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Differential Expression in *Phanerochaete chrysosporium* of Membrane-Associated Proteins Relevant to Lignin Degradation▽†

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Fungal lignin-degrading systems likely include membrane-associated proteins that participate in diverse processes such as uptake and oxidation of lignin fragments, production of ligninolytic secondary metabolites, and defense of the mycelium against ligninolytic oxidants. Little is known about the nature or regulation of these membrane-associated components. We grew the white rot basidiomycete *Phanerochaete chrysosporium* on cellulose or glucose as the carbon source and monitored the mineralization of a ^{14}C-labeled synthetic lignin by these cultures to assess their ligninolytic competence. The results showed that the cellulose-grown cultures were ligninolytic, whereas the glucose-grown ones were not. We isolated microsomal membrane fractions from both types of culture and analyzed tryptic digests of their proteins by shotgun liquid chromatography-tandem mass spectrometry. Comparison of the results against the predicted *P. chrysosporium* proteome showed that a catalase (Joint Genome Institute *P. chrysosporium* protein identification number [I.D.] 124398), an alcohol oxidase (126879), two transporters (137220 and 132234), and two cytochrome P450s (5011 and 8912) were upregulated under ligninolytic conditions. Quantitative reverse transcription-PCR assays showed that RNA transcripts encoding all of these proteins were also more abundant in ligninolytic cultures. Catalase 124398, alcohol oxidase 126879, and transporter 137220 were found in a proteomic analysis of partially purified plasma membranes from ligninolytic *P. chrysosporium* and are therefore most likely associated with the outer envelope of the fungus.

White rot basidiomycetes make an essential contribution to global carbon cycling by efficiently degrading the recalcitrant aromatic biopolymer lignin, which encases the cellulose and hemicelluloses of vascular plants and is second only to these polysaccharides as a repository of terrestrial biomass. It is generally thought that the lignin is first oxidatively depolymerized outside the fungal hyphae by the combined action of lignin peroxidases, manganese peroxidases, reactive oxygen species, and secreted secondary metabolites, after which the resulting lignin fragments are taken up and mineralized intracellularly (7, 9, 11, 14). Proteins associated with fungal membranes probably have a major role in many steps of this process, including biosynthesis and secretion of secondary metabolites, uptake and intracellular oxidation of lignin fragments, and protection of the mycelium against ligninolytic oxidants.

Now that the genome of *Phanerochaete chrysosporium* has been sequenced and annotated (20, 34), proteomic work has begun on this intensively researched white rot fungus. Recent studies have shown variation in the expression patterns of many extracellular and cytoplasmic enzymes relevant to ligninolysis (1, 21, 26, 28, 30, 35) but have so far revealed little about the regulation of membrane-associated components. In part, this lack of information likely reflects the poor behavior of some membrane proteins during the electrophoretic separations that are generally employed (27). An alternative approach is to analyze proteolytic digests of whole membrane fractions by shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) and then to assign the observed peptide fragments to the predicted *P. chrysosporium* proteome (18, 37). By this method, we have now identified some membrane-associated proteins that are upregulated during ligninolytic metabolism.

MATERIALS AND METHODS

Culture conditions. *P. chrysosporium* RP-78, the monokaryotic strain used previously for genome sequencing (20), was obtained from the Forest Mycology Center of the USDA Forest Products Laboratory. It was grown in medium containing carbon and nitrogen sources as specified below, together with the basal level of Kirk's mineral salts and trace elements solution (16), sodium 2,2-dimethylsuccinate buffer (20 mM, pH 4.5), thiamine (10 mg/liter), and Tween 20 (0.05%, vol/vol). Nonligninolytic cultures were produced by using glucose (1%, wt/vol) as the carbon source and ammonium tartrate (9 mM) as the nitrogen source. Ligninolytic cultures were produced by using microcrystalline cellulose (Sigmacell 50; Sigma-Aldrich, St. Louis, MO; 0.4%, wt/vol) as the carbon source and ammonium tartrate (20 mM) as the nitrogen source.

The cultures were grown in 2.8-liter Fernbach flasks that each contained 600 ml of sterile medium. Each flask was inoculated with 50 ml of a *P. chrysosporium* conidiospore suspension that had an absorbance of 0.9 at 600 nm. The conidia had been produced beforehand by cultivating the fungus on yeast extract-malt extract-peptone-glucose agar plates for several weeks. The inoculated cultures were incubated at 37°C with rotary shaking at 200 rpm for glucose-grown cultures and 150 rpm for cellulose-grown cultures. Cultures intended for measurements of lignin mineralization were stoppered with rigs designed for headspace flush-

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ing, whereas those intended for enzyme assays or membrane preparation were covered with aluminum foil.

Assays. To assess lignin mineralization, 1.0×10^5 dpm of a synthetic syringyl/guaiacyl β-^{14}C-labeled lignin, labeled in its syringyl substructures (0.01 mCi/mmol), was added to each culture in a small volume of *N*,*N*-dimethylformamide. This lignin, prepared as described earlier (29), had an initial molecular mass of greater than 1,000 Da, as shown by gel permeation chromatography. The stoppered flasks were flushed with sterile, humidified air every 12 h for 5 days and then every 24 h for 2 more days. Vented $^{14}CO_2$ was trapped for quantification by scintillation counting (15).

To assess the production of ligninolytic enzymes, samples were taken every 12 h and clarified by centrifugation. The supernatant fractions were assayed for manganese peroxidase activity by measuring the H_2O_2- and Mn(II)-dependent oxidation of 2,6-dimethoxyphenol to 3,3′,5,5′-tetramethoxydiphenoquinone spectrophotometrically at 469 nm as described previously (36). Lignin peroxidase activity was assayed by measuring the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde spectrophotometrically at 310 nm (31).

Preparation of microsomes. Mycelia from two flasks were harvested by filtration through cheesecloth, pooled, and washed twice with distilled, deionized water. The biomass (50 g, wet weight) was suspended in 50 ml of 1.2 M sorbitol that contained 50 mg of Novozyme 234 (Batch 1989; Novo Biolabs, Bagsvaerd, Denmark) (32). The mycelium was incubated for 30 min at room temperature with gentle stirring. The resulting suspension was centrifuged at $1,500 \times g$ for 5 min, and the pellet was washed twice by centrifugation in 1.2 M sorbitol. This pellet was divided into five approximately equal portions, each of which was mixed with 20 ml of homogenization medium and disrupted with five to seven slow thrusts in a Tenbroeck glass homogenizer. The homogenization medium consisted of 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and one tablet of protease inhibitor per 100 ml (Complete Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany).

The crude homogenate was centrifuged at $5,000 \times g$ for 5 min at 4°C to remove debris, and the resulting supernatant fraction was centrifuged at $10,000 \times g$ for 10 min at 4°C to remove mitochondria. Crude microsomes were then recovered by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C. The microsomes were suspended in 2 ml of resuspension medium (5 mM potassium phosphate buffer [pH 7.8], 1 mM EDTA, 1 mM dithiothreitol, 330 mM sucrose) and held at 4°C. They were assayed for protein with a Coomassie blue dye-binding kit (Bio-Rad, Hercules, CA) and assayed for vanadate-inhibited plasma membrane ATPase activity by the method of Müller et al. in the presence of 0.01% Triton X-100 (22). One unit of activity was defined as the amount of enzyme that liberated 1 μmol of inorganic phosphate from ATP per min.

Preparation of a plasma membrane-enriched fraction. Six grams of a mixture containing 6.0% (wt/wt) polyethylene glycol 3350 (PEG; Sigma-Aldrich), 6.0% (wt/wt) Dextran T 500 (Amersham Biosciences, Uppsala, Sweden), 5 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, 330 mM sucrose, and 3 mM KCl was mixed by inversion 20 times and cooled to 4°C. After the mixture separated into two phases, 2 g of microsomal suspension was added and mixed in by vigorous inversion. The phases were then separated again by centrifugation at $2,000 \times g$ for 5 min at 4°C. The upper phase was collected, diluted with 3 volumes of resuspension medium, centrifuged at $100,000 \times g$ for 30 min at 4°C, and brought up in 200 μl of resuspension medium. The resulting plasma membrane-enriched fraction was assayed for protein and for vanadate-inhibited ATPase activity as described above.

LC-MS/MS analysis. Microsomes and partially purified plasma membranes intended for proteomic analysis were lyophilized. These samples were resuspended in 7 M guanidine HCl and reduced with tris(2-carboxyethyl)phosphine (Pierce Biotechnology, Rockford, IL) by following the manufacturer's instructions. Iodoacetamide (50 mM in 500 mM ammonium bicarbonate) was then added to the samples, which were incubated at room temperature in the dark for 1 h to alkylate cysteine residues. Peptides were next produced by digesting the samples with trypsin (Promega) at a ratio of 1 μg/100 μg of protein extract. Peptide mixtures were isolated on a reverse-phase C_{18} cartridge (Sigma-Aldrich) in accordance with the manufacturer's instructions, lyophilized, and redissolved in 0.1% formic acid at a concentration of 1.8 μg original undigested protein per μl.

A 1.0-μl portion of each sample was then injected onto a Jupiter C_{18} reverse-phase high-performance liquid chromatography column (38-cm length by 150-μm inner diameter, 5-μm particle size; Phenomenex, Torrance, CA). The peptides were eluted at 2 μl/min with an Agilent (Santa Clara, CA) 1100 high-performance liquid chromatography apparatus and with mixtures of H_2O-formic acid at 1,000:1 (solvent A) and acetonitrile-H_2O-formic acid at 800:200:1 (solvent B) according to the following program: 0 to 15 min, isocratic at 100% A; 15 to 20 min, linear gradient to 20% B; 20 to 75 min, linear gradient to 50% B; 75 to 80 min, linear gradient to 95% B; 80 to 85 min, isocratic at 95% B; 95 to 100 min, linear gradient to 100% B; 100 to 140 min, isocratic at 100% B. The peptides were eluted and introduced into an LTQ mass spectrometer (Thermo Fisher, Waltham, MA) by electrospray ionization. Spectra were collected in a data-dependent mode, with the five most intense ions within each sampling window selected for dissociation.

The raw data were analyzed by using the SEQUEST program (6) with a *P. chrysosporium* protein database (http://genome.jgi-psf.org/Phchr1/Phchr1.download.html) derived from the Joint Genome Institute v2 genome assembly (20, 34). Peptides obtained from this analysis with accepted scoring criteria (38) were used to generate peptide abundance measurements. Detailed information on protein models cited here can be retrieved from the Joint Genome Institute web browser by appending the protein I.D. to the following string: http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Phchr1&id=.

Isolation of nucleic acids and cDNA. For total RNA purification, 100 mg of mycelium was ground with a mortar and pestle in liquid nitrogen and RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The resulting RNA samples were then treated with RNase-free DNase (Promega, Madison, WI) as outlined by the manufacturer. RNA concentration was measured by microspectrophotometry with a Nanodrop NT1000 instrument (Thermo Fisher Scientific, Waltham, MA).

First-strand cDNA was prepared from RNA by using avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's instructions with 5 μg of RNA and oligo(dT) primers. The reaction mixture was then diluted 1:5 with RNase-free, distilled, deionized water, and a 5-μl sample was used for quantitative reverse transcription-PCR (qRT-PCR) analyses as described below.

RNA transcript analysis. Primers (Table 1) for the internal reference glyceraldehyde-3-phosphate dehydrogenase gene (protein I.D. 132198) and for the target genes (catalase 124398, alcohol oxidase 126879, transporter 137220, transporter 132234, cytochrome P450 5011, and cytochrome P450 8912) were designed by using PrimerExpress software from Applied Biosystems (Foster City, CA). The glyceraldehyde-3-phosphate dehydrogenase gene was used as the reference because it is generally regarded as constitutively expressed in *P. chrysosporium* (5, 19).

Relative quantifications of gene expression were obtained by qRT-PCR by using Sybr green supermix with ROX reference dye (Bio-Rad) on an ABI Prism 7000 sequence detection system according to the manufacturer's instructions. Standard curves were generated by using five serial dilutions in triplicate. The cycle threshold (C_T) was plotted against the $\log_{10}$ of the cDNA dilution factor. For all of the genes, linear standard curves were obtained with $R^2 = >0.98$, calculated amplification efficiencies of 101 to 107%, and good consistency among the replicates. Control reactions in which the RNA was not reverse transcribed showed no detectable contamination by genomic DNA.

PCR mixtures contained 300 nM each primer, 5 μl of cDNA, and 12.5 μl of Sybr green Supermix in a final volume of 25 μl. The samples were amplified in 96-well plates. Amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, and then 40 cycles that consisted of 15 s at 95°C and 1 min at 58°C. The amplification reactions were shown to be target specific by using dissociation curves with a temperature range of 60 to 95°C. Three biological replicates were included for each sample, and each replicate was analyzed three times. C_T values for each target gene were normalized to the C_T value of the reference gene for all samples. The normalized values for gene expression in cellulose-grown cultures were then expressed as *n*-fold differences relative to the expression values for the glucose-grown cultures, which were set to 1.

RESULTS AND DISCUSSION

Establishment of culture conditions. Our first goal was to find conditions that elicit ligninolytic activity in *P. chrysosporium* while simultaneously providing large quantities of mycelia suitable for membrane isolation. We used a variation of the method developed by Zacchi et al. (42), who reported that lignin peroxidases were produced by submerged, rotary-shaken cultures grown on cellulose, a natural carbon source for *P. chrysosporium*, under air and in the presence of nonlimiting nitrogen. To produce nonligninolytic mycelium for comparison, we used glucose instead of cellulose as the carbon source, again under air and with nonlimiting nitrogen. Although lig-

TABLE 1. Sequences of the qRT-PCR primers used in this study

Model name	Protein I.D.	Primer	Sequence (5′ → 3′)	Length (bp)
e_gww2.9.328.1	132198	Forward	GTTTGTCTGCGGTGTCAACCT	21
		Reverse	TGCCGAACTTGTCGTGGAT	19
e_gwh2.12.114.1	124398	Forward	TTCGACAGAGAGCGTATCCCAG	22
		Reverse	AAGAACGAAGCGCAAGTGAGG	21
e_gwh2.7.116.1	126879	Forward	AGCGCGAGCTTTTCACAGAGT	21
		Reverse	CCCATCTCCTTCAGCTCTTCCT	22
fgenesh1_pg.C_scaffold_8000186	5011	Forward	AAGATCACGGATGAGGCACTG	21
		Reverse	GCGGCCCATTTGATGATATC	20
fgenesh1_pg.C_scaffold_20000002	8912	Forward	AACTCTCCATCTGCAACGTGG	21
		Reverse	GGATTGGCTGCTCTAATCCCT	21
e_gww2.9.144.1	132234	Forward	TACCTCAATCTGCCCATCACC	21
		Reverse	CGCTTCATAGGCAAAGAATGC	21
e_gww2.3.250.1	137220	Forward	AGCAGCTACCTCGACTGCTTCA	21
		Reverse	GCCGTGCCGTAGTAGAAGATGA	21

ninolytic activity by *P. chrysosporium* is known to be nutritionally regulated on defined medium (17), the proteomic studies done so far under various culture conditions have not included tests of whether the full capacity to degrade lignin was, in fact, induced. To fill this gap, we added a high-molecular-weight, β-^{14}C-labeled, synthetic lignin to our cultures and monitored the evolution of $^{14}CO_2$ periodically to determine the onset of ligninolytic metabolism.

The results showed that the cellulose-grown cultures expressed a fully competent ligninolytic system, with mineralization of the [^{14}C]lignin commencing between 60 and 84 h after inoculation (Fig. 1). We could not detect lignin peroxidase activity in these cultures, but all of them produced extracellular manganese peroxidase activity at 72 h and approximately half of them did so at 60 h (data not shown). Accordingly, we grew additional cultures without [^{14}C]lignin in the same cellulose-based medium and harvested them at 66 h to obtain membrane preparations typical of ligninolytic mycelium. By contrast, the glucose-grown cultures mineralized very little [^{14}C]lignin (Fig. 1) and did not express detectable peroxidase activity. These cultures also grew faster than those on cellulose. Accordingly, we grew additional cultures on glucose without [^{14}C]lignin and harvested them at 42 h to obtain membrane preparations typical of nonligninolytic mycelium.

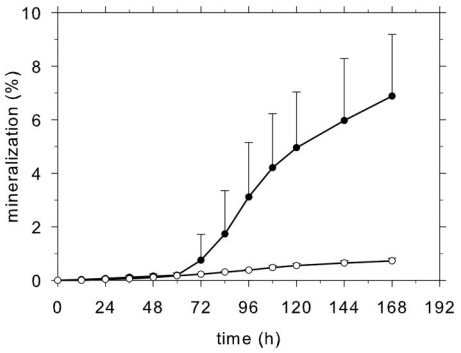

FIG. 1. Mineralization of ^{14}C-labeled synthetic lignin by glucose-grown cultures (open circles) and cellulose-grown cultures (closed circles) of *P. chrysosporium*. The error bars indicate the standard deviation of the sample for six replicates of each culture type.

Preparation and analysis of membranes. Mycelia from both types of culture were treated with a glycosylhydrolase mixture to weaken their cell walls, disrupted by gentle homogenization, and fractionated by differential centrifugation. The mitochondria, which have already been the subject of proteomic research (30), were not retained for analysis. Each supernatant fraction was pelleted by ultracentrifugation to yield crude microsomes, which were lyophilized, treated with trypsin, and analyzed by LC-MS/MS. SEQUEST analysis of the data (6) using the predicted *P. chrysosporium* proteome showed that a typical sample contained 1,200 to 1,800 detectable peptides (see Table S1 in the supplemental material). The resulting data sets, typically comprising 300 to 400 proteins per sample, were then inspected for likely transporters, cytochrome P450s, and any enzymes previously reported to have an outer membrane location in lignocellulolytic fungi. Table 2 shows the number of MS/MS spectra (i.e., the number of peptides) attributable to each of the proteins found in microsomes from triplicate cultures grown on cellulose or glucose. Spectral counting provides a useful, semiquantitative estimate of relative protein abundance between samples, provided the differences being assessed are clear-cut and can be validated by some independent method such as RNA transcript analysis (24).

In an additional experiment, we partitioned the microsomes from one set of cellulose-grown cultures in a PEG-dextran two-phase system to obtain a fraction enriched in plasma membranes from ligninolytic *P. chrysosporium*. We included this sample because interesting functions such as lignin fragment uptake and antioxidant defense are likely to be associated with the outer envelope of the hyphae. Typically, plasma membranes partition to the upper PEG-dextran phase and can be purified three- to sixfold in preparations from filamentous fungi, as assessed by measurements of vanadate-inhibited

TABLE 2. Selected proteomic data for membranes from ligninolytic and nonligninolytic *P. chrysosporium*

Protein I.D.	Protein description	Swiss-Prot alignment score	Peptide count[a]						
			Nonligninolytic glucose-grown cultures			Ligninolytic cellulose-grown cultures			
			G1M	G2M	G3M	C1M	C2M	C3M	C1PM
124398	Catalase	1,439	0	0	0	32	8	13	15
126879	Alcohol oxidase	1,582	0	0	0	40	17	24	18
26782	MFS transporter	494	0	0	0	0	1	6	0
9336	MFS transporter	515	2	2	1	0	0	0	0
137220	MFS transporter	1,022	4	0	2	5	6	11	4
132234	MFS transporter	657	0	0	0	4	10	14	0
138934	ABC transporter	2,573	6	0	1	0	0	0	0
134856	ABC transporter	1,470	0	0	0	0	0	4	0
3654	Cytochrome P450 (family 64)	530	2	0	2	0	0	0	0
5011	Cytochrome P450 (family 64)	623	0	0	0	3	3	3	0
8912	Cytochrome P450 (family 67)	563	0	0	0	2	5	3	0
131706	Cytochrome P450 (family 52)	346	3	1	0	0	0	0	0
138737	Cytochrome P450 (family 67)	506	2	0	11	0	1	2	0
140188	Cytochrome P450 (family 52)	1,180	16	0	12	0	0	1	0
126811	Plasma membrane ATPase	2,384	71	6	62	38	71	85	6

[a] Three cultures of each type were grown. G, glucose; C, cellulose; M, microsomes; PM, plasma membranes.

plasma membrane ATPase specific activity (22, 32, 33). From 8.3 mg of microsomes with a specific activity of 0.055 U/mg protein for this ATPase, we obtained 81 μg of partially purified plasma membranes with a specific activity of 0.337 U/mg. The fraction enriched in plasma membranes was processed and analyzed by LC-MS/MS by following the same procedure used for microsomes and yielded 226 peptides that were assigned to 46 proteins. Spectral counts for proteins of interest in this preparation are shown in Table 2. The complete data set (see Table S2 in the supplemental material) shows that cell wall proteins were present in addition to plasma membrane proteins, and therefore we also refer to this material as an "outer envelope" fraction. Proteins originating from intracellular membranes, although detectable, were relatively infrequent. For example, the plasma membrane ATPase was found both in the microsomes and in the enriched fraction, as expected, whereas cytochrome P450s, which are predominantly located in the endoplasmic reticulum, were detectable only in the microsomes (Table 2).

Enzymes involved in H_2O_2 metabolism. The LC-MS/MS data (Table 2) showed that a catalase in the microsomal fraction (protein 124398) was detectable only in ligninolytic, cellulose-grown mycelium. In addition, the qRT-PCR results showed that transcripts encoding this catalase were increased more than 35-fold in ligninolytic cultures relative to nonligninolytic cultures (Table 3). Catalase 124398 also appeared in the partially purified plasma membranes and thus appears to be associated with the outer envelope of the fungus. Our result agrees with past work based on cytochemical staining of *P. chrysosporium* hyphae, which provided evidence that catalase activity is present in the periplasmic space and is upregulated during ligninolytic metabolism (3, 8). Although the *P. chrysosporium* genome encodes four catalases that could have been responsible for the activity seen in these earlier electron microscopic studies (20, 34), our results suggest that protein 124398 is the major isozyme associated with the plasma membrane under our experimental conditions. *P. chrysosporium* protein 124398 is 58% identical to a *Candida albicans* protein (National Center for Biotechnology Information [NCBI] accession no. O13289) that has been shown to have catalase activity (39). It has a likely role in surveillance against extracellular reactive oxygen species, which are simultaneously inducers, agents, and by-products of fungal ligninolysis (2, 9, 11, 12).

A protein in the glucose-methanol-choline oxidoreductase superfamily (protein 126879) was present in both microsomes and partially purified plasma membranes of ligninolytic cultures, whereas it was undetectable in membranes from nonligninolytic cultures (Table 2). Moreover, transcript levels for the gene encoding protein 126879 were markedly higher in ligninolytic cultures than in nonligninolytic ones (Table 3). Protein 126879 is very likely an alcohol oxidase associated with the periplasm and extrahyphal membranes of *P. chrysosporium*, because it is 88% identical to an enzyme with this location and activity from the wood decay basidiomycete *Gloeophyllum trabeum* (NCBI accession no. ABI14440) (4). Although *G. trabeum* causes brown rot of wood rather than white rot, both types of decay employ H_2O_2 to generate lignocellulolytic oxidants (9, 11). Extracellular alcohol oxidases associated with the fungal mycelium likely produce some of this H_2O_2 when they oxidize methanol that is derived from the aromatic methoxyl groups

TABLE 3. qRT-PCR results for elevated transcripts encoding *P. chrysosporium* membrane-associated proteins

Protein I.D.	Protein description	Fold increase in cellulose-grown cultures[a]
124398	Catalase	35.9 ± 5.4
126879	Alcohol oxidase	361.3 ± 188.2
137220	MFS transporter	12.1 ± 4.0
132234	MFS transporter	5.0 ± 1.2
5011	Cytochrome P450 (family 64)	4.2 ± 1.6
8912	Cytochrome P450 (family 67)	1.7 ± 0.3

[a] Mean for triplicate cultures ± standard deviation of the sample.

of lignin (4). It is not clear why a catalase and an H_2O_2-producing oxidase are simultaneously present on the outer envelope of *P. chrysosporium*, but perhaps the two enzymes are somehow compartmentalized (3).

Transporters. Our microsomal preparations contained six detectable proteins that can be assigned to either the major facilitator superfamily (MFS) or the ATP-binding cassette (ABC) superfamily (Table 2). One of these, protein 137220, was more abundant in microsomes from ligninolytic cellulose-grown mycelium and also appeared in the partially purified plasma membrane fraction from this type of culture. Although the LC-MS/MS result for protein 137220 is not clear-cut, its importance is corroborated by a 12-fold transcript increase in the cellulose-grown cultures relative to the glucose-grown cultures (Table 3). Protein 137220 is 63% identical to the *Amanita muscaria* AmMst1 protein (NCBI accession no. CAB06078), which has a documented role in hexose transport (23), and it is 42% identical to the *Saccharomyces cerevisiae* SNF3 glucose sensor (NCBI accession no. EDV08455) (25). Accordingly, protein 137220 has a likely role in the detection or uptake of extracellular metabolites by ligninolytic *P. chrysosporium*. Another probable transporter, protein 132234, was detected only in ligninolytic cultures (Table 2), and transcripts encoding it were also higher under these conditions (Table 3). However, this protein did not appear in the plasma membrane-enriched fraction. Protein 32234 is 48% identical to a putative transporter from *Coprinus cinereus* (NCBI accession no. AAF01426), but its location and function remain to be established.

Cytochrome P450s. We detected six cytochrome P450s in *P. chrysosporium* microsomes out of approximately 150 encoded in the genome (Table 2) (20, 34). Two of these enzymes, protein 5011 and protein 8912, were detectable only in ligninolytic, cellulose-grown cultures. For protein 5011, the proteomic data were corroborated by the qRT-PCR results, which showed that transcripts were elevated about fourfold on cellulose (Table 3). A small increase was also found for transcripts encoding protein 8912 under these conditions, but this result should be viewed cautiously because it could have been affected by minor fluctuations in the expression of the glyceraldehyde-3-phosphate dehydrogenase reference gene (5).

The expression of enzymes 5011 and 8912 has not been noted in previous work, but it has been shown that many other cytochrome P450s are nutritionally regulated in *P. chrysosporium* (5, 40). The large number of cytochrome P450s in this fungus likely reflects, in part, its high requirement for monooxygenases that hydroxylate aromatic rings. Ring hydroxylations occur in the biosynthesis of the *P. chrysosporium* secondary metabolite veratryl alcohol, an essential cofactor for ligninolysis by lignin peroxidases (10, 13), and similar intracellular monooxygenations are presumably required to prepare low-molecular-weight products of ligninolysis for ring fission prior to mineralization. Our results do not exclude a role for nonmicrosomal hydroxylases in lignin fragment oxidation by *P. chrysosporium* (41), but cytochrome P450s 5011 and 8912 are good candidates for participation in lignin biodegradation.

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