditions in defined culture. Enzymes from other sources are described to indicate advances that may contribute to the understanding of *P. chrysosporium*

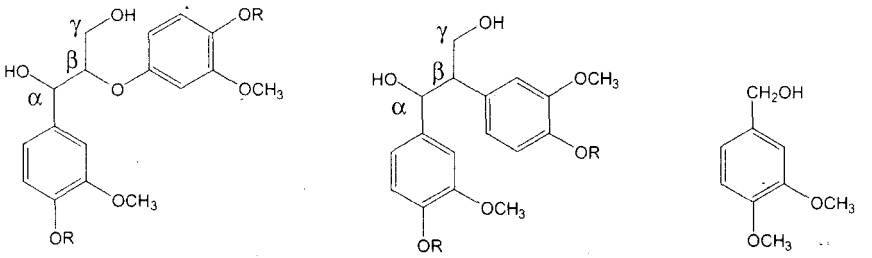

Fig. 1. Lignin model substructures. Example structures of ß-*O*-4 and ß-1 guaiacyl lignin substructures are indicated. R = H, CH_3, or continuing lignin polymer. Veratryl alcohol is a naturally occurring metabolite in *P. chrysosporium* cultures and a substrate for LiP

physiology, especially as genetic characterizations allow detailed analysis of newly discovered transcripts during lignin decay in complex medium, such as wood.

A. Peroxidases

1. Lignin Peroxidase

Lignin peroxidase (LiP) was first discovered based on the H_2O_2-dependent C_a-$C_ß$ cleavage of lignin model compounds and subsequently shown to catalyze depolymerization of methylated lignin in vitro (Glenn et al. 1983; Tien and Kirk 1983, 1984; Gold et al. 1984). Multiple isozymes of LiP are secreted by *P. chrysosporium*, and they have been categorized by their pI and order of elution from a Mono Q anion exchange column (Renganathan et al. 1985; Kirk et al. 1986; Leisola et al. 1987). Ten peroxidases are separated by Mono Q chromatography and designated H1 through H10 (Farrell et al. 1989). Six of these catalyze the prototypical reaction for LiP, the peroxide-dependent oxidization of veratryl alcohol to veratraldehyde. Growth conditions (e.g., N vs. C starved), purification methods, and storage affect relative isozymic levels. Isozymic multiplicity can be explained, at least in part, through dephosphorylation by an extracellular mannose-6-phosphatase (Rothschild et al. 1997, 1999).

LiPs are glycoproteins with molecular weights estimated at 38–46 kDa. Enzyme intermediates in the catalytic cycle of lignin peroxidase are analogous to other peroxidases; steady-state and transient-state kinetics have been studied in detail (Renganathan and Gold 1986; Tien et al. 1986; Andrawis et al. 1988; Marquez et al. 1988; Harvey et al. 1989; Wariishi and Gold 1990). The interaction of lignin peroxidase with its substrates is by a ping-pong mechanism, i.e., H_2O_2 oxidizes ferric enzyme by two electrons to give compound I (one oxidizing equivalent as an oxyferryl center and the other in the porphyrin cation radical); compound I oxidizes aromatic substrates by one electron to give compound II (a one-electron oxidized intermediate), which again oxidizes aromatic substrates to return the enzyme to resting state.

$$\text{Native (ferric) peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + S \rightarrow \text{Compound II} + S^{+\cdot}$$

$$\text{Compound II} + S \rightarrow \text{Native (ferric) peroxidase} + S^{+\cdot}$$

Although the assortment of reactions catalyzed by LiP is very complex, the initiation of these reactions is simple. LiP oxidizes the aromatic substrates (indicated as S above) by one electron; the resulting aryl cation radicals (indicated as $S^{+\cdot}$) degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactants. Production of cation radical intermediates from methoxybenzenes was conveniently detected by ESR because of the relatively long half-lives of the cation radicals (Kersten et al. 1985). Using more lignin-related compounds, Hammel et al. (1986) showed the involvement of radical intermediates by identifying radical-dimer products, as well as carbon-centered and peroxyl radical intermediates. Lip-catalyzed reactions include C_α-$C_ß$ cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and even aromatic cleavage of nonphenolic lignin model compounds (Tien and Kirk 1984; Hammel et al. 1985; Leisola et al. 1985; Renganathan et al. 1985, 1986; Umezawa et al.

1986). Detailed reviews on the radical chemistry of LiP-catalyzed reactions are provided elsewhere (Higuchi 1990; Schoemaker 1990).

The secondary metabolite veratryl alcohol is thought to play an important role as a mediator of the oxidations (Chung and Aust 1995; Goodwin et al. 1995; Koduri and Tien 1995; Khindaria et al. 1997) or maintaining an effective catalytic cycle (Koduri and Tien 1994) in oxidations of both nonphenolic and phenolic substrates by LiP. The role of the veratryl alcohol cation radical intermediate as a diffusible redox mediator is controversial and an enzyme-bound mediator is argued to be a more likely scenario (Schick and Tien 1997).

The oxidation of nonphenols by LiP produces phenolics. This explains both the depolymerization of lignin (Tien and Kirk 1983) and also the repolymerization of phenolic lignin fragments in vitro (Haemmerli et al. 1986; Odier et al. 1988). Dilute lignin dispersions and low steady-state H_2O_2 concentrations are thought to be important in minimizing bimolecular coupling of phenoxy radicals that would lead to polymerization in vitro (Hammel and Moen 1991). Glycosylation of lignin breakdown products may also be important in favoring the depolymerization reactions (Kondo et al. 1990). The importance of lignin peroxidase in depolymerization of lignin in vivo was convincingly demonstrated by Leisola et al. (1988). Addition of exogenous lignin peroxidase to carefully washed mycelial pellets greatly stimulated the conversion of ^{14}C-lignin to $^{14}CO_2$. When veratryl alcohol was added, a further stimulatory effect was observed. Horseradish peroxidase had no effect. These results suggest that the presence of mycelia may play an important role in favoring overall depolymerization by removing lignin fragments as they are released.

The crystal structure of LiP is strikingly similar to that of cytochrome c peroxidase (CCP), even though sequence identity is only approximately 20% (Edwards et al. 1993; Piontek et al. 1993). In both cases, the proximal heme ligand is a histidine that is hydrogen-bonded to a buried aspartic acid residue; the peroxide pocket is also similar with distal histidine and arginine. In contrast to CCP, which has tryptophans contacting the distal and proximal heme surfaces, LiP has phenylalanines. Furthermore, the hydrogen bonding of the heme propionate of LiP to Asp-183 (in contrast to Asn with CCP) may explain the low pH optimum of LiP (Edwards et al. 1993). Crystal structure reveals a hydroxy group on the C_β of tryptophan 171 (Choinowski et al. 1999) that is formed by autocatalysis (Blodig et al. 1998). Substitution of the Trp171 surface residue abolishes the ability of the enzyme to oxidize veratryl alcohol suggesting that Trp171 may be involved in long-range electron transfer between the natural substrates and the heme cofactor (Blodig et al. 1999; Johjima et al. 1999).

2. Manganese Peroxidase

The principal function of manganese peroxidase (MnP) is to oxidize Mn^{2+} to Mn^{3+}, using H_2O_2 as oxidant (Kuwahara et al. 1984; Paszczynski et al. 1985). Enzyme activity is typically followed colorimetrically using phenolics which are both substrates for the enzyme and are readily oxidized by Mn^{3+}. Activity of the enzyme is stimulated by simple organic acids which stabilize the Mn^{3+}, thus producing diffusible oxidizing chelates (Glenn and Gold 1985; Glenn et al. 1986). As with lignin peroxidase, the prosthetic group of MnP is iron protoporphryn IX and several isozymic forms of MnP are detected in culture (Paszczynski et al. 1986; Leisola et al. 1987; Mino et al. 1988; Wariishi et al. 1988). The 46-kDa glycoproteins do not cross-react with polyclonal antibodies raised against LiP, and the peptide mapping patterns are different from those observed with lignin peroxidase (Leisola et al. 1987). Consistent with the nomenclature used for the LiP isozymes (Farrell et al. 1989), specific MnPs identified in cultures are H3 (pI = 4.9), H4 (pI = 4.5), and H5 (pI = 4.2; Pease and Tien 1992).

Manganese peroxidase enzyme intermediates are analogous to other peroxidases (Wariishi et al. 1988, 1989). Native manganese peroxidase is oxidized by H_2O_2 to compound I, which can then be reduced by Mn^{2+} and phenols to generate compound 11. Compound II is then reduced back to resting state by Mn^{2+}, but not by phenols (Wariishi et al. 1989). Therefore, Mn^{2+} is necessary to complete the catalytic cycle and shows saturation kinetics (Wariishi et al. 1988; Pease and Tien 1992).

The oxidation of phenolics by MnP bring into question the role of the enzyme in lignin depolymerization. The biomimetic oxidation of lignin model compounds by Mn^{3+} suggests that it may play a role in oxidizing both phenolic and nonphenolic residues of lignin (Hammel et al. 1989). More recently, the in vitro partial depolymeriza-

tion of synthetic lignin by manganese peroxidase has been demonstrated (Wariishi et al. 1991). Rate constants of dimer, trimer, and tetramer phenolic lignin oligomers with compound I of MnP and LiP dramatically decreased with increasing substrate size, the effect being most dramatic with MnP (Banci et al. 1999). This suggests that Mn^{2+} is the significant physiological substrate for MnP, whereas lignin can be effectively oxidized by LiP directly.

Kinetic studies with Mn^{2+} chelates support a role for oxalate in reduction of MnP compound II by Mn^{2+}, and physiological levels of oxalate in *P. chrysosporium* cultures stimulate manganese peroxidase activity (Kuan and Tien 1993b; Kishi et al. 1994). In addition to the oxidases (reviewed below), extracellular H_2O_2 may also be generated by the oxidation of organic acids secreted by white rot fungi. Specifically, Mn-dependent oxidation of glyoxylate and oxalate generates H_2O_2 (Kuan and Tien 1993a, b; Urzua et al. 1998a, b). In the presence of Mn^{2+}, MnP also promotes the peroxidation of unsaturated lipids. Transient lipoxyradical intermediates are generated and these have been shown to oxidize nonphenolic lignin model compounds. The MnP/lipid peroxidation system depolymerizes phenolic and phenol-blocked (methylated) synthetic lignins (Bao et al. 1994; Kapich et al. 1999). The identity of the substrate lipids is under investigation, but currently unknown.

The crystal structure of manganese peroxidase shows similarity with lignin peroxidase; the active site has a proximal His ligand H-bonded to Asp, and a distal side peroxide-binding pocket consisting of a catalytic His and Arg (Sundaramoorthy et al. 1994b). In contrast to LiP which has four disulfide bonds, manganese peroxidase has five. Kinetic studies of MnP variants, derived by site-specific mutagenesis, indicate that the manganese-binding site involves Asp-179, Glu-35, Glu-39 (Kusters et al. 1995; Whitwam et al. 1997; Sollewijn et al. 1999; Youngs et al. 2001), and a heme propionate, consistent with X-ray crystallographic analysis (Sundaramoorthy et al. 1997). Site-directed mutations at F190 and D242 of MnP indicate they influence the electronic environment around the heme (Kishi et al. 1997; Whitwam et al. 1999). Calcium also plays a role in maintaining the heme environment critical for catalysis (Sutherland and Aust 1996, 1997; Sutherland et al. 1997; Timofeevski and Aust 1997).

3. Other Peroxidases

A consequence of studies on the fundamental structure—function relationships of both LiP and MnP is the engineered modification of the enzymes to catalyze new reactions. For example, a manganese-binding site has been engineered into LiP H8 (Mester and Tien 2001). Similarly, veratryl alcohol oxidizing activity has been engineered into MnP by a single amino acid change S168W (Timofeevski et al. 1999). In addition to demonstrating the importance of Trp171 in LiP, this also demonstrates that the functional distinctions between LiP and MnP may result from very minor structural features. Indeed, an enzyme with both LiP and MnP activity is secreted by *Pleurotus* and *Bjerkandera* and given the abbreviated name VP for versatile peroxidase (reviewed Martinez 2002). This suggests new possibilities for the range of peroxidases expressed by *P. chrysosporium* that may have so far gone undetected.

B. Laccase

Laccases are blue copper oxidases that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with the concomitant reduction of O_2 to H_2O (Malmström et al. 1975). Like Mn(III) chelates, they oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-C_α cleavage (Kawai et al. 1988,1989). Laccase can also oxidize nonphenolic substrates in the presence of certain auxiliary substrates such as 2,2´-azino-bis-3-ethylthiazoline-6-sulfonate (Youn et al. 1995; Bourbonnais et al. 1997, 1998; Call and Muncke 1997). Most white rot fungi produce laccases, but some do not, indicating that laccase is not absolutely required in lignin degradation. *P. chrysosporium* is one of those fungi that traditionally has been thought not to have laccase. This view has come into question with the report of laccase production in cellulose-grown cultures of *P. chrysosporium* (Srinivasan et al. 1995) and in cultures with high Cu^{2+} (Dittmer et al. 1997). Conditions are also reported where both MnP and laccase are produced (Rodriguez et al. 1999). However, the identification of laccase activity in *P. chrysosporium* cultures remains inconclusive (Podgornik et al. 2001), and very recent studies show that the genome does not contain laccase-encoding sequences (Larrondo et al. 2003; see below).

C. Glyoxal Oxidase, a Copper Radical Oxidase

An important component of the ligninolytic system of *P. chrysosporium* is the H_2O_2 that is required as oxidant in the peroxidative reactions. A number of oxidases have been proposed to play a role in this regard. However, the only one that appears to be secreted in ligninolytic cultures in liquid medium is glyoxal oxidase (GLOX). The temporal correlation of GLOX, peroxidase, and oxidase substrate appearances in cultures suggests a close physiological connection between these components (Kersten and Kirk 1987; Kersten 1990). The oxidase is a glycoprotein of 68 kDa with two isozymic forms (pI 4.7 and 4.9). Glyoxal oxidase is produced in cultures when *P. chrysosporium* is grown on glucose or xylose, the major sugar components of lignocellulosics. The physiological substrates for GLOX, however, are not these growth-carbon compounds, but apparently intermediary metabolites. A number of simple aldehyde-, a-hydroxycarbonyl-, and α-dicarbonyl compounds are oxidized by GLOX. Lignin itself is a likely source of GLOX substrates. Oxidation of a ß-O-4 model compound (representing the major substructure of lignin) by lignin peroxidase releases glycolaldehyde (Hammel et al. 1994). Glycolaldehyde is a substrate for GLOX and sequential oxidations yield oxalate and multiple equivalents of H_2O_2. The oxalate may, in turn, be a source of chelate required for the manganese peroxidase reactions described above.

The reversible inactivation of GLOX is a property perhaps of considerable physiological significance (Kersten 1990; Kurek and Kersten 1995). Glyoxal oxidase becomes inactive during enzyme turnover in the absence of a coupled peroxidase system. The oxidase is reactivated, however, by lignin peroxidase and nonphenolic peroxidase substrates. Conversely, phenolics prevent the activation by lignin peroxidase. This suggests that GLOX has a regulatory mechanism that is responsive to peroxidase, peroxidase substrates, and peroxidase products (e.g., phenolics resulting from ligninolysis). Notably, lignin will also activate GLOX in the coupled reaction with LiP.

Detailed spectroscopic studies of recombinant GLOX confirmed the redox nature for the interconversion of active and inactive forms of the enzyme (Whittaker et al. 1996). The spectroscopic studies on GLOX demonstrate that it has a free radical-coupled copper active site remarkably similar to that of galactose oxidase. The native (inactive) enzyme is activated by oxidants leading to the elimination of the cupric EPR signal consistent with the formation of an antiferromagnetically coupled radical-copper complex. An estimate of the redox potential of the GLOX radical forming site was made using absorption/potential data analyzed in terms of the Nernst equation. A midpoint potential $E_{1/2}$ = 0.42 V vs. NHE was determined. This is consistent with the requirement of relatively high potential oxidants for the activation of GLOX, such as the substrate cation radicals produced by lignin peroxidase secreted by *P. chrysosporium* (Kersten et al. 1985; Kurek and Kersten 1995). Theoretical sequence comparison of the GLOX and galactose oxidase structures, for which there are X-ray crystal data, has allowed four potential catalytic residues to be targeted for site-directed mutagenesis in recombinant protein. Biochemical and spectroscopic characterizations support the structural correlations with galactose oxidase and clearly identifies the catalytic residues in GLOX (Whittaker et al. 1999).

D. Flavin Adenine Dinucleotide Enzymes

1. Pyranose 2-Oxidase

In early studies of peroxide production by *P. chrysosporium*, two glucose oxidases were identified; glucose 1-oxidase from *P. chrysosporium* ME-446 (Kelley and Reddy 1986), and glucose 2-oxidase or pyranose 2-oxidase from *P. chrysosporium* K3 (Eriksson et al. 1986). Volc et al. (1996) addressed the question of whether the *P. chrysosporium* strains produced distinctly different glucose oxidases. They grew ME-446 and K-3 strains under three different culture conditions and found only pyranose 2-oxidase. Although the peroxide-generating enzyme pyranose oxidase is predominantly intracellular in liquid cultures of *P. chrysosporium*, there is evidence that the oxidase plays an important role in wood decay (Daniel et al. 1994). The oxidase is preferentially localized in the hyphal periplasmic space and the associated membraneous materials. Similar ultrastructural distribution is observed with manganese peroxidase, suggesting a cooperative role.

Many other wood-decay fungi in addition to *Phanerochaete* are reported to produce pyranose 2-oxidase. These include *Oudemansiella mucida*,

Trametes versicolor (Daniel et al. 1994), *Polyporus obtusus* (Ruelius et al. 1968), *Phlebiopsis gigantea* (Schäfer et al. 1996), and *Trametes multicolor* (Volc et al. 1999). In general, pyranose oxidase is a flavin adeine dinucleotide (FAD) homotetramer with subunit MW of 68–76kDA (Machida and Nakanishi 1984; Volc and Erikksson 1988; Danneel et al. 1993; Volc et al. 1999). Substrates include D-glucose, L-sorbose, and D-glucono-1,5-lactone. Pyranose 2-oxidase from *T. versicolor* oxidizes both alpha and beta anomers of glucose essentially equally well (Taguchi et al. 1985). One apparent function for intracellular pyranose 2-oxidase is the synthesis of cortalcerone involving pyranosone dehydratase (Baute and Baute 1984; Volc et al. 1991; Koths et al. 1992; Gabriel et al. 1993, 1994).

2. Aryl Alcohol Oxidase

Another strategy for peroxide generation is observed with *Bjerkandera* sp. Strain BOS55 which produces extracellular aryl alcohol oxidase (AAO), an FAD enzyme (de Jong et al. 1994). The preferred substrates are chlorinated anisyl alcohols which the fungus synthesizes de novo from glucose. The oxidation products are reduced and recycled by the fungal mycelia. LiP does not oxidize the chlorinated anisyl alcohols and thus the redox system is protected. Similarly, various *Pleurotus* species support a redox cycle supplying extracellular peroxide using AAO (or veratryl alcohol oxidase) coupled to intracellular aryl alcohol dehydrogenase (Guillén et al. 1990; Guillén and Evans 1994; Marzullo et al. 1995; Varela et al. 2000a). Studies with *Pleurotus ostreatus* indicate veratryl alcohol oxidase participates not only in lignin degradation by supplying peroxide, but also reduces quinones and phenoxy radicals and therefore may also inhibit the repolymerization of lignin degradation products (Marzullo et al. 1995). Highest affinities of the AAOs from *Pleurotus* and *Bjerkandera adusta* are against p-anisyl alcohol. In contrast, the intracellular AAO of *P. chrysosporium* has best activity with m-anisyl alcohol (Asada et al. 1995b).

3. Cellobiose Dehydrogenase

Cellobiose dehydrogenase (CDH) is widely distributed among white rot and brown rot fungi, and may play a role in carbohydrate metabolism, but also lignin degradation. The enzyme has two domains containing FAD or heme prosthetic groups; the two domains can be cleaved by *P. chrysosporium* proteases. CDH binds to cellulose and oxidizes cellodextrins, mannodextrins, and lactose. Suitable electron acceptors include quinones, phenoxy radicals, and Fe^{3+}. The biological function of CDH is uncertain. One model suggests that CDH generates hydroxyl radicals by Fenton-type reactions, thus oxidizing wood components including lignin. The possible roles of CDH has been reviewed (Henriksson et al. 2000).

E. Auxiliary Enzymes

No doubt the complete degradation of lignin requires many intracellular enzymes both for the complete mineralization of monomers to CO_2 and H_2O and for the generation of secondary metabolites (e.g., veratryl alcohol), supporting extracellular metabolism. Examples of enzymes that have been characterized from *P. chrysosporium* include methanol oxidase (Asada et al. 1995a), 1,4-benzoquinone reductase (Brock et al. 1995; Brock and Gold 1996), methyltransferases (Harper et al. 1990; Jeffers et al. 1997), a cytochrome P450 (Kullman and Matsumura 1997), L-phenylalanine ammonia-lyase (Hattori et al. 1999), 1,2,4-trihydroxybenzene 1,2-dioxygenase (Rieble et al. 1994), glutathione transferases (Dowd et al. 1997), superoxide dismutase (Ozturk et al. 1999) and catalase (Kwon and Anderson 2001). Whole genome sequence of *P. chrysosporium* should allow rapid progress in making protein—genecorrelations useful for studying the regulation of genes involved in lignin degradation.

IV. Molecular Genetics

A. Experimental Systems

Advances on the molecular genetics of white rot fungi have been made possible by an array of experimental tools. For *P. chrysosporium*, methodology has been established for auxotroph production (Gold et al. 1982), recombination analysis (Alic and Gold 1985; Raeder et al. 1989b; Krejci and Homolka 1991; Gaskell et al. 1994), rapid DNA and RNA purification (Haylock et al. 1985; Raeder and Broda 1985), differential display (Birch 1998; Kurihara et al. 2002; Assmann et al. 2003), pulsed field electrophoretic karyotyping (Gaskell et al. 1991;

D'Souza et al. 1993; Orth et al. 1994), and genetic transformation by auxotroph complementation (Alic et al. 1989,1990,1991; Alic 1990; Randall et al. 1991; Akileswaran et al. 1993; Zapanta et al. 1998) and by drug resistance markers (Randall et al. 1989,1991; Randall and Reddy 1992; Gessner and Raeder 1994; Ma et al. 2003). Transformation efficiencies are relatively low and gene disruptions are difficult (Alic et al. 1993), but reporters for studying gene expression have been described (Gettemy et al. 1997; Birch et al. 1998; Ma et al. 2001). Beyond *P. chrysosporium*, *P. ostreatus* is probably the next best white rot experimental system offering transformation protocols (Yanai et al. 1996; Honda et al. 2000; Irie et al. 2001a; Sunagawa and Magae 2002) and methodology for physical (Larraya et al. 1999) and genetic mapping (Eichlerova and Homolka 1999; Eichlerova-Volakova and Homolka 1997; Larraya et al. 2000, 2002). *T. versicolor* has also been transformed with drug resistance vectors (Bartholomew et al. 2001; Kim et al. 2002), and gene disruptions have been demonstrated (Dumonceaux et al. 2001). Aspects of the molecular biology of *P. chrysosporium* have been reviewed (Alic and Gold 1991; Pease and Tien 1991; Gold and Alic 1993; Cullen and Kersten 1996; Cullen 1997).

In a major research advance, the US Department of Energy's Joint Genome Institute (JGI) has completed whole genome shotgun sequencing of *P. chrysosporium* to 10.5 X coverage. A draft assembly of the 30 Mbp genome is freely available on an interactive annotated browser (www.jgi.doe.gov/whiterot). A homokaryotic derivative (Stewart et al. 2000) of the widely used dikaryotic laboratory strain, BKM-F-1767, was sequenced. In rough agreement with comparably sized fungal genomes (e.g., *Neurospora crassa;* Kupfer et al. 1997), approximately 8500 predicted proteins give one or more significant Smith-Waterman alignments. Thus, along with the ascomycete *N. crassa* (Galagan et al. 2003) *P. chrysosporium* is among the first available filamentous fungal genomes. In addition to their importance in understanding eukaryotic genomes and evolutionary processes, the data open whole new areas of exploration related to lignocellulose degradation. To be as current as possible, this review describes gene models recently "mined" from the current database maintained on the Joint Genome Institute's web portal. However, proteins predicted from genomic sequence should be considered tentative until verified by cDNA analysis.

B. Gene Structure and Organization

1. Peroxidases

Soon after Tien and Tu (1987) first cloned and sequenced the *P. chrysosporium* cDNA encoding LiP isozyme H8, several structurally related clones were characterized (de Boer et al. 1987; Asada et al. 1988; Brown et al. 1988; Holzbaur and Tien 1988; Smith et al. 1988; Walther et al. 1988). Initially, the exact number of genes was obscured by allelism and inconsistent nomenclature. However, subsequent analyses of single basidiospore cultures (Alic et al. 1987; Sihalch et al. 1989; Gaskell et al. 1992) allowed discrimination of allelic variants and a family of at least ten closely related genes were identified and designated *lipA* through *lipJ* (Gaskell et al. 1994). Pair-wise amino acid sequence comparisons range from 64 to 96% similarity. The gene encoding isozyme H8, *lipA*, has a predicted secretion signal cleavage site at residues 21–22 (ANA-AA) and a putative propeptide (residues 22–28; Schalch et al. 1989). Experimental support for the propeptide was provided by in vitro translation of LiP2 (=*lipE;* Ritch et al. 1991). Residues essential to peroxidase activity are conserved, i.e., the proximal heme ligand (His176 in mature *lipA* product H8) and the distal arginine (Arg43) and histidine (His47). Many of the *P. chrysosporium* genes feature a proline-rich carboxy terminus, although its significance is unknown. Recent analysis of genome data failed to identify any additional LiP genes.

Five *P. chrysosporium* MnP genes are known, two of which were recently revealed by genome sequencing. cDNAs and genomic clones had been reported for genes *mnp1*, *mnp2*, and *mnp3* (Pease et al. 1989; Pribnow et al. 1989; Orth et al. 1994; Alic et al. 1997). Gene model pc.9.126.1 corresponds to the N-terminal amino acid sequence of an MnP purified from *P. chrysosporium*-colonized wood pulp (Datta et al. 1991). Designated *mnp4*, the pc15.18.1 gene is located only 5.7kb from *mnp1*, and the two genes have nearly identical sequences. Interestingly, a cytochrome P450 gene lies in the *mnp1-mnp4* intergenic region.

Several LiP and MnP genes have been characterized from other fungal species, including *T. versicolor* (Black and Reddy 1991; Johansson 1994; Jonsson and Nyman 1992, 1994), *B. adusta* (Asada et al. 1992), and *Phlebia radiata* (Saloheimo et al. 1989). On the basis of Southern blot hybridization to the *P. chrysosporium* genes, LiP-like sequences

also appear to be present in the genomes of *Fomes lignosus* (Saloheimo et al. 1989), *Phlebia brevispora*, *C. subvermispora* (Ruttimann et al. 1992), and several other white rot fungi (Varela et al. 2000b). Beyond *P. chrysosporium*, MnP genes have been characterized in white rot fungi such as *T. versicolor*, *Dichomitus squalens*, *Pleurotus* spp., *B. adusta* and *Ganoderma applanata* (Forrester et al. 1990; Ruttimann-Johnson et al. 1994; Johansson and Nyman 1996; Perie et al. 1996; Lobos et al. 1998; Mester and Field 1998; Tello et al. 2000; Larrondo et al. 2001; Maeda et al. 2001; Johansson et al. 2002). Using degenerate primers, MnP and LiP gene fragments have been PCR-amplified from a wide range of basidiomycetes (Rajakumar et al. 1996; Chen et al. 2001). Curiously, some liplike sequences have been amplified from species producing no detectable LiP activity such as *C. subvermispora* (Rajakumar et al. 1996). Whether such sequences encode a functional LiP remains to be established, and if so, under what conditions.

Multiple alignments reveal substantial sequence conservation among the white rot peroxidase genes (Cullen and Kersten 1996; Cullen 1997; Martinez 2002). All contain 5–15 short introns (approx. 40–90nt), the number and position of which have been used to delineate families (Brown et al. 1988; Schalch et al. 1989; Ritch and Gold 1992; Gold and Alic 1993; Alic et al. 1997; Stewart and Cullen 1999). (As an aside, introns are often positioned near the N- or COOH-termini, and this has complicated gene predictions from genomic sequence.) Cladistic analysis by Martinez (2002) shows >50 invariant residues among approximately 30 known peroxidases. In general, the MnP and LiP genes fall within clearly defined clades and can be discriminated by certain key residues. As to be expected by its role in catalysis, Trp171 is common to LiPs, and Mn-binding residues (Glu35, Glu39, Asp179 in *mnp1*) are found in MnP sequences. Several *mnps* can be distinguished from *lips* by a 7–11 amino acid surface loop (Sundaramoorthy et al. 1994a; e.g., numbers 228–234 in *mnp1*) and an extended carboxy terminus. The latter insertion contains a fifth disulfide bond, not found in *lips*.

Certain peroxidases defy simple classification. Structurally unusual sequences of *Pleurotus eryngii* encode "versatile peroxidases", which have both LiP-like activities (oxidation of veratryl alcohol and an array of phenols) and MnP-like activities (Mn^{2+} oxidation; Ruiz-Duenas et al. 1999, 2001; Camarero et al. 2000). A similar enzyme has been characterized from *B. adusta* cultures although the corresponding clone has not yet been isolated. Consistent with LiP and MnP oxidations, the *P. eryngii* genes have both Trp171 and the residues involved in Mn-binding. Other unusual sequences include the *T. versicolor* LiP7 gene, which encodes lignin peroxidases isozyme LP7, but features apparent Mn-binding sites (Johansson and Nyman 1995). A structurally unique *T. versicolor* peroxidase clone, PGV, is most closely related to LiPs, but certain residues are characteristic of MnPs (Jonsson et al. 1994). The significance of the PGV sequence remains uncertain until its encoded product is identified and characterized. Another interesting *T. versicolor* clone, NPR, has characteristic Mn-binding residues, but is otherwise quite distinct from all other MnP sequences (Collins et al. 1999).

2. Laccases

As mentioned above, scant biochemical evidence supports a significant role for laccases in lignin degradation by *P. chrysosporium.* Genome data further challenge the importance of such blue copper phenol oxidases. Specifically, no conventional laccase sequences have been detected in the genome database. Instead, several distantly related sequences with weak overall similarity to iron transport ferroxidase (Fet3), ascorbate oxidase, and laccase are observed. Recently, a multicopper oxidase gene, designated *mco1*, has been shown to encode an extracellular ferroxidase (Larrondo et al. 2003). The role of these oxidases remains to be established.

The absence of conventional laccases from *P. chrysosporium* does not exclude a role in lignin degradation in related fungi. As described above, laccases oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-C, cleavage (Kawai et al. 1988). In the presence of certain mediators, the enzyme can depolymerize synthetic lignin (Kawai et al. 1999) and delignify wood pulps (Bourbonnais et al. 1997; Call and Muncke 1997), suggesting a role in lignin biodegradation. Beyond this, laccase genes, often occurring as multigene families (reviewed in Cullen 1997), are widely distributed among lignin-degrading fungi (Thurston 1994; Youn et al. 1995; Mayer and Staples 2002). White rot fungi such as *Pycnoporus cinnabarinus* efficiently degrade lignin, and in contrast to *P. chrysosporium*, secrete laccases but not peroxidases. Two laccase genes, closely related

to sequences derived from other white rot fungi, have been characterized from *P. cinnabarinus* (Eggert et al. 1998; Temp et al. 1999). Also consistent with an important role for laccase in *P. cinnabarinus,* "lac⁻" mutants are impaired in their ability to degrade ^{14}C-labeled DHP (Eggert et al. 1997).

3. Copper Radical Oxidases

Glyoxal oxidase of *P. chrysosporium* is encoded by a single gene with two alleles (Kersten and Cullen 1993; Kersten et al. 1995). The deduced amino acid sequences of allelic variants differ by a single residue (Lys308 Thr308), possibly explaining the two isozyme forms observed on isoelectric focusing gels (Kersten and Kirk 1987; Kersten 1990). Database searches indicated no striking homology with any other genes/proteins or copper-binding domains, but Bork and Doolittle (1994) identified a 50-residue "kelch" motif in glyoxal oxidase and galactose oxidase. On the basis of catalytic similarities with *Dactylium dendroides* galactose oxidase, potential copper ligands were tentatively identified at Tyr377 and His378 (Kersten and Cullen 1993). Subsequent studies also implicated Tyr135, Tyr70, and His471 in the active site (Whittaker et al. 1999). Surprisingly, Blast analysis of the genome has revealed six sequences with low overall sequence homology to *glx* (<50% amino acid similarity), but with highly conserved residues surrounding the catalytic site.

4. Flavin Adenine Dinucleotide Oxidases

Genes encoding CDH have been cloned from several fungi including the white rot fungi *P. chrysosporium* (Raices et al. 1995; Li et al. 1996), *T. versicolor* (Dumonceaux et al. 1998), and *P. cinnabarinus* (Moukha et al. 1999). Sequences are highly conserved. All share a common architecture with separate FAD, heme, and cellulose binding domains (CBD), although the latter domain has no obvious similarity to functionally similar bacterial or fungal CBDs. (Interestingly, the CBD domain of a CDH from ascomycete *Sporotrichum thermophile* shows sequence similarity to the CBDs of *Trichoderma* and *Phanerochaete* cellobiohydrolases and endoglucanases; Subramaniam et al. 1999). Li et al. (1997) characterized both allelic variants of *P. chrysosporium cdh.* No evidence for CDH gene multiplicity has been reported in any fungi. The heme ligands of *P. chrysosporium* CDH have been confirmed by site-specific mutagenesis (Rotsaert et al. 2001). As mentioned above, the role of CDH in lignin degradation remains uncertain, and CDH⁻ gene disruptions are unaffected in their ability to degrade synthetic lignin (Dumonceaux et al. 2001).

Genes encoding FAD oxidases include aryl alcohol oxidases (AAO) of *Pleurotus* (Marzullo et al. 1995; Varela et al. 1999, 2000a, c) and a pyranose oxidase from *Coriolus* (*Trametes*) *versicolor* (Nishimura et al. 1996). With the exception of the oxidase domain of the CDH gene, extracellular FAD-dependent oxidases have not been characterized in I! *chrysosporium.* Nevertheless, at least three separate AAO-like sequences, all with predicted secretion signals, have been identified in the genome database. The role of these genes in lignin degradation remain to be determined (Ander and Marzullo 1997), but when viewed together with the copper radical oxidase genes, it is clear that *P. chrysosporium* possesses an impressive array of genes encoding extracellular oxidative enzymes.

5. Other Enzymes

Posttranslational processes regulate extracellular enzyme activity and contribute to isozyme multiplicity, but to date little progress has been made at the genetic level. Proteolytic processing of LiP has been shown in *P. chrysosporium* (Eriksson and Pettersson 1982; Dosoretz et al. 1990a,b; Datta 1992; Dass et al. 1995; Feijoo et al. 1995) and *T. versicolor* (Staszczak et al. 2000) cultures, and extracellular dephosphorylation of certain *P. chrysosporium* LiP isozymes is well established (Kuan and Tien 1989; Rothschild et al. 1997, 1999). Proteases have also been implicated in regulating cellulase (Eriksson and Pettersson 1982) and CDH (Eggert et al. 1996) activity. Although protease or phosphatase genes have not yet been reported in the literature, the genome database offers substantial opportunities for rapid progress. Current gene models include several proteases with likely secretion signals and at least one corresponds to an EST clone similar to aspartyl protease of the basidiomycetous yeast *Phaffia rhodozyma* (Bang et al. 1999). Based on previously published N-terminal sequence of a pulp-derived protease (Datta 1992), our lab has cloned the corresponding cDNA. Similarly, we have recently isolated a cDNA encoding a mannose-6-phosphatase possibly involved in dephosphorylation.

C. Genomic Organization

Genetic linkage in *P. chrysosporium* has been established by restriction fragment length polymorphisms (RFLPs; Raeder et al. 1989a, b) and later refined by PCR-based segregation analysis (Gaskell et al. 1994). Genetic maps are entirely consistent with physical distances established by Southern blotting of CHEF gels (Gaskell et al. 1991; Covert et al. 1992a; Stewart et al. 1992; Kersten et al. 1995), walking in genomic libraries (Huoponen et al. 1990; Gaskell et al. 1991; Stewart and Cullen 1999) and recent genome assemblies.

Gene multiplicity and gene clustering are common features of the *P. chrysosporium* genome. Detailed maps of cellobiohydrolases (*cbh1*; Covert et al. 1992a, b) and *lip* (Stewart and Cullen 1999) clusters have been constructed. The genome data have revealed additional multiplicity and clusters such as the two *mnps* mentioned above. In addition, four multicopper oxidases distantly related to laccases lie on the same scaffold (Larrondo et al. 2003). Most surprisingly, three copper radical oxidase genes (*cro*) were uncovered within a cluster of LiP genes (Fig. 2). The clustering of *lip* and cro genes seem consistent with a physiological connection between peroxidases and peroxide-generating oxidases.

All ten *lips* and *glx* reside on chromosomes which are dimorphic with respect to CHEF gel migration: (1) *lipA, lipB, lipC, lipE, lipG, lipH, 1ipI,* and *lipJ* hybridize to a 3.5/3.7-mb pair; (2) *lipD* hybridizes to a 4.8/4.4-mb pair; (3) *lipF* hybridizes to a 1.8/2.0-mb pair; and *glx* hybridizes to a 3.7/3.9-mb pair (Gaskell et al. 1991, 1994; Stewart et al. 1992; Gaskell and Cullen 1993; Kersten et al. 1995). These dimorphisms are mitotically stable in strain BKM-F-1767, but banding patterns rearrange substantially following meiosis, presumably due to recombination. Simultaneous resolution of all bands has not been achieved under a single set of electrophoretic parameters (Gaskell et al. 1991; Covert et al. 1992a; D'Souza et al. 1993).

Homologous chromosomes differing in electrophoretic mobility have been observed in numerous eukaryotes such as *Plasmodium falciparum* (Corcoran et al. 1986, 1988), dozens of fungi (reviewed in Zolan 1995), and most recently in the related white rot fungus *P. ostreatus* (Larraya et al. 1999). Mechanism(s) giving rise to these chromosome length polymorphisms (CLPs) and their possible role in genetic variability are not well understood. Intrachromosomal recombination between repetitive sequences of the same orientation would generate shortened chromosomes, as would unequal exchange between homologues. In her review, Zolan (1995), describes translocation models in which repeated sequences on nonhomologous chromosomes might recombine.

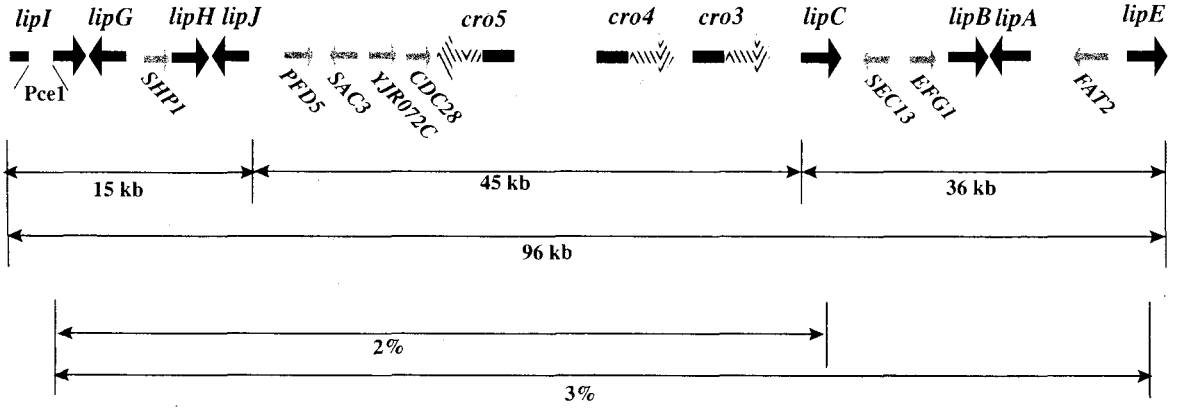

Fig. 2. Gene models and transcriptional orientation within the major lignin peroxidase gene (*lip*) cluster. Physical (kb) and genetic (% recombination) distances are shown below. Genes encoding copper radical oxidases (*cro3, cro4, cro5*) feature repeated N-terminal WSC domains. Lignin peroxidase gene nomenclature is as described (Gaskell et al. 1994). *lipI* is transcriptionally inactivated by repeat element Pcel (Gaskell et al. 1995). *Gray arrows* indicate gene models with substantial similarity (Blast E value <10−5) to *S. cerevisiae* genes: *SHP1* SHP1 protein (regulator of phosphoprotein phosphatase 1); *PFD5* prefoldin subunit 5; *SAC3* leucine permease transcriptional regulator; *YJR072* hypothetical protein (ATP binding); *CDC28* cell division control protein (kinase); *SEC13* protein transport protein (component of COPII); *EFG1* elongation factor G; *FAT2* peroxisomal coenzyme A synthetase

Repetitive elements of *P. chrysosporium* have been associated with several genes encoding extracellular enzymes, but to date there is no clear evidence for a role in generating CLPs. The most thoroughly studied element is Pcel, a nonautonomous class II repeat inserted within LiP allele lip12 (Gaskell et al. 1995). The 1747-nt sequence transcriptionally inactivates lip12 and three copies are distributed on the same chromosome. The sequence flanking these copies shows no evidence of recombination (Stewart et al. 2000). In addition to Pcel-like elements, a broad array of noncoding repetitive sequences and putative mobile elements have been identified in the genome database. Short repeats (<3kb) not clearly associated with transposons vary in copy number from >40 (GenBank accession number 231724) to 4 (AF134289-AF134291). Several putative transposase-encoding sequences resemble class II transposons of Ascomycetous fungi such as *Aspergillus niger* Ant, *Cochiobolus carbonum* Fot1, *Nectria* "Restless", *Fusarium oxysporum* Tfo1, and *Cryphonectria parasitica* Crypt1 (for review see Kempken and Kuck 1998). Additional transposase-encoding sequences include EN/Spm- and TNP-like elements that are common in higher plants, but previously unknown in fungi. Fungal class II elements often exceed 50–100 copies per genome, but interestingly, the corresponding *P. chrysosporium* transposases are represented by only one to four copies of each.

A substantial number of multi-copy retrotransposons are identifiable in the database, some of which seem likely to impact expression of genes related to lignin degradation. Typical of these elements, they often appear truncated and/or rearranged, and the long terminal repeats, typical of retroelements, often lie apart as "solo LTRs" (Kim et al. 1998; Goodwin and Poulter 2000). Several non-LTR retrotransposons, similar to other fungal LINE-like retroelements, were also identified. Copia-like retroelements are particularly abundant, and in one case, the element interrupts a cytochrome P450 gene within its seventh exon (gene model 24.16.1). A similar situation was observed for an extracellular phenol oxidase gene, where a *Skippy*-like gypsy retroelement has inserted within the twelfth exon. Coding regions flanking these inserts seem intact suggesting recent transpositions and/or splicing of the elements. Another *gyspy*-like element is inserted 100 nt upstream of the hybrid peroxidase gene mentioned above.

D. Gene Regulation

1. Peroxidases

Steady state transcript levels of *P. chrysosporium* LiP genes are dramatically altered by culture conditions. Northern blot analysis by Holzbaur and Tien (1988) showed that under carbon limitation, *lipD* transcripts dominated and *lipA* transcripts were not detected. Under nitrogen limitation, *lipA* was the most abundant transcript and *lipD* expression was relatively low. Extending these early studies, competitive RT-PCR and nuclease protection assays were employed for quantitative differentiation of the closely related transcripts (Stewart et al. 1992; Reiser et al. 1993). Transcript levels of the ten known LiP genes have been measured in defined media (Stewart et al. 1992; Reiser et al. 1993; Stewart and Cullen 1999), organopollutant contaminated soils (Bogan et al. 1996a) and in colonized wood (Janse et al. 1998). These investigations have shown that differential regulation can exceed five orders of magnitude and that transcript profiles in defined media poorly predict profiles in complex substrates. Patterns of expression show no clear relationship with genome organization. A report suggesting that nitrogen limitation regulates LiP expression post-translationally by heme processing (Johnston and Aust 1994) has been contradicted by Li et al. (1994).

Manganese peroxidase production in *P. chrysosporium* is dependent upon Mn concentration (Bonnarme and Jeffries 1990; Brown et al. 1990). Quantitative transcript analyses of the three known *P. chrysosporium* MnP genes generally show coordinate regulation in colonized soil and wood (Bogan et al. 1996c; Janse et al. 1998). Putative metal response elements (MREs) have been identified upstream of *P. chrysosporium mnp1* and *mnp2* and their transcript levels increase substantially in response to Mn^{2+} supplementation of low nitrogen media (Pease and Tien 1992; Gettemy et al. 1998). Transcript levels of *P. chrysosporium* genes lacking paired MREs, *mnp3,* are not influenced by addition of Mn^{2+} (Brown et al. 1990,1991; Gettemy et al. 1998). In aggregate, these observations suggest an important role for MREs in transcriptional regulation of *P. chrysosporium* MnP gene (Alic et al. 1997; Gettemy et al. 1998). In contrast, *T. versicolor* MnP regulation appears not to involve MREs. Putative MREs have been identified in *T. versicolor mnp1* (Johansson and Nyman 1993), but not in *T. versicolor mnp2*. Mn-

dependent upregulation of *mnp2* (Johansson et al. 2002) must be governed by other means. Another exceptional *T. versicolor* gene, *npr*, appears to be repressed by Mn even though putative Mn-binding residues are present in the sequence (Collins et al. 1999).

2. Laccases

Laccase genes are often differentially regulated and the patterns of regulation differ substantially between species (Wahleithmer et al. 1995; Yaver and Golightly 1996; Yaver et al. 1996; Smith et al. 1998; Palmieri et al. 2000; Soden and Dobson 2001). Transcripts of *P. radiata* laccase are readily detected under N-limited, ligninolytic conditions (Saloheimo and Niku-Paavola 1991). In *Trametes villosa, lcc1* is strongly induced by 2,5-xylidine addition to cultures, while *lcc2* transcript levels remain unchanged. In contrast, Northern blots failed to detect *lcc3, lcc4,* and *lcc5* transcripts under any conditions (Yaver and Golightly 1996; Yaver et al. 1996). Three *Rhizoctonia solani* laccases (*lcc1, lcc2, 1cc3*) are transcribed at low constitutive levels which can be further repressed by the addition of p-anisidine to cultures. However, *R. solani lcc4* is expressed at much higher levels and induced by additions of p-anisidine. In *R. solani, lcc1, lcc2, lcc3* are clustered, but separate from *lcc4* suggesting a relationship between genomic organization and transcriptional regulation (Wahleithmer et al. 1995). Transcriptional induction by copper and other metals is well established (Karahanian et al. 1998; Palmieri et al. 2000; Soden and Dobson 2001; Galhaup et al. 2002).

3. Copper Radical Oxidases

Consistent with a close physiological relationship between GLOX and LiP, *glx* transcript appearance in defined media (Stewart et al. 1992; Kersten and Cullen 1993), soil (Bogan et al. 1996b) and in wood chips (Janse et al. 1998) is coincident with *lip* and *mnp.* Transcript profiles of the newly discovered *cro* genes (Fig. 1) have not been systematically examined.

4. Cellobiose Dehydrogenase and Other Flavin Adenine Dinucleotide Oxidases

Northern blots show upregulation of *cdh* in cellulose-containing media (Li et al. 1996; Moukha et al. 1999), and competitive RT-PCR revealed transcripts in *P. chrysosporium* colonized wood (Vallim et al. 1998). Transcripts of *cdh* are not detectable in N- or C-limited defined media commonly used to induce peroxidases and glyoxal oxidase. Culture conditions for production of pyranose-2-oxidase, veratryl alcohol oxidase and glucose oxidase have been described, but nothing is known of their transcriptional regulation (Muheim et al. 1990; Daniel et al. 1994; Volc et al. 1996; Ander and Marzullo 1997; Varela et al. 1999). Genes encoding extracellular FAD-dependent oxidases have only been recently identified in the *P. chrysosporium* genome and their regulation has not been studied.

E. Expression in Heterologous Hosts

Fundamental biochemical investigations have been hampered in some instances by difficulties purifying native isozymes and/or development of efficient heterologous expression systems. The fungal peroxidases have been particularly problematic. *Saccharomyces cerevisiae* expression systems yield no extracellular and little or no intracellular apoprotein (Pease and Tien 1991). Attempts to express *P. radiata* LiPs in *Trichoderma reesei* gave only transcripts and no protein when placed under the control of the highly expressed and inducible *cbh1* promoter (Saloheimo et al. 1989). Recovery and reconstitution of active peroxidases from *Escherichia coli* initially met with little success (reviewed in Pease and Tien 1991), but techniques are now available for recovery of MnP (Whitwam et al. 1995; Miyazaki and Takahashi 2001; Reading and Aust 2000, 2001) and LiP (Doyle and Smith 1996; Nie et al. 1998, 1999) from inclusion bodies.

Baculovirus systems have been used to produce active recombinant MnP isozyme H4 (Pease et al. 1991) and LiP isozymes H2 (Johnson et al. 1992) and H8 (Johnson and Li 1991). Although yields are relatively low, improvements have been made (Lin et al. 1997) and baculovirus production may be useful for experiments requiring limited quantities of recombinant protein,, e.g., site-specific mutagenesis. In contrast, highly efficient secretion of active *P. chrysosporium* MnP isozyme H4 has been demonstrated in *Aspergillus oryzae* (Stewart et al. 1996). Expression was under the control of the *A. oryzae* TAKA amylase promoter, and like the baculovirus system, addition of hemin to the cultures increased yields substantially

(Stewart et al. 1996). The secreted MnP is fully active, and the physical and kinetic properties of the recombinant protein were similar to the native protein. Attempts to express *P. chrysosporium* LiP genes in *Aspergillus* has not yielded active enzyme (Stewart et al. 1996; Conesa et al. 2000). Most recently, a *Pichia pastoris* system has been successfully used to produce active MnP (Gu et al. 2003), although some glycosylation was observed.

A "homologous expression" system, in which *mnp* or *lip* transcriptional control is placed under the glyceraldehyde-3-phosphate dehydrogenase promoter, temporally separates production of the recombinant protein from other peroxidases (Mayfield et al. 1994; Sollewijn Gelpke et al. 1999). Homologous expression can also be driven under the control of the promoter of the translational elongation factor (Ma et al. 2003). The approach has been successfully employed in various biochemical investigations including structure function studies of MnP (Kusters-van Someren et al. 1995; Sollewijn Gelpke et al. 2000) and LiP (Sollewijn Gelpke et al. 2002). A similar system of "homologous expression" has been developed for production of MnP in *Pleurotus* using a native *P. ostreatus* promoter (Irie et al. 2001b). An MnP gene from *D. squalens* (Li et al. 2001) has also been expressed in *P. chrysosporium* under the control of the *gpd* promoter.

In contrast to peroxidases, the heterologous expression of fungal laccases has been straightforward. The *A. oryzae* TAKA amylase system has been successfully used for the production of *T. villosa, R. solani,* and *Coprinus cinereus* laccases (Wahleithmer et al. 1995; Yaver et al. 1996, 1999). *T. versicolor* laccases have been produced using *P. pastoris* (Jonsson et al. 1997; O'Callaghan et al. 2002) and S. *cerevisiae* (Cassland and Jonsson 1999) systems. Good yields of a *P. cinnabarinus* laccase were obtained in both *A. niger* (Record et al. 2002) and *P. pastoris* (Otterbein et al. 2000), although the latter system tends to overglycosylate the product. The *P. radiata* laccase was efficiently expressed in *T. reesei* under the control of the *T. reesei cbh1* promoter (Saloheimo and Niku-Paavola 1991). The *Coriolus* (*Trametes*) *hirsutus* laccase gene was expressed in S. *cerevisiae* (Kojima et al. 1990).

Glyoxal oxidase is efficiently expressed in *Aspergillus nidulans* under the control of the *A. niger* glucoamylase promoter (Kersten et al. 1995). Under maltose induction, fully active GLOX was secreted by *A. nidulans* at levels 50-fold greater than optimized *P. chrysosporium* cultures, and subsequent yield improvements were obtained using *P. pastoris* (Whittaker et al. 1999). Site-specific mutagenesis enabled production of recombinant GLOX isozymes corresponding to the native allelic variants (Kersten et al. 1995). FAD-dependent enzymes have been successfully expressed in *Aspergillus* (Varela et al. 2001) as well as the "homologous" *P. chrysosporium* system (Li et al. 2000; Rotsaert et al. 2001).

V. Conclusions

Given their pivotal role in the carbon cycle, it is perhaps surprising that the mechanism(s) of lignin degradation remain unsettled. This is especially remarkable considering the demonstrated potential of white rot fungi in environmentally benign bioprocesses such as fiber bleaching, biopulping, and organopollutant degradation. Major obstacles to progress include difficulties working with recalcitrant substrates such as lignin and deficiencies in white rot fungi as experimental systems.

Nevertheless, considerable progress has been made over the past 10 years. The development of heterologous expression systems, site-specific mutagenesis and crystallography have substantially advanced our understanding of structure—function relationships among the peroxidases. Further contributions include progress in understanding the number, structure, genomic organization and transcriptional regulation of genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase.

The prospects for rapid progress are encouraging particularly with the completion of the *P. chrysosporium* genome. Future investigations will undoubtedly focus on systematic transcript profiling using microarray approaches. Large scale proteomics projects are imminent (Hernandez-Macedo et al. 2002). As these studies continue to elucidate the genes and enzymes potentially involved in the degradation of lignin and related organopollutants, future investigations will focus on their functionality. The precise roles and interactions of these genes will be established by gene disruption, heterologous expression, and subcellular localization experiments.

In addition to addressing long-standing questions regarding lignin degradation, functional

genomics will illuminate fundamental aspects of the molecular biology of fungi. The origin and nature of gene multiplicity and chromosome length polymorphisms, well-established features of the *P. chrysosporium* genome, are of general interest in eukaryotic systems. Global comparisons of *P. chrysosporium*, *S. cerevisiae* and *N. crassa* may offer clues on the factors governing filamentous growth as well as the specific genes defining the major phylogenetic division between ascomycetes and basidiomycetes.

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