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Recent advances on the molecular genetics of ligninolytic fungi

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Abstract

This review highlights significant recent advances in the molecular genetics of white-rot fungi and identifies areas where information remains sketchy. The development of critical experimental tools such as genetic mapping techniques is described. A major portion of the text focuses on the structure, genomic organization and transcriptional regulation of the genes encoding peroxidases, laccases and glyoxal oxidase. Finally, recent efforts on heterologous expression of lignin-degrading enzymes are discussed. © 1997 Elsevier Science B.V.

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1. Introduction

1.1. Microbial degradation of lignin

The white-rot basidiomycetes degrade lignin more extensively and rapidly than any other known group of organisms. In contrast to other fungi and bacteria, white-rot fungi are capable of completely degrading lignin to carbon dioxide and water. The species are widely distributed. occurring in tropical and temperate environments. White-rot fungi are also well adapted for utilizing other plant components and the species vary substantially with regard to their relative cellulolytic versus ligninolytic efficiency. The microbiology of lignin degradation has been reviewed (Kirk and Shimada, 1985; Buswell and Odier, 1987; Kirk and Farrell, 1987; Eriksson et al., 1990).

The most extensively characterized white-rot fungus is *Phanerochaete chrysosporium,* previously known as *Chysosporium lignorum* and *Sporotrichum pulverulentum* (Burdsall and Eslyn, 1974; Raeder and Broda, 1984). Four classes of extracellular enzymes have been implicated in lignin degradation: lignin peroxidases (LiPs), manganese peroxidases (MnPs), laccases and the H_2O_2-generating enzyme glyoxal oxidase (GLOX).

Lignin peroxidase was first discovered based on the H_2O_2-dependent C*x* -Cβ cleavage of lignin model compounds and subsequently shown to catalyze the partial depolymerization of methy-

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lated lignin in vitro (Glenn et al., 1983; Tien and Kirk, 1983, 1984; Gold et al., 1984). Several isozymic forms have been detected in *P. chrysosporium* cultures and a number of other white-rot fungi, e.g. *Trametes versicolor, Bjerkandera adusta, Phlebia radiata.* The principle criteria for identifying *P. chrysosporium* LiP isozymes are their pI and their 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 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 characteristic of LiP (Farrell et al., 1989). Growth conditions (e.g. N vs. C starved), purification methods and storage can affect relative isozymic levels.

Lignin peroxidase catalyzes a variety of oxidations, all of which are dependent on H_2O_2. These include C*x* -Cβ 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 ring cleavage of non-phenolic lignin model compounds (Tien and Kirk, 1984; Leisola et al., 1985; Renganathan et al., 1985; Umezawa et al., 1986; Umezawa and Higuchi, 1987). Relative to other peroxidases, LiPs exhibit low pH optima (< pH 3) and high redox potential. Comprehensive reviews on the biochemistry of lignin peroxidase are provided elsewhere (Kirk and Farrell, 1987; Higuchi, 1990; Shoemaker, 1990; Hammel, 1992).

Another heme peroxidase first found in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* is MnP (Kuwahara et al., 1984; Paszczynski et al., 1985). The enzyme is now known to be widely distributed among lignin-degrading fungi (Orth et al., 1993; Hatakka, 1994). The principle function of MnP is to oxidize Mn^{2+} to Mn^{3+} using H_2O_2 as oxidant. Activity is stimulated by simple organic acids which stabilize the Mn^{3+}, thus producing diffusible oxidizing chelates (Glenn and Gold, 1985; Glenn et al., 1986). Physiological levels of oxalate in *P. chrysosporium* cultures have been shown to stimulate MnP activity (Kuan et al., 1993). As with LiP, the prosthetic group is iron protoporphryn IX and several isozymes can be detected (Paszczynski et al., 1986; Leisola et al., 1987; Mino et al., 1988; Wariishi et al., 1988). 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).

The role of MnP in lignin depolymerization has been questioned because, in contrast to LiP, non-phenolic lignin is inefficiently depolymerized by MnP. However, the biomimetic oxidation of lignin model compounds by Mn^{3+} suggests that it may play a role in oxidizing both phenolic and non-phenolic residues of lignin (Hammel et al., 1989). Partial depolymerization of synthetic lignin by MnP has been demonstrated in vitro (Wariishi et al., 1991). Recently, MnP has been shown to promote peroxidation of unsaturated fatty acids. This lipid peroxidation system oxidatively cleaved phenanthrene (Moen and Hammel, 1994), non-phenolic synthetic lignin and a non-phenolic lignin model compound (Bao et al., 1994).

Both MnP and LiP are common in white-rot fungi. However, several efficient lignin-degrading species (e.g. *Phanerochaete sordida, Ceriporiopsis subvermispora, Dichomitus squalens)* produce MnP but apparently lack LiP activity (Perie and Gold, 1991; Ruttiman et al., 1992; Orth et al., 1993; Ruttimann-Johnson et al., 1994). The mechanism(s) of lignin degradation by these fungi is uncertain. Potentially unidentified enzyme mechanism(s) or a MnP-promoted lipid peroxidation system may play a role. Alternatively, LiP may be involved but concentrations are relatively low and/or detection is complicated by interfering substances, particularly in woody substrates. Supporting the latter view, Orth et al. (1993) demonstrated the presence of inhibitors to LiP activity in colonized sawdust substrates. Also consistent with a role for LiP is the presence of LiP-like genes in these 'LiP-deficient' species as shown by Southern blot hybridizations (Ruttiman et al., 1992). In any event, the failure to detect enzyme activity should be viewed with caution. This point was recently, and unexpectedly, illustrated by a report of laccase activity in *P. chrysosporium* cultures (Srinivasan et al., 1995).

Laccases are copper-containing enzymes which reduce molecular oxygen to water and oxidize phenolic compounds. In the presence of certain mediators, non-phenolic compounds may be oxidized and this has generated considerable interest as an approach to the enzymatic bleaching of kraft pulps (Bourbonnais et al., 1995). The possible role of laccases in lignin degradation has been recently reviewed (Youn et al., 1995). Like the peroxidases, laccases are widely distributed among ligninolytic basidiomycetes (Orth et al., 1993; Hatakka, 1994).

Several oxidases have been proposed as the source of H_2O_2 in peroxidative reactions but GLOX is the only oxidase secreted under standard ligninolytic conditions (Kirk and Farrell, 1987). The temporal correlation of glyoxal oxidase, peroxidase and oxidase substrates in cultures suggests a close physiological connection between these components (Kersten and Kirk, 1987; Kersten, 1990). Substrates for GLOX are simple aldehyde-, *x*-hydroxycarbonyl-, and *x*-dicarbonyl compounds. Among these substrates is glucoaldehyde, which is produced by the action of LiPs on lignin (Hammel et al., 1994).

A physiologically significant property of GLOX is its reversible inactivation in the absence of the peroxidase system (Kersten, 1990; Kurek and Kersten, 1995). The enzyme is reactivated, however, by reconstituting the complete peroxidase system, including both lignin peroxidase and non-phenolic peroxidase substrates. This suggests that H_2O_2 supplied by glyoxal oxidase is responsive to the demand of the peroxidases, thereby providing an extracellular regulatory mechanism for control of the coupled enzyme systems. GLOX is produced by several white-rot fungi (Orth et al., 1993), and two isozymic forms have been detected in *P. chrysosporium* (Kersten and Kirk, 1987).

2. Molecular genetics

Knowledge concerning the molecular genetics of white-rot fungi. particularly *P. chrysosporium,* has been advanced considerably over the past decade. Standard procedures have been established for auxotroph production (Gold et al., 1982), recombination analysis (Alic and Gold, 1985; Raeder et al., 1989a; Krejci and Homolka, 1991; Gaskell et al., 1994), rapid DNA and RNA purification (Haylock et al., 1985; Raeder and Broda, 1985) and genetic transformation by auxotroph complementation (Alic et al., 1989, 1990, 1991, 1993) and by drug resistance markers (Randall et al., 1989, 1991; Randall and Reddy, 1992; Gessner and Raeder, 1994). Aspects of the molecular biology of *P. chrysosporium* have been recently reviewed (Alic and Gold, 1991; Pease and Tien, 1991; Cullen and Kersten, 1992; Gold and Alic, 1993; Reddy and D'Souza, 1994; Broda et al., 1996; Cullen and Kersten, 1996).

2.1. Gene structure

Soon after Tien and Tu (1987) first cloned and sequenced the *P. chrysosporium* cDNA encoding LiP H8, the cDNAs and genomic clones of several structurally-related genes were reported (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). The existence of allelic variants complicated the identification of LiP genes, but analysis of single basidiospore cultures allowed alleles to be differentiated from closely-related genes. Alic et al. (1987) showed that single basidiospores of the widely used strain BKM-F-1767 contain two identical haploid nuclei which are the products of meiosis. The presence of specific alleles in these haploid segregants could be determined by restriction polymorphisms (Schalch et al., 1989) or by allele-specific probes (Gaskell et al., 1992). It is now clear that *P. chrysosporium* LiPs are encoded by a family of at least ten closely related genes which have been designated *lip*A through *lip*J (Gaskell et al., 1994).

Recently, *P. chrysosporium* strain BKM-F-1767 was shown to harbor a 1747 bp insertion within LiP gene *lip*I (Gaskell et al., 1995). The element. Pcel, lies immediately adjacent to the fourth intron of *lip*I2 and features several transposon-like features including inverted terminal repeats and a dinucleotide (TA) target duplication. Southern blots revealed the presence of Pcel in other, but not all, *P. chrysosporium* strains. Pcel is present at very low copy numbers and lacks homology to

known transposons or transposases. The element is inherited in a simple 1:1 Mendelian fashion among single basidiospore progeny. The distribution of Pcel or other transposon-like elements in lignin degrading species is unknown.

Several LiP genes have been characterized from other fungal species, including four *Trametes versicolor* clones LPGI (Jonsson and Nyman, 1992), LPGII (Jonsson and Nyman, 1994), VLGI (Black and Reddy, 1991), LPGVI (Johansson, 1994)), *Bjerkandera adusta* clone LPO-1 (Asada et al., 1992) and *Phlebia radiata lpg*3 (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* (Huoponen et al., 1990), *Phlebia brevispora* and *Ceriporiopsis subvermispora* (Ruttiman et al., 1992).

Sequence conservation is high among the LiP genes. Pairwise amino acid sequence comparisons range from 53 to 98.9% similarity (Table 1, sequences labeled 1 – 16). Residues believed essential to peroxidase activity are conserved, i.e. the proximal heme ligand (His204 in *lip*A) and the distal arginine (Arg71) and histidine (His75). The gene encoding isozyme H8. *lip*A, also features a putative propeptide (residues 22–28; 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). Many of the *P. chrysosporium* genes feature a proline rich carboxy terminus, although its significance is unknown. The *P. chrysosporium* clones also 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).

The crystal structure of LiP is now known (Edwards et al., 1993; Poulos et al., 1993). Most striking is the similarity of the overall three-dimensional structure to that of cytochrome c peroxidase (CCP). even though sequence identity is only approximately 20%. 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 (in contrast to Asn with CCP) may explain the low pH optimum of LiP (Edwards et al., 1993). Other notable features of LiP are four disulfide bonds (CCP has none) and a C-terminal region without regular extended secondary structure, for which there is no equivalent in CCP.

Relative to LiPs, less is known concerning the number and structure of the MnP genes. Four distinct sequences have been reported: *P. chrysosporium* MP-1 (Pease et al., 1989), MnP-1 (Pribnow et al., 1989), MnP-2 (Orth et al., 1994) and *Trametes versicolor* MPG1 (Johansson, 1994). The total number of MnP genes in any of these species remains to be established. The N-terminal amino acid sequences of *P. chrysosporium* isozymes (Datta et al., 1991; Pease and Tien, 1992) indicate the existence of at least two more genes.

Recently, a structurally unique *Trametes versicolor* peroxidase clone, PGV, was discovered (Jonsson et al., 1994). Overall, the sequence is most closely related to LiPs, but certain residues are characteristic of MnPs (Fig. 1). The significance of the PGV sequence is uncertain. No PGV encoded protein has been identified, nor is it clear whether the gene is transcribed.

Multiple sequence alignments reveal conserved residues which help distinguish *mnp*s from *lip*s (Fig. 1). Three residues adjacent to the proximal His (e.g. Asp193, Trp199, Ala203 based on *lip*A numbering) as well as Leu115 and Ile113 are invariant in all LiP genes, but differ in MnP genes. Useful residues, but less reliable, are those purportedly involved in Mn^{2+} binding (Sundaramoorthy et al., 1994) (e.g. Glu59, Glu63, Asp203 based on MP-1 numbering). Excluding *T. versicolor* clone MPGI, *mnp*s can be distinguished from *lip*s by a 7-11 amino acid surface loop (Sundaramoorthy et al., 1994) (e.g. nos. 52-58 in MP-1) and an extended carboxy terminus. The latter insertion contains the fifth disulfide bond. not found in *lip*s. Although *T. versicolor* MPGI lacks the insertions found on *P. chrysosporium* genes, the sequence clearly encodes MnP isozyme MP2 (Johansson, 1994).

Table 1
Percent sequence similarity among known LiP and MnP genes

		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
*P.c.lip*A[2]	1	98.9	80.6	70.2	89.8	87.6	90.6	89.5	96.0	84.5	60.2	59.1	61.0	59.7	56.3	58.4	55.6	55.9	38.1	41.3	38.3
*P.c.lip*B	2		80.9	70.2	90.0	87.9	90.9	89.8	96.2	84.5	60.2	59.1	61.0	59.7	56.3	58.4	55.6	55.9	38.3	41.6	38.3
*P.c.lip*C	3			64.4	76.8	80.1	77.6	77.6	79.2	82.6	56.1	54.4	57.4	56.4	55.2	53.0	53.4	53.7	40.4	42.0	40.2
*P.c.lip*D	4				69.5	70.9	70.2	68.4	68.9	78.3	56.4	59.1	62.9	61.6	59.6	62.2	60.3	60.8	36.7	42.1	37.5
*P.c.lip*E	5					81.9	86.8	84.6	88.7	83.9	59.1	58.5	59.0	57.2	55.7	56.5	54.0	54.2	36.4	39.9	37.5
*P.c.lip*F	6						86.5	88.4	86.8	85.7	59.4	59.0	59.6	58.6	55.7	56.0	56.4	58.4	39.9	43.9	39.9
*P.c.lip*G	7							92.8	89.8	83.2	56.9	58.3	58.6	57.2	53.3	55.7	55.1	55.3	36.7	41.3	37.5
*P.c.lip*H	8								89.0	82.6	57.7	58.1	58.1	56.9	52.7	55.7	54.2	55.1	37.5	41.3	38.1
*P.c.lip*I	9									83.9	59.9	59.4	60.8	60.2	56.8	57.6	55.6	55.6	37.8	41.6	38.3
*P.c.lip*J	10										66.5	67.7	69.6	70.8	65.2	61.5	59.6	59.6	46.6	46.6	44.1
*P.r.lpg*3	11											57.5	59.4	59.7	57.5	57.7	53.9	56.4	38.1	43.1	41.4
B.a.LPO-1	12												58.6	57.2	56.0	57.9	57.3	56.4	34.7	36.3	36.0
*T.v.LPG*I	13													86.1	73.8	73.4	66.6	68.2	37.9	39.2	36.6
*T.v.LPG*II	14														75.1	72.2	64.1	66.3	38.4	40.3	38.4
*T.v.VLG*I	15															65.6	57.5	58.9	36.9	38.3	37.2
*T.v.LPG*VI	16																67.9	68.5	37.8	39.4	37.0
T.v.PGV	17																	79.2	43.0	48.5	45.8
*T.v.MPG*I	18																		40.0	44.4	43.8
P.c.MP-1	19																			74.1	69.3
P.c.MnP-1	20																				77.3
P.c.MnP-2	21																				—

Deduced amino acid sequences aligned by Clustal method (Higgins and Sharp, 1989) and percent similarities computed as previously described (Lipman and Pearson, 1985). Except for *lip*J (Schalch et al., 1989), all sequences were full-length, including secretion signals. *P. chrysosporium lips* (*P.c.lip*A through *P.c.lip*J) (primary references and synonyms listed by Gaskell et al., 1994); *Phlebia radiata lip P.r.lpg*3, (Saloheimo et al., 1989)); *Bjerkandera adusta lip*, *B.a.LPO*-1 (Asada et al., 1992); *Trametes versicolor lips*: *T.v.LPG*I (Jonsson and Nyman, 1992), *T.v.LPG*II (Jonsson and Nyman, 1994), *T.v.VLG*I (Black and Reddy, 1991) and *T.v.LPG*VI (Johansson, 1994); *T. versicolor mnp*, *T.v.MPGI* (Johansson, 1994); *P. chrysosporium mnp*s, *P.c.MP*-1 (Pease et al., 1989), *P.c.MnP*-1 (Pribnow et al., 1989), and *P.c.MnP*-2 (Orth et al., 1994). The deduced sequence of *T. versicolor* PGV (*T.v.PGV*) is most closely related to LiPs, but features certain MnP-like residues (Jonsson et al., 1994).

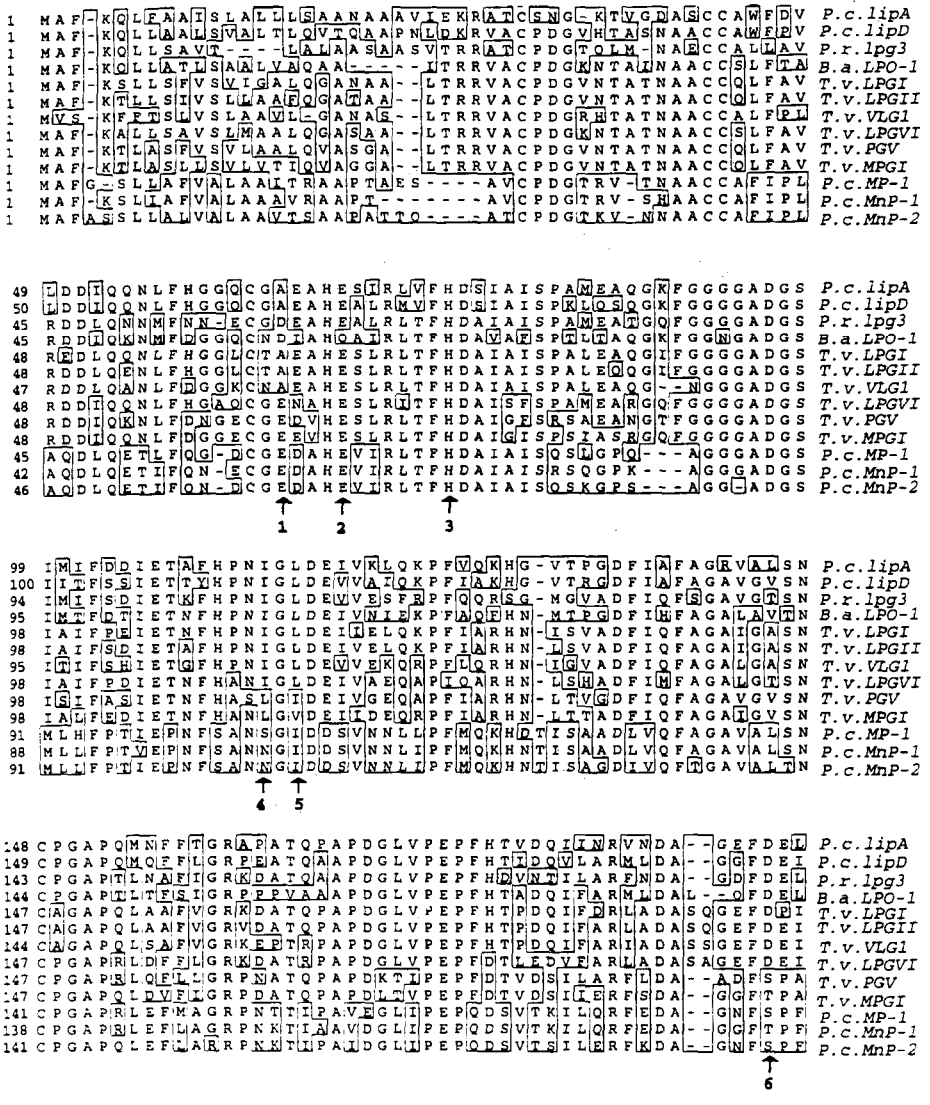

Fig. 1. Alignment of LiP and MnP amino acid sequences by Clustal method (Higgins and Sharp, 1989). Residues differing from the consensus are boxed. Lignin peroxidase sequences included two divergent *P. chrysosporium lips* (*P.c.lip*A, *P.c.lip*D) (Table 1; reviewed by Cullen and Kersten, 1996)); *Phlebia radiata P.r.lpg*3, (Saloheimo et al., 1989)); *Bjerkandera adusta*, *B.a.LPO*-1 (Asada et al., 1992) and *Trametes versicolor* clones *T.v.LPG*I (Jonsson and Nyman, 1992), *T.v.LPG*II (Jonsson and Nyman, 1994), *T.v.VLG*I (Black and Reddy, 1991), T.v. LPGVI (Johansson, 1994). The four known MnP gene sequences are: *T. versicolor T.v.MPGI* (Johansson, 1994); *P. chrysosporium P.c.MP*-1 (Pease et al., 1989); *P.c.MnP*-1 (Pribnow et al., 1989); and *P.c.MnP*-2 (Orth et al., 1994). The deduced sequence of *T. versicolor* PGV (*T.v.PGV*) is most closely related to LiPs, but features certain MnP-like residues (Jonsson et al., 1994). Vertical arrows 3, 9 and 11 indicate residues common to all peroxidases. Vertical arrows 4, 5, 6, 7 and 8 indicate amino acids invariant in LiP genes, but different in MnP genes. Vertical arrows 1, 2 and 10 indicate putative Mn^{2+} binding site (Sundaramoorthy et al., 1994).

The crystal structure of MnP shows similarities with LiP: the active site has a proximal His ligand H-bonded to an Asp and a distal side peroxide-binding pocket consisting of a catalytic His and Arg. However, there is also a proposed manganese-binding site involving Asp203, Glu59,

Glu63 (numbering based on *P. chrysosporium* MP-1, Fig. 1) and a heme propionate (Sundaramoorthy et al., 1994). Recent experimental support for Asp203 in Mn^{2+} binding has been provided by site-directed mutagenesis of the residue (Kusters-van Someren et al., 1995).

Like the peroxidases, laccases appear to be encoded by complex families of structurally related genes. At least 17 fungal laccase genes have been cloned and sequenced. These include four from *Rhizoctonia solani* (Wahleithmer et al., 1995), five from *Trametes villosa* (Yaver and Golightly, 1996; Yaver et al., 1996) two from *Agaricus bisporus* (Perry et al., 1993) and single genes from *Neurospora crassa* (Germann et al., 1988), *Coriolus hirsutus* (Kojima et al., 1990), *Cryphonectria parasitica* (Choi et al., 1992), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Pleurotus ostreatus* (Giardina et al., 1995) and an unidentified basidiomycete (Coll et al., 1993). Overall sequence identity between certain pairs of fungal laccases may be low, but conservation is high within regions involved in copper binding (Fig. 2). Also, Yaver and Golightly (1996) noted conservation of the intron position in the laccase genes of *T. villosa* and other basidiomycetes. Three *R. solani* laccase genes each contain 11 introns at identical positions (Wahleithmer et al., 1995).

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 sequence lacks significant homology with any other known proteins (Kersten and Cullen, 1993). 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). 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).

2.2. Genomic organization

Meiotic recombination analysis in *P. chrysosporium* (Alic and Gold, 1985; Krejci and Homolka, 1991) has been useful for rudimentary identification of several linkage groups. While adequate for mapping simple nutritional markers, the mulitplicity of genes involved in the degradation of lignin and organopullutants are not amenable to such 'classical' genetic analysis.

Genetic linkage based on restriction fragment length polymorphisms (RFLPs) has been useful for identifying gene clusters and for creating recombinant genotypes. Using a set of 53 homokaryotic basidiospores and 38 RFLP markers, at least six linkage groups were recognized (Raeder et al., 1989a,b). Probes included randomly chosen clones, a cellobiohydrolase (CBH1) gene, and several clones containing LiP-like sequences. Two clusters of LiP genes were identified and one of these was linked to the CBH1 gene (Raeder et al., 1989b). Lignin mineralization was not correlated with the segregation of any individual RFLP marker (Wyatt and Broda, 1995).

In agreement with genetic linkage, analysis of cosmid and λ libraries has provided detailed physical maps of gene clusters. In *P. chrysosporium*, *lip*A and *lip*B are transcriptionally convergent and their translational stop codons separated by 1.3 kb (Huoponen et al., 1990; Gaskell et al., 1991), *lip*C lies approximately 15 kb upstream of *lip*B (Gaskell et al., 1991). Clustering of CBHI genes has also been shown in *P. chrysosporium* (Covert et al., 1992b). In *T. versicolor*, a MnP and two LiP genes are clustered within a 10 kb region (Johansson, 1994; Johansson and Nyman, 1996). The genes have the same transcriptional orientation and they are separated by approximately 2.4 kb. Three laccase genes of *R. solani* are also clustered and share the same transcriptional orientation (Wahleithmer et al., 1995).

Pulsed field electrophoresis has provided a rapid means of localizing cloned genes to chromosomes. In the dikaryotic *P. chrysosporium* strain BKM-F-1767, clamped homogeneous electrical field (CHEF) gels reveal seven or more chromosome bands. The ten known LiP genes are distributed on three separate chromosomes. two of which are dimorphic with respect to length (Gaskell et al., 1991, 1994; Stewart et al., 1992). Thus. eight *lip*s, including the *lip*A, *lip*B, *lip*C cluster, have been localized to a dimorphic chro-

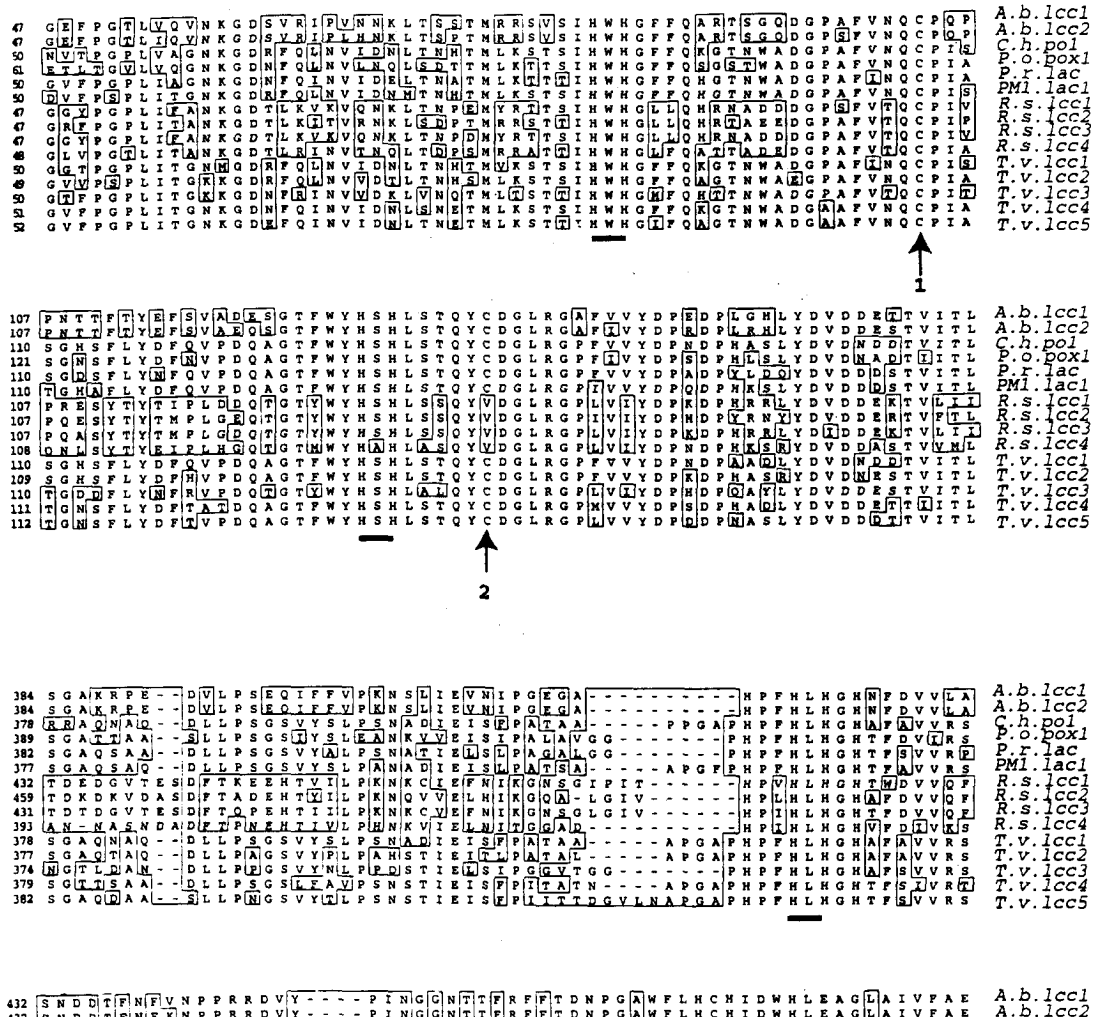

Fig. 2. Alignment of laccase amino acid sequences surrounding conserved copper binding sites. Residues differing from the consensus are boxed. Horizontal bars indicate positions of copper coordination sites. With the exception of a single conservative change (Ser↔Ala) in *R. solani lcc*4, sites are identical. Disulfide binding is believed to involve Cys103 (*A. bispsorus* numbering, arrow 1) to Cys509 (not shown) and Cys135 (arrow 2) to Cys229 (not shown). *A.b.lcc*1 and *A.b.lcc*2, *A. bisporus* (Perry et al., 1993); *C.h.po*1, *C. hirustus*; *P.o.pox*1 (Kojima et al., 1990), *P. ostreatus*; *P.r.lac* (Giardina et al., 1995), *P. radiata* (Saloheimo and Niku-Paavola, 1991); *PM*1.*lac*1, unidentified basidiomycete (Coll et al., 1993); *R.s.lcc*1–4, *R. solani* (Wahleithmer et al., 1995); *T.v.lcc*1–5, *T. villosa* (Yaver and Golightly, 1996; Yaver et al., 1996).

mosome of 3.5/3.7 mb (Gaskell et al., 1991). The LiP gene encoding isozyme H2, *lip*D, was localized to another dimorphic pair of 4.4/4.8 mb (Stewart et al., 1992), along with the CBHI cluster (Covert et al., 1992a). In contrast to many of the CBHI and LiP genes, Southern blot analysis of CHEF gels suggests that MnP and GLOX genes are unlinked to each other and to LiP genes (Orth et al., 1994; Kersten et al., 1995). Recently, CHEF gel blots have shown the five known *T. villosa* laccase genes to be distributed on three separate chromosome bands (Yaver and Golightly, 1996).

Although convenient, pulsed field gel blots have disadvantages. The common occurrence of dimorphic chromosomes necessitates the simultaneous analysis of parent dikaryon and several homokaryotic progeny. Electrophoresis run times are often quite long (> 5 days) and several chromosomes co-migrate. Resolution is poor as most basidiomycete chromosomes are quite large, greater than 1000 kb.

Genetic linkage relationships in *P. chrysosporium* have been further refined by monitoring the segregation of specific alleles in single basidiospore progeny (Gaskell et al., 1994). The method involves PCR amplification of genomic DNA from single basidiospore cultures and then probing with allele-specific oligonucleotides. Using these techniques, the ten LiP genes of *P. chrysosporium* were mapped to three separate linkage groups. Entirely consistent with pulsed field gel blots. no *lip*s were linked to *mnp*s or *glx*, and *lip*D was distantly linked to a CBHI cluster. Eight *lip*s, including the *lip*A, *lip*B, *lip*C cluster, were closely linked. Four genes, *lip*G, *lip*H, *lip*I and *lip*J, showed 100% cosegregation. This gene clustering was recently confirmed by cosmid mapping (unpublished data). An integrated physical and genetic linkage map is shown in Fig. 3.

Preliminary data suggest a relationship between genetic linkage of LiP genes and their intron/exon structure. Gold and coworkers delineated four subfamilies on the basis of intron conservation (Ritch and Gold, 1992; Gold and Alic, 1993). The LiP genes unlinked to all others, *lip*D and *lip*F, were also assigned to separate subfamilies. Not all genomic *lip* clones have been sequenced and until the intron structure of all LiP genes is determined it remains to be seen if the relationship between genomic organization and gene structure holds.

2.3. Gene expression

Transcription of *P. chrysosporium* strain BKM-F-1767 LiP genes is dramatically modulated by culture conditions. Holzbaur and Tien (1988) examined transcript levels of the genes encoding isozymes H8 *(lip*A*)* and H2 *(lip*D*)* by Northern blot hybridization. Under carbon limitation, *lip*D transcripts dominated and *lip*A transcripts were not detected. Under nitrogen limitation, *lip*A was the most abundant transcript and *lip*D expression was relatively low. Quantitative RT-PCR and nuclease protection assays have been developed to detect all known LiP transcripts (Stewart et al., 1992; Reiser et al., 1993). The genes *lip*A, *lip*B and *lip*I were expressed at similar levels in both C- and N-limited cultures. In contrast, *lip*C and *lipJ* transcript levels were substantially increased under N-limitation. Consistent with Holzbaur and Tien (Holzbaur and Tien, 1988), *lip*D transcripts were the most abundant under C-limitation. A recent report suggests that nutrient nitrogen limi-

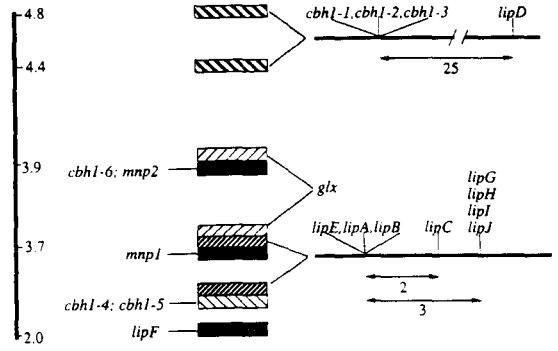

Fig. 3. Integrated physical and genetic map of P. *chrysosporium* dikaryon BKM-F-1767 (Gaskell et al., 1994). Approximate chromosome size in mb is based on CHEF migration and shown on the vertical bar. Dimorphic chromosome pairs have identical shading. Genetic linkage, established by monitoring the segregation of specific alleles in single basidiospore cultures, is presented on horizontal lines to the right. Thus, the *cbh*1-1, *cbh*1-2, *cbh*1-3 cluster shows 25% recombination with *lip*D, but their relative orientation is unknown. Positions of *mnp*1, *mnp*2, *cbh*1-4 and *cbh*1-5 were established solely by CHEF blots. Four LiP genes *(lip*G, *lip*H, *lip*I, *lipJ)* show *100%* cosegregation.

tation may regulate LiP expression post-translationally by heme processing (Johnston and Aust, 1994), but this view has been contradicted by Li et al, (1994).

Strain variation plays an important role in transcriptional regulation of *P. chrysosporium* LiP genes. In contrast to the above mentioned studies with BKM-F-1767, *lip*D (= LIG5) transcripts of strain ME446 appear to dominate in N-limited defined media (James et al., 1992) as well as in more complex substrates such as ball-milled straw (Broda et al., 1995). In strain OGC1O1, *lip*E (= LG2) is expressed at highest levels (Ritch and Gold, 1992).

Manganese peroxidase production in *P. chrysosporium* is dependent upon Mn concentration (Bonnarme and Jeffries, 1990; Brown et al., 1990). Regulation by Mn is at the transcriptional level (Brown et al., 1990; 1991). Potential transcriptional control elements have been identified in 5′-untranslated regions of MnP genes (Godfrey et al., 1990; Alic and Gold, 1991; Brown et al., 1993) and reporter gene constructions may be useful in confirming these putative regulating elements (Godfrey et al., 1994). Pease and Tien (1992) showed differential regulation of MnP isozymes in C- and N-limited cultures. Each isozyme also responded differently to Mn concentration (Pease and Tien, 1992).

GLOX is also transcriptionally regulated in *P. chrysosporium* (Kersten and Cullen, 1993). Consistent with a close physiological relationship between GLOX and LiP, *glx* transcript appearance is coincident with those of *lip*s and *mnp* (Stewart et al., 1992).

The regulation of laccase expression differs substantially among species. Transcripts of *P. radiata* laccase are readily detected under N-limited, lignin olytic conditions. In *T. villosa*, *lcc*1 is strongly induced by 2,5-xylidine addition to cultures, while *lcc*2 transcript levels remained unchanged. In contrast, Northern blots failed to detect *lcc*3, *lcc*4 and *lcc*5 transcripts under any conditions. Three *R. solani* laccases *(lcc*1, *lcc*2, *lcc*3*)* are transcribed at low constitutive levels which can be further repressed by the addition of *p-anisidine* to cultures. However. *R. solani lcc*4 is expressed at much higher levels and induced by additions of *p*-anisidine. In *R. solani, lcc*1. *lcc*2. *lcc*3 are clustered but are separate from *lcc*4 suggesting a relationship between genomic organization and transcriptional regulation.

No obvious relationship exists between genomic organization and transcriptional control in *P. chrysosporium.* The eight closely linked LiP genes show dramatic differences in their response to media composition. For example *lip*C resides less than 15 kb upstream of *lip*B, but *lip*B transcript levels remain unchanged in C- vs N-limited medium while *lip*C transcript levels are greatly increased in N-limited medium (Gaskell et al., 1991; Stewart et al., 1992). As a further example, *glx* is unlinked to peroxidase genes (Gaskell et al., 1994; Kersten et al., 1995) regardless of the close physiological connections (Kersten, 1990; Kurek and Kersten, 1995) and coordinate transcription of *glx* and *lip*s (Stewart et al., 1992; Kersten and Cullen, 1993). In contrast to the situation in *P. chrysosporium,* a cluster of two *lip*s and one *mnp* have been detected in *T. versicolor* and all three genes may be coordinately expressed under certain culture conditions (Johansson, 1994; Johansson and Nyman, 1996).

Elucidating the role and interaction of individual isozymes in lignin degradation and organopollutant degradation requires the identification of specific isozymes in complex substrates. Immunogold labeling experiments have identified LiPs and MnPs in *P. chrysosporium* colonized wood (Blanchette et al., 1989; Daniel et al., 1989, 1990) and Datta et al. (1991) partially purified MnP, LiP, and GLOX from colonized Aspen pulp. However, enzyme yields from solid wood samples are too low for precise isozyme identification.

Recently, quantitative RT-PCR has been applied to *lip* expression in *P. chrysosporium* colonized soil during organopollutant degradation (Lamar et al., 1995). Transcript analysis in such complex substrates has been made possible by the use of magnetic capture techniques for the rapid purification of poly(A) RNA. Initially designed for the detection of *lip*A, *lip*C, *lip*D, *lip*E, *cbh*1-1 and *cbh*1-4 transcripts the methodology has been extended to quantitative analysis of *glx* and all known *lip* and *mnp* transcripts (Bogan et al., 1996a,b). To summarize, MnP genes are coordinately expressed and the patterns of LiP gene transcription are unlike those observed in defined media. Extreme sensitivity and specificity is pro-

vialed by RT-PCR, but caution is warranted as transcript and enzyme levels are not necessarily directly correlated.

Peroxidases are needed for a variety of biochemical investigations, but yields of purified isozymes from fungal cultures are low and heterologous expression has been problematic. In *E. coli*, relatively low levels of aggregated LiP apoprotein are produced in inclusion bodies. Attempts to recover and reconstitute active LiP from *E. coli* have had limited success (reviewed by Pease and Tien, 1991), although recently Whitwam et al. (1995) developed techniques for recovery of active *P. chrysosporium* MnP isozyme H4 from inclusion bodies. *S. cerevisiae* expression systems yield no extracellular and little or no intracellular apoprotein (Pease and Tien, 1991). Expression of the *P. radiata* LiPs in *T. reesei* has been limited to transcripts even when placed under the control of the highly expressed and inducible *cbh*1 promoter (Saloheimo et al., 1989).

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, 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*. Expression was under the control of the *A. oryzae* TAKA amylase promoter and like the baculovirus system, addition of herein 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.

A 'homologous expression' system, in which *mnp* 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). The system has been successfully used in site-directed mutagenesis experiments (Kusters-van Someren et al., 1995).

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* and *R. solani* laccases (Wahleithmer et al., 1995; Yaver et al., 1996). The *P. radiata* laccase has been efficiently expressed in *Trichoderma reesei* under the control of the *T. reesei cbh*1 promoter (Saloheimo and Niku-Paavola, 1991). The *C. hirsutus* laccase gene was expressed in *S. cerevisiae* (Kojima et al., 1990).

Glyoxal oxidase is efficiently expressed in *A. 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. Site specific mutagenesis enabled production of recombinant GLOX isozymes corresponding to the native allelic variants. Several recent biochemical investigations have been aided by *A. nidulans*-produced GLOX (Hammel et al,, 1994; Kurek and Kersten, 1995; Whittaker et al., 1996).

3. Conclusions

Microbial degradation of lignin plays a pivotal role in carbon cycling. Ligninolytic fungi are also critical to emerging biotechnologies such as biomechanical pulping (Wegner et al., 1991; Akhtar et al., 1992), enzymatic bleaching of pulps (Eriksson and Kirk, 1985; Eriksson et al., 1990; Bourbonnais et al., 1995), and organopollutant degradation (Hammel, 1992; Lamar, 1992). Progress has been made. but the genetics and physiology of lignin-degrading fungi is still poorly understood.

A central unanswered question is the role of individual genes in the degradation of lignin and/or xenobiotics. Rapidly developing experimental techniques such as transcript analyses in complex substrates, gene replacements and heterologous expression will help establish the importance of specific genes. In this regard, there continues to be a critical need for an efficient expression system for LiPs.

In addition to basic mechanism(s) of ligninolysis, *P. chrysosporium* is a model system for investigating eukaryotic genome organization. The

species offers interesting and challenging features such as complex families of structurally related genes, differential transcriptional regulation among related genes. polymorphic chromosomes and transposon-like elements. A variety of powerful experimental tools are available including techniques for physical/genetic mapping and for transformation and gene replacement. By studying *P. chrysosporium*. fundamental questions relating to the mechanisms controlling genetic variation can be addressed.

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