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PCR-Mediated Analysis of Lignocellulolytic Gene Transcription by *Phanerochaete chrysosporium*: Substrate-Dependent Differential Expression within Gene Families

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We compare the kinetics of appearance of supernatant enzyme activities (lignin peroxidase, manganese peroxidase, and cellulase) and gene expression (*LIG*, *mnp*, and *cbhI* gene families and the unique *cbhII* gene) in *Phanerochaete chrysosporium* ME446 when grown on four different carbon sources: ball-milled straw, representing the natural substrate lignocellulose; Avicel as a crystalline cellulose; and high and low concentrations of glucose, in all cases with limiting nitrogen. PCR-based technology utilizing pairs of primers specific for particular genes showed that there is differential expression between and within the families. There were a number of instances of mRNA species being present only on a single day, implying tight regulation of lignocellulose degradation at the mRNA level. The patterns of extracellular enzyme activities and *mnp* and *cbh* gene expression are similar whereas *LIG* gene expression can be detected when no corresponding enzyme activity is observed in the extracellular supernatant. The enzyme produced under these conditions is presumably sequestered by the mycelium and is likely to be functionally significant. Another striking result is that cellulose, in the form of Avicel, elicits the expression of three *LIG* genes for which there is no expression under the same conditions with the other carbon sources.

The white rot fungus *Phanerochaete chrysosporium* degrades all three components of lignocellulose: lignin, cellulose, and hemicellulose (12). Lignin degradation is regarded as a stress response to nutritional depletion for carbon, nitrogen, or sulfur (24), resulting in access to nutrients located within lignified cells. In the past decade, the primary emphasis of studies of *P. chrysosporium* has been on the lignin and manganese peroxidases (LiP and MnP) that are proposed as the primary enzymes for lignin degradation (17, 25). Much effort has been directed towards obtaining high yields of extracellular preparations of these enzymes, because of their perceived value for biotechnology. However, attempts to reconstruct lignin degradation as an in vitro process have had only limited success. The main reason for this is that the oxidative activity of these peroxidases results in radicals that allow repolymerization via processes that resemble lignin biosynthesis. Thus, although in vitro depolymerization of model lignin has been reported with both LiP (19) and MnP (47), a net increase in molecular weight has also been reported (18). The mechanism by which the fungus shifts the equilibrium of the reaction towards depolymerization is not well understood, but it is likely that the intact fungus is required to achieve this. A number of authors have pointed out that significant proportions of the peroxidase enzymes can be associated with mycelium (14, 15, 26, 37, 42).

It has been shown that for both LiPs and MnPs there are families of genes which are differentially expressed (2, 4, 33, 35, 43). Cellobiohydrolases have been characterized and show synergy (46), and whereas a complex family of CBHI-encoding genes showing differential expression exists (8, 9, 41), only a single CBHII-encoding gene exists (44). Other enzymes that are being studied are xylanases (10, 11) and those concerned with the generation of hydrogen peroxide (1, 3, 13, 20, 23).

Where enzymes are not easily obtained because they are either immobilized within or associated with the mycelium and where there are families of closely related genes showing differential expression, accurate assay of gene expression is difficult. We have proposed previously (4) that PCR technology is particularly appropriate for the study of gene expression under such conditions, because it is sensitive to the mRNA level and because it allows the monitoring of expression of individual gene sequences within closely related families. In the present paper, we apply this technique to 11 genes from strain ME446 and ask about their patterns of expression during growth on four different carbon sources. Two of these conditions, high and low glucose, are identical to those used previously to monitor performance with respect to mineralization of synthetic lignin (48). The others are more complex substrates, ball-milled straw (BMS) as a model for whole lignocellulose and Avicel as the model for the cellulose component of lignocellulose. At the same time, we have followed supernatant enzyme activities for both classes of peroxidase and for cellulase. The PCR methodology reveals cases of differential expression within gene families, of differences in gene expression profiles with different substrates and of expression of a *LIG* gene without concomitant appearance of LiP activity in the culture supernatant.

MATERIALS AND METHODS

Organism. *P. chrysosporium* ME446 (ATCC 34541) was maintained on slopes of 2% (wt/vol) malt extract.

Culture medium. Culture medium comprised 0.01 M dimethylsuccinate buffer (pH 4.5) containing (in grams liter^{-1}): KH_2PO_4 (0.2), $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2 \cdot 2H_2O$ (0.013), thiamine (0.001), $NH_4H_2PO_4$ (0.23; low N), and veratryl alcohol (0.068). The medium also included minerals, 7 ml liter^{-1}, containing (in grams liter^{-1}): nitrolotriacetate (1.5), $MgSO_4 \cdot 7H_2O$ (3.0), $MnSO_4 \cdot 7H_2O$ (0.5), NaCl (1.0), $FeSO_4 \cdot 7H_2O$ (0.1), $CoSO_4$ (0.1), $CaCl_2 \cdot 2H_2O$ (0.082), $ZnSO_4 \cdot 7H_2O$ (0.1), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01), H_2BO_3 (0.01), $NaMoO_4$ (0.01), and $CuSO_4 \cdot 5H_2O$ (0.05). Carbon source added was either glucose (0.2 or 2.0% [low or high, respectively]), Avicel (0.2%), or BMS (0.2%). Two hundred milliliters was transferred into a 2-liter Erlenmeyer flask, and triplicate cultures were each inoculated with approximately 10^6 conidiospores scraped from agar slopes

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TABLE 1. Gene-specific sequences and annealing temperatures of upstream (u) and downstream (d) primers for PCR amplification

Gene	Sequence	Annealing temp (°C)
cbhI.1u	ACA ATG TTC CGC ACT GCT ACT T	61
cbhI.1d	AGG GTG CCC GCG GAG GTG CC	61
cbhI.2u	CAC TCC TCG CAT TCA CTT GTC T	61
cbhI.2d	CTG CCG GTC TCG GTC CAG TTG C	61
cbhIIu	CCT CAG CCC TTA CTA CGC	55
cbhIId	CCA ATC TAC CTC TAC AGC	55
LIG1u	GCC GCA ATT TCT CTT GCT CTT TCC A	68
LIG1d	TAC ATC GAA CCA CGC GCA CGA TGA TT	68
LIG2u	CAT CGC AAT TTC GCC CGC CAT GGA GGC A	70
LIG2d	ACC TTC TGA ACG AAT GGC TTC TGG AGC	70
LIG3u	TAT TGC CAT CTC TCC TGC TAT GGA GGC C	70
LIG3d	ATG TTA GGG TGG AAG TTG GGC TCG ATG	70
LIG4u	GTG CGC CTG GTT CCC CAT TCT GCA G	63
LIG4d	AAT TGG TCT CGA TAG TAT CGA AGA C	63
LIG5u	GGT CTC GAT CGA GGA GAA GGT AAT GAT C	68
LIG5d	TTG CCC CGA CGG CGT GCA CAC	68
LIG6u	GAC CTG CTC GAA CGG CAA GGT CGT CC	68
LIG6d	CAT GAT AGA ACC ATC GGC GCC TCG C	68
mnp1u	TCC GGT CAA CGG CTT GGT ATT CCA G	64
mnp1d	GCG ATC GTC TTG TTC GGG CGG CCA G	64
trpCu	CAC GGG CAT CGT GAC GGA TAC	63
trpCd	TGG GTC TTG AGT GTG TAG TGG	63

into sterile distilled water. Each flask was stoppered with a bung with inlet and outlet tubes. Flasks were incubated without agitation at 37°C and flushed with oxygen every 3 days.

Assays for enzyme activity. General cellulase activity was measured by a cellulose-azure method (36). Release of dye was measured at 595 nm and calibrated against commercial cellulase (Sigma C-0901). The method used for the assay of LiP activity was that involving the oxidation of veratryl alcohol to veratryl aldehyde (45). MnP activity was assayed via the oxidation of vanillylacetone (Aldrich) in the presence of Mn(II) ions (31).

RNA preparation and cDNA synthesis. RNA was prepared by the method of James et al. (22) and was precipitated with LiCl. Poly(A^+) mRNA was prepared from this with Dynal's Dynabeads mRNA extraction kit according to the manufacturer's recommendations. cDNA was synthesized from poly(A^+) mRNA according to the manufacturer's protocol with Pharmacia's First Strand cDNA synthesis kit.

Design of PCR primers. The sequences of the primers and the annealing temperatures at which they were used are listed in Table 1. The following have been described previously: *trpC* (38); *cbhI.1*, *cbhI.2*, and *cbhII* (44); *LIG1* and *LIG5* (4). Criteria for the design of the other gene-specific primer pairs were those of Brooks et al. (4). In all cases, absolute specificity was confirmed by diagnostic restriction analysis of amplification products derived from genomic DNA (gDNA) templates. Furthermore, under the amplification conditions described below, only template DNA derived from clones containing the cognate sequences yielded such products. Thus, for instance, the *LIG1*-specific primer pair was unable to amplify templates derived from any of the other *LIG* sequences. As a final check of specificity, the products from three primer pairs chosen at random were sequenced and found to consist of the single targeted region.

PCR amplification. Approximately 200 ng of genomic DNA in 1 μl of distilled water or 50 ng of cDNA (see above) was subjected to 30 cycles of amplification by PCR. In addition to template DNA, the amplification mixture contained (in a final volume of 100 μl) 2.5 U of *Taq* polymerase (Promega), 10 mM Tris · HCl (pH 8.3), 50 mM KCl, optimized amounts of $MgCl_2$ within the range of 0.6 to 1.5 mM, 100 μg of gelatin per ml or 0.5% Tween 20 and 0.5% Nonidet P-40, 100 μmol of each deoxynucleoside triphosphate, and 100 ng of each of the primers for a given gene (Table 1).

RESULTS

Experimental strategy. The overall strategy of the experiment was to grow replicate cultures of strain ME446, in low-nitrogen medium buffered with dimethyl succinate at pH 4.5, by using each of four carbon sources, namely, BMS representing whole lignocellulose, Avicel as a crystalline cellulose, and both high (2%) and low (0.2%) concentrations of glucose. The replicate cultures were incubated at 37°C over a period of 8 days from inoculation. On each day from day 3, three of the replicate cultures of ME446 growing with each of the carbon sources were used to provide supernatants for enzyme assays and mycelium for mRNA preparations. The supernatants were assayed for cellulase, LiP, and MnP activities, and the replicate mRNA preparations were used for the independent synthesis of cDNA populations. These cDNA populations were stored at −80°C, and aliquots were subsequently used as template material for PCR experiments. The results of the enzyme activity determinations (Fig. 1) are described first.

Supernatant enzyme activities. The relationship between a particular protein sequence and the precise nature of the cellulase activity that it will elicit (exo- or endo-) is complex and hard to predict; proteins classified on the basis of activity as falling into one class show significant amino acid similarity to those from the other. Moreover, such activity-based classification is always an oversimplification: all exocellulases show measurable and often significant endocellulase activity. Rather than prejudging the precise cellulase activity likely to be expressed from the genes studied, we chose to use a general cellulase assay. Cellulase activities were detected in the supernatants of both the Avicel- and the BMS-grown cultures. Activity on Avicel was highest, but in each case, the cultures produced two peaks of activity, on days 4 and 6. As has been previously shown with both strains ME446 and BKM-F-1767 (references 40 and 9, respectively), cellulase activities were absent in the presence of glucose.

Significant LiP activities were elicited by only two of the substrates, Avicel and BMS. The latter showed the highest activity from days 3 to 6, but from day 7, activity in the Avicel-grown cultures rose sharply to a level that was approximately twice that seen on BMS. The very low activities measured with the high-glucose cultures are consistent with previous results obtained under the same growth conditions which show mineralization of synthetic lignin in the absence of supernatant LiP (48).

Levels of MnP activities rose steadily during days 3 to 7 in the Avicel-grown cultures, whereas BMS elicited levels of activities that were intermediate and variable. The levels produced on low glucose were much lower than the activities initially seen in the high-glucose cultures, but in the former

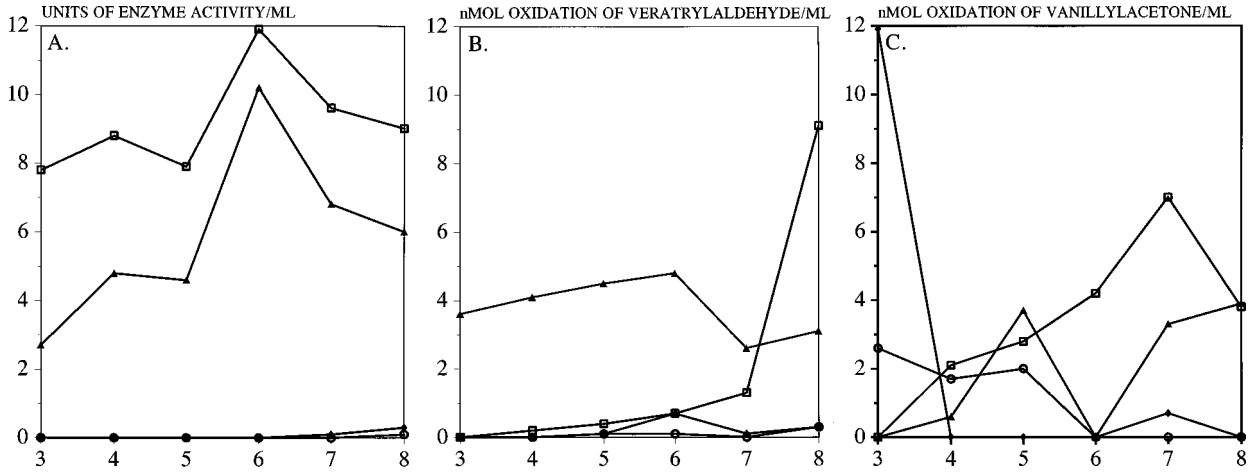

FIG. 1. Graphical representation of the extracellular cellulase (graph A), LiP (graph B), and MnP (graph C) activities in the culture supernatant of *P. chrysosporium* ME446 when grown under nitrogen-limiting conditions and with either Avicel (0.2%), BMS (0.2%), or two levels of glucose, high (2%) and low (0.2%), as the sole carbon source. In each graph, □, ▲, ◆, and ○ represent the activity when grown with either Avicel, BMS, high glucose, or low glucose, respectively. Each time point (days 3 to 8 of culture growth) is the average of the three replicate cultures. Standard deviations were within the ranges ±9% to ±21% for cellulase, ±49% to ±78% (the low-glucose 6-day point) for LiP, and ±16 to ±44% for MnP activities.

case, they were maintained for 2 days longer. The highest MnP activities were seen with the high-glucose cultures on day 3; however, 24 h later no such activity was detected in any of the three replicate cultures.

Origin of gene sequence information used for PCR primer design. Our complementary aim was to study the expression of as many ME446 genes encoding different isozymes relevant to lignocellulose degradation as possible. For this strain, five LiP sequences have been described; of these, *LIG5* (22) encodes a protein analogous to the H2 isozyme of strain BKM-F-1767. The other four, *LIG1*, *LIG2*, *LIG3*, and *LIG4* (6), all encode H8-like isozymes, but until now, none of these has been shown to be expressed in strain ME446 (22). In order to extend this study further, to include any ME446 genes encoding proteins analogous to H10 from BKM-F-1767, we designed PCR primers based on the sequence of the corresponding gene *GLG2* (49). When used in PCR experiments with ME446 genomic DNA as template, these amplified a fragment of 315 bp. Six independent clones were subsequently analyzed, and all were found to contain the same H10-encoding sequence. Interestingly, the region sequenced included two putative introns, and in both cases, their positions and sequences were identical to those found for their BKM-F-1767 counterparts. This is the first instance of absolute conservation of intron sequences from equivalent genes of these two strains. We show below that the PCR-amplified products from a cDNA template were of a size expected from the removal of both sequences by mRNA splicing (Fig. 2).

In order to examine the expression of MnP in ME446, we designed PCR primers (Table 1) based on the single published sequence available at that time, *mnp1* (16), from a derivative strain, OGC101. Cellulase gene expression was investigated by using two pairs of primers designed to differentiate between the genes encoding two of the known cellobiohydrolase I isozymes, *cbhI.1* and *cbhI.2*, of ME446 (41) and a third pair specific for its unique cellobiohydrolase II gene, *cbhII* (44).

Endoglucanase genes analogous to those of *Trichoderma reesei* have not been included in this analysis because, despite considerable efforts, such sequences have not been identified in this organism (41). A primer pair specific for the amplification of the constitutively expressed *trpC* gene (38) was also used as a positive control for gene expression in all cultures.

PCR analysis of gene expression. Table 2 summarizes the data obtained for the replicate cultures grown with each of the four carbon sources with respect to the expression of each of the 11 genes under analysis. Samples of cDNA preparations were analyzed by competitive PCR (39). Low but significant levels of gDNA were found to be present in all samples, confirming earlier observations (38). These have the potential to produce serious artifacts that could fatally compromise results. However, in the analyses presented here, the presence of this gDNA has been exploited as the competitive species. The level of gDNA within any particular cDNA preparation has been used to relate the amount of cDNA-derived amplification product from any particular primer pair to that derived from any other pair on the same template preparation. By using a semiquantitative notation that compares the yield of product from cDNA to that derived from gDNA, we alleviate concerns about the relative efficiencies of the different primer pairs. Such analysis is valid since all of the genes studied are single copy, and we assume that all genomic sequences are present in equimolar amounts.

From control experiments, the level of gDNA in samples of the cDNA preparations was calculated and found to be similar, approximately 10^4 copies per 50 ng of cDNA (5). This is further confirmed by the *trpC* results which also show that the level of gDNA is relatively constant across the range of cDNA preparations (Table 2; also illustrated for Avicel-grown cultures in Fig. 2). We have therefore also been able to relate the yield of cDNA product derived from a particular primer pair on one cDNA preparation with that derived from the same primer pair on a different cDNA preparation. In addition, the presence of amplified cDNA found by using the *trpC*-specific primer pair, under all conditions and for each replicate, demonstrates that in each case intact mRNA had been extracted from the cultures and converted to cDNA.

Exhaustive control experiments (5) using different ratios of known amounts of gDNA and cloned cDNA templates showed that, for all primer pairs, amplification, under the conditions used here, maintained the initial ratio of target to competitor. Nevertheless, we have adopted only a semiquantitative notation which classifies the level of expression into four categories. As well as there being clear positive and negative results for the presence of cDNA (++ and −), there are the additional

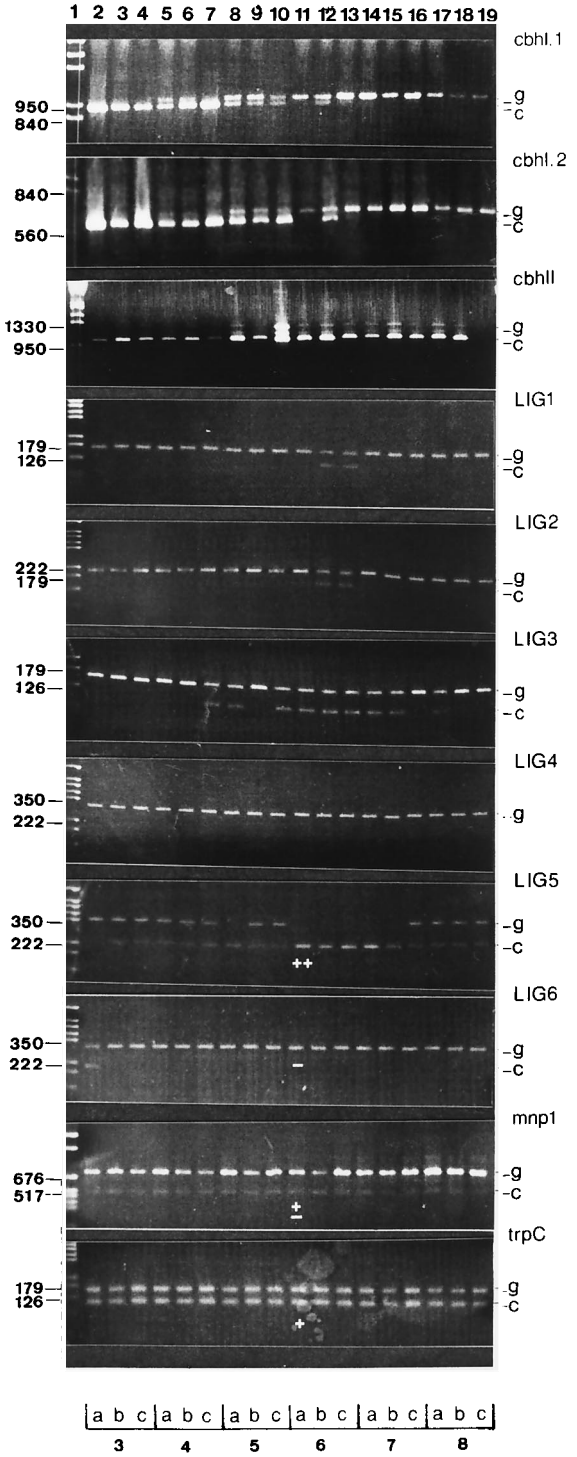

FIG. 2. Temporal PCR analysis of gene expression in *P. chrysosporium* ME446 when grown under nitrogen-limiting conditions and with Avicel (0.2%) as the sole carbon source. From top to bottom, respectively, each panel represents PCR amplification reactions using primer pairs specific for the *cbhI.1*, *cbhI.2*, *cbhII*, *LIG1*, *LIG2*, *LIG3*, *LIG4*, *LIG5*, *LIG6*, *mnp1*, and *trpC* genes or sequences of *P. chrysosporium* ME446. Within each panel, lane 1 was loaded with an appropriate size marker: λ DNA digested with *Eco*RI and *Hin*dIII for panels containing the *cbhI.1*, *cbhI.2*, and *cbhII* amplification products or pGEM DNA marker (Promega) for all the other panels. The sizes of marker DNA closest in size to the amplification products within each panel are given in base pairs. Lanes 2 to 19 in each panel represent the amplification products of cDNA prepared from mycelia grown for 3 days (lanes 2 to 4), 4 days (lanes 5 to 7), 5 days (lanes 8 to 10), 6 days (lanes 11 to 13), 7 days (lanes 14 to 16), and 8 days (lanes 17 to 19). For each time point, cDNA samples were prepared from triplicate cultures (a, b, and c). PCR amplification products derived from genomic and cDNA templates are indicated by the letters g and c, respectively. The notation adopted to classify the level of gene expression for construction of Table 2 is depicted in the final four panels and is represented by one of four symbols: ++ (product derived from cDNA only), + (gDNA and cDNA products both present in approximately similar amounts), ± (weak cDNA product, strong gDNA product), and − (product derived from gDNA only).

two categories in which amplified gDNA is present in amounts approximately equal to those of the cDNA (+) and as the predominant species (±). We consider that these categories (++, +, ±, and −) correspond to high, medium, low, and undetectable levels of expression. The final four panels of Fig. 2, representing the expression of the *LIG5*, *LIG6*, *mnp1*, and *trpC* genes in Avicel-grown cultures, illustrate each of these categories. The cDNA- and gDNA-derived species can be distinguished on the basis of size because, in all cases, the primer pairs span at least one intron.

Relationship between gene expression and supernatant enzyme activities. There was expression of all three cellulase genes in both Avicel- and BMS-grown cultures until day 6. Thereafter, expression of neither *cbhI.1* nor *cbhI.2* was detectable whereas that of *cbhII* continued throughout the experiment. Cellulase activities also declined from day 6 (Fig. 1). The *cbhI.1* gene was the only cellulase-encoding gene for which any expression was detected in the presence of glucose; such expression was confined to days 7 and 8 of the low-glucose cultures. Thus, there is a reasonable correspondence between the cDNA levels for these *cbh* genes and the overall levels of supernatant cellulase activity.

We have shown above that BMS and Avicel both elicit significant extracellular LiP activity, whereas both high- and low-glucose-grown cultures do not. How does this correlate with the expression of the six LiP genes for which such analysis is possible? The *LIG5* gene was not expressed in high-glucose-grown cultures, confirming earlier suggestions that this concentration of glucose prevents expression of this gene (4) and its equivalent in BKM-F-1767 (21). Also as reported previously, the *LIG5* gene was expressed in the low-glucose-grown cultures throughout the period examined. This contrasts with the finding that in the same low-glucose-grown cultures supernatant LiP activity was negligible at all times (Fig. 1). That is, expression of *LIG5* is not reflected in supernatant enzyme activity. No other *LIG* genes studied here were expressed in either the low- or high-glucose-grown cultures.

LIG5 is also expressed throughout the period of study in BMS-grown cultures; in this case, the only other *LIG* gene expressed is *LIG1* on day 8. In previous work (22), a cDNA library produced from mRNA isolated from BMS-grown mycelium on day 5 contained *LIG5*-derived sequences but not *LIG1*-, *LIG2*-, *LIG3*-, and *LIG4*-derived sequences. However, in contrast to the result found with low glucose, in BMS-grown cultures there was also supernatant LiP activity throughout the period examined.

It was noted above that in Avicel-grown cultures there was a late and dramatic rise in supernatant LiP levels (Fig. 1). The pattern of *LIG5* expression in this case was similar to those for low-glucose- and BMS-grown cultures, i.e., cDNA was detected throughout the period from 3 to 8 days. In this case, however, the highest levels of expression were at days 6 and 7, preceding the peak in enzyme activities. A more striking observation was that four of the other five *LIG* genes were also expressed on Avicel. The exception is *LIG4*, which cannot yet formally be recognized as a gene in strain ME446 since con-

TABLE 2. Gene expression by gene-specific PCR amplification of cDNA populations[a]

Gene/ sequence	Avicel at day:						BMS at day:						Low glucose at day:						High glucose at day:					
	3	4	5	6	7	8	3	4	5	6	7	8	3	4	5	6	7	8	3	4	5	6	7	8
cbhI.1	++	++	+	±	−	−	++	++	+	±	−	−	−	−	−	−	±	±	−	−	−	−	−	−
cbhI.2	++	++	+	±	−	−	++	++	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
cbhII	++	++	++	+	++	+	++	++	++	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
LIG1	−	−	−	+	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−
LIG2	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LIG3	−	±	±	±	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LIG4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LIG5	+	+	+	++	++	+	±	+	+	+	+	±	±	+	+	++	+	±	−	−	−	−	−	−
LIG6	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
mnp1	±	±	±	±	±	±	±	±	±	±	±	±	−	−	−	−	−	−	±	−	−	−	−	−
trpC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Replicate cultures were grown in medium containing Avicel, BMS, or a high or low concentration of glucose as substrate. Each value represents the average of results of three cDNA populations derived from independent cultures. ++, product derived from cDNA only; +, gDNA and cDNA products both present in approximately equal amounts; ±, weak cDNA product and strong gDNA product; −, product derived from gDNA only.

ditions for its expression have yet to be identified. However, sequence comparisons show it to be closely related to a gene which has been shown to be expressed in strain BKM-F-1767 (43). Three of the genes, *LIG1*, *LIG2*, and *LIG3*, all produced their maximum levels on day 6 as was the case for *LIG5*. Expression of *LIG6* was confined to a single replicate culture on day 3.

The polymeric substrates, Avicel and BMS, had elicited MnP activity throughout the experiment, whereas with both the high- and low-glucose samples activity was highest at the earlier times. As expected, *mnp1* expression in Avicel- and BMS-grown cultures was also continuous, and it was also seen on day 3 with high-glucose-grown cultures. However, there was no evidence of expression of *mnp1* in the low-glucose-grown cultures.

DISCUSSION

The natural substrate for *P. chrysosporium* is lignocellulose, and therefore, we need to understand how each of its components is degraded by the fungus, presumably as a synergistic process. This is the first attempt at simultaneous description of the enzyme activities produced by this fungus against lignin and cellulose and their comparison with the profiles of expression of *cbh*, *LIG*, and *mnp* gene families. We also seek to relate these profiles of enzymes and expressed genes to the use of four different substrates. Of these substrates, BMS approximates to lignocellulose whereas Avicel represents cellulose alone. Because the same conditions of nitrogen and of buffering were used throughout, any observed differences must be related to the differences in substrate. The use of the constitutively expressed *trpC* gene as a control allowed us to be confident that, in all replicates of all four sets of cultures, mRNA had been extracted and successfully used to make cDNA in comparable yields.

Under one of these conditions (high glucose), ME446 degrades synthetic lignin efficiently without the appearance of supernatant LiP activity (48). Thus, either LiP is not produced or that which is produced does not appear in the supernatant. These alternatives can be tested by looking for gene expression. Until recently the standard method was the use of Northern (RNA) blotting. However, we have shown that PCR of cDNA preparations can be used for more sensitive analysis that also allows the resolution of genes coding for isozymes (4). This is important since it has been shown that there are gene families with differential gene expression (2, 4, 9, 21, 33, 34, 43, 44).

As expected, the independently inoculated cultures that constituted the replicates behaved similarly with respect to cellulase and MnP activities and also in respect to expression of genes encoding these activities. For example, PCR analysis of *cbhI.1* and *cbhI.2* after growth on Avicel clearly showed expression at times earlier than day 6. On day 6 itself, expression of these two genes was observed in only one of the replicate cultures, possibly reflecting slightly different growth rates (Fig. 2). The PCR results for *mnp1* and *trpC* were also consistent between replicates. *LIG* gene expression also showed clear trends but with significant differences between replicates. Figure 1 shows that the variation in enzyme activities was also greatest with the LiPs.

In a number of cases, the appearance of an amplified cDNA species on a particular day with no such amplified cDNA apparent prior to or following that time point indicates rather tight regulation of mRNA production and suggests that such mRNA turns over in hours rather than days. If the corresponding proteins appear in the supernatant, there should be a good correlation between gene expression and appearance in the supernatant of the corresponding enzyme activity. An apparent example of such a case is the simultaneous appearance of both *mnp1* mRNA and MnP activity on day 3 in the high-glucose cultures. This particular case demonstrates the importance of developing technology for isolating adequate amounts of cDNA from 1- and 2-day cultures as well.

The results obtained with BMS as the substrate were broadly as expected. That is, cellulase, MnP, and LiP enzyme activities were all present. Also, genes for cellulases and both types of peroxidase were expressed, and as shown previously for this strain (22), *LIG5* is the predominantly expressed *LIG* gene. However, the gene expression study reveals a number of points of interesting detail. Thus, of the six *LIG* genes, *LIG5* is expressed throughout, *LIG1* is expressed late, and the others are not expressed at all. *LIG1* is a member of the *LIG1*, *LIG2*, *LIG3*, and *LIG4* gene cluster (34), and so this shows that expression of genes within this cluster is not coordinate. Stewart et al. (43), using strain BKM-F-1767, have also demonstrated independent expression of LiP-encoding genes within a cluster.

In contrast, the kinetics of *cbh* expression show that expression of *cbhI.1* and *cbhI.2* is coordinate, ceasing on day 6. This was expected since the corresponding enzymes are known to

act synergistically in a different strain, K3 (46). However, *cbhII* is expressed throughout. What is seen as continuing supernatant cellulase activity after day 6 can be sufficiently explained either by the continued presence of *cbhII* gene expression or by stability of enzyme produced earlier. These alternatives are not mutually exclusive. We also know that *cbhII* differs from *cbhI.1* and *cbhI.2* in being expressed on xylan (44), suggesting that this gene is either coordinately expressed with a xylanase or has some xylanase activity itself.

Xylanase activity was assayed in all cultures used in this study (results not shown). The observed abundance of xylanase activity in Avicel-grown cultures supports the above hypothesis that *cbhII* has a role in the xylanolytic system as well as in the cellulolytic system. However, it can only be one component since a number of proteins have been implicated in this system (11), and moreover, xylanase activity was detected in the low-glucose case, in which the three *cbh* genes studied here are not expressed.

With respect to the series of cultures grown on Avicel, we suppose that cellulose degradation is initially likely to produce only low amounts of glucose. This view is supported by the observations that cellulase activity is present (Fig. 1) and that the *cbh* genes that we have studied are expressed (Table 2); we have shown previously that expression of all three genes is strongly repressed by glucose (40, 41, 44). The presence of LiP and MnP activities in addition to cellulase activities suggests that cellulose alone provides a sufficient signal to induce all components of the lignocellulolytic system. This is reasonable since in nature these will presumably act synergistically.

Nevertheless, there are interesting differences between the BMS and Avicel cases with respect to the *LIG* family. Thus, with Avicel alone, there is the appearance of *LIG1*, *LIG2*, and *LIG3* gene expression centered on day 6; this is evidence of coordinate expression. Of these, only *LIG1* was also produced on BMS, and in this case, it was produced later. *LIG6* expression was seen for the first time and only on day 3. Thus, Avicel is able to elicit expression of three *LIG* genes that are not expressed on BMS. Nor does glucose elicit such expression. Therefore, it seems that within the period of the experiment some component of BMS represses expression of these three *LIG* genes or else that pure cellulose or a product of its degradation is a specific inducer of their expression.

Cultures grown on high and on low glucose have been shown to mineralize synthetic lignin (48). We can therefore ask which of the genes under examination are expressed. As has been noted, in both glucose conditions there is negligible supernatant LiP activity. In each case, there were unexpected results.

With low glucose, as discussed above, *LIG5* is the only *LIG* gene of those studied that is expressed. Since under these conditions there is no supernatant activity, this supports the hypothesis (14, 15, 26, 29, 37, 42) that the protein product remains associated with the mycelium. The other important observation here is the presence of MnP activity but the absence of *mnp1* gene expression. Two possible explanations are prior gene expression of *mnp1* (for which we have preliminary evidence) or the possibility that in this case the enzyme activity being measured is due to the product of another gene such as the recently described *mnp2* (28) that we have not yet studied. Again, the two explanations are not mutually exclusive.

High levels of glucose must be absent from cultures grown on Avicel and BMS since the *cbhII* gene is expressed throughout the experiment on these substrates (Table 2) and this gene is highly glucose repressed (44). The *mnp1* gene is also expressed continuously on these substrates. However, we can conclude that this gene is not glucose repressed as it is also expressed early in cultures grown on high glucose (Table 2). A feature common to all of these conditions is the presence of low nitrogen concentrations, and this has previously been found to be necessary for expression of this gene (7). The early pulse of MnP activity on high-glucose, low-nitrogen conditions prior to the cessation of growth has also been noted previously by other authors (7). The time of occurrence of this pulse from inoculation varies and has been observed as early as 3 days for *P. chrysosporium* (32, 33) and at 2 days for *Trametes versicolor* (30). The variation in the occurrence of this peak of activity may reflect differences in growth rates and consequent depletion of nitrogen; we observed growth to be considerably more rapid in glucose medium than in either Avicel or BMS medium (results not shown).

In high glucose, none of the *LIG* genes examined in this study was found to be expressed. However, previous work from this laboratory has shown that under exactly the same high-glucose, low-nitrogen conditions, lignin degradation, as monitored by mineralization of synthetic lignin, certainly does occur (48). It is possible that LiP is not essential for such degradation but more likely that such activity results from the expression of LiP genes not studied in this work. Other workers (27) have shown that strain OGC101, which is derived from ME446, contains an LiP gene that is nitrogen regulated, and as such, we would expect it to be expressed on our high-glucose medium. However, since no supernatant LiP was detected in our experiments, any products derived from this gene must be efficiently sequestered by the mycelium under our growth conditions. The sequestration and possible subsequent release of LiP, associated with senescence of the mycelium, may well account for the poor correlation that we and others have observed between lignin mineralization and supernatant LiP levels, as determined by the veratryl alcohol assay. Further direct observations on the expression of specific genes of the type that we have described are likely to clarify the mechanism of this organism's contribution to nature's most important recycling process.

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