5 Molecular Genetics of Lignin-Degrading Fungi and Their Applications in Organopollutant Degradation

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I. Introduction

This chapter provides an overview of the physiology and associated molecular genetics of wood-decaying fungi as they relate to organopollutant degradation. White-rot fungi are characterized by an ability to fragment all major structural polymers of wood including lignin. More poorly understood are the brown-rot fungi, which rapidly depolymerize cellulosic materials while only modifying lignin. Collectively, these wood- and litter-degrading fungi play a pivotal role in the carbon cycle. In fact, white-rot fungi are the only microbes known to efficiently mineralize fully lignified tissue.

The wood-rotting fungi are obligate aerobes, deriving their nourishment from the biological combustion of wood and associated materials, using molecular oxygen as a terminal electron acceptor. The focus here is on the non-specific extracellular enzyme systems of white-rot fungi. These oxidative enzymes have been shown to transform and degrade an array of organopollutants in addition to depolymerizing lignin. Intracellular metabolism of small molecular weight products is also covered, although relatively little is known of these processes. This chapter does not comprehensively review the voluminous literature on fungal degradation of lignin and related organopollutants. Recent developments are emphasized, and the reader is referred to previous review articles (Kirk and Farrell 1987; Eriksson et al. 1990; Gold and Alic 1993; Hammel 1995b; Cullen and Kersten 1996; Cullen 1997; Kirk and Cullen 1998; Cameron et al. 2000; Pointing 2001) for more detailed information. Areas of uncertainty are highlighted.

II. Physiology of Wood-Decaying Fungi

Lignin is a formidable substrate. The polymer is large and initial depolymerization must be extracellular. The structure is comprised of inter-unit carbon—carbon and ether bonds that demand oxidative rather than hydrolytic degradative mechanisms. In addition, the polymer's lack of stereoregularity requires **ligninolytic agents** that are less specific relative to the hydrolytic enzymes attacking cellulose. White-rot fungi have met these challenges through the evolution of extracellular peroxidases and oxidases that act non-specifically via the generation of unstable lignin free radicals, which undergo a variety of spontaneous cleavage reactions. The major enzymes acting directly or indirectly on lignin are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Fig. 5.1). All three enzymes can act with low molecular weight mediators to

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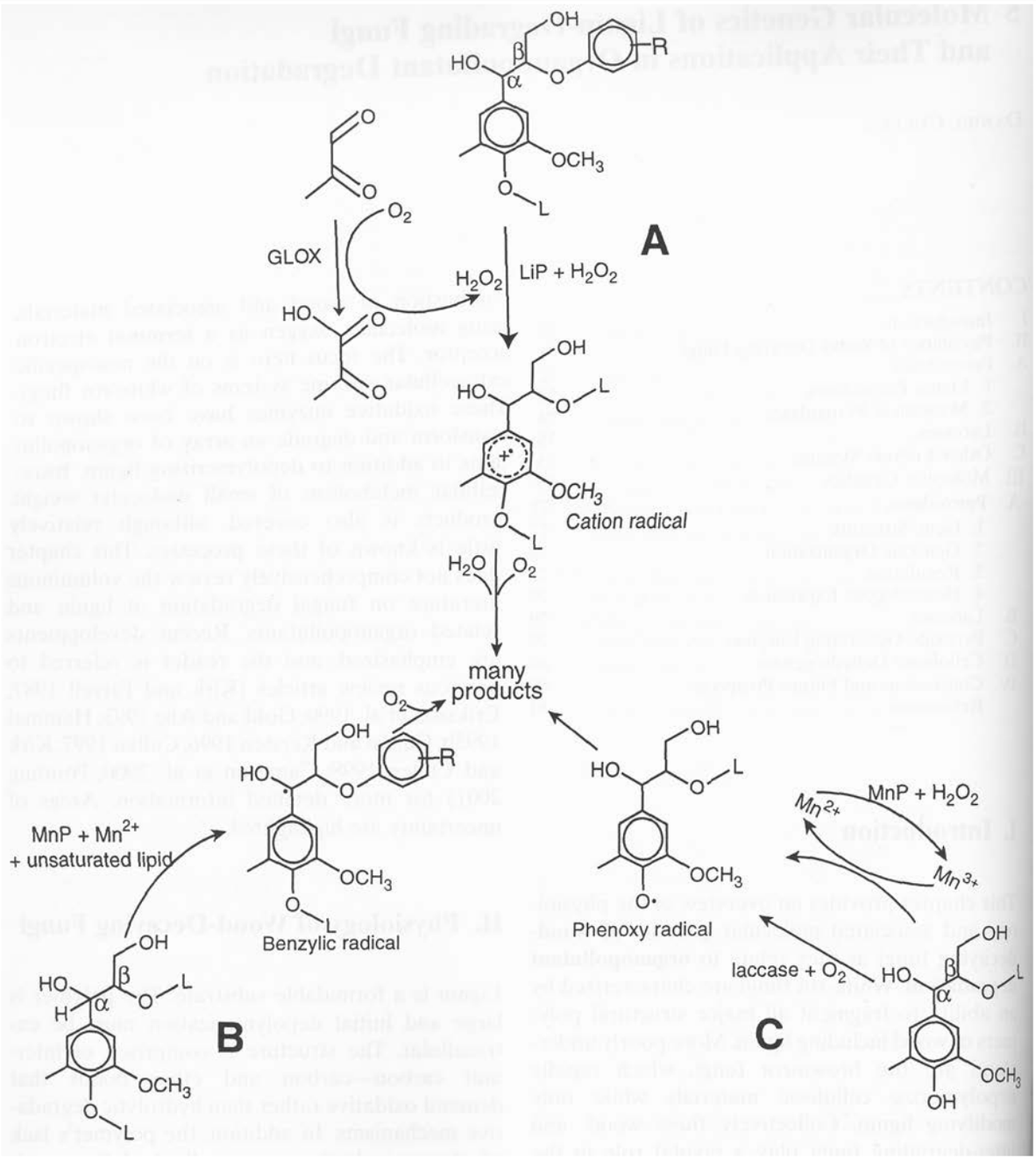

Fig. 5.1. Extracellular oxidative enzyme systems of *P. chrysosporium* and related white-rot fungi. Lignin peroxidase (LiP) and manganese peroxidase (MnP) coupled lipid peroxidation oxidize *ß*-O-4 models as shown in reactions **A** and **B,** respectively. Laccases and Mn(III) oxidize phenols (C)

bring about oxidation of lignin as well as an impressive array of xenobiotics (Table 5.1). The microbiology and physiology of ligninolytic fungi have been reviewed (Kirk and Farrell 1987; Eriksson et al. 1990; Gold and Alic 1993; Higuchi 1993; Tuor et al. 1995; Cullen and Kersten 1996).

A. Peroxidases

1. Lignin Peroxidases

LIP was first discovered based on H2O2-dependent $C\alpha—C\beta$ cleavage of lignin model compounds and subsequently shown to catalyze the partial

Table 5.1. Examples of organopollutants degraded by wood-decaying fungi PCBs polychlorinated biphenyls; PAHs polycyclic aromatic hydrocarbons

Species	Organopollutant	References(s)
Berkandera adusta	Azo dyes	Heinfling et al. (1998)
	Phenyl urea herbicides	Khandrani et al. (1999)
	PCBs	Beaudette et al. (1998)
	PAHs	Field et al. (1992); Kotterman et al. (1998a,b)
Cerioporiopsis subvermispora	PCBs	Ferrey et al. (1994)
	Anthracene	Krivobok et al. (1998)
	Fluorene	Garon et al. (2000)
Coriolus versicolor	4-Methyldibenzothiophene	Ichinose et al. (1999)
	Textile dyes	Kapdan et al. (2000)
Corolopsis gallica	PAHs	Pickard et al. (1999)
Corolopsis polyzona	PCBs	Novotny et al. (1997)
Gloeophyllum striatum[a]	2,4,-Dichlorophenol	Schlosser et al. (2000)
	Pentachlorophenol	Fahr et al. (1999)
Gloeophyllum trabeum[a]	Pentachlorophenol	Fahr et al. (1999)
	Polyethylene glycol	Kerem et al. (1998); Kerem and Hammel (1999)
Irpex lacteus	2,4,6-Trinitrotoluene	Kim and Song (2000)
Kuehneromyces mutabilis	Phenathrene and pyrene	Sack and Fritsche (1997); Sack et al. (1997a)
Laetiporus sulphureus[a]	Phenanthrene and pyrene	Sack and Gunther (1993); Sack et al. (1997a)
Nematoloma frowardii	PAHs	Sack and Gunther (1993); Sack et al. (1997 a-c)
	2,4,6-Trinitrotoluene	Scheibner and Hofrichter (1998)
Phanerochaete chrysosporium	Alkyl halide insecticides	Kennedy et al. (1990)
	Chloroanilines	Sandermann et al. (1998)
	DDT	Bumpus and Aust (1987)
	2,4-Dichlorophenol	Valli and Gold (1991)
	Heterocyclic dyes	Cripps et al. (1990)
	Endosulfan (cyclodiene)	Kullman and Matsumura (1996)
	Various PAHs	Bumpus 1989; Hammel et al. (1991); Vazquez-Duhalt et al. (1994); Bogan et al. (1996a); May et al. (1997)
	PCBs	Bumpus et al. (1985); Eaton (1985; Thomas et al. (1992); Dietrich et al. (1995); Yadav et al. (1995); Novotny et al. (1997)
	Pentachlorophenol	Hammel and Tardone (1988); Mileski et al. (1988); Lamar et al. (1990); Aiken and Logan (1996); Reddy and Gold (1999, 2000)
	Trichloroethylene	Yadav et al. (2000)
	2,4,5-Trichlorophenol	Joshi and Gold (1993)
	2,4,6-Trichlorophenol	Armenante et al. (1994); Reddy et al. (1998)
	2,4,6-Trinitrotoluene	Fernando et al. (1990); Sublette et al. (1992); Hodgson et al. (2000)
Phanerochaete laevis	PAHs	Bogan and Lamar (1996)
Phanerochaete sordida	Creosote	Davis et al. (1993); Lamar et al. (1994)
	Penta chlorophenol	Lamar and Dietrich (1990); Lamar et al. (1990a,b,1993)
	Dioxins	Takada et al. (1996)
Phlebia radiata	2,4,6-Trinitrotoluene	Van Aken et al. (1999)
Phlebia tremellosa	PCBs	Ferrey et al. (1994)
Pleurotus eryngii	Azo dyes	Heinfling et al. (1998)
Pleurotus ostreatus	Industrial dyes	Vyas and Molitoris (1995); Rodriguez et al. (1999)
	PCBs	Beaudette et al. (1998)
	Phenanthrene	Bezalel et al. (1996)
	Phosphorothiolates	Amitai et al. (1998)
	Various PAHs	Martens and Zadrazil (1998); Novotny et al. (1999); Baldrian et al. (2000)
Pleurotus pulmonarius	Benzo[a]pyrene	Maspahy et al. (1999)
Pycnoporus cinnabarinus	Triclosan	Hundt et al. (2000)
Trametes hirsutus	Lindane	Singh and Kuhad (1999)
Trametes hispida	Industrial dyes	Rodriguez et al. (1999)
Trametes versicolor	PAHs	Majcherczyk et al. (1988); Collins et al. (1996); Johannes et al. (1996); Johannes and Majcherczyk (2000)
	PCBs	Novotny et al. (1997); Beaudette et al. (1998)
	Triclosan	Hundt et al. (2000)

[a]Brown-rot fungi. All others are classified as white-rot fungi.

depolymerization of methylated lignin in vitro (Glenn et al. 1983; Tien and Kirk 1983,1984; Gold et al. 1984; Fig. 5.1A). Lignin peroxidase catalyzes a variety of oxidations, all of which are dependent on H_2O_2. Simply stated, **LiP oxidizes aromatic nuclei of substrates** by one electron and the resulting aryl cation radicals degrade spontaneously via many reactions dependent on substrate structure and on the presence of reactants (Harvey et al. 1985; Kersten et al. 1985; Shoemaker et al. 1985; Hammel et al. 1986b). Reactions subsequent to cation radical formation are complex. The chemistry of Lip-catalyzed reactions has been reviewed (Higuchi 1990).

Direct involvement of LiP has been demonstrated in the degradation of a wide range of organopollutants. **Polycyclic aromatic hydrocarbons** (PAHs) with ionization potentials below approximately 7.6 eV, including benzo[a]pyrene, anthracene, and pyrene, are substrates for LiP (Hammel et al. 1986a). Similarly, free radical mechanisms involving purified LiP have been shown for chlorinated phenols (Hammel and Tardone 1988; Mileski et al. 1988; Valli and Gold 1991; Reddy and Gold 2000), tetrahydrofurans (Vazquez-Duhalt et al. 1994), dioxins (Hammel et al. 1986a; Valli et al. 1992b), methoxybenzenes (Kersten et al. 1985), and various chloro- and nitromethoxybenzenes (Valli and Gold 1991; Valli et al. 1992a,b; Teunissen et al. 1998). The biochemistry of organopollutant degradation has been reviewed (Higson 1991; Hammel 1995a,b).

Isozymic forms of *Phanerochaete chrysosporium* LiPs are commonly distinguished by their pI and order of elution from a MonoQ anion-exchange column. Ten peroxidases have been separated by this method and are designated H1 through H10. Six of these, H1 (pI 4.7), H2 (pI 4.4), H6 (pI 3.7), H7 (pI 3.6), H8 (pI 3.5), and H10 (pI 3.3), have veratryl alcohol oxidation activity (Renganathan et al. 1985; Kirk et al. 1986; Leisola et al. 1987; Farrell et al. 1989). Analytical isoelectric focusing resolved 15 proteins with LiP activity (Leisola et al. 1987). The number of isozymes observed depends on the growth conditions (e.g. nitrogen- versus carbon-limited), the means of purification, and storage conditions. Proteolysis, dephosphorylation and other post-translational modifications contribute to isozyme multiplicity (Eriksson and Pettersson 1982; Kuan and Tien 1989; Dosoretz et al. 1990a,b; Datta 1992; Dass et al. 1995; Feijoo et al. 1995; Rothschild et al. 1997, 1999). Although the various LiPs have similar substrate specificities towards aromatic substrates, their kinetic behavior varies more clearly when *tert*-butyl hydroperoxide is substituted for H_2O_2 (Farrell et al. 1989; Glumoff et al. 1990). The processing, biological roles and interactions among isozymes are poorly understood.

The precise role of LiPs in complex substrates such as wood and organopollutant-contaminated soils remains uncertain. As mentioned above, extracellular peroxidases are physically too large to enter the pores of the cell wall (reviewed by Blanchette et al. 1997). Thus, LiP has been proposed to act indirectly by oxidizing low molecular weight substrates, which in turn penetrate the wall and oxidize the polymer (reviewed by Hammel 1996). Specifically, the secondary metabolite **veratryl alcohol,** which is synthesized and secreted by *P. chrysosporium* and which is oxidized by LiP to a cation radical (Khindaria et al. 1995), was proposed to play this role (Harvey et al. 1986). However, the veratryl alcohol cation radical is too short-lived to function as a diffusible mediator (Khindaria et al. 1995). Recent studies show LiP is capable of oxidizing **ferrocytochrome c** and synthetic lignin at the protein surface by a long-range electron transfer mechanism (Wariishi et al. 1994; Johjima et al. 1999).

2. Manganese Peroxidases

Low molecular weight diffusible oxidants could be provided by **manganese peroxidases** (MnPs; Glenn et al. 1986; Paszczynski et al. 1986). Like LiPs, MnPs are glycosylated and have acidic pIS and pH optima. Also like LiPs, they have a conventional peroxidase catalytic cycle, but with Mn(II) as the substrate. The Mn(II) must be chelated by bidentate organic acid chelators such as glycolate or oxalate, which stabilize the product Mn(III) and promote its release from the enzyme. The resulting Mn(III) chelates are **diffusible oxidants** that can act at some distance from the enzyme. However, they are not strong oxidants and cannot attack the non-phenolic units of lignin. Instead, they oxidize phenolic structures (Fig. 5.1C), which make up about 10% of the units in lignin. The phenoxy radicals resulting from the one-electron oxidation undergo a variety of reactions, some of which result in polymer cleavage within certain units, e.g., between the aromatic rings and $C\alpha$ (Wariishi et al. 1991; Tuor et al. 1992; Fig. 5.1C). Purified MnPs from cultures of *P. chrysosporium, Nematoloma frowardi,* and

Phlebia radiata oxidize **xenobiotics** such as pentachlorophenol and 2,4,6-trinitrotoluene (TNT) in an Mn-dependent manner (Scheibner and Hofrichter 1998; Van Aken et al. 1999; Reddy and Gold 2000). In contrast, azo dye decolorization by *Pleurotus eryngii* and *Bjerkandera adusta* MnP isozymes is Mn-independent (Heinfling et al. 1998).

It seems unlikely that the MnP-catalyzed reaction should result in the extensive depolymerization of lignin or the efficient oxidation of organopollutants of relatively high ionization potential. However, certain efficient **lignin degraders,** including *Ceriporiopsis subvermispora, Phanerochaete sordida,* and *Dichomitus squalens,* lack detectable LiP but produce MnP (Perie and Gold 1991; Ruttimann et al. 1992, Ruttimann-Johnson et al. 1993, 1994; Hatakka 1994; Orth et al. 1994). Also, phenanthrene and fluorene are not LiP or MnP substrates and yet both are efficiently degraded by *P. chrysosporium* cultures (George and Neufield 1989; Hammel et al. 1992; Vazquez-Duhalt et al. 1994; Bogan et al. 1996a). These studies point to **alternative mechanisms,** and recent research shows that the production of diffusible oxyradicals by MnP can occur by a mechanism other than via chelates of Mn(III). In the presence of Mn(II), MnP promotes the peroxidation of unsaturated lipids. Transient lipoxyradical intermediates are generated and these have been shown to oxidize non-phenolic lignin model compounds (Fig. 5.1B). The **MnP/lipid peroxidation system** depolymerizes phenolic- and phenol-blocked (methylated) synthetic lignins (Bao et al. 1994), and oxidizes fluorene (Bogan et al. 1996a), phenanthrene (Moen and Hammel 1994), and ß-O-4 linkages within lignin model compounds (Bao et al. 1994; Kapich et al. 1999). Taken together, these data suggest that lipid peroxidation may be a component of the lignin-degrading system of certain fungi. The identity of the substrate lipid(s) is under investigation (Enoki et al. 1999), but currently unknown.

MnP/lipid peroxidation models aside, LiP remains the only fungal oxidant known that can efficiently mimic, in vitro, the $C\alpha$—$C\beta$ cleavage and extracellular cleavage of aromatic rings that is characteristic of ligninolysis by white-rot fungi. Recently identified peroxidases defy simple classification as MnP or LiP, and can oxidize Mn(II) as well as non-phenolic substrates (e.g., veratryl alcohol) in the absence of manganese (Mester and Field 1998; Camarero et al. 1999).

B. Laccases

Laccases are **blue copper oxidases** that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates. Like Mn(III) chelates, they oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl–$C\alpha$ cleavage (Kawai et al. 1988; Fig. 5.1C). Most white-rot fungi produce laccase but some do not, indicating that laccase is not absolutely required in lignin degradation. Isozyme multiplicity is common among basidiomycetes including *C. subvermispora* (Fukushima and Kirk 1995; Salas et al. 1995), *P. chrysosporium* (Srinivasan et al. 1995; Dittmer et al. 1997; Rodriguez et al. 1997), and *D. squalens* (Perie et al. 1998).

Laccase can oxidize non-phenolic lignin-related substrates in the presence of certain auxiliary substrates such as ABTS **(2,2'-azino-bis-3-ethylthiazoline-6-sulfonate;** Collins et al. 1996: Bourbonnais et al. 1997, 1998). For example, purified *P. ostreatus* laccase efficiently degrades organophosphorus insecticides and related nerve agents in the presence of ABTS (Amitai et al. 1998). High ionization potential PAHs are also oxidized by *C. gallica and T. versicolor* laccases in the presence of synthetic mediators (Johannes et al. 1996; Pickard et al. 1999). *Pycnoporus cinnabarinus* and *Trametes versicolor* cultures produce small molecular weight compounds that may act as natural intermediaries for oxidation of non-phenolic lignin substructures (Eggert et al. 1996) and PAHs (Johannes and Majcherczyk 2000). The structure and function of fungal laccases have been reviewed (Thurston 1994; Youn et al. 1995).

C. Other Enzyme Systems

Although numerous laboratory studies show extracellular peroxidases and laccases capable of oxidizing various xenobiotics, it is clear that a **multitude of additional extracellular and intracellular processes** must be involved in the transformation and/or mineralization of these compounds. For examples, O-methylation of PCP and triclosan has been observed in *P. chrysosporium* (Lamar and Dietrich 1990) and *P. cinnabarinus* (Hundt et al. 2000) cultures, respectively. Glycosyl conjugation of triclosan has also been shown in *T. versicolor* cultures (Hundt et al. 2000). Membrane-bound and intracellular enzymes have

received relatively little attention to date, but **cytochrome P-450 monooxygenases** have been implicated in bioconversions of phenanthrene by *P. ostreatus* (Bezalel et al. 1996), benzo[a]pyrene by *Pleurotus pulmunoarius* (Maspahy et al. 1999), endosulfan by *P. chrysosporium* (Kullman and Matsumura 1996), and 4-methyldibenzothiophene by *Coriolus versicolor* (Ichinose et al. 1999). In the case of 2,4,6-trichlorophenol (Reddy et al. 1998) and PCP (Hammel and Tardone 1988; Mileski et al. 1988; Reddy and Gold 1999,2000) degradation by *P. chrysosporium*, extracellular LiP or MnP may intitially catalyze oxidative dechlorination and the products subsequently undergo various intracellular reductive dechlorination and hydroxylation reactions.

Organopollutant degradation by **brown-rot** fungi may involve extracellular oxidative mechanisms that are **fundamentally different** from white-rot species. *Gloeophyllum* spp. and related brown-rot fungi lack detectable LiP, MnP, or laccase activities but nevertheless efficiently degrade 2,4 dichlorophenol (Fahr et al. 1999; Schlosser et al. 2000), PCP (Fahr et al. 1999), and polyethylene glycol (PEG; Kerem et al. 1998; Kerem and Hammel 1999). Hydroxyl radical, formed by a Fenton-type reaction of Fe(II) and H_2O_2, has long been suspected as the powerful oxidizing agent needed to rapidly depolymerize cellulose during brown rot (Koenigs 1974a,b). Analysis of metabolites and the identification of Fe(II) and H_2O_2 in *Gleophyllum* cultures support a Fenton-type degradation (Hyde and Wood 1997; Kerem et al. 1998; Kerem and Hammel 1999; Paszczynski et al. 1999; Schlosser et al. 2000).

Several systems have been suggested as potential sources of extracellular H_2O_2 for peroxidase activity and hydroxyl generation (Fig. 5.1A). One likely source of H_2O_2 for *P. chrysosporium* LiPs is glyoxal oxidase (GLOX; Kersten and Kirk 1987). GLOX is a **radical-copper oxidase** (Whittaker et al. 1996) that utilitizes a wide variety of small aldehydes such as glyoxal and methylglyoxal (extracellular metabolites of *P. chrysosporium*) and transfers the electrons to O_2, generating H_2O_2. Another substrate, glycolaldehyde, is produced by the action of LiP on *ß*-O-4 lignin substructures. Perhaps the most interesting property of GLOX, and of considerable physiological significance, is that in the absence of a peroxidase system, the oxidase is reversibly inactivated (Kersten 1990). The enzyme is reactivated, however, by reconstituting the complete peroxidase system, including both LiP and substrate. This suggests that the supply of H_2O_2 by GLOX is responsive to the demand of the peroxidases, thereby providing an extracellular regulatory mechanism for control of the coupled enzyme systems. GLOX is also produced by other white-rot fungi, although apparently not by all (Hatakka 1994; Orth et al. 1994).

Another likely candidate for generating H_2O_2 in some white-rot fungi, including *P. chrysosporium* and *B. adusta*, is **aryl alcohol oxidase** (AAO; Muheim et al. 1990; Asada et al. 1995b). This enzyme oxidizes benzyl alcohols to aldehydes, transferring the electrons to O_2, producing H_2O_2. Interestingly, *B. adusta* secretes chlorinated benzyl alcohols that are not substrates for LiP but are for the AAO. Because *B. adusta* also secretes LiP, this strategy circumvents the non-productive oxidation of the AAO substrate by LiP. *P. ostreatus* secretes a mixture of benzyl alcohols, including anisyl alcohol, and an AAO that oxidizes them (Sannia et al. 1991).

Various **sugar oxidases** have also been suggested to be producers of the required extracellular H_2O_2. Most are intracellular enzymes. Pyranose oxidase, however, which oxidizes various monosaccharides at C-2, with transfer of electrons to O_2 to produce H_2O_2, has been located in the extracellular polysaccharide matrix that coats the lumens of cells during white-rot. The enzyme has been studied in *P. chrysosporium*, *T. versicolor*, *Oudemansiella mucida*, and *Agaricus bisporus* (Daniel et al. 1994; Artolozaga et al. 1997; Volc et al. 1997). Glucose 1-oxidase activity has been reported in *P. chrysosporium* mycelium (Kelley and Reddy 1986, 1988) but appears to be less common than pyranose 2-oxidase (reviewed by Ander and Marzullo 1997).

In addition to the oxidases, extracellular H_2O, may also be generated by the oxidation of organic acids that are secreted by many white-rot fungi. Specifically, Mn(II)-dependent oxidation of glyoxylic and oxalic acids in *C. subvermispora* cultures generates H_2O_2 (Urzua et al. 1995, 1998a,b; Aguilar et al. 1999).

Cellobiose dehydrogenase (CDH) is widely distributed among white-rot and brown-rot fungi, and may also play an important role in organopollutant degradation (Westermark and Eriksson 1974a,b, 1975; Henriksson et al. 2000a). The enzyme has two domains containing FAD or heme prosthetic groups, and these 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+}. (CDH can use O_2 as a poor electron acceptor, but it is still classified as a dehydrogenase, not an oxidase.)

The biological function of CDH is uncertain (Eriksson et al. 1990). One model suggests that CDH generates hydroxyl radicals by Fenton-type reactions [Fe(II) and H_2O_2 reactants] as described above (Kremer and Wood 1992; Mansfield et al. 1997; Henriksson et al. 2000b). The model is consistent with many of the properties of CDH and entails the concerted efforts of additional enzymes for efficient lignin depolymerization. In addition to Fe(II), the Fenton mechanism requires a system for generating extracellular H_2O_2, and enzymes such as GLOX may be involved. Possibly, the function(s) of CDH differ among species. This issue has been recently reviewed (Henriksson et al. 2000a).

III. Molecular Genetics

The **molecular genetics** of lignin-degrading fungi has advanced considerably over the past 15 years. *Phanerochaete chrysosporium* has emerged as the model system, in large part due to the development of experimental procedures. Methodologies for auxotroph production, recombination analysis, rapid DNA and RNA purification, pulsed-field electrophoretic separation of chromosomes, and **genetic transformation** by auxotroph complementation and by drug resistance markers have been described (Alic et al. 1989, 1990, 1991, 1993; Alic 1990; reviewed by Cullen and Kersten 1996; Cullen 1997). Another auxotroph complementation system was recently reported (Zapanta et al. 1998). No other white-rot fungi provide a range of experimental tools comparable to *P. chrysosporium,* although genetic recombination analysis (Eichlerova and Homolka 1999; Larraya et al. 1999a), pulsed field **electrophoretic karyotyping** (Larraya et al. 1999b), and genetic transformation (Yanai et al. 1996; Kim et al. 1999; Honda et al. 2000) are now available for *Pleuotus ostreatus.* Most recently, transformation of *Trametes versicolor* has been achieved using **phleomycin resistance** as a dominant selectable marker (Bartholomew et al. 2001). Virtually nothing is known of the molecular genetics of brown-rot fungi. The molecular biology of *P. chrysosporium* has been reviewed (Alic and Gold 1991;Pease and Tien 1991;Gold and Alic 1993;Cullen and Kersten 1996; Cullen 1997; Kirk and Cullen 1998).

A. Peroxidases

1. Gene Structure

Tien and Tu (1987) were first to clone and sequence a *P. chrysosporium* cDNA encoding a **LiP.** It is now clear that *P. chrysosporium* LiPs are encoded by a **family of at least ten closely related genes** that have been designated *lipA* through *lipJ* (reviewed by Cullen and Kersten 1996). One allele, *lipI2,* is transcriptionally inactivated by an unusual class II non-autonomous transposon (Gaskell et al. 1995). Residues believed essential to peroxidase activity are conserved (Tien and Tu 1987; Schalch et al. 1989). The gene-encoding isozyme H8, *lipA,* also features a putative propeptide (Schalch et al. 1989). A similar sequence was shown in the LiP2 gene of strain OGC101, and the proenzyme was identified as an in vitro translation product (Ritch et al. 1991). The *P. chrysosporium* clones contain 8–9 short introns, and the positions of six of these are highly conserved (Brown et al. 1988; Schalch et al. 1989; Ritch and Gold 1992; Gold and Alic 1993;Stewart and Cullen 1999).The **crystal structure of LiP** is similar to the overall three-dimensional structure of **cytochrome c peroxidase** (CCP), even though sequence identity is only approximately 20% (Edwards et al. 1993; Poulos et al. 1993).

Several LiP genes have been characterized from other fungal species, including *Trametes versicolor* genes *LPGI/LPGIV* (Jonsson and Nyman 1992,1994; Johansson and Nyman 1996), *LPGII/LPGIII* (Jonsson and Nyman 1994; Johansson and Nyman 1996), *VLG1* (Black and Reddy 1991), *LPGVI* (Johansson and Nyman 1995),*Bjerkandera adusta* clone LPO-1 (Asada et al. 1992), and *Phlebia radiata lpg3* (Saloheimo et al. 1989). Further, PCR amplification of LiP-like sequences also suggests the existence of LiP genes in *Ceriporiopsis subvermispora* and *Phanerochaete sordida* (Rajakumar et al. 1996). These results appear to contradict numerous biochemical investigations that failed to detect LiP activity in *C. subvermispora* (Ruttimann et al. 1992; Orth et al. 1993) and *P. sordida* (Ruttimann-Johnson et al. 1994) cultures.

Multiple MnP genes have been sequenced from several white-rot fungi including *P.*

chrysosporium (Pease et al. 1989; Pribnow et al. 1989; Orth et al. 1994; Alic et al. 1997), *T. versicolor MPGI* (Johansson 1994), *T. versicolor PGVII* (Johansson and Nyman 1996), *C. subvermispora* (Lobos et al. 1998; Tello et al. 2000), and *P. ostreatus* (Asada et al. 1995a). The actual number of MnP genes in these or other species remains to be established. The N-terminal amino acid sequences of *P. chrysosporium* isozymes indicate the existence of at least two more genes (Datta et al. 1991; Pease and Tien 1992).

Multiple sequence alignments reveal features that help to distinguish MnP and LiP genes (reviewed by Cullen 1997). The crystal structure of MnP shows similarities with LiP. Experimental support for Asp203 in Mn^{2+} binding has been provided by site-directed mutagenesis of the residue (Kusters-van Someren et al. 1995). Consistent with its ability to oxidize Mn^{2+} and its Mn-independent activity on aromatic substrates, the gene encoding *Pleurotus eryngii* "Mn-independent" peroxidase features putative Mn-binding ligands (Ruiz-Duenas et al. 1999b). Analogous genes have not yet been identified in *P. chrysosporium.*

2. Genomic Organization

Genetic linkage in *P. chrysosporium* has been demonstrated by restriction fragment length polymorphisms (RFLPs; Raeder et al. 1989a,b) and later refined by PCR-based segregation analysis (Gaskell et al. 1994). Recombination frequencies are based on analyses of single basidiospore cultures, which are the homokaryotic products of meiosis. **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) and by walking in genomic libraries (Huoponen et al. 1990; Gaskell et al. 1991).

Gene clustering and chromosome length polymorphisms are common *in P. chrysosporium.* For example, *lipA, lipB, lipC, lipE, lipG, lipH, lipI,* and *lipJ* are clustered within 3% recombination and hybridize to a 3.5/3.7mb chromosome pair on CHEF gel Southern blots (see Stewart and Cullen 1999 and refs. cited therein). In contrast, *lipD* and *lipF* are unlinked and hybridize to 4.8/4.4mb and 1.8/2.0mb pairs, respectively (Stewart et al. 1992; D'Souza et al. 1993). The gene encoding glyoxal oxidase, *glx,* hybridizes to a 3.7/3.9mb pair (Kersten et al. 1995). These length dimorphisms are mitotically stable in strain BKM-F-1767, but the number and sizes of bands 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. 1992b; D'Souza et al. 1993). Beyond *P. chrysosporium,* little is known of the organization of peroxidase genes, although a cluster containing two LiP- and one MnP-encoding genes has been described for *T. versicolor* (Johansson and Nyman 1996).

Homologous chromosomes differing in electrophoretic mobility have also been observed in numerous eukaryotes such as *Plasmodium falciparum* (Corcoran et al. 1988), dozens of fungi (reviewed in Zolan 1995), and, most recently, in the related white-rot fungus *P. ostreatus* (Larraya et al. 1999a,b). 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 or unequal exchange between homologues could generate shortened chromosomes (Zolan 1995; Gray 2000). The presence of repetitive elements in *P. chrysosporium* (Gaskell et al. 1995), and their apparent restriction to a single allelic homologue, has some impact on length differences. However, recent sequence analyses showed no recombination between elements (Stewart et al. 2000).

3. Regulation

LiP genes are differentially transcribed and their **expression** is dramatically **modulated by culture conditions** (Holzbaur and Tien 1988; Stewart et al. 1992; Reiser et al. 1993; Janse et al. 1998; Vallim et al. 1998; Stewart and Cullen 1999). Although the *P. chrysosporium* LiP genes are tightly clustered and structurally similar, no clear relationship between genomic organization and transcriptional regulation has been observed (Stewart et al. 1992; Stewart and Cullen 1999). A report suggesting that nutrient nitrogen limitation regulates LiP expression post-translationally by heme processing (Johnston and Aust 1994) has been directly contradicted (Li et al. 1994).

Quantitative transcript analyses by competitive RT-PCR support a role for *P. chrysosporium* peroxidases in organopollutant-contaminated soils. Consistent with an MnP-dependent lipid peroxidation mechanism, depletion of high ionization potential PAHs generally coincided with increased transcripts of *mnp1, mnp2,* and *mnp3* as well as MnP activity (Bogan et al. 1996c). In con-

trast to *mnps*, the transcript patterns among LiP genes varied dramatically during anthracene transformation in soils (Bogan et al. 1996b). Comparison of anthracene and PCP-contaminated soils suggests differential regulation of LiP genes in response to the particular organopollutant (Lamar et al. 1995; Bogan et al. 1996b).

Manganese peroxidase production in *P. chrysosporium* is generally **dependent on Mn concentration** (Bonnarme and Jeffries 1990; Brown et al. 1990, 1991). Differential regulation of MnP isozymes has been observed in response to media composition for *C. subvermispora* and *P. chrysosporium* (Pease and Tien 1992; Lobos et al. 1994). Transcriptional control of MnP expression is complex. Possible transcriptional control elements, particularly metal response elements (MREs), have been identified in 5'-untranslated regions (Godfrey et al. 1990,1994; Alic and Gold 1991; Brown et al. 1993; Alic et al. 1997). Consistent with the occurrence of upstream MREs, Gettemy et al. (1998) observed upregulation of *P. chrysosporium mnp1* and *mnp2* in response to Mn^{2+} concentration in defined media, but transcript levels of *mnp3* were unaffected. In contrast, *T. versicolor mnp2* does not contain any MREs but is dramatically upregulated in response to Mn (T. Johansson and D. Cullen, unpubl.). Differential display analysis of *Coriolus versicolor* cultures exposed to PCP identified upregulated genes encoding a heat shock protein, but not MnP genes (Iimura and Tatsumi 1997).

4. Heterologous Expression

Basic biochemical investigations have been hampered by low yields and purification difficulties associated with LiP/Mnp isozymes. As a result, considerable research effort has been expended on the development of **heterologous expression systems.** Baculovirus systems have been used to produce active recombinant MnP isozyme H4 (Pease et al. 1991) and LiP isozyme H8 (Johnson and Li 1991; Johnson et al. 1992; Lin et al. 1997). Yields are relatively low, but baculovirus production may be useful for experiments requiring limited quantities of recombinant protein, e.g., site-specific mutagenesis. Techniques have also been developed for recovering active *P. chrysosporium* isozymes MnP H4 (Whitwam et al. 1995; Whitwam and Tien 1996), LiP H8 (Doyle and Smith 1996), and LiP H2 (Nie et al. 1998) from *E. coli* inclusion bodies. *P. chrysosporium* MnP and LiP can also be produced in a **"homologous expression" system** in which *mnp* transcriptional control is placed under the glyceraldehyde-3-phosphate dehydrogenase promoter (Mayfield et al. 1994; Sollewijn Gelpke et al. 1999). The system temporally separates the recombinant protein from other peroxidases, and it has been successfully used in site-directed mutagenesis (Kusters-van Someren et al. 1995; Kishi et al. 1997). **Efficient secretion of fully active MnP isozyme** H4 has been achieved in *A. oryzae* (Stewart et al. 1996) and *A. niger* (Conesa et al. 2000). The recombinant enzyme has served mechanistic studies (Jensen et al. 1996; Kapich et al. 1999). and *Aspergillus* expression systems have been extended to peroxidases from *C. subvermispora* [Larrondo et al. 2001, #1418], *P. eryngii* (Ruiz-Duenas et al. 1999a), and *Geotrichym candidum* (Sugano et al. 2000). Aifa et al. (1999) has recently reported low-level secretion of *P. chrysosporium* LiP by *Aspergillus niger.*

B. Laccases

Laccases of white-rot fungi are also encoded by complex families of structurally related genes (Kojima et al. 1990; Saloheimo et al. 1991; Coll et al. 1993; Yaver and Golightly 1996; Yaver et al. 1996; Karahanian et al. 1998; Giardina et al. 1999; Temp et al. 1999). Although laccase activity has been demonstrated in *P. chrysosporium* cultures (Srinivasan et al. 1995; Dittmer et al. 1997; Rodriguez et al. 1997), the corresponding genes have not yet been isolated (D'Souza et al. 1996). **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). Little information is available on the genomic organization of laccases, but *Trametes villosa* pulsed field gels suggest the possible linkage of certain laccase genes (Yaver and Golightly 1996). Basidiomycete laccases have been expressed in several systems including *A. oryzae* (Yaver et al. 1999) and *Pichia pastoris* (Otterbein et al. 2000).

C. Peroxide-Generating Enzymes

Glyoxal oxidase (GLOX) of *P. chrysosporium* is encoded by a single gene with two alleles (Kersten and Cullen 1993; Kersten et al. 1995). The deduced amino acid sequence lacks clear homology with

any other known proteins (Kersten and Cullen 1993), although Bork and Doolittle (1994) have identified a 50-residue 'kelch' motif in a variety of database sequences including glyoxal oxidase and galactose oxidase. On the basis of catalytic similarities with *Dactylium dendroides* galactose oxidase, potential copper ligands were tentatively identified at Tyr135, Tyr377, His378, and His 471(Whittaker et al. 1996). The deduced amino acid sequences of allelic variants differ by a single residue (Lys308<<Thr308), which may explain the two isozyme forms observed on isoelectric focusing gels (Kersten and Kirk 1987; Kersten 1990). Southern blot analysis of CHEF gels and segregation analysis show that *glx* is unlinked to MnP or LiP genes (Gaskell et al. 1994; Orth et al. 1994; Kersten et al. 1995). The GLOX gene (*glx*) is transcriptionally regulated in *P. chrysosporium* (Kersten and Cullen 1993). 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*. Production of mutant GLOX in *Aspergillus nidulans* and *Pichia pastoris* has confirmed the identity of catalytic residues (Kersten et al. 1995; Whittaker et al. 1999).

Beyond GLOX, relatively little is known of the molecular genetics of white-rot oxidases. Although numerous studies implicate pyranose 2-oxidase in lignin degradation, the corresponding gene has only been isolated from *Coriolus versicolor* (Nishimura et al. 1996). The sequence shows weak similarity to a short region within the FAD domain of several cellobiose dehydrogenase genes. Glucose 1-oxidase genes have not been isolated from any basidiomycete. Martinez and coworkers have cloned and sequenced **aryl alcohol oxidase** (AAO) encoding genes from *Pleurotus pulmunarius* (Varela et al. 2000) and *Pleurotus eryngii* (GenBank accession No. AF064069). The predicted proteins are over 90% identical, and show substantial sequence similarity to *A. niger* glucose 1-oxidase (35% identity). Blast searches clearly show that the *P. pulmunarius* sequence is related to an array of fungal "oxidases" including *P. pastoris* alcohol oxidase (24%), and the FAD domain of cellobiose dehydrogenases from *P. chrysosporium* (25%), *Trametes versicolor* (25%), and *Pyconporus cinnabarinus* (24%). We have recently cloned and partially sequenced cDNAs from *P. chrysosporium* colonized wood that exhibit striking similarity to these FAD oxidases and to GLOX (T. Johansson and D. Cullen, unpubl.). Taken together, these results suggest involvement of a wide range of sugar oxidoreductases, aryl alcohol oxidases, and glyoxal oxidase.

D. Cellobiose Dehydrogenase

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 structural similarity to functionally similar bacterial or fungal CBDs. Li et al. (1997) characterized both allelic variants of *P. chrysosporium cdh.* 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). Renganathan and coworkers (1985) temporally altered expression of *P. chrysosporium* CDH by fusing *cdh* with the promoter of the highly expressed glyceraldehyde-3-phosphate dehydrogenase gene (Li et al. 2000). No evidence for CDH gene multiplicity has been reported.

IV. Conclusions and Future Prospects

Experiments with purified enzymes, analysis of metabolites in whole cultures, and field trials demonstrate efficient degradation of a wide range of organopollutants by wood-decaying fungi. Oxidative mechanisms involving extracellular peroxidases and laccases have been repeatedly shown, although their relative importance in complex substrates such as organopollutant-contaminated soils remains uncertain. Fenton-type reactions and an array of membrane-bound and intracellular enzyme systems probably augment or supersede peroxidases and laccases in certain circumstances.

Establishing the role of specific genes and enzymes in degradation pathways requires ex-

perimental tools that have only recently become available. Biochemical analysis in cell-free systems is greatly facilitated by the increasing availability of pure recombinant enzymes. Quantitative analysis of transcripts and organopollutant transformations in complex substrates provides further evidence for the involvement of specific genes. A more direct experimental approach might be disruption or replacement of individual genes, and transformation systems are now available for *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor*. Gene replacement through homologous recombination occurs at low frequency in *P. chrysosporium* (Alic et al. 1993), but the technique has not yet been brought to bear on questions of organopollutant degradation. *Agrobacterium*-mediated gene transfer systems may facilitate gene disruption experiments in related wood-decaying fungi (Covert et al. 2001 #1538; Chen et al. 2000 #531; de Groot et al. 1998 #1537).

Current genomics research offers significant opportunities for elucidating the roles and interactions of known genes and for identifying new and novel genes involved in degrading lignin and organopolluntants. The US Department of Energy's JointGenome Institute (JGI) has completed whole genome shotgun sequencing of *P. chrysosporium*. A draft assembly is currently available for Blast searchingat<url>http://www.jgi.doe.gov/programs/whiterot/whiterot_mainpage.html</url>. Our laboratory and the JGI are collaborating on sequencing ESTs from *P. chrysosporium* colonized wood, and all sequences should be publicly available by 2002. Clearly, these efforts will reveal an enormous number of genes of potential relevance to organopollutant degradation. When coupled to transcript profiling, heterologous expression, and gene disruptions, these investigations will elucidate majordegradation pathways, and answer fundamental questions concerning lower eukaryotic genome organization.

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The Mycota

A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research

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XI

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