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ScienceDirect
Fungal Genetics and Biology 44 (2007) 77–87
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Review

Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*

Phil Kersten, Dan Cullen *

Forest Products Laboratory, USDA, One Gifford Pinchot Drive, Madison, WI 53705, USA

Received 29 June 2006; accepted 20 July 2006
Available online 12 September 2006

Abstract

The US Department of Energy has assembled a high quality draft genome of *Phanerochaete chrysosporium*, a white rot Basidiomycete capable of completely degrading all major components of plant cell walls including cellulose, hemicellulose and lignin. Hundreds of sequences are predicted to encode extracellular enzymes including an impressive number of oxidative enzymes potentially involved in lignocellulose degradation. Herein, we summarize the number, organization, and expression of genes encoding peroxidases, copper radical oxidases, FAD-dependent oxidases, and multicopper oxidases. Possibly relevant to extracellular oxidative systems are genes involved in posttranslational processes and a large number of hypothetical proteins.
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Keywords: *Phanerochaete chrysosporium*; Oxidases; Copper radical oxidases; Multicopper oxidases

1. Introduction

Lignin degrading basidiomycetes, collectively referred to as white rot fungi, are common inhabitants of forest litter and fallen trees. These are the only microbes that have been convincingly shown to efficiently depolymerize, degrade, and mineralize all components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin. As such, white rot fungi play an important, if not pivotal, role in the carbon cycle.

Extracellular oxidative enzymes thought to be involved in lignin depolymerization include an array of oxidases and peroxidases. These enzymes are responsible for generating highly reactive and nonspecific free radicals that affect lignin degradation. The nonspecific nature and extraordinary oxidation potential of the peroxidases have attracted considerable interest in the development of bioprocesses such as fiber bleaching and the remediation of organopollutant contaminated soils and effluents.

The ~30-Mb haploid genome of the most intensively studied white rot fungus, *Phanerochaete chrysosporium*, has been sequenced using a pure whole genome shotgun strategy. Initial analysis of the high quality draft assembly (Martinez et al., 2004) revealed features of general importance to lower eukaryotic gene structure and organization, identified hundreds of genes involved in lignocellulose degradation, and provided a framework for future investigations of degradative processes. More recently, a substantially improved assembly and gene models have been released (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html; Vanden Wymelenberg et al., 2006a). This review focuses on extracellular oxidative enzymes of *P. chrysosporium*, with emphasis on recent advances made possible by the genome sequence. Readers are referred to previous reviews for information on the microbiology and physiology of lignocellulose degradation (Kirk and Farrell, 1987; Eriksson et al., 1990; Blanchette, 1991; Kirk and Cullen, 1998) and on the molecular biology of *P. chrysosporium* (Alic and Gold, 1991; Pease and Tien, 1991; Gold and Alic, 1993; Cullen and Kersten, 1996; Cullen, 1997, 2002; Cullen and Kersten, 2004; Larrondo et al., 2005b).

* Corresponding author. Fax: +608 231 9262.
E-mail address: dcullen@facstaff.wisc.edu (D. Cullen).

1087-1845/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.fgb.2006.07.007

2. General features of the *P. chrysosporium* genome

The current assembly (v.2.0) is considerably improved relative to earlier versions (Martinez et al., 2004). Excluding gaps, the assembly contains 32.5 Mb of sequence with a total of 232 scaffolds. Ninety percent of the assembled sequence is contained on the longest 21 scaffolds, compared to 161 scaffolds in assembly v1.0.

A total of 10,048 v2.1 gene predictions, supporting evidence, annotations, and analyses are available through the Department of Energy's Joint Genome Institute's Genome Portal (www.jgi.doe.gov/whiterot). Predictions were based on *ab initio* methods Fgenesh (Salamov and Solovyev, 2000), homology-based methods, Fgenesh+ (www.softberry.com) and Genewise (Birney and Durbin, 2000). Fgenesh was trained on a set of available mRNAs, wood-derived ESTs and reliable homology gene models. Approximately 75% of v2.1 models contain recognizable functional domains or show homology to known proteins in other genomes. The v2.1 models are generally much improved compared to v1.0, particularly with respect to defining intron-exon junctions and N-terminal completeness. These refinements have facilitated computational predictions of the 'secretome' (Vanden Wymelenberg et al., 2006a), but it should be emphasized that automated predictions are often partially incorrect. Accordingly, proteins predicted from genomic sequence should be considered tentative until verified by cDNA analysis. All v2.1 gene predictions presented in this review can be easily accessed by appending the model number to the following URL: http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Phchr1&id=.

A distinguishing feature of the *P. chrysosporium* genome is the occurrence of large and complex families of structurally related genes including cytochrome P450s, peroxidases, glycoside hydrolases, proteases, copper radical oxidases, and multicopper oxidases. In some instances, close linkage has been observed, but there is scant evidence relating transcriptional regulation and such clustering. The role of genetic multiplicity in lignocellulose degradation remains unclear. Structurally related genes may encode proteins with subtle differences in function, and such diversity may provide the flexibility needed in changing environmental conditions (pH, temperature, ionic strength), substrate composition and accessibility, and wood species. Alternatively, some or all of the genetic multiplicity may merely reflect redundancy.

3. Lignin peroxidases and manganese-dependent peroxidases

Lignin peroxidase (LiP) and manganese peroxidase (MnP) have been the most intensively studied extracellular enzymes of *P. chrysosporium*, and several reviews summarize their biochemistry (Kirk and Farrell, 1987; Kirk, 1988; Higuchi, 1990; Schoemaker and Leisola, 1990) and genetics (Alic and Gold, 1991; Gold and Alic, 1993; Cullen and Kersten, 1996; Cullen, 1997, 2002). These protoporphyrin IX peroxidases are encoded by families of structurally related genes and further modified posttranslationally.

Reactions catalyzed by LiP 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 aromatic cleavage of nonphenolic lignin model compounds (Tien and Kirk, 1984; Hammel et al., 1985; Leisola et al., 1985; Renganathan et al., 1985; Renganathan and Gold, 1986; Umezawa et al., 1986) (Fig. 1). Leisola et al. (1988) demonstrated the importance of lignin peroxidase in the depolymerization of lignin *in vivo*, and *in vitro* depolymerization of lignin has been shown 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). Organic acids such as oxalic acid stimulate MnP activity by stabilizing the Mn^{3+}, and produce diffusible oxidizing chelates (Glenn and Gold, 1985; Glenn et al., 1986). Physiological levels of oxalate occurring in *P. chrysosporium* cultures stimulate manganese peroxidase activity (Kuan et al., 1993; Kishi et al., 1994), and extracellular H_2O_2 may also be generated by the oxidation of glyoxylate and oxalate (Kuan et al., 1993; Urzua et al., 1998).

Crystal structures of LiP (Edwards et al., 1993; Piontek et al., 1993, 2001) and MnP (Sundaramoorthy et al., 1997) show similarities in the active site with a proximal His ligand H-bonded to Asp, and a distal side peroxide-binding pocket consisting of a catalytic His and Arg. Site specific mutagenesis has implicated Asp179, Glu35, and Glu39 in Mn binding (Kusters-van Someren et al., 1995; Whitwam et al., 1997; Sollewijn Gelpke et al., 1999; Youngs et al., 2001).

Genome analyses (Martinez et al., 2004) located the 10 known LiP genes (Gaskell et al., 1994), eight of which reside within a 96 kb region of scaffold 19 (Martinez et al., 2004). In addition to previously characterized MnP genes *mnp1, mnp2*, and *mnp3* (Pease et al., 1989; Pribnow et al., 1989; Orth et al., 1994; Alic et al., 1997), two new MnP genes were revealed by BLAST searches of the genome (Martinez et al., 2004). Unexpectedly, the gene designated *mnp4* (model 8191) (Martinez et al., 2004) was found to lie approximately 5 kb upstream from *mnp1* (model 140708), and a cytochrome P450 gene is located in the *mnp4–mnp*1 intergenic region. *mnp1* and *mnp4* differ by a single amino acid. Gene model 4636, *mnp5*, corresponds to the N-terminal amino acid sequence of a MnP purified from *P. chrysosporium*-colonized wood pulp (Datta et al., 1991). In addition to *mnp4* and *mnp5*, a partial *mnp*-like sequence of 267 nt was located 85 kb downstream of *mnp5* on scaffold 9. No evidence for *mnp6* transcription could be obtained by RT-PCR (unpublished).

Analyses of LiP and MnP genes from different white rot species show >50 invariant residues (Martinez, 2002). Further, the MnP and LiP genes fall within clearly defined clades discriminated by certain key residues. For example,

Trp171, which is hydroxylated at Cβ (Blodig et al., 1998; Doyle et al., 1998), is essential in LiP catalysis by long range electron transfer, whereas Mn-binding residues (Glu35, Glu39, Asp179 in *mnp1*) are found in MnP sequences.

However, certain peroxidases defy easy classification. A novel *Pleurotus eryngii* sequence encodes "versatile peroxidase," an enzyme exhibiting LiP-like activities (oxidation of veratryl alcohol and an array of phenols) and MnP-like activities (Mn^{+2} oxidation) (Ruiz-Duenas et al., 1999, 2001; Camarero et al., 2000). The *P. eryngii* gene has Trp171 and residues involved in Mn-binding. A *P. chrysosporium* extracellular peroxidase gene, designated *nop* (Larrondo et al., 2005a; GenBank accession AY727765), is distantly related to the *Pleurotus* hybrid peroxidase (Ruiz-Duenas et al., 1999), but lacks catalytic and Mn-binding residues (Martinez et al., 2004).

As initially demonstrated by Holzbaur and Tien (1988), the peroxidase genes of *P. chrysosporium* are differentially regulated by culture conditions. Transcript patterns differ sharply in defined media (Stewart et al., 1992; Reiser et al., 1993; Stewart and Cullen, 1999), in organopollutant contaminated soils (Bogan et al., 1996a,b), and in colonized wood (Janse et al., 1998). Regulation by post translational heme processing has been suggested (Johnston and Aust, 1994) but contradicted by the results of Li et al. (1994). Recent identification of LiP isozymes by mass spectroscopy (Vanden Wymelenberg et al., 2006a) is generally consistent with previous transcript analysis (Stewart and Cullen, 1999). For example, isozyme H2 peptides, encoded by *lipD*, were observed in carbon-limited cultures, whereas peptides corresponding to *lipC* were identified in nitrogen-limited cultures.

MnP production is dependent upon Mn concentration (Bonnarme and Jeffries, 1990; Brown et al., 1990), and putative metal response elements (MREs) have been identified upstream of *mnp1* and *mnp2*, but not *mnp3*. Supporting a role for MREs in transcriptional regulation, expression of *mnp3* is not influenced by Mn^{2+} addition, whereas *mnp1* and *mnp2* transcripts increase in response to Mn^{2+} supplements in low nitrogen media (Pease and Tien, 1992; Gettemy et al., 1998). Reporter fusions support the existence of additional Mn-responsive *cis* acting sequences upstream of *mnp*1 (Ma et al., 2004). MREs are not involved in MnP regulation in related white rot fungi *Tramates versicolor* (Johansson and Nyman, 1993; Johansson et al., 2002) and *Ceriporiopsis subvermispora* (Manubens et al., 2003).

4. Copper radical oxidases

Peroxide is required as oxidant in peroxidative reactions, and several oxidases have been proposed to play a role in this regard. Glyoxal oxidase (GLX) is temporally correlated with peroxidase and with oxidase substrates in ligninolytic cultures suggesting a close physiological connection (Kersten and Kirk, 1987; Kirk and Farrell, 1987; Kersten, 1990). Glyoxal oxidase is a glycoprotein of 68 kDa with two isozymic forms (pI 4.7 and 4.9). Several simple aldehyde-, α-hydroxycarbonyl-, and α-dicarbonyl compounds are oxidized by GLX, and lignin itself is a likely source of GLX substrates. Oxidation of a β-O-4 model compound (representing the major substructure of lignin) by LiP releases one such substrate, glycolaldehyde (Hammel et al., 1994). Sequential oxidations of glycolaldehyde yield oxalate and multiple equivalents of H_2O_2. The oxalate may act as a chelate required for the manganese peroxidase reactions described above.

Sequence comparisons of GLX with galactose oxidase from *Dactylium dendroides*, together with spectroscopic characterizations of GLX and site-specific mutants, show that GLX is a copper radical oxidase (CRO) (Kersten and Cullen, 1993; Whittaker et al., 1999; Whittaker, 2002). Although the overall sequence similarity is low, and the catalytic specificities are different, the two enzymes have nearly identical active sites. Copper radical oxidases have two distinct one-electron acceptors, a Cu(II) metal center and an internal Cys-Tyr radical forming a metalloradical complex (reviewed by Whittaker, 2002). Mature galactose oxidase has three structural domains; Domain I or N-terminal domain has a metal-binding center and a carbohydrate-binding site; Domain II or catalytic domain is based on a four-stranded antiparallel beta sheet repeat element giving rise to a "seven-petalled flower" or "super-barrel" structure belonging to the *kelch* superfamily of protein folds; Domain III or C-terminal domain caps the super-barrel domain and supplies a His ligand by threading a loop down the core to access the copper center. Sequence alignments, conservation of active site ligands and similarity in protein folds suggest that GLX tertiary structure is similar to that of galactose oxidase, except that the N-terminal domain of galactose oxidase is absent in GLX. The super-barrel catalytic domain of GLX supplies copper ligands Tyr135 Tyr377 and His378. Cys70 is cross-linked with Tyr135 as part of the internal radical cofactor. His471 of the C-terminal domain is the fourth amino acid directly coordinated to the mononuclear copper center.

GLX has a regulatory mechanism responsive to peroxidase, peroxidase substrates, and peroxidase products (e.g., phenolics resulting from ligninolysis), supporting evidence for close physiological connections between GLX and the extracellular peroxidases of *P. chrysosporium* (Kersten, 1990; Kurek and Kersten, 1995). The oxidase becomes inactive in the absence of a coupled peroxidase system but is reactivated by lignin peroxidase and non-phenolic peroxidase substrates. Conversely, phenolics prevent the activation by lignin peroxidase. GLX is also activated by lignin in the coupled reaction with LiP (Kersten, 1990; Kurek and Kersten, 1995). The basis for this modulation in GLX activity is probably related, at least in part, to the redox interconversion ($E_{1/2} = 0.64$ V versus NHE, pH7) of active and inactive forms of the enzyme (Whittaker et al., 1996).

Phanerochaete chrysosporium genome searches have revealed six additional copper radical oxidase genes.

Overall sequence homology to *glx* is relatively low (<50% amino acid similarity) but residues coordinating copper and constituting the radical redox site are conserved (Martinez et al., 2004; VandenWymelenberg et al., 2006b). Clustered within a larger cluster of LiP genes, the copper radical oxidase genes *cro3, 4* and *5* show remarkable conservation of sequence (71–86% amino acid identity) and exon/intron positions. These three genes form a subfamily of sequences, all containing an N-terminal WSC domain potentially involved in carbohydrate binding (IPR288, www.ebi.ac.uk/interpro/). 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 the Badisiomycete, *Coprinus cinereus* (www.broad.mit.edu/annotation/fungi/coprinus_cinereus/).

Cro6 is most closely related to *glx* (47% amino acid identity), but features a ~200 amino acid N-terminal region absent from the other copper radical oxidases and distantly related to functionally disparate sequences such as *Propionibacterium acnes* β-galactosidase fused to β-N-acetylhexosaminidase (gi50843277, score 63.5) and *Solibacter usitatus* family 2 glucosyl transferase (gi67932023, score 59.7). TBLASTN searches using the predicted CRO6 protein against the *C. cinereus* genome (www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) reveal a homologous translation on scaffold 5, contig 1.103 (coordinates 529357 to 531543). Thus, this N-terminal domain is conserved in these two filamentous Basidiomycetes together with the super-barrel catalytic, and C-terminal domains of the copper radical oxidases.

The predicted mature *cro2* protein is only 28% identical to GLX, but 47% identical to *Ustilago maydis glo1* (gi71013128). The membrane-bound Glo1 protein is involved in filamentous growth and pathogenicity of *U. maydis* (Leuthner et al., 2005). The *cro2* gene encodes a secreted protein which has been identified in culture filtrates by mass spectroscopy. The *cro2* cDNA has been expressed in *Aspergillus nidulans* and the enzyme shown to oxidize glycolaldehyde dimer, but not methylglyoxal, the prototypical substrate for GLX. Therefore, the two oxidases are distinguished by catalytic differences (VandenWymelenberg et al., 2006b).

Consistent with a role in lignin degradation, *glx* transcripts are coincident with *lip* and *mnp* in defined media (Stewart et al., 1992; Kersten and Cullen, 1993), soil (Bogan et al., 1996a), and wood (Janse et al., 1998). The regulation of *cro1–cro6* has not been systematically studied, but all transcripts are detectable in colonized wood wafers and transcript patterns vary over time (VandenWymelenberg et al., 2006b).

5. Flavin and cytochrome enzymes

Cellobiose dehydrogenase (CDH) has been intensively studied for potential roles in carbohydrate metabolism and in lignin degradation. Functional domains containing FAD and heme prosthetic groups can be proteolytically cleaved. CDH binds cellulose via a binding module in the flavin domain and oxidizes cellodextrins, mannodextrins, and lactose. Electron acceptors include quinones, phenoxy radicals, molecular oxygen, and Fe^{3+}. A possible role for CDH may be enhancement of cellulases by relieving product inhibition (Igarashi et al., 1998; Cameron and Aust, 2001).

CDH may also be involved in generating highly reactive hydroxyl radicals that participate in lignocellulose depolymerization. Several investigations (Kremer and Wood, 1992b; Kremer and Wood, 1992a; Mason et al., 2003a,b) have emphasized the FeIII reductase activity of the heme domain and its implications in generating hydroxyl radicals via a Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$). The possibility of such a reactive oxygen species was long ago suggested in *P. chrysosporium* (Forney et al., 1982; Kutsuki and Gold, 1982; Bes et al., 1983; Kirk and Nakatsubo, 1983; Evans et al., 1984), 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. chrysosporium* (Wood, 1994; Tanaka et al., 1999), particularly via CDH (for review see Henriksson et al., 2000a; Henriksson et al., 2000b; Cameron and Aust, 2001).

A single gene encodes CDH in *P. chrysosporium* (Raices et al., 1995; Li et al., 1996), and in related white rot fungi *T. versicolor* (Dumonceaux et al., 1998), and *Pycnosporus cinnabarinus* (Moukha et al., 1999). Structurally, these genes share a common architecture with separate FAD, heme, and cellulose binding module (CBM), although the latter domain has no obvious similarity to other fungal CBMs. Recently, the electron transfer chain mechanism of CDH with cytochrome c as the electron acceptor was studied using site-specific mutants (Igarashi et al., 2005).

Interestingly, genome analysis has identified a separate gene (GenBank AY682742) encoding a similar heme domain fused to a highly conserved family 1 CBM (http://afmb.cnrs-mrs.fr/CAZY/fam/acc_CBM.html). The predicted cytochrome b562 protein is 46% identical to the corresponding region of CDH, and the purified recombinant enzyme has the expected electron transfer activity (Yoshida et al., 2005). The structure and regulation of the cytochrome b562 are compatible with a role in cellulose degradation via Fenton chemistry. Specifically, cellulose binding, iron reduction, and its relatively small molecular weight seem well suited to generating Fe(II) on or near the substrate. In the presence of peroxide, highly reactive hydroxyl radicals would be spontaneously generated at a distance from the advancing hyphae, an important consideration in any plausible Fenton system (Hyde and Wood, 1997; Henriksson et al., 2000a; Cameron and Aust, 2001; Mason et al., 2003b).

Two glucose oxidases have been reported 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). While predominantly intracellular in liquid cultures of *P. chrysosporium*, evidence supports an important role for pyranose 2-oxidase in wood decay, with no evidence for glucose 1-oxidase (Daniel et al., 1994). The pyranose 2-oxidase is preferentially localized in the periplasmic space and the associated membranous materials. The *P. chrysosporium* pyranose 2-oxidase transcript patterns are similar to lignin peroxidases and glyoxal oxidase, supporting a role in lignocellulose degradation (de Koker et al., 2004). A *P. chrysosporium* putative glucose oxidase gene, designated *gox1*, is related (Smith-Waterman score = 480) to *Aspergillus niger* glucose-1-oxidase (Martinez et al., 2004). Using mass spectrometry (LC-MS/MS), peptides corresponding to *gox1* have been identified in media containing avicel as sole carbon source (Vanden Wymelenberg et al., 2005) but a role for GOX1 is yet to be determined.

Structurally related to glucose oxidases (Varela et al., 2000), several putative aryl alcohol oxidases have been identified in *P. chrysosporium*. Specifically, gene models 135972, 37188, and 6199 are 46.3%, 43.8% and 39.0% identical to the aryl alcohol oxidases from *Pleurotus eryngii* (gi3851524); (Varela et al., 2001). The precise role(s) of these enzymes remain uncertain, but they may support a redox cycle by supplying extracellular peroxide, perhaps coupled to intracellular aryl alcohol dehydrogenase (Guillen and Evans, 1994; Marzullo et al., 1995; Varela et al., 2000). In *Pleurotus ostreatus*, veratryl alcohol oxidase may participate in lignin degradation by supplying peroxide and by reducing quinones and phenoxy radicals and thereby inhibiting the repolymerization of lignin degradation products (Marzullo et al., 1995).

In aggregate, *P. chrysosporium* possesses an impressive repertoire of genes encoding putative peroxide-generating and electron-transfer enzymes. The role of multiple copper radical oxidases and FAD oxidoreductases in lignocellulose degradation remains to be established through transcript profiling, sub-cellular localization, gene disruption, and heterologous expression.

6. Multicopper oxidases

In contrast to several other white rot fungi, it is generally accepted that *P. chrysosporium* produces LiP and MnP, but no laccase (Kirk and Farrell, 1987; Eriksson et al., 1990; Hatakka, 1994; Thurston, 1994). Contradictory findings have occasionally been published (Srinivasan et al., 1995; Dittmer et al., 1997; Rodriguez et al., 1997), but laccase identification based on ABTS oxidation may be misleading due to an artifact caused by Mn^{3+} (Podgornik et al., 2001). Exhaustive searches of the *P. chrysosporium* strain RP-78 genome provide overwhelming evidence that conventional laccases are absent in this efficient lignin degrading fungus. (This observation should not be construed to exclude laccase involvement in lignin degradation by other species. Indeed, considerable evidence supports laccase in ligninolysis (Bourbonnais et al., 1997; Call and Muncke, 1997; Eggert et al., 1997; Kawai et al., 1999).) A recent report (Gnanamani et al., 2006) of laccase activity in another *P. chrysosporium* strain cannot, at this time, be verified as the isolate is not publicly available.

While conventional laccases are absent from the *P. chrysosporium* genome, four sequences with homology to distantly related multicopper oxidases (MCOs), were identified within a 25 kb region. All four are transcribed, but only *mco1* has a classical secretion signal (Larrondo et al., 2004). Multiple alignments show significant overall similarity to Fet3 proteins, key enzymes in iron homeostasis (Larrondo et al., 2003; Larrondo et al., 2004; Larrondo et al., 2005b). Further, an essential residue (Glu-185) for oxidation of Fe^{2+} by *Saccharomyces cerevisiae* Fet3 (Askwith and Kaplan, 1998; Bonaccorsi di Patti et al., 2000, 2001) is present in *mco1*, but absent in laccase sequences. Regardless of these similarities, the *mco1* gene seems unlikely to function as a Fet3-like ferroxidase because a transmembrane anchor, typical of Fet3 proteins, is absent. Moreover, a membrane-anchored ferroxidase, highly homologous to *S. cerevisiae fet3*, has been identified at a separate locus (gene model 26890).

The *mco1* cDNA was expressed in *A. nidulans* and the substrate specificity of the secreted protein shown to be different from laccases (Larrondo et al., 2003). Commonly used laccase substrates, such as 2,6-dimethoxyphenol (2,6-DMP), syringaldazine, and ABTS, were poor substrates as were other phenolic compounds. Instead, high ferroxidase activity was observed, with a K_m of the same order of magnitude as that described for Fet3 (de Silva et al., 1997). Certain aromatic amines were also oxidized, a property common among Fet3 proteins (de Silva et al., 1997; Bonaccorsi di Patti et al., 2000). In short, *mco1* encodes a novel fungal multicopper oxidase with strong ferroxidase activity but lacking the canonical domains of Fet3 proteins.

The precise role of *mco1* remains uncertain. One intriguing possibility is the modulation of Fenton reactions, through the Fe^{2+} oxidation (Fig. 1). If appropriately regulated, an extracellular ferroxidase-like protein such as MCO1 might reduce otherwise toxic levels of hydroxyl radical. A similar function was proposed for *Cryptococcus neoformans* laccase (Williamson, 1994; Liu et al., 1999) and *S. cerevisiae* Fet3 (Shi et al., 2003; Stoj and Kosman, 2003).

7. Related extracellular enzymes

Several proteases have been partially characterized from submerged cultures, but it remains uncertain whether extracellular peroxidases are substantially degraded under ligninolytic culture conditions (Dosoretz et al., 1990a,b; Bonnarme et al., 1993; Dass et al., 1995) or in colonized wood (Datta, 1992). Proteases in cellulolytic cultures have been implicated in the activation of cellulase activity (Eriksson and Pettersson, 1982) and in the cleavage of

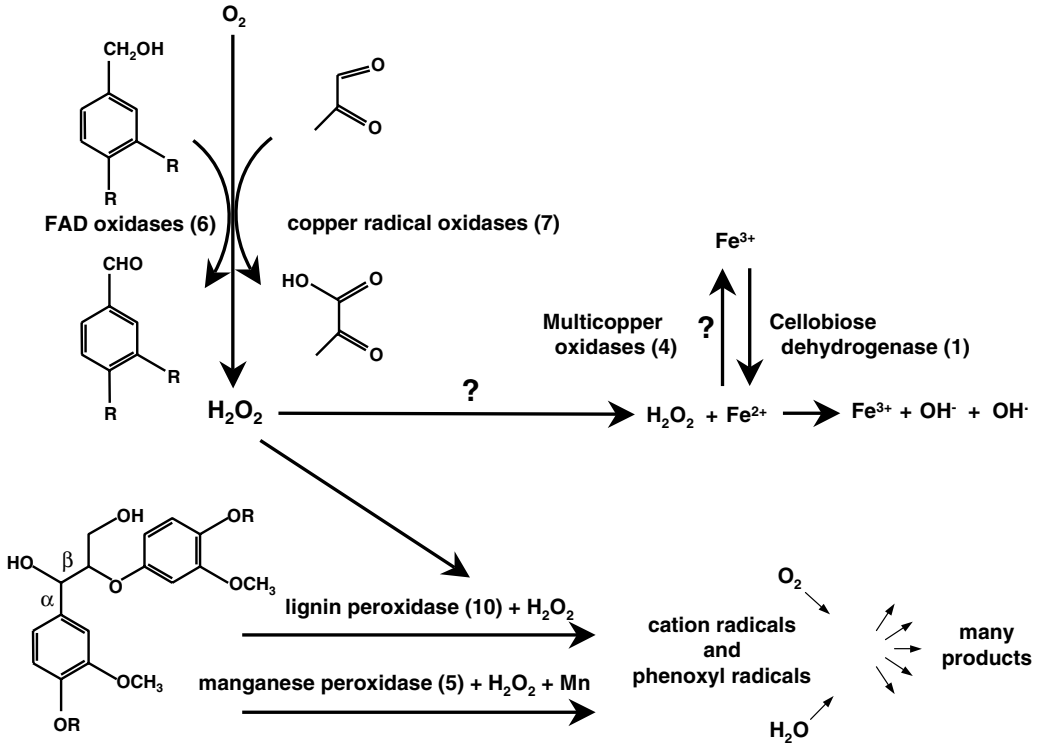

Fig. 1. Proposed extracellular oxidative system of *P. chrysosporium*. Substantial evidence supports physiological connections between H_2O_2 and peroxidases. Benzyl alcohol derivatives (R = H or OCH3) are substrates for FAD-dependent oxidases such as aryl alcohol oxidase. Methylglyoxal is a substrate for glyoxal oxidase, a copper radical oxidase; additional copper radical enzymes (CROs 1–6) may have a similar role in peroxide production. The peroxidase substrate shown typifies the major β-O-4 linkage of lignin (R = H or ether linkage to additional monomeric units). Less certain, peroxide may also serve as a reactant in the spontaneous (non-enzymatic) generation of hydroxyl radical via Fenton's chemistry. Reduction of Fe^{3+} and oxidation of Fe^{2+} have been demonstrated for CDH and MCO1, respectively, but relatively less is known about the biochemistry and regulation of MCO1. The numbers of structurally related sequences are in parentheses. SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP-1.0/) supports non-classical signal peptides in *mco2*, *mco3*, and *mco4*. All other secretory predictions are based on signalP (http://www.cbs.dtu.dk/services/SignalP/).

cellobiose dehydrogenase functional domains (Habu et al., 1993; Eggert et al., 1996). In addition to CDH, LC-MS/MS peptide identification demonstrated the expression of 3 aspartyl protease family A1 (http://merops.sanger.ac.uk/) genes in a medium containing cellulose as sole carbon source (Vanden Wymelenberg et al., 2005). Extracellular proteases increased in ligninolytic cultures with peptides assigned to 6 family A1 genes, 2 family S10 genes, and 5 S53 genes (VandenWymelenberg et al., 2006b).

Extracellular dephosphorylation of *P. chrysosporium* LiP isozyme H2 has been attributed to a mannose-6-phosphatase (Kuan and Tien, 1989; Rothschild et al., 1997, 1999). Based on experimentally determined amino acid sequence, the corresponding gene model (No. 3383) has been identified and the *mpa1* cDNA sequenced (GenBank DQ242647). Consistent with isozyme H2 dephosphorylation, *mpa1*-encoded peptides have been identified by LC-MS/MS in ligninolytic cultures (VandenWymelenberg et al., 2006b).

8. Hypothetical proteins

Analysis of N-terminal regions of the 10,048 current gene models predicts 769 sequences encoding secreted proteins, of which 359 were similar only to hypothetical proteins (Vanden Wymelenberg et al., 2006a). Likely, some of these proteins are not truly secreted but instead cell wall bound or residing in vacuoles or ER associated. On the other hand, the N-termini of many gene models are often inaccurate and incomplete. (Of a total of 10,048 v2.1 models, only 8545 sequences feature an ATG start codon.) Not surprisingly then, up to 25% of the soluble extracellular proteins identified by mass spectroscopy were not predicted to be secreted. Accurate gene model predictions are often complicated by multiple introns and short exons within N-terminal regions. Accordingly, computational 'secretomes' generally underestimate the number of secreted proteins.

By definition, functions of these 'hypothetical' genes are unknown, but it is reasonable to assume some may be involved in lignin degradation. Peptides corresponding to hypothetical genes have been detected in defined media under ligninolytic (models 6845, 3328) and cellulolytic (models 139777, 138739, 131440, 5607, 2035, 2036) conditions (Vanden Wymelenberg et al., 2005, 2006a). Clearly, establishing the function of these genes presents a major challenge for future research.

9. Conclusions

The *P. chrysosporium* genome sequence represents an important advance in the molecular genetics of Basidiomycetes and provides a framework for future investigations on lignocellulose degradation. Fundamental questions regarding mechanisms of lignin degradation, particularly the identity of small molecular weight oxidants, remain unresolved. In this connection, the impressive repertoire of extracellular oxidases implies complex relationships with peroxidases, and with lignin and carbohydrate metabolism. Peroxide may play a central role, and while considerable biochemical evidence argues against a significant role for Fenton chemistry in lignin degradation, hydroxyl radicals may be important in attacking other cell wall polymers (e.g. cellulose).

Genetic multiplicity, a distinguishing feature of the *P. chrysosporium* genome, has not been adequately explained. Among the extracellular oxidative systems discussed above, families of structurally related genes encode peroxidases, copper radical oxidases, multicopper oxidases, and FAD-dependent oxidases. In the case of the copper radical oxidases it seems likely that these differentially expressed genes encode enzymes with similar catalytic mechanisms but divergent substrate specificities. Such oxidative diversity may be necessary for efficient colonization and cell wall degradation as the physical state, composition, and accessibility of polymers varies with the extent of decay. In contrast, lignin peroxidase isozymes are generally viewed as non-specific and the potential advantages of gene multiplicity and differential expression are less clear.

Addressing these issues has been hampered by the limited experimental tools available for *P. chrysosporium*. Genetic transformation systems are available, but efficiencies and targeting are inadequate for gene disruptions/replacements. Recently reported *Agrobacterium* mediated transformation holds promise in this regard (Sharma et al., 2006). Other significant developments related to experimental approaches include mass spectrometry-aided proteomics (Abbas et al., 2004; Shimizu et al., 2005; Vanden Wymelenberg et al., 2005, 2006a) and transcript profiling using oligo-based microarrays (Doddapaneni and Yadav, 2005).

Comparative genome analysis offers another promising approach to establishing the role of individual genes. The soon to be released genome of the brown rot fungus, *Postia placenta*, is of particular interest. The morphology, mating type systems, broad substrate preferences (conifers vs hardwood), and molecular phylogeny suggest repeated shifts in modes of decay between white rot and brown rot (Hibbett and Donoghue, 2001). Do *P. chrysosporium* and *P. placenta* share a similar inventory of genes encoding oxidative enzymes? If not, what genes have been lost or gained? Assuming genes have been generally retained in the brown rot fungus, how have expression patterns been altered such that cellulose is rapidly depolymerized and lignin relatively unaffected?

The *P. ostreatus* genome, recently selected by the Joint Genome Institute's Community Sequencing Program, also provides important opportunities for comparative analysis. In contrast to *P. chrysosporium, P. ostreatus* secretes laccases but no liginin peroxidases. Nevertheless, *P. ostreatus* is an efficient and highly selective lignin degrading fungus. Broad comparisons of gene number and organization as well as more detailed examination of orthologous sequences in these white rot species may answer fundamental questions related to mechanism(s) of lignin degradation.

Acknowledgment

This research was supported by U.S. Department of Energy grant DE-FG02-87ER13712.

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