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Phanerochaete chysosporium Genomics

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A high quality draft genome sequence has been generated for the lignocellulose-degrading basidiomycete *Phanerochaete chrysosporium* (Martinez et al. 2004). Analysis of the genome in the context of previously established genetics and physiology is presented. Transposable elements and their potential relationship to genes involved in lignin degradation are systematically outlined. Our current understanding of extracellular oxidative and hydrolytic systems is described. Areas of uncertainty are highlighted and future prospects discussed in light of the newly available genome data.

1. INTRODUCTION

Numerous microorganisms participate in the global conversion of organic carbon to CO_2 with the concomitant reduction of molecular oxygen. The most abundant source of carbon is plant biomass, composed primarily of cellulose, hemicellulose, and lignin. Many fungi and bacteria are capable of degrading and utilizing cellulose and hemicellulose as carbon and energy sources, but a much smaller group has evolved with the ability to breakdown lignin, the most recalcitrant component of plant cell walls. Collectively referred to as white rot fungi, these filamentous basidiomycetes possess the unique ability to degrade lignin completely to CO_2 in order to gain access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources. Such wood-decay fungi are common inhabitants of forest litter and fallen trees.

The enzymes from white rot fungi that catalyze degradation of lignin are extracellular and unusually nonspecific. A constellation of oxidases, peroxidases, and hydrogen peroxide are responsible for generating highly reactive and nonspecific free radicals that can affect depolymerization and degradation of lignin. The nonspecific nature and extraordinary oxidation potential of the enzymes from white rot fungi have attracted considerable interest for industrial applications such as biological pulping of

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wood, fiber bleaching, and remediation of soils and effluents contaminated with a wide range of organopollutants.

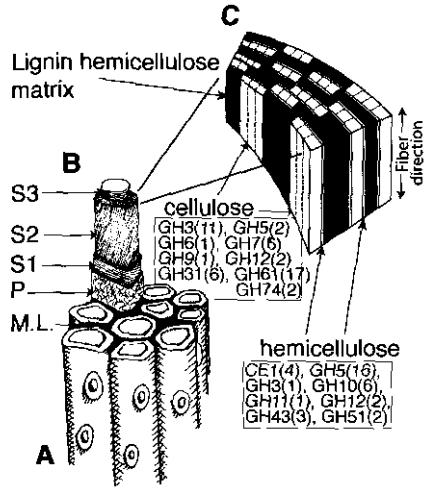

Fig. 1. Schematic illustration of wood tissue showing A. tracheid bundle; B. cell wall layers; and C. arrangement of carbohydrates and lignin within the S2 layer of the secondary wall (based on (Goring, 1977)). The bulk of the wall is in the S2 layer. In the model shown, lignin and hemicellulose form a matrix encrusting cellulose. The cellulose makes up approximately 45% of the weight of wood and is arranged in microfibril spirals along the long axis of the cell. Lignin is distributed throughout the S2 and middle lamella (M.L.). On th basis of their catalytic domains, enzymes hydrolysing the glycosidic bonds of cellulose and hemicellulose were assigned to glycosyl hydrolase (GH) families (http:://afmb.cnrs-mrs.fr/CAZY/index.). Within families, the number of sequences detected by Martinez et al (2004) is indicated parenthetically. The precise function of genes within families is often unclear. Certain families, e.g. GH3, GH5, GH31 are quite diverse with respect to the biological function of members. Lignin depolymerization is believed to involve free radicals generated through the combined action of peroxidases, possibly small molecular weight mediators, and peroxide-generating oxidases (see Figure 2). P, primary wall; S1-S3, secondary wall layers.

The most intensively studied white rot organism is *P. chrysosporium.* Using a pure whole genome shotgun strategy, a high quality draft genome sequence has been assembled (www.jgi.doe.gov/whiterot). Initial analysis (Martinez et al. 2004) of the *P. chrysosporium* genome revealed features of importance to our understanding of lower eukarotic gene structure and organization, identified hundreds of genes involved in lignocellulose degradation, and provided a framework for achieving a deeper understanding of degradative processes. In the following pages, we briefly summarize the microbiology and physiology of ligniocellulose degradation. For more detail, readers are referred to previous reviews (Blanchette, 1991; Eriksson et al. 1990; Kirk and Farrell, 1987; Kirk and Cullen, 1998). Emphasis will be on the molecular genetics of *P. chrysosporium*. Other fungi are mentioned only as points of reference.

2. MICROBIOLOGY AND PHYSIOLOGY OF WOOD DECAY

The hyphae of white rot fungi rapidly invade wood cells and from within the lumen (Figure 1) secrete an array of enzymes and metabolites that depolymerize hemicelluloses, cellulose, and lignin. Constituting approximately 40% of the weight of wood, cellulose is a linear polymer of cellobiose units linked by β-1,4-glycosidic bonds. Individual cellulose molecules are arrayed in bundles known as microfibrils. The bulk of the cell wall is within the secondary wall (Figure 1; S1, S2 and S3 layers), and within these layers microfibrils have different parallel orientations with respect to the axis of the cell. Cellulose appears highly crystalline in diffraction measurements. Like cellulose, hemicelluloses are linear β-1,4-linked monosaccharide polymers. However hemicelluloses have mono-, di- or trisaccharides branches that may include sugars, sugar acids, acetylated sugars and sugar acid esters. Hemicellulose makes up 25 to 30% of the weight of wood and is covalently bound through infrequent linkages to lignin.

In contrast to the glycosidic linkages within cellulose and hemicellulose, lignin is comprised of carbon-carbon and ether bonds between phenylpropanoid residues (Higuchi, 1990; Lewis and Sarkanen, 1998). Consequently, lignin degradation involves oxidative mechanisms, as opposed to hydrolytic mechanisms. The polymer is stereoirregular, and the ligninolytic agents are generally assumed to be less specific relative to cellulases and hemicellulases. 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. It is generally believed that lignin depolymerization is necessary to gain access to cellulose and hemicellulose. No microbe, including any white rot species, is known to utilize lignin as a sole carbon source.

Only white rot basidiomycetes have been convincingly shown to efficiently mineralize lignin, although gross patterns of decay can differ substantially among species and strains (for review see Blanchette, 1991; Daniel, 1994; Eriksson et al. 1990). Microscopic analysis show *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. How such selective degradation occurs is puzzling because enzymes are too large to penetrate sound, intact wood (Blanchette et al. 1997; Cowling, 1961; Flournoy et al. 1993; Srebotnik et al. 1988b; Srebotnik and Messner, 1991) 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. Because lignin-depolymerizing enzymes and many cellulases are in the same size range as ovalbumin, it has been proposed that small molecular weight oxidants penetrate from the lumens into the walls. Various diffusible oxidative species have been proposed.

Brown rot fungi, another category among homobasidiomycete wood decay fungi, do not degrade lignin but may be relevant to lignocellulose degradation by *P. chrysosporium*. 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 see Gilbertson, 1981; Worral et al. 1997). Recent molecular phylogeny suggests they have been repeatedly derived from white rot fungi (Hibbett and Donoghue, 2001). Depolymerization of crystalline cellulose proceeds long before wood porosity would admit cellulases, suggesting the participation of small molecular weight oxidants.

Hydroxyl radical, generated via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + .OH$), has been strongly implicated as a diffusible oxidant in brown rot (Cohen et al. 2002; Cohen et al. 2004; Xu and Goodell, 2001), and to a lesser extent, in white rot wood degradation. The possibility of such a reactive oxygen species was long ago suggested in *P. chysosporium* (Bes et al. 1983; Evans et al. 1984; Forney et al. 1982; Kirk and Nakatsubo, 1983; Kutsuki and Gold, 1982), but subsequent studies showed that Fenton reactions with lignin model compounds yielded products unlike those produced in ligninolytic cultures or by isolated peroxidases (Kirk et al. 1985). Still, some evidence supports Fenton system involvement in lignocellulose depolymerization by *P. chysosporium* (Henriksson et al. 1995; Tanaka et al. 1999; Wood, 1994). particularly via cellobiose dehydrogenase (see below; for review see Henriksson et al. 2000a; Henriksson et al. 2000b). Current models for hydroxyl radical participation have been reviewed (Goodell, 2003) and typically involve generation of the highly reactive oxidant at or near the substrate. This might include small molecular weight chelators transferring iron along extracellular pH gradients (Xu and Goodell, 2001) or through cellulose binding as in the case of cellobiose dehydrogenase (Henriksson et al. 2000a; Henriksson et al. 2000b).

3. EXPERIMENTAL SYSTEMS

3.1. Experimental Tools

Advances on the molecular genetics of white rot fungi have been made possible by an array of experimental tools. For *P. chysosporium*, methodology has been established for auxotroph production (Gold et al. 1982), recombination analysis (Alic and Gold, 1985; Gaskell et al. 1994; Krejci and Homolka, 1991; Raeder et al. 1989a). rapid DNA and RNA purification (Haylock et al. 1985; Raeder and Broda, 1985), differential display (Birch, 1998; Kurihara et al. 2002). pulsed field electrophoretic karyotyping (D'Souza et al. 1993; Gaskell et al. 1991; Orth et al. 1994). and genetic transformation by auxotroph complementation (Akileswaran et al. 1993; Alic et al. 1989; Alic et al. 1990; Alic et al. 1991; Alic, 1990; Randall et al. 1991; Zapanta et al. 1998) and by drug resistance markers (Gessner and Raeder, 1994; Randall et al. 1989; Randall et al. 1991; Randall and Reddy, 1992). Transformation efficiencies are relatively low and gene disruptions are difficult (Alic et al. 1993), but reporters for studying gene expression have been described (Birch et al. 1998; Gettemy et al. 1997; Ma et al. 2001). One of the most promising experimental approaches currently being adapted to *P. chysosporium* is the use of two dimensional gel electrophoresis followed by mass spectrometry-based protein identification (Abbas et al. 2004; Shimizu et al. 2004).

As is common for basidiomycetes, the vegetative mycelium of *P. chrysosporium* is dikaryotic. However, clamp connections are absent and the cells are coenocytic

(Burdsall and Eslyn, 1974; Stalpers, 1984). The most widely studied strain, BKM-F-1767, produces abundant asexual spores, all of which are multinucleate and dikaryotic. Difficulties differentiating allelic variants from closely related genes and the lack of an accepted standardized nomenclature has complicated studies of gene families (Gaskell et al. 1994). Pulsed field gel electrophoresis identified 7-9 chromosomes with a haploid genome size of approximately 30 Mbp. Most allelic chromosomes differ in length (Gaskell et al. 1991; Gaskell et al. 1994; Kersten et al. 1995; Orth et al. 1994; Stewart et al. 1992). The underyling structure of such chromosome length polymorphisms (CLPs) is unknown although they are a common feature of fungal genomes (Zolan, 1995).

Single basidiospores of *P. chrysosporium* are fully viable and generally homokaryotic. Analyses of single basidiospore cultures have been used to differentiate alleles, and to create genetic and physical maps of *P. chrysosporium* (Covert et al. 1992a; Gaskell et al. 1992; Gaskell et al. 1994; Kersten et al. 1995; Li et al. 1997; Li and Renganathan, 1998; Raeder et al. 1989b; Schalch et al. 1989; Stewart et al. 1992; Stewart and Cullen, 1999). However, single basidiospore strains typically exhibit reduced sporulation, growth rate, and enzyme yields relative to the parental strain (Raeder et al. 1989b; Wyatt and Broda, 1995). Further, CLPs and other aspects of genome organization are not maintained through meiotic recombination, limiting the experimental value of basidiospores (Covert et al. 1992a; Gaskell et al. 1991; Kersten et al. 1995; Stewart et al. 1992; Zolan, 1995). A homokaryon of non-meiotic origin, RP-78, circumvents the disadvantages incurred by recombination and has greatly simplified assembly of genome sequence (Martinez et al. 2004; Stewart et al. 2000).

Beyond *P. chrysosporium*, *Pleurotus ostreatus* is probably the next best white rot experimental system offering transformation protocols (Honda et al. 2000; Irie et al. 2001; Sunagawa and Magae, 2002; Yanai et al. 1996) and methodology for physical (Larraya et al. 1999) and genetic mapping (Eichlerova and Homolka, 1999; Eichlerova-Volakova and Homolka, 1997; Larraya et al. 2000; Larraya et al. 2002). *Trametes 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; Cullen and Kersten, 1996; Cullen, 1997; Gold and Alic, 1993; Pease and Tien,1991).

3.2. Genome Sequencing

In a major research advance, the U.S. Department of Energy´s Joint Genome Institute (JGI) has completed whole genome shotgun sequencing of *P. chrysosporium* strain RP-78 to 10.5X coverage. The draft assembly and interactive annotated browser are freely available at www.jgi.doe.gov/whiterot. The 30 Mb genome is distributed on 383 scaffolds greater than 2 kb, and the largest 165 scaffolds contain 90% of the assembled sequence. Contiguity of the largest scaffolds has been validated by genetic segregation analysis of terminal markers (Gaskell et al. 1994). Further support for the long-range structure of the assembly came from end sequencing cosmid clones. Of 1390 unique

ESTs derived from colonized wood, 98% were identified in the assembly and their positions noted on the browser.

Gene modeling predicted over 11,222 genes of which 8486 gave significant Smith-Waterman scores to known GenBank proteins. The taxonomic distribution and identification of conserved Interpro (Mulder et al. 2003) domains were reported and compared to the other published fungal genomes, *Saccharomyces cerevisiae* (Goffeau et al. 1996), *Schizosaccharomyces pombe* (Wood et al. 2002). and *Neurospora crassa* (Galagan et al. 2003; Mannhaupt et al. 2003; Schulte et al. 2002). Among other features, this analysis revealed a major expansion of the cytochrome P450s (below). Models not showing significant similarity to known proteins correspond to highly divergent, previously unrecognized genes perhaps unique to filamentous fungi, basidiomycetes, or white rot fungi, or spurious gene predictions.

One of the more distinguishing features of *P. chysosporium* genome is the occurrence of large and complex families of structurally related genes. As described more fully below, families include cytochrome P450s, peroxidases, cellulases, copper radical oxidases and multicopper oxidases. In some cases, but not all, clustering is observed. The role of gene families in lignocellulose degradation remains uncertain. Structurally related genes may encode proteins with subtle differences in function, and such diversity may be needed to meet the challenges of changing environmental conditions (pH, temperature, ionic strength), substrate composition and accessibility, and wood species. Alternatively, some or all of the genetic multiplicity may simply reflect redundancy. Evidence against the latter view, albeit indirect, is that certain closely related genes are differentially regulated in response to substrate composition It should also be mentioned that the genetic multiplicity of *P. chysosporium* stands in stark contrast to *N. crassa,* where a repeat-induced point mutation system is believed to have greatly restricted the number and size of gene families. Providing further insight into these issues, analyses of the basidiomycetes *Filobasidiella neoformans* (= *Cryptococcus neoformans*) (http://www-sequence.stanford.edu/group/C.neoformans/index.html), *U. maydis* (http://www-genome.wi.mit.edu/seq/fgi/candidates.html), and *C. cinereus* (http://www-genome.wi.mit.edu/seq/fgi/candidates.html) will soon be published.

To be as current as possible, this review describes gene models recently 'mined' from the current database. However, we emphasize that these are generally automated predictions many of which are partially incorrect. This is a common problem in eukaryotic genomes, particularly among genes with multiple introns and short exons. Accordingly, proteins predicted from genomic sequence should be considered tentative until verified by cDNA analysis. Another qualification concerning the genome relates to inclusion of repeats. In short, whole genome shotgun assemblies such as *P. chrysosporium* typically exclude telomeres, rRNA clusters, and many repeats.

3.3. Repeats

Repetitive elements of *P. chrysosporium* have been associated with several genes encoding extracellular enzymes. The most thoroughly studied element had been Pce1, a repeat inserted within LiP allele *lipI2* (Gaskell et al. 1995). The 1747 nt sequence

transcriptionally inactivates *lipI2* and three copies are distributed on the same chromosome. Sequence flanking these copies showed no evidence of recombination (Stewart et al. 2000). In addition to Pce1-like elements, a broad array of non-coding repetitive sequences and putative mobile elements have been identified by systematic examination of the genome database. Short repeats (<3kb) not clearly associated with transposons vary in copy number from >40 (GenBank accession number Z31724) to 4 (AF134289-AF134291)(Table 1).

Table 1. Simple non-coding repeats identified in *P. chrysosporium*

Location[1]	Type[2]	Accession	Probable copies	Comment
85:13061-14631	Pce1	L40593		Element inserted within lignin peroxidase gene *lipI*. Co-segregates with Pce2, Pce3, and Pce4.
223:16314-17591	Pce2	AF134289		Truncated at REND of scaffold.
57:144178-142237	Pce3	AF134290		
10:259013-257364	Pce4	AF131291		
91:115464-117260		Z31724	Portions distributed on >40 scaffolds Mort at termini, e. g. LEND s384, s28, s141; REND s292, s260, s136, s72.	Often associated with retroelements as direct or inverted repeats First 350 nt of the 731724 not located by blast.

[1]Location is defined by genome scaffold number : nucleotide coordinates on current assembly of the Joint Genome Institute´s interactive browser (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html)

[2]The sequence, transcriptional impact, and genetic linkage of Pce elements have been reponed (Gaskell et al. 1995; Stewart et al. 2000).

Several putative Class II elements, or DNA transposons, were identified in the *P. chrysosporium* genome (Table 2). Similar ascomycetous elements include *Aspergillus niger* Ant, *Cochiobolus carbonum* Fot1, *Nectria* "Restless", *Fusarium oxysporum* Tfo1, and *Cryphonectria parasitica* Crypt1 (Kempken and Kuck, 1998). Atypical of fungi but common in higher plant genomes, EN/Spm- and TNP-like elements were also found (Martinez et al. 2004). Interestingly, the *P. chrysosporium* DNA transposons are present in low copy numbers (1-4 copies) relative to Ascomycetes, where class II elements often exceed 50-100 copies.

A substantial number of multi-copy retrotransposons were identified in the database, some of which seem likely to impact expression of genes related to lignin degradation (Table 3). Typical of these elements, they often appear truncatcd and/or rearranged, and the long terminal repeats, characteristic of retroelements, often lie apart as "solo LTRs" (Goodwin and Poulter, 2000; Kim et al. 1998). Several non-LTR retrotransposons, similar to other fungal LINE-like retroelements, were also found by blast searches, Unusual for a fungal genome (Daboussi and Capy, 2003), *copia*-like retroelements are particularly abundant. In one case, the *copia* element gx.24.14.1 interrupts a cytochrome

P450 gene (models pc.24.16.1 + pc.24.17.1) within its seventh exon (gene model gx.24.14.1 (Martinez et al. 2004). A multicopper oxidase gene, *mco3* also has an inserted element (unpublished), and gypsy element pc.91.4.1 lies immediately adjacent to a gene encoding an extracellular peroxidase (gx.91.10.1).

Table 2 Putative class II elements identified[1] in *P. chrysosporium*

Gene model/ location[2]	Type	Related[3] to:	Comments[4]
pc.197.6.1	Ac/hAT	*F. oxysporum* Folyt1 (AF057141)	Poor model. Possible ITRs.
pc.88.61.1	Ac/hAT	*F. oxysporum* Tfo1 (T00208)	Poor model. (210aa). Copy:pc.14.47.1 + pc.14.45.1
gw.65.44.1	Ac/hAT	*F. oxysporum* Tfo1 (T00208)	Poor model. (210aa).
pc.247.3.1+ pc.247.4.1	Ac/hAT	*C. parasitica* Crypt1 (AF2283502)	Disjoint models. (76aa + 115aa).
pc.112.17.1	Tc1/Mariner	*A. niger* Ant1 (AF283502)	Partial model (320aa). Possible imperfect 65nt ITRs.
s213.13484-17985 (pc.213.8.1)	Tc1/Mariner	*Aspergillus niger* Ant1 (AF283502)	213 nt ITRs. Model overlaps dehydrogenase.
s128.64902-68226 (pc.128.43.1)	Tc1/Mariner	*Aspergillus niger* Ant1 (AF283502)	80 nt ITRs. Copy:pc.234.7.1 (REND; no ITRs)
pc.270.1.1(N-teminal) + gx.224.2.1(COOH region)	Fot1/Pogo	*Cochliobolus carbonum* Fot1-like. (JC5096)	N-terminus (239aa) & COOH (149aa) terminus on LEND & REND, respctively, of 2 scaffolds.
pc.25.43.1+gx.25.15.1	TNP	Putative *A. thliana* TNP2 (AC005897). No fungal examples.	Poor models. Similar to higher plant TNP- & En/Spm-like elements Copies:pc.15.120.1; pc.125.4.1
pc.90.8.1	TNP	Hypothetical carrot Tdc1(AB001569). No fund examples.	Similar to higher plant TNP- and En/Spm-like elements.Copies:pc.125.4.1; pc.249.91.1

[1]Searches of Joint Genome Institute's interactive browser (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) by keywords (e.g. transposase, transpos, retroelement, transposon, retrotransposon) and by blast with known fungal class I and II elements; [2]Computer generated gene model designations (pc., gx., gw.) or, in case of elements with terminal repeats, scaffold coordinates (s.nucleotide positions). Models are often truncated at either termini: [3]Following initial screening, trimmed sequences were identified by blast searches of NCBI database. Short sequences at scaffold termini and those located on short scaffolds were generally ignored; [4]Abbreviations: REND, right end of scaffold; LEND, left end of scaffold; ITRs, inverted terminal repeats.

Viewed together with the Pce1 mutant of *lip1*, it seems *P. chrysosporium* transposons have a proclivity for insertions within gene families. Recombination among these repetitive elements may be involved in the evolution of these families as well as in chromosome length polymorphisms (for review see Zolan, 1995). A novel class of eukaryotic DNA transposons, Helitrons, were recently identified in *P. chrysosporium*

(Poulter et al. 2003). None of the three *P. chrysosporium* Helitrons appear to be within or adjacent to functional genes. Tyrosine recombinase-encoding retrotransposons appear more abundant in the *P. chysosporium* genome (Goodwin and Poulter, 2004), but again, the elements and their remnants are not inserted within recognizable structural genes.

Table 3. Identification[1] of class I elements in the *P. chrysosporium* genome.

Location or gene model[2]	Related[3] to:	LTRs/copies (c)/ comments (cm)
Copia-like retrotransposons (LTRs):		
s84.4772-9223 (gx.84.1.1)	*T. tabacum* retrotransposon TNT1 (P10978)	LTR: 333 nt c: pc.142.16.1; pc.209.1.1; pc.214.3.1: LTR at REND s23 cm: Few fungal examples, e.g. *Candida* (AF065434).
s24.47199-52675 (gx.24.14.1)	T. tabacum retrotransposon TNT1 (P10978)	LTR:420 nt c: LTR at REND s302 and s277; LTR at LEND s114 cm: Few fungal examples e.g. *S. cerevissiae* Ty2.
s235.5494-10983 (pc.235.3.1)	Copia type pol polypetptide rice (AC092553) and tobacco (T02206)	LTR: 127 nt c: gx.224.1.1 (w/LTR); gx.253.1.1 (w/LTR): gx.247.1.1 (w/LTR); pc.265.3.1 (w/LTR); gx.290.1.1(w/1LTR); gx.268.1.1; gx.84.1.1; gx.217.1.1(w/LTR); gx.293.1.1 cm: Few fungal examples t.g. *S. cervisiae* Ty1 protein B (T29093).
gx.173.6.1	Gag protein of insects and higher plants. *Anopheles gambiae* (AF387862)	LTR: ~200 nt c: gw.14.1.1 cm: No pol polypeptide detected.
s24.202590-205821	*A. thaliana* pol polypeptide (AC006841)	LTR: 190 nt c: pc.324.2.1 + pc. 324.1.1 (w/LTR) cm: LTR at REND s145
Gypsy-like retrotransposons (LTRs):		
s166.34077-42356 (pc.166.16.1 + pc.166.17.1)	*Yarrowia lipolytica* retrotransposon Ylt1 (AJ310725)	LTR: 327 nt cm: Also similar to other fungal elements (e.g. MAGGY, CfT-1)
pc.233.1.1	*Yarrowia lipolytica* retrotransposon Ylt1 (AB310725)	c: s183.13150-27198 (w/LTR) pc.84.26.1; pc.141.13.1; s250. REND; gw.269.1.1; s152.LEND; gx.264.1.1; pc.65.56.1 + pc.65.57.1
s91.3017-11324 pc.91.4.1	*Magnaporthe grisea* MAGGY (L35053) and *Tricholoma matsuke* marY1 (AB028236)	LTR: 1023 nt c: gx.311.1.1; gx.241.2.1;pc.241.2.1; gx.219.2.1; s242.3501-8500 cm: Polymerase and integrase (91.4.1) similar to MAGGY and Tricholoma marY1. Gag polypeptide (pc.91.5.1) closer to *Glomeralla* and *Aspergillus.*

s21.40796-50402 (gx.21.9.1)	*Tricholoma matsuke* marY1 (AB028236)	LTR: 209 nt c:pc.14.4.1 cm: LTR flanking tandem duplication.
s91.69200-78439 (pc.91.37.1 + pc.91.36.1)	Most closely related to higher plant elements. Best fungal hit = *Glomerella* Cgret (AF264028).	LTR: 327 nt c: gw.195.3.1; gx.248.2.1; gx.204.1.1 + pc.204.3.1; gx.308.1.1; pc.98.1.1 cm: More distantly related to *S. pombe* Tf2.
Possible Gypsy-like Retrotransposon (LTRs not detected):		
gx.305.1.1	*Tricholoma matsuke* marY1 (AB028236)	LTR: probably lost c: pc.291.1.1; gx.329.1.1; gx2.266.1.1; gx.170.1.1; gx.328.1.1 cm: No gag detected.
Retroposons (LINE-like):		
gx.214.3.1 + gw.214.5.1	gx.214.3.1 most like *Candida albicans* RT AF139376.	cm: gw.214.5.1 encodes RnaseH with no fungal hits. Z31724 simple repeat located near COOH terminus.
gx.419.1.1	*Tricholoma matsuke* marY2 (AB047280	cm: Similar to other fungal LINEs. Truncated at COOH terminus (LEND scaffold) & missing polyA.
pc.36.36.1(RnaseH) + pc.36.37.1(pol2)+ pc.36.38.1(pol1)+ pc.36.39.1(gag)	*Tricholoma matsuke* marY2 (AB047280	c: pc.47.81.1 (pol2) + pc.47.80.1 (pol1); pc.44.90.1 (pol2); pc.206.5.1 (pol1) + pc.206.6.1 (pol2) + pc.206.7.1 (pol2) cm: Similar to other fungal LINEs, especially *N. crassa* Tad1. Appears complete (gag, pol, polyA)

[1]Searches of joint Genome Institute's interactive browser (http://genome.jgi/psf.org/whiterot1/whiterot1.home.html) by keywords (e.g. transposase, transspos, retroelement, transposon, retrotransposon) and by blast with known fungal class I elements (e.g. MAGGY, Tad1); [2]Computer generated gene model designations (pc., gw., gx. prefixes) or, in cases of elements with extended terminal repeats, scaffold (s) coordinates. Models are often truncated at either termini; [3]Following initial screening, trimmed sequences were blasted against NCBI databases; [4]Following initial screening, trimmed sequences were used to blastn and tblastn search the *P. chrysosporium* database. Short sequences at scaffold termini and those located on short scaffolds were generally ignored.

4. EXTRACELLULAR OXIDATIVE SYSTEMS

As mentioned above, the polymers constituting plant cell walls are too large to be taken up by fungal hyphae and extracellular depolymerization must occur. Accordingly, the vast majority of research on white rot fungi has focused on the secreted enzymes.

4.1. Lignin Peroxidases and Manganese-dependent Peroxidases

Since their discovery (Glenn et al.1983; Gold et al. 1984; Paszczynski et al. 1985; Tien and Kirk, 1983, 1984), lignin peroxidase (LiP) and manganese peroxidase (MnP) have been the most intensively studied extracellular enzymes of *P. chrysosporium*. Review articles summarize the biochemistry (Higuchi, 1990; Kirk and Farrell, 1987; Kirk, 1988; Shoemaker and Leisola, 1990) and genetics (Alic and Gold, 1991; Cullen and Kersten,

1996; Cullen, 1997, 2002; Gold and Alic, 1993) of these enzymes. Both are protoporphyrin IX peroxidases. Isozymic forms are encoded by families of structurally related genes and further modified posttranslationally.

LiP catalyzed reactions include C_α-C_β 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 (Hammel et al. 1985; Leisola et al. 1985; Renganathan et al. 1985; Renganathan and Gold, 1986; Tien and Kirk, 1984; Umezawa et al. 1986)(Figure 2). The importance of lignin peroxidase in the depolymerization of lignin *in vivo* was convincingly shown by Leisola et al. (Leisola et al. 1988). Partial depolymerization of lignin *in vitro* has been demonstrated for LiP (Hammel and Moen, 1991) and MnP (Wariishi et al. 1991).

MnP oxidizes Mn^{2+} to Mn^{3+}, using H_2O_2 as oxidant (Gold et al. 1984; Paszczynski et al. 1985). 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). 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 (Kishi et al. 1994; Kuan et al. 1993). 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(II)-dependent oxidation of glyoxylate and oxalate generates H_2O_2 (Kuan et al. 1993; Urzua et al. 1998).

The crystal structures of LiP (Edwards et al. 1993; Piontek et al. 1993; Piontek et al. 2001) and MnP (Sundaramoorthy et al. 1997) show similarities; 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. Kinetic studies of MnP variants indicate that manganese-binding involves Asp-179, Glu-35, Glu-39 (Kusters-van Someren et al. 1995; Sollewijn Gelpke et al. 1999; Whitwam et al. 1997; Youngs et al. 2001) and a heme propionate, consistent with x-ray crystallographic analysis (Sundaramoorthy et al. 1997).

Recent genome analyses (Martinez et al. 2004) located the 10 known LiP genes previously designated *lipA* through *lipJ* (Gaskell et al. 1994). Eight of the LiP genes were found clustered within 3% recombination (Gaskell et al. 1994; Stewart and Cullen, 1999), which corresponds to 96 kb (Martinez et al. 2004). cDNAs were previously reported for genes *mnp1, mnp2,* and *mnp3* (Alic et al. 1997; Orth et al. 1994; Pease et al. 1989; Pribnow et al. 1989), and two new MnP genes were revealed by Blast searches of the genome (Martinez et al. 2004). One of these new genes has been designated *mnp4* (gene model, 15.18.1) (Martinez et al. 2004). Unexpectedly, mnp4 was found to lie approximately 5 kb upstream from mnpl (model 15.23.1). and a cytochrome P450 gene is located in the *mnp4-mnp1* intergenic region. Recent data shows that mnp4 is actively transcribed when *P. chrysosporium* is grown on wood-containing soil samples (Stuardo et al. 2004). Gene model 9.126.1, *mnp5,* (Martinez et al. 2004) corresponds to the N-terminal amino acid sequence of an MnP purified from *P. chrysosporium*-colonized wood pulp (Datta et al.

1991). Future work requires a more detailed analysis of the expression profile of these newly identified *mnp* genes. The five *mnp* sequences are remarkably conserved (Figure 3). The number and positions of introns are also conserved, particularly *mnp1* and *mnp4*, and this suggests a recent duplication (Figure 4) (Stuardo et al. 2004).

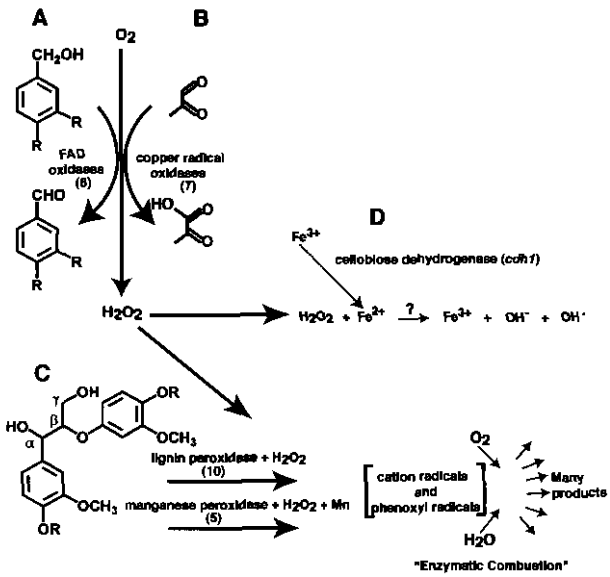

Fig. 2. Schematic representation of major extracellular oxidative enzymes produced by lignin degrading fungi. Generation of H_2O_2 is physiologically coupled to peroxidases. Benzyl alcohol derivatives (A) are substrates for FAD-dependent oxidases such as aryl alcohol oxidase (R= H or OCH_3). Methyl gloxal (B) is a substrate for glyoxal oxidase and possibly for related copper radical oxidases Peroxidase substrate C is a lignin model featuring the major β-O-4 linkage (R= H or ether linkage to additional monomeric units). Peroxidases abstract one electron from aromatic substrates which then undergo spontaneous degradation reactions or "enzymatic combustion" (Kirk and Farrell, 1987). The resulting small molecular weight fragments are further metabolized intracellularly to CO_2 and H_2O. Peroxide might also be a reactant in the spontaneous (non-enzymatic) generation of hydroxyl radical via Fenton's chemistry (D). Reduction of Fe^{3+} has been demonstrated for cellobiose dehydrogenase. Parenthetical numbers indicate structurally related sequences identified to date.

In addition to *mnp4* and *mnp5*, a partial *mnp*-like sequence encoding just the COOH-terminus was discovered. This partial 274 nt *mnp* sequence (gw.9.92.1), named *mnp6*, is located 85 kb downstream from *mnp5* (scaffold 9), and as observed for the latter, it is encoded by the minus strand. PCR amplification and sequencing verified *mnp6*, and excluded the possibility of assembly error (unpublished data). In addition, we confirmed that this partial sequence is present in both nuclei, and also in *P. chrysosporium* strain ME446 (unpublished data). When manually translated, this

sequence shows the highest homology (65%) to aminoacids 299 to 370 of MnP5. An intron splits the codon for aa 355 in *mnp5,* and *mnp6* also possesses an 58 nt intervening sequence at that position. However, splicing of the intron adheres to the GT-AG rule only in its 5´; the 3´ splice seems to be less well defined.

No evidence for the transcription of *mnp6* could be obtained. This, in addition to the presence of a stop codon in the middle of its sequence, and the low conservation of its intronic region suggest that mnp6 is inactive and the possible product of an aberrant recombination or duplication event.

LiP and MnP genes have been identified in other species, and recent cladistic analysis by Martinez (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 expected by its role in catalysis by long range electron transfer, Trp171 is common to LiPs, whereas Mn-binding residues (Glu35, Glu39, Asp179 in *mnp1*) are found in MnP sequences.

Certain peroxidases cannot be easily classified. Unusual *Pleurotus eryngii* sequences 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) (Camarero et al. 2000; Ruiz-Duenas et al. 1999; Ruiz-Duenas et al. 2001). The *P. eryngii* genes have both Trp171 and the residues involved in Mn-binding. Searches of *P. chrysosporium* genome revealed a putative extracellular peroxidase related to the Pleurotus hybrid peroxidase (Ruiz-Duenas et al. 1999), but catalytic and Mn-binding residues are not conserved (Martinez et al. 2004). Designated *nop*, the *P. chrysosporium* gene (GenBank accession AY727765), shows novel structural features, which distinguish it from any of the aforementioned peroxidases (unpublished results).

It is now well established that the peroxidase genes of *P. chrysoporium* are differentially regulated by culture conditions. Holzbaur and Tien (Holzbaur and Tien, 1988) showed that steady state levels of *lipD* transcripts were far more abundant than those of *lipA* under carbon starvation. The situation was reversed under nitrogen starvation, i.e *lipA* transcripts dominated. Beyond these early Northern blot results, competitive RT-PCR and nuclease protection assays have been used for quantitative differentiation of the closely related transcripts (Reiser et al. 1993; Stewart et al. 1992) in defined media (Reiser et al. 1993; Stewart et al. 1992; Stewart and Cullen, 1999), in organopollutant contaminated soils (Bogan et al. 1996c). and in colonized wood (Janse et al. 1998). These studies have shown that differential regulation can exceed five orders of magnitude and that transcript profiles in defined media poorly predict profiles in complex substrates. Importantly, the observed patterns of expression show no clear relationship with genome organization. Post translational regulation by heme processing has been suggested (Johnston and Aust, 1994) but contradicted by the results of Li et al. (1994).

In addition to nutrient conditions, MnP production in *P. chrysosporium* is dependent upon Mn concentration (Bonnarme and Jeffries, 1990; Brown et al. 1990). The *P. chysosporium* MnP genes *mnp1, mnp2,* and *mnp3* generally show coordinate regulation in colonized soil and wood (Bogan et al. 1996a; Janse et al. 1998). Putative metal

response elements (MREs) have been identified upstream of *mnp1* and *mnp2* and their transcript levels respond to Mn^{2+} supplements in low nitrogen media (Pease and Tien 1992a; Gettemy et al. 1998). In contrast, *mnp3*, lacks paired MREs and its transcript levels are not influenced by addition of Mn^{2+} (Gettemy et al. 1998; Pease and Tien, 1992). Taken together, these observation support a possible role for MREs in transcriptional regulation of *P. chrysosporium* MnP genes (Alic et al. 1997; Gettemy et al. 1998). However, MnP regulation appears not to involve MREs in *T. versicolor* (Johansson and Nyman, 1993; Johansson et al. 2002) or in *C. subvermispora* (Manubens et al. 2003). Another interesting *T. versicolor* gene, *npr*, appears repressed by Mn even though putative Mn-binding residues are present in the sequence (Collins et al. 1999).

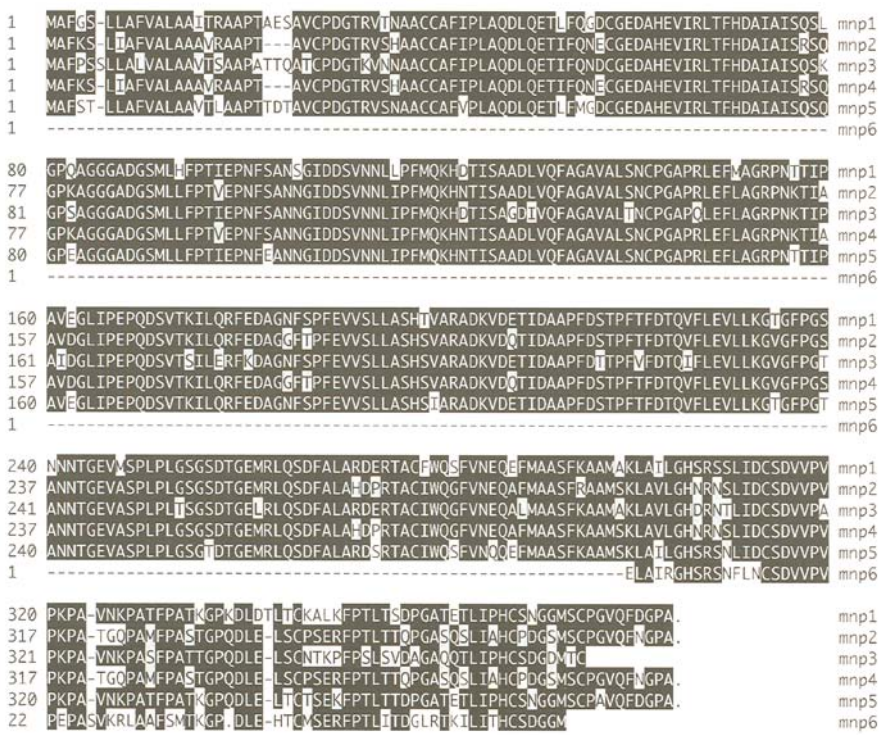

Fig. 3 Clustal W alignment of predicted manganese peroxidase proteins of *P. chrysosporium.* Genes *mnp1* through *mnp5* are supported by cDNA evidence. cDNAs corresponding to *mnp6* sequence have not been detected.

The role of MREs in Mn-regulated transcription of the *mnp* genes was recently examined by Ma et al (2004). Using a green fluorescent protein reporter system, a 48-bp

sequence containing at least one Mn^{2+}-responsive *cis* element was identified. Further characterization suggests that a 33-nt portion is responsible for the observed regulation. None of the 6 putative MREs present in *mnp1* is contained in the aforementioned region, and functional evaluation of 4 of these MREs, show no significant effect in the Mn^{2+} response (Ma et al. 2004). Similar regulatory sequences were identified upstream of *mnp2* and *mnp3* (Ma et al. 2004). The new data suggest that in the absence of Mn, a negative control is exerted at that sequence, which is released in the presence of the metal. The existence of additional Mn-responsive *cis* acting sequences in the *mnp1* promoter is supported by the residual responsiveness to Mn when this *bona fide* sequence is absent (Ma et al. 2004).

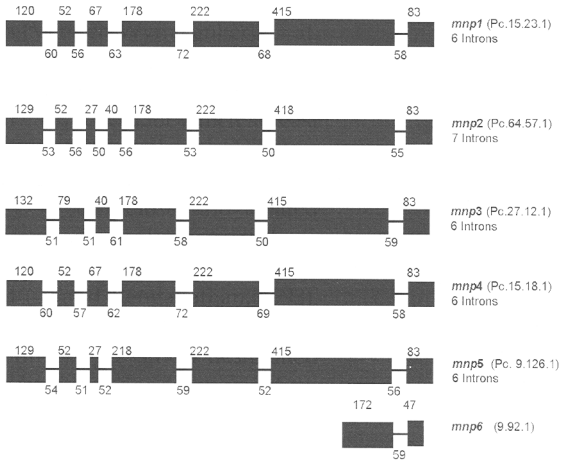

Fig. 4. Schematic representation of intro-exon composition of *P. chrysosporium* manganese peroxidase genes.

4.2. Copper Radical Oxidases

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, only one appears to be secreted in ligninolytic cultures; glyoxal oxidase (Kirk and Farrell, 1987). The temporal correlation of glyoxal oxidase (GLOX), peroxidase, and oxidase substrate appearances in cultures suggests a close physiological connection between these components (Kersten and Kirk, 1987; Kersten, 1990). Glyoxal oxidase is a glycoprotein of 68 kDa with two isozymic forms (pI 4.7 and 4.9). A number of simple aldehyde-, α-hydroxycarbonyl-, and α-dicarbonyl compounds are oxidized by GLOX. Lignin itself is a likely source of GLOX substrates. Oxidation of a β-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. Oxalate may be a source of chelate required for the manganese peroxidase reactions described above. Biochemical and spectroscopic investigations show structural similarities between GLOX and glactose oxidase and the correponding catalytic residues have been clearly identifed (Kersten et al. 1985; Kurek and Kersten, 1995; Whittaker, 2002; Whittaker et al. 1999).

The reversible inactivation of GLOX is of considerable physiological significance (Kersten, 1990; Kurek and Kersten, 1995). During enzyme turnover, GLOX becomes inactive in the absence of a coupled peroxidase system. The oxidase is reactivated, however, by lignin peroxidase and non-phenolic peroxidase substrates. Conversely, phenolics prevent the activation by lignin peroxidase. These observation show that glyoxal oxidase has a regulatory mechanism responsive to peroxidase, peroxidase substrates, and peroxidase products (e.g., phenolics resulting from ligninolysis). Also, lignin will also activate glyoxal oxidase in the coupled reaction with LiP (Kersten, 1990; Kurek and Kersten, 1995).

Glyoxal oxidase of *P. chysosporium* is encoded by a single gene with two alleles (Kersten and Cullen, 1993; Kersten et al. 1995). On the basis of the 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 6 sequences with low overall sequence homology to *glx* (<50% amino acid similarity) but with highly conserved residues surrounding the catalytic site. Extended N-terminal domains of unknown function are present in new copper radical oxidase genes *cro3, cro4,* and *cro5.* On the basis of similarities to galactose oxidase, *cro1, cro2,* and possibly *cro6* may have propeptides which play a role in self-catalytic processing (Firbank et al. 2001; Rogers and Dooley, 2003; Xie and van der Donk, 2001). Our recent blast searches have found structurally related genes in a diverse array of fungi including, the ascomycete *Magnaporthe grisea* (www.broad.mit.edu/annotation/fungi/ ustilago_maydis/) and in the Badisiomycete, *Coprinus. cinereus.* (www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). Recently, one of these *M. grisea glx*-like sequences, *glo1,* was shown to be required for filamentous growth and pathogenicity (Leuthner et al. 2004). Elucidating the biological function of these copper radical oxidases remains a major challenge for future research.

One of the unexpected findings in the *P. chrysosporium* genome project was the identification of the aforemnentioned family of copper radical oxidases In particular, copper radical oxidase genes *cro3, cro4,* and *cro5* were located within the cluster of LiP genes (Cullen and Kersten, 2004). The clustering of *lip* and *cro* genes seems consistent with a physiological connection between peroxidases and peroxide-generating oxidases. Of the seven copper radical oxidascs of *P. chrysosporium,* only *glx* has been the focus of transcript analysis. Again consistent with a role in lignin degradation, *glx* transcripts are coincident with *lip* and *mnp* in defined media (Kersten and Cullen, 1993;

Stewart et al. 1992), soil (Bogan et al. 1996b), and wood (Janse et al. 1998)

4.3. FAD-dependant oxidases

Cellobiose dehydrogenase (CDH) is widely distributed among white-rot fungi. Its precise function is uncertain, but it may play a role in carbohydrate metabolism and in lignin degradation. The enzyme has two domains containing FAD and heme prosthetic groups, respectively. The two domains can be cleaved by *P. chrysosporium* proteases. CDH binds cellulose via a binding module in the flavin domain and oxidizes cellodextrins, mannodextrins, and lactose. Suitable electron acceptors include quinones, phenoxy radicals, molecular oxygen and Fe^{3+}. Several studies have emphasized the FeIII reductase activity of the heme domain and its implications in generating hydroxyl radicals via a Fenton reaction (Kremer and Wood, 1992a; Kremer and Wood, 1992b; Mason et al. 2003). One possible role for CDH may be enhancement of cellulases by relieving product inhibition (Cameron and Aust, 2001; Igarashi et al. 1998). Another possibility is that CDH generates hydroxyl radicals via Fenton type reactions thus oxidizing wood components including lignin. Potential CDH roles have been reviewed (Cameron and Aust, 2001; Henriksson et al. 2000a).

Genes encoding CDH have been cloned from several fungi including the white-rot fungi *P. chrysosporium* (Li et al. 1996; Raices et al. 1995). *Trametes versicolor* (Dumonceaux et al. 1998). and *Pycnosporus 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 analogous bacterial or fungal CBDs. 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 unsettled, but CDH gene disruptions in *T. versicolor* do not affected the ability to degrade synthetic lignin (Dumonceaux et al. 2001). A single CDH gene is present in *P. chrysosporium* as well as related white rot fungi, and full length sequences with non-trivial Smith-Waterman scores ($<e^{-20}$) are obvious in *A. nidulans, N. crassa,* and *M. grisea* genomes (http://www-genome.wi.mit.edu/annotation/fungi/).

Two glucose oxidases have been identified in *P. chrysosporium* cultures; 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). The peroxide-generating enzyme pyranose-2-oxidase is predominantly intracellular in liquid cultures of *P. chrysosporium,* but evidence supports 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. Pyranose 2-oxidase sequences have been reported from *C. versicolor* (Nishimura et al. 1996). related *Trametes* strains (Acc. Nos. P59097 and AAP40332) and most recently *P. chrysosporium* (de Koker et al. 2004). Transcript patterns for the *P. chrysosporium* pyranose 2-oxidase are similar to lignin peroxidases and glyoxal oxidase supporting a role in lignocellulose degradation (de Koker et al. 2004). A *P. chrysosporium* sequence highly similar (Smith-Waterman score = 480) to *A. niger* glucose-1-oxidase has been identified in the genome (Martinez et al. 2004).

Another strategy for peroxide generation may involve aryl alcohol oxidase (AAO) which is secreted by *Bjerkandera sp.* (de Jong et al. 1994). Chlorinated anisyl alcohols, synthesized *de novo* from glucose, are the preferred substrates. The oxidation products are reduced and recycled by the fungal mycelia, LiP does not oxidize the chlorinated anisyl alcohols. Various *Pleurotus* species may support a redox cycle supplying extracellular peroxide using AAO (or veratryl alcohol oxidase) coupled to intracellular aryl alcohol dehydrogenase (AAD) (Guillen and Evans, 1994; Marzullo et al. 1995; Varela et al. 2000b). In *Pleurotus ostreatus,* veratryl alcohol oxidase may participate in lignin degradation by supplying peroxide and by reducing quinones and phenoxy radical and thereby inhibiting the repolymerization of lignin degradation products (Marzullo et al. 1995). Genes encoding aryl alcohol oxidases have been characterized from *Pleurotus* spp. (Marzullo et al. 1995; Varela et al. 1999; Varela et al. 2000a; Varela et al. 2000b), and multiple AAO-like sequences ([3]4) have been identified in the *P. chrysosporium* genome (Martinez et al. 2004).

4.4. Multicopper Oxidases

Analysis of the genome has shown that, unlike other white rot fungi, *P. chrysosporium* does not have any sequence encoding conventional laccases. Instead, it produces a multicopper oxidase possessing strong ferroxidase activity with catalytic parameters similar to those of yeast Fet3p (Larrondo et al. 2003). The physiological function of this protein (MCO1) is uncertain. The gene (*mco1*) is part of a cluster of 4 structurally related sequences located within 25 kb region. All 4 are transcribed, but only *mco1* has a clear secretion signal (Larrondo et al. 2004).

Multiple alignments of a large collection of fungal multicopper oxidase sequences, as well as structural comparison of MCO1, show that these MCOs are closer to Fet3 proteins, than to conventional laccases (Larrondo et al. 2003; Larrondo et al. 2004). Together with iron permease Ftr1 (Stearman et al. 1996), Fet3 ferroxidase (Askwith et al. 1994) plays a key role in iron homeostasis. Our recent clustal analysis of multicopper oxidases sequences in *P. chrysosporium, N. crassa* and *M. grisea,* databases (unpublished results) supports a new branch of the multicopper oxidase family in which the *P. chrysosporium mco* encoded sequences are in close association with two *M. grisea* sequences (MG00551.1, MG07771.1, www-genome.wi.mit.edu/ annotation/fungi/ magnaporthe/) and with *C. neoformans* ´laccase". This branch is in close proximity to another harboring all known Fet3 proteins (Figure 5). Like *mco1,* the *C. neoformans* ´laccase´ exhibits Fe^{2+} oxidation activity (Liu et al. 1999; Williamson, 1994).

Because of the intriguing similarity between *mco1* and *Fet3,* the *P. chrysosporium* genome database was searched for related sequences. A single gene highly homologous to *S. cerevisiae fet3* was identified at a separate locus less than 1 Kb from a *Ftr1*-like iron permease gene model. Transcripts of both are detected by RT-PCR. The *P. chrysosporium fet3* encodes a protein of 628 amino acids, 69 residues larger than *mco1* (unpublished results). Typical of ferroxidases, but unlike *mco1,* the COOH terminus has a predicted transmembrane domain.

Structural determinants that confer multicopper oxidases with ferroxidase activity have been identified (Askwith and Kaplan, 1998; Bonaccorsi di Patti et al. 2000; Bonaccorsi di Patti et al. 2001). Glu-185 and Tyr-354 are essential for the oxidation of Fe^{2+} by Fet3 from *S. cerevisiae.* These two residues are conserved in all known Fet3 proteins, whereas they are absent in ascorbate oxidases and laccases. The equivalent Glu residue is conserved in all but one of the MCO-like ferroxidase (Figure 6). However, the Tyr-354 is absent in most of these sequences, suggesting that Glu-185, but not Tyr, is essential for Fe^{2+} oxidation.

Our analysis supports the importance of the Glu-185 residue. The *C. neoformans* ´laccase´ lacks this residue, which may explain its weak ferroxidase activity relative to MCO1 (Liu et al. 1999; Williamson, 1994). Supporting a new subfamily of multicopper oxidases, the abovementioned *M. grisea* sequences have the essential ferroxidase residues, as well as putative secretion signals. In addition, all these MCO-like sequence lack a COOH terminal transmembrane domain, which are common to Fet3 proteins. Consistent with separate subfamilies, blast analysis of the *M. grisea* genome reveals a *fet3* othologue (MG02156.1) at a locus separate from MCO1-like sequences. Thus, like *P. chrysosporium, M. grisea* seems to have extracellular ferroxidases, distinct from Fet3. Possibly, the similarities reflect the common requirements for attacking plant cell walls. In this context, it is interesting to note that both *P. chrysosporium* and *M. grisea* genome features an impressive number of glycosyl hydrolases. Our recent analysis of plant pathogens *Ustilago maydis* and *Fusarium graminearum* identified Fet3s, laccases, as well as MCO-like sequences. The saprophytes *Aspergillus nidulans* and *Coprinus cinereus* possess the first types of multicopper oxidases but lack MCO-like sequences in their genomes (unpublished results). A recently deposited multicopper oxidase sequence (GenBank AAR82933) from the basidiomicete *Auricularia auricula* falls directly into the MCO-like clade, posseses the discussed Glu residue, and does not have a COOH-terminal anchor.

The role of *mco1* remains unclear, although we have hypothesized that it might be involved in the control of Fenton-based chemical reactions in the extracellular medium (Larrondo et al. 2003). Recently, a similar function has been attributed to yeast Fet3p (Shi et al. 2003; Stoj and Kosman, 2003).

It should be noted that the absence of conventional laccases in *P. chrysosporium* does not exclude a role in lignin degradation in related fungi. Laccase genes, often occurring as multigene families, are widely distributed among lignin-degrading fungi (Cullen, 1997; Mayer and Staples, 2002; Thurston, 1994; Youn et al. 1995). Laccases oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-Cα 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. 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. 1977).

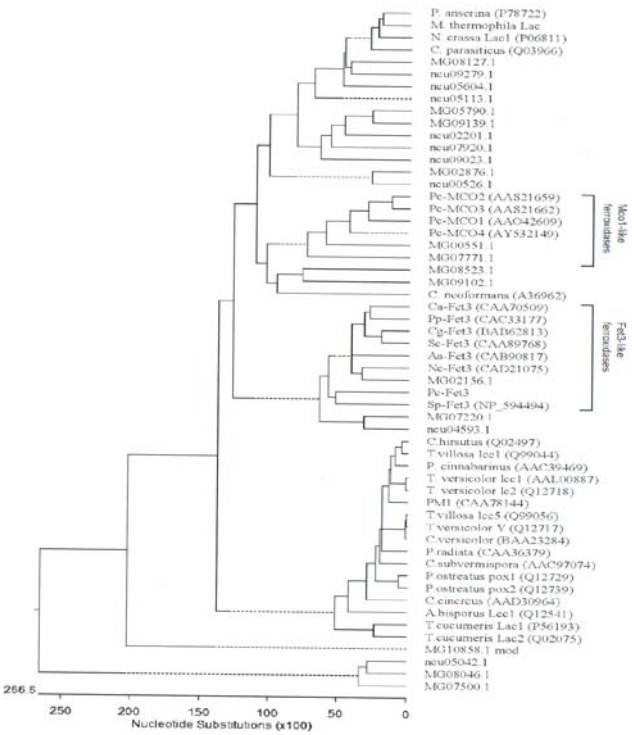

Fig. 5. Clustal W analysis of multicopper oxidases. GenBank accessions, where available, are given parenthetically. All other sequences are derived from publicly accessible databases for *N.crassa* (ncu), *M. grisea* (MG), *P. chrysosporium* (Pc).

5. EXTRACELLULAR CARBOHYDRATE ACTIVE ENZYMES

Relative to ligninolysis, the degradation of cellulose, hemicellulose and pectin by *P. chrysosporium* has received less attention. Enzyme activities imply a degradative strategy similar, but not identical, to other microbes, especially the intensively studied Ascomycete *Trichoderma reesei* (*Hypocrea jecorina*). Components of the cellulolytic system include multiple exocellobiohydrolase I (CBHI) isozymes, as well as an exocellobiohydrolase II (CBHII), and a β-glucosidase (reviewed by Kirk and Cullen, 1998). From a genetic point of view, the degradation of cellulose, hemicellulose and pectin is rather complicated in *P. chrysosporium.* More than 240 sequences encode putative carbohydrate active enzymes, and this includes a minimum of 50 cellulases

(Martinez et al. 2004). As in the case of peroxidases, many of these sequences are distributed within complex gene families.

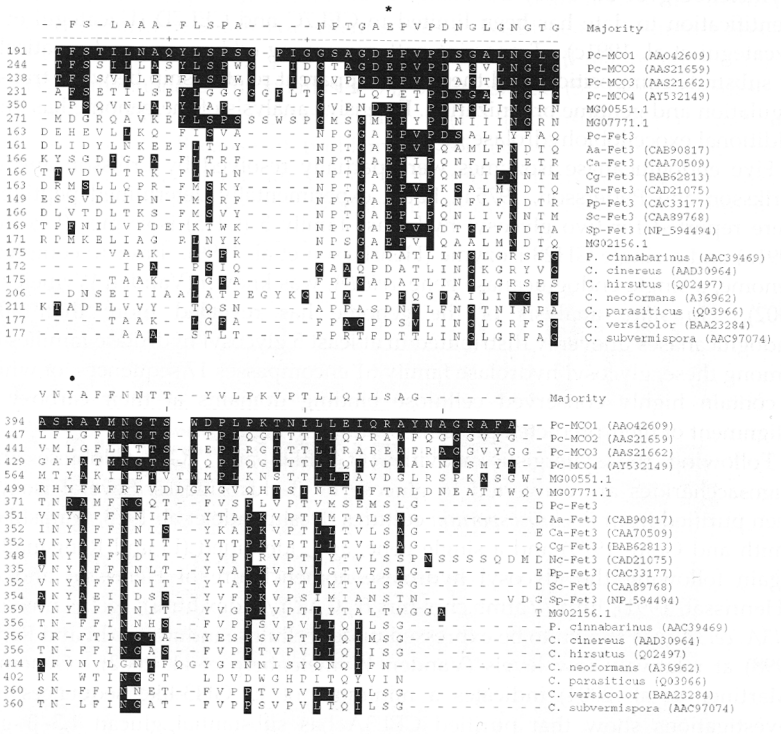

Fig. 6. Clustal W alignment of predicted multicopper oxidases: Selected region of the multiple alignment shows the location of Sc-Fet3 Glu185 (*) and Tyr354 (●), which are essential for ferroxidase activity in *S. cerevisiae* Fet3. Genebank accession numbers are provided. *M. grisea* (MG) sequences were obtained from NCBI. Numbering of the MG sequences might not correspond to the actual length of the proteins. Shaded residues match Pc-MCO1.

Eriksson and coworkers (Eriksson and Pettersson, 1975a, 1975b) characterized multiple endoglucanase (EG) and exocellobiohydrolase (CBH) isozymes in *P. chrysosporium* cultures containing cellulose as sole carbon source. Two CBHI isozymes designated CBH62 and CBH58, and a single CBHII, designated CBH50, were later purified (Uzcategui et al. 1991c). Genetic analysis identified the corresponding genes, and following the glycosyl hydrolase nomenclature of Henrissat and coworkers ((Henrissat, 1991); http://afmb.cnrs-mrs.f/CAZY/ index.html), these have been named *cel7C*, *cel7D*, and *cel6A*. Four additional CBH1 cDNAs have been sequenced, and all six

have been the subject of molecular modeling (Munoz et al. 2001). Transcripts of the *cel7* genes are present in cellulose-containing media (Covert et al. 1992b; Vanden Wymelenberg et al. 1993) and in colonized wood (Vallim et al. 1998), but protein identification to date has been limited to CEL7C and CEL7D (Uzcategui et al. 1991b; Uzcategui et al. 1991c). Transcript patterns among *cel7* genes are dramatically altered by substrate composition, and there is no apparent relationship between transcriptional regulation and genome organization. Extensive analysis of the genome has not revealed additional exocellobiohydrolase genes.

Five endoglucanase isozymes were partially resolved by Eriksson and colleagues (Eriksson and Pettersson, 1975a). Later, experimentally determined peptide sequence were reported for two glycosyl hydrolase family 5 (GH5) isozymes (Uzcategui et al. 1991a) and a single GH28 isozyme (Henriksson et al. 1999). Prior to completion of the genome, a single endoglucanase gene, *cel61A*, was known (Vanden Wymelenberg et al. 2002). *In silico* analysis of the genome has revealed more than 40 putative endoglucanases unevenly distributed in at least 5 glycosyl hydrolase families (Figure 1). Among these, glycosyl hydrolase family 61 encompasses 17 sequences, of which at least 5 contain highly conserved cellulose binding domains at their carboxy terminus. Alignment of their predicted proteins is shown in Figure 7.

Following the synergistic activities of CBHs and EGs, cellobiose and related oligosaccharides are converted to glucose by β-glucosidases. Several isozymes have been purified from *P. chrysosporium* cultures (Deshpande et at. 1978; lgarashi et al. 2003; Smith and Gold, 1979) and a single gene, *bgl1,* identified (Li and Renganathan, 1998). Again following the glycosyl hydrolase nomenclature of Henrissat and coworkers ((Henrissat, 1991); http://afmb.cnrs-mrs.fr/CAZY/index.html), *bgl1* is now designated *cel3A. cel3A* was shown to be expressed under cellulose induction (Li and Renganathan, 1998) at relatively low levels (Vanden Wymelenberg et al. 2002). Genome analysis (Martinez et al. 2004) indicates a minimum of 12 GH3-like sequences and recent investigations show that purified CEL3A has substantial glucan 1,3- β -glucosidase activity (Igarashi et al. 2003). Uncertainty regarding CEL3A substrate preference highlights the difficulties assigning function based solely on structure and family membership.

Relatively little is known about the enzymes involved in hemicellulose and pectin degradation in *P. chrysosporium* (Castanares et al. 1995; Copa-Patino et al. 1993; Kirk and Cullen, 1998). The complete conversion of major hemicelluloses of wood, glucuronoxylans and galactoglucomannans, requires the combined activities of numerous enzymes including endoxylanase, acetylxylan esterase, a-glucuronidase, β-xylosidase, α-arabinosidase, endomannanase, α-galactosidase, acetylglucomannan esterase, β -mannosidase, and β-glucosidase (Kirk and Cullen, 1998). Very few of these enzymes have been characterized from *P. chrysosporium* (Castanares et al. 1995; Copa-Patino et al. 1993). Two cDNAs encoding endoxylanases, *xyn10A* and *xyn11A,* were sequenced (GenBank accessions AAG44993, AAG44995) and recently expressed in a heterologous system (Decelle et al. 2004). An α-galactosidase and corresponding gene (*aga27A*) have been characterized (Brumer et al. 1999; Hart et al. 2000). Analysis of the

genome reveals many putative genes involved in hemicellulose and pectin degradation (Martinez et al. 2004). Among probable xylanases, no additional members of glycoside family 10 were recognized, but a total of 6 family 11 sequences were identified. Sequences with significant Smith-Waterman scores to NCBI genes of known function include acetylxlan esterases (3), pectin methylesterase (1), β-mannosidases (2), β-xylosidase (1), β-mannanase (3). xylanases (7). β-glucosidase (5), α-galactosidase (3). polygalacturonases (4), rhamnogalacturonase (1), β-xylosidase (1), and a-L-arabinofuranosidase (1). Because of problems associated with accurate gene model predictions, the relatively small number of NCBI representatives of certain enzyme classes, and sequence divergence, our assignments no doubt grossly underestimate the total number of genes.

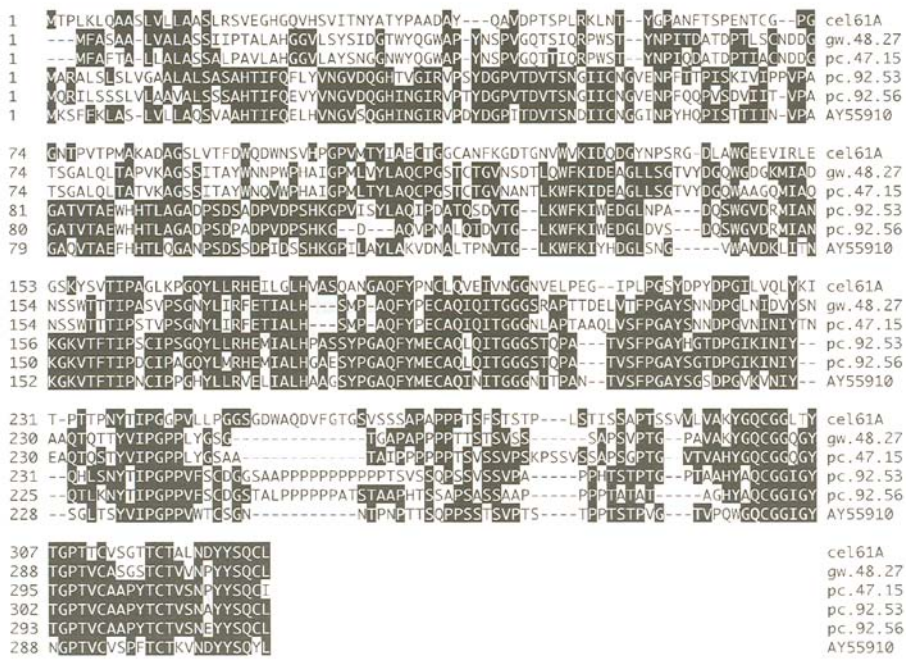

Fig. 7. Clustal W alignment of predicted Cel61 proteins of *P. chrysosporium* Only *cel61A* is supported by cDNA sequence (Vanden Wymelenberg et al. 2002). Others correspond to gene models available on the Joint Genome Institutes web browser (http//genome.jgi-psf.org/whitcrot1/whiterot1.home.html) Models were manually adjusted to provide full length.

While our chapter focuses an genes involved in wood decay, it should be noted that numerous other sequences encoding carbohydrate active enzymes have been identified in the *P. chrysosporium* genome database. Among these are at least 25 putative enzymes

involved in the degradation of β-1,3-glucan and mixed-linkage β-1,3-1,4-glucans. These polysaccharides are common constituents of cell walls of certain cereal grains, grasses and related plants as well as the cell walls of yeasts and fungi. Also present in the genome are hydrolase-encoding sequences probably involved in the degradation of starch and glycogen (amylase, glucoamylase, β-glucosidase), mutan (β-1, 3-glucanase), and chitin (chitinase). Chitin is an important structural component of fungal cell walls, and the 10 putative chitinases (GH family 18) may be involved in cell wall morphogenesis. In addition, a minimum of 57 putative glycosyltransferase encoding genes were identified in the genome of *P. chrysosporium.* Their precise function is unkown, but some are likely candidates for the biosynthesis of chitin, β-1,3-glucan glycogen, cell wall mannan and N-glycans.

6. OTHER EXTRACELLULAR 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* (Dass et al. 1995; Datta, 1992; Dosoretz et al. 1990a; Dosoretz et al. 1990b; Eriksson and Pettersson, 1982; 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; Rothschild et al. 1999). Proteases have also been implicated in regulating cellulase (Eriksson and Pettersson, 1982) and CDH (Eggert et al. 1996) activity. Dozens of putative extracellular protease genes are predicted from genome analysis (Martinez et al. 2004) including one that corresponds to the published N-terminal sequence of a pulp-derived protease (Datta, 1992). Recently, an unusual cluster of glutamic proteases have been identified in the *P. chrysosporium* genome database (Sims et al. 2004).

7. INTRACELLULAR ENZYMES RELATED TO LIGNOCELLULOSE DEGRADATION

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. 1995). 1.4-benzoquinone reductase (Brock and Gold, 1996). methyltransferases (Jeffers et al. 1997), cytochrome P450s (Kullman and Matsumura, 1997; Van Hamme et al. 2003; Yadav and Loper, 2000; Yadav et al. 2003), 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). catalase (Kwon and Anderson, 2001) and aryl alcohol dehydrogenase (Reiser et al. 1994).

Genome mining identified a large number of cytochrome P450s, 14 of which are located on a single scaffold (number 24). The startling enumeration of >148 partial or complete cytochrome P450 gene models (Martinez et al. 2004) represents a promising, if

not daunting, framework for future investigation. The genetic complexity is reflected in recent studies demonstrating an impressive array of potential substrates and transformation products (Matsuzaki and Wariishi, 2004; Miura et al. 2004; Teramoto et al. 2004). In another recent study, Yadav and coworkers (Doddapaneni and Yadav, 2004) demonstrated differential regulation of two cytochrome P450 genes in response to various xenobiotics.

8. CONCLUSION

Analysis of the *P. chrysosporium* genome presents challenges and opportunities for future research. The biological role of impressive gene multiplicity among glycosyl hydrolases, cytochrome P450s. peroxidases and oxidases remains one of the most pressing issues. Are these closely dated genes merely redundant or do they encode enzymes with subtle but important functional differences? Other significant questions revolve around the abundant transposable elements identified. Do these elements impact gene expression and/or the emergence of gene families?

The genome database provides a framework for addressing these and other questions. Transcript profiling using microarrays may provide indirect evidence for the role(s) of many genes. Tandem mass spectrometry and MALDI analysis, already begun in several laboratories, will also help identify key genes and enzymes. A major concern for such high throughput approaches centers on difficulties inherent in gene prediction, a problem especially common in fungal genomes where introns are present in most genes. As gene models are corrected, databases will need continual updating. A powerful approach to determining gene function would be gene knockouts, but unlike well established experimental systems, e.g. *S. cerevisiae*, gene disruptions are currently very difficult in *P. chrysosporium.* Hopefully, more efficient methodology will be forthcoming. A productive route for establishing the role of individual genes in lignocellulose degradation will continue to involve biochemical characterization of pure recombinant enzymes. Toward this end, more efficient heterologous expression systems are needed. Finally, whole genome comparisons, particularly among filamentous Ascomycetes and Basidiomycetes, may provide valuable information concerning gene function.

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