JOURNAL OF BACTERIOLOGY, Aug. 1992, p. 5036–5042
0021-9193/92/155036-07$02.00/0

Vol. 174, No. 15

Lignin Peroxidase Gene Family of *Phanerochaete chrysosporium*: Complex Regulation by Carbon and Nitrogen Limitation and Identification of a Second Dimorphic Chromosome

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Received 13 March 1992/Accepted 18 May 1992

Lignin peroxidases (LiP) of *Phanerochaete chrysosporium* are encoded by a family of six closely related genes. Five LiP genes have been localized to the same dimorphic chromosome. In this investigation, relative transcript levels of the LiP genes were determined. Transcripts of the LiPA, LiPB, and O282 genes were at similar levels in both carbon- and nitrogen-limited cultures. In contrast, transcription of the GLG5, V4, and GLG4 genes was dramatically altered by culture conditions. Under carbon-limited conditions, GLG4 transcripts were, by far, the most abundant. Southern blot analyses of clamped homogeneous field gels were used to map the GLG4 gene to a dimorphic chromosome separate from the other LiP genes.

Lignin depolymerization is catalyzed by extracellular enzymes of white rot basidiomycetes such as *Phanerochaete chrysosporium* (19). Major components of this system include lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and a peroxide-generating enzyme, glyoxal oxidase (GLOX). Recent evidence supporting the central role of LiP include the depolymerization of lignin in vitro by LiP (14) and the identification of LiP in decayed wood (6).

In culture, multiple LiP isozymes are present. Isozymes differ in physical characteristics, substrate specificity, and stability (8, 12), but how these differences relate to lignin degradation is unknown. Production of LiP is derepressed under carbon and nitrogen limitation (17), and the pattern of isozymes formed is influenced by growth conditions (21).

The LiP isozymes are encoded by a family of structurally related genes, but the situation has been complicated by inconsistencies in nomenclature and misidentified *P. chrysosporium* strains (Table 1). In the widely used strain BKM-1767, six genes plus allelic variants have been identified (7, 9, 16, 30, 31, 33). Four LiP genes (LiPA, LiPB, GLG5, and O282) are closely related with respect to nucleotide sequence, intron positions, and codon preference. Deduced amino acid sequences exceed 80% identity in any pairwise comparison within this subfamily. In contrast, LiP genes CLG4 (7) and V4 (30) are distinctly different from each other and from members of the subfamily.

Five LiP genes (LiPA, LiPB, GLG5, O282, and V4) have been localized to a single dimorphic chromosome, and three genes (LiPA, LiPB, and GLG5) are within a 30-kb region (9). The chromosomal location of the CLG4 gene has not been determined, but a restriction fragment length polymorphism map of *P. chrysosporium* ME446 predicted the existence of two unlinked clusters of LiP-like genes (27).

The regulation of LiP gene expression is poorly understood, in large part because of technical difficulties in discriminating closely related transcripts. However, it is clear that LiP-like transcripts appear during secondary metabolism (1, 33). Holzbaur and Tien (15) investigated expression of the CLG4 and LPOA genes in carbon- and nitrogen-starved cultures. On the basis of Northern (RNA) blots probed with nick-translated clones, they concluded that the CLG4 gene was the only LiP gene expressed under carbon limitation and that both the CLG4 and CLG5 genes were expressed under nitrogen starvation.

In this paper we demonstrate a complex pattern of LiP gene expression. Additionally, we identify a second dimorphic chromosome which contains LiP gene GLG4.

MATERIALS AND METHODS

Plasmids and strains. *P. chrysosporium* BKM-1767 was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis., and used throughout the study. Cosmid pQQ24 is a pWE15 derivative (35) containing LiP genes LiPA, LiPB, and GLG5 (9). Single LiP genes are contained in cosmid pKBY2 (38) derivatives and in pO282 and pV4 (30). Subclone pO282::910 contains a 910-bp *Xho*I fragment of O282 in Bluescript KSII (Stratagene Inc., La Jolla, Calif.). MnP and GLOX cDNAs were cloned from a BKM-1767 λgt11 library and subcloned into pKSII. Partial sequence analyses showed the MnP clone to be identical or allelic to a previously described clone (25). The identity of the GLOX clone was established by reactions with antibodies and by comparisons of the deduced amino acid sequence with an experimentally determined N-terminal sequence (18a). Plasmid pGLG4::340 was constructed to provide a GLG4-specific probe for clamped homogeneous field gels. It contains a 340-bp region of genomic DNA which was polymerase chains reaction (PCR) amplified and subcloned into pCR1000 (22). The amplified region corresponds to bp 484 to 680 on the cDNA sequence (7). For use in competitive PCR experiments, the full-length genomic copy of the CLG4 gene was obtained by PCR amplification. The amplified region is approximately 1,600 bp and corresponds to the CLG4 gene bp 15 to 1148 (7). A pCR1000 subclone was

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TABLE 1. Nomenclature of lignin peroxidase clones of *P. chrysosporium*[a]

Designation(s)	Identity	Reference(s)
LiPA, ML-4	Genomic DNA and corresponding cDNA	1, 33
LPOA (=H8), ML-1	Genomic DNA and cDNA, allelic variant of LiPA	9, 31
LiPB	Genomic clone closely linked to LiPA	9
LPOB	Genomic clone, allelic variant of LiPB	9, 16
GLG5, CLG5	Genomic DNA and cDNA	7, 9
GLG2, LIP6	Genomic clones, allelic variant of GLG5	39
O282	Genomic clone	30
GLG4, CLG4	Genomic DNA and corresponding cDNA	7
V4	Genomic clone	30

[a] Clones from strains other than BKM-1767 are not presented. Genomic clone GLG3 (24) was recently shown not to have originated from BKM-1767 (10).

designated pGLG4. Both strands of pGLG4::340 and the 5′ and 3′ termini of the pGLG4 insert were sequenced by the dideoxy method (29). The nucleotide sequence within the coding regions was identical to that of the CLG4 gene (7). Two introns were identified within pGLG4::340 (data not shown).

Culture conditions. Nitrogen- and carbon-limited cultures were grown statically at 39°C as previously described (3, 20) and harvested on days 6 and 5, respectively. Mycelia were harvested by filtration through Miracloth (Calbiochem, La Jolla, Calif.), immediately immersed in liquid nitrogen, and stored at −90°C. MnP activities of filtrates were measured as the change in A_{415} (3) and were 0.8 and 0.9 min^{-1} ml^{-1} for nitrogen- and carbon-starved cultures, respectively. LiP activities were assayed by veratryl alcohol oxidation (32) and were 12.8 and 7.3 nmol min^{-1} ml^{-1} in N- and C-starved cultures, respectively.

Northern blots. Total RNA was extracted from frozen mycelium and pelleted in CsCl (28, 34). Approximately 20 μg of RNA was size fractionated on formaldehyde gels and blotted to Hybond N (Amersham Inc., Arlington Heights, Ill.).

Conditions described by Wallace and Miyada (36) were used for the oligonucleotide hybridization and washing of blots. Oligonucleotide 5′-GTACGTGGTCTCGATCGAGG-3′ corresponds to the reverse complement of CLG4 gene (7) bp 343 to 362, a region of little homology to related LiP genes. Oligonucleotide 5′-GAAATTGGTCTCGATAGTAT-3′ is V4 specific, lacking significant homology with all LiP genes except the reverse complement of the V4 gene at a position 37 bp downstream of the third intron (30). Probes were synthesized by the β-cyanoethyl phosphoramidite method on an Applied Biosystems (Foster City, Calif.) model 391 without further purification and then ^{32}P end labeled with T4 polynucleotide kinase (28). Unincorporated label was removed by size exclusion chromatography (Push Columns; Stratagene Inc.). Probe-specific activity was approximately 3×10^8 dpm μg^{-1}, and 3×10^6 dpm/ml of hybridization buffer was used. The final wash was for 1 min at 48°C with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS).

Northern blots were also probed with MnP and GLOX cDNAs by hybridization in 5× SSC–5× Denhardt's solution–0.5% SDS–100 μg of denatured calf thymus DNA ml^{-1} overnight at 55°C. The MnP cDNA probe was random primed to a specific activity of 1.5×10^9 dpm μg^{-1}. The GLOX cDNA was nick translated to a specific activity of 6×10^8 dpm μg^{-1}. Approximately 2×10^7 dpm of the labeled probes was used per ml of hybridization buffer. Blots were washed successively for 15 min in 2×, 1×, and 0.1× SSC at 55°C.

Competitive PCR. Relative transcript levels of four closely related LiP genes (LiPA, LiPB, GLG5, and O282) were established by modifying previously described methods (4, 11, 23). In essence, first-strand cDNAs for all four genes were prepared by reverse transcription with a conserved downstream primer (Fig. 1). Then, cDNA of the individual genes was PCR amplified with gene-specific upstream primers. Included in the PCRs was a competitive template in the form of genomic DNA. The competitor was added in a series of dilutions of known concentration. Introns within the competitive template allowed the target cDNA and genomic product to be size fractionated on agarose gels. Finally, the identity of the amplified products was then ensured by probing with oligonucleotides specific for individual LiP genes.

As described above, primers and probes were synthesized

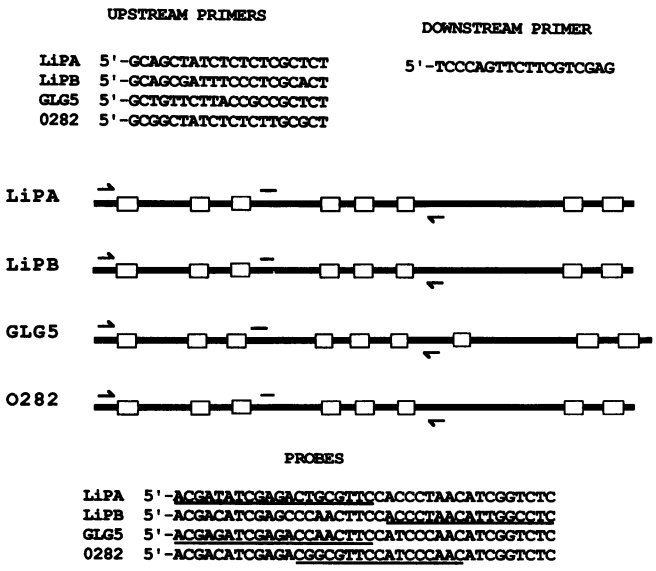

FIG. 1. Schematic representation of four closely related LiP genes and strategy for PCR amplification. Coding regions are shown as solid lines, and introns are shown as open boxes. Positions of downstream primers (←), upstream primers (→), and probes (—) are indicated.

by the β-cyanoethyl phosphoramidite method and used without further purification. The 18-mer downstream primer was selected from a highly conserved region approximately 95 bp downstream of intron 6. The 20-mer upstream primers were selected from a variable region within secretion signals. Probes were 17-mers (for the LiPB and O282 genes) or 20-mers (for the LiPA and GLG5 genes) chosen from a variable region 38-bp downstream of intron 3 (Fig. 1).

First-strand syntheses and subsequent PCR amplifications were as previously described (11) with minor modifications. Reverse transcription reactions were for 15 min at 42°C with 50 U of Moloney murine leukemia virus enzyme (GIBCO BRL, Gaitherburg, Md.). Each 20 μl of reaction mixture also included 40 U of RNasin (Promega Biotech Inc., Madison, Wis.), 21 pmol of downstream primer, and 250 ng of total RNA. PCRs were in a 100-μl total volume with 2.5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.) and 21 pmol of each primer. Competitive templates in the form of cosmid pQQ24 or p0282 were present at concentrations ranging from 5×10^{-10} to 1×10^{-13} g/100 μl of reaction mixture. Reaction mixtures were subjected to an initial cycle of denaturation (6 min, 94°C), annealing (2 min, 54°C), and prolonged extension (40 min, 72°C) followed by 35 cycles of denaturation (1 min, 94°C), annealing (2 min, 54°C), and extension (5 min, 72°C). A final 15-min extension at 72°C was also included.

Following amplification, 10-μl quantities of the PCR mixtures were size fractionated on 1.0% agarose gels, ethidium bromide stained, blotted to Nytran (Schleicher & Schuell, Inc., Keene, N.H.), and then UV cross-linked (UV Stratalinker; Stratagene Inc.). Minor modifications of the Southern blot protocols of Wallace and Miyada (36) were then used. Blots were prehybridized for 1 h in 6× SSC–5× Denhardt's reagent–0.1% SDS–100 μg of denatured calf thymus DNA ml^{-1}. Hybridizations were overnight in a mixture containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's, and 0.1% SDS. Probes were ^{32}P end labeled with T4 polynucleotide kinase. Unincorporated label was not removed. Blots were washed in 6× SSC–0.1% SDS three times for 5 min each time at room temperature. For each probe, the temperatures of prehybridization, hybridization, and a final 15-min wash were adjusted to $T_d - 12$°C, where $T_d = 2(A + T) + 4(G + C)$. Blots were exposed to Kodak XAR film without amplifying screens overnight.

Competitive PCR was also used to assess GLG4 and V4 transcript levels. For GLG4 gene amplification, the above-mentioned downstream primer was used with 5′-CAGCC CTCTCCGTCGCCCTG-3′ as the upstream primer. The latter aligns to a position within the secretion signal of the CLG4 gene, the cDNA equivalent of GLG4 (7). The competitive template, pGLG4, was required in relatively high concentrations for RNA derived from C-starved cultures. From 5×10^{-7} to 1×10^{-11} g/100 μl of PCR mixture was used. For V4 gene amplification, both upstream (5′-GCC ATCGCGATCTCTCCC-3′) and downstream (5′-GACAAA GAATTGCGTATC-3′) primers were synthesized. These correspond to positions 13 and 76 bp downstream of introns 2 and 6, respectively. With these primers, the expected lengths of amplified cDNA and genomic targets were 479 and 694 bp, respectively, for the V4 gene. The predicted length of amplified GLG4 cDNA was 685 bp, and the genomic equivalent was estimated to be approximately 1,000 bp.

Clamped homogeneous field Southern blot. Gels and blots were prepared as previously described (9). Size-fractionated chromosomes of the parental dikaryon (BKM-1767) and a single basidiospore derivative, SB1, were probed with the 340-bp insert of pGLG4::340. The purified fragment was random primed to a specific activity of $>10^9$ dpm μg^{-1}. Hybridization buffer was 50% formamide, 7% SDS, 0.25 M Na^+, and 1 mM EDTA with 5×10^6 dpm of probe ml^{-1}. Hybridization and subsequent 0.25 M Na^+ washes were at 49°C. The blot was exposed to Kodak XAR film with an amplifying screen for 5 days.

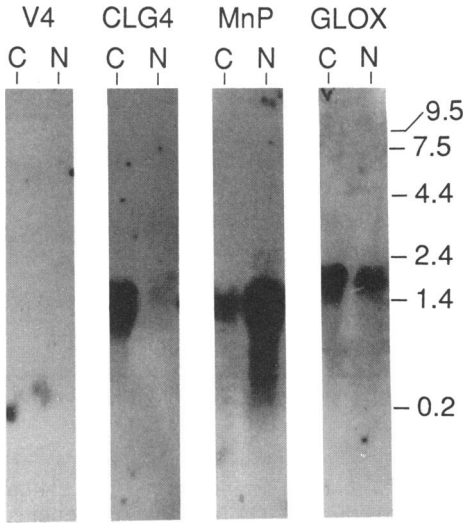

FIG. 2. Northern blot of total RNA derived from C- and N-limited cultures of *P. chrysosporium*. Probes were end-labeled oligonucleotides specific for LiP genes V4 and CLG4 or full-length cDNAs encoding MnP and GLOX. (The CLG4 gene is the cDNA equivalent of the GLG4 gene; Table 1.) Blots were exposed to Kodak XAR film with amplifying screens overnight or for three days (V4 gene). Molecular size markers in kilobases are shown on the right.

RESULTS

Transcripts of three components of the lignin-degrading system were readily detected by Northern blots of total RNA (Fig. 2). GLOX and MnP transcripts were observed in both C- and N-limited cultures. In contrast, GLG4 transcript levels appeared to be far more abundant in C-limited than in N-limited cultures. Some GLG4 gene hybridization was evident in the N-starved RNA lane, particularly after prolonged exposures. Transcripts of the V4 gene were not detected in either culture even after prolonged exposures or when poly(A) mRNA was used (data not shown). When blots were probed with any of the four closely related LiP clones (LiPA, LiPB, GLG5, and O282) or with gene-specific oligonucleotides (Fig. 1), interpretation was complicated by low signals and the possibility of cross hybridization. To solve this problem, competitive PCR was used to quantitate specific transcripts.

The strategy involved reverse transcription of total RNA with a primer common to all four genes (Fig. 1). First-strand cDNAs were then PCR amplified with gene-specific upstream primers. Several dilutions of competitive genomic templates were added to these PCR amplifications. The initial concentration of specific cDNAs was determined by estimating the dilution points at which target cDNA and competitive template are equivalent.

By this approach, cDNA levels of the four LiP genes ranged from 50 to 500 pg for the LiPA gene to 2.5 to 5.0 pg

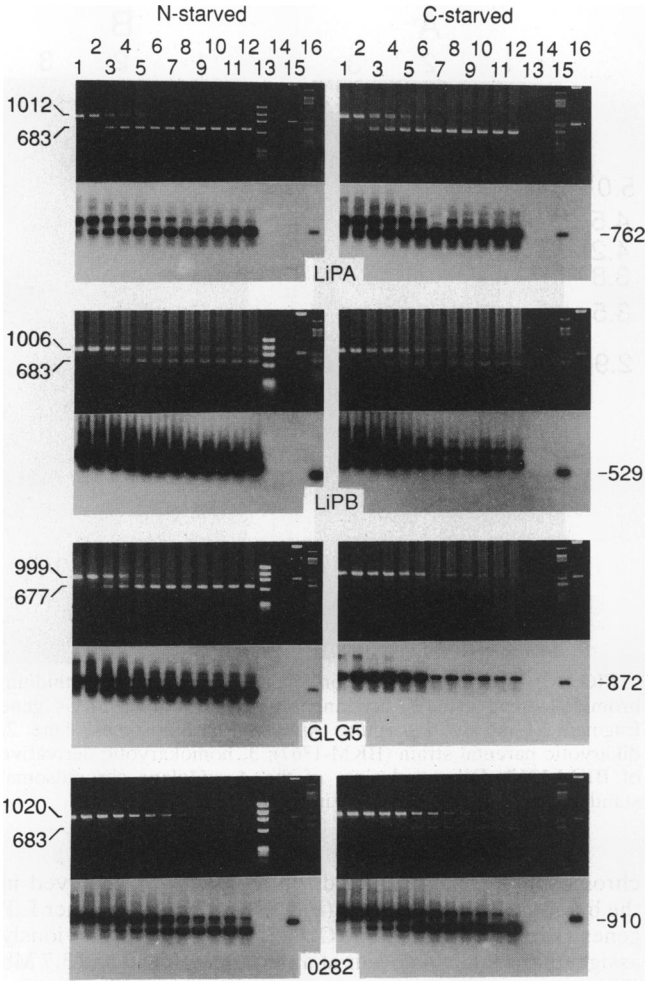

FIG. 3. Competitive PCR quantitation of message of four closely related LiP genes. Lanes 1 through 11 contain decreasing amounts of competitive template in the form of pQQ24 (LiPA, LiPB, and GLG5) and pO282. The 11 100-μl PCR mixtures received 500, 100, 50, 25, 10, 5, 2.5, 1.0, 0.5, 0.25, and 0.1 pg of the cosmids. No competitive template was added to reaction 12 (lane 12). In each case oligonucleotide-probed blots are shown immediately below corresponding ethidium bromide-stained gels. For RNA derived from N-limited cultures (left panels), lanes 13 to 16 are as follows: molecular weight standard φX174 digested with *Hae*III (lane 13), blank (lane 14), 0.1 μg of pO282::910 digested with *Xho*I (lane 15), and 0.6 μg of pQQ24 digested with *Pst*I-*Sph*I-*Xho*I (lane 16). For C-limited cultures, lanes 13 to 16 are as follows: blank (lanes 13 and 14), 0.6 μg of the pQQ24 digest (lane 15), and 0.1 μg of the pO282::910 digest (lane 16). As indicated on the right, the pQQ24 digestion yields LiPA, LiPB, and GLG5 gene fragments of 762, 529, and 872 bp, respectively (9). The pO282::910 *Xho*I digest yields a 910-bp fragment. Hybridization of the expected fragments and the exclusion of any other bands confirm the specificity of the oligonucleotide probes. Predicted sizes of competitive template and cDNAs are indicated on the left (in base pairs). A minor band in lane 12 above the cDNA is attributable to the presence of low levels of genomic DNA in RNA samples.

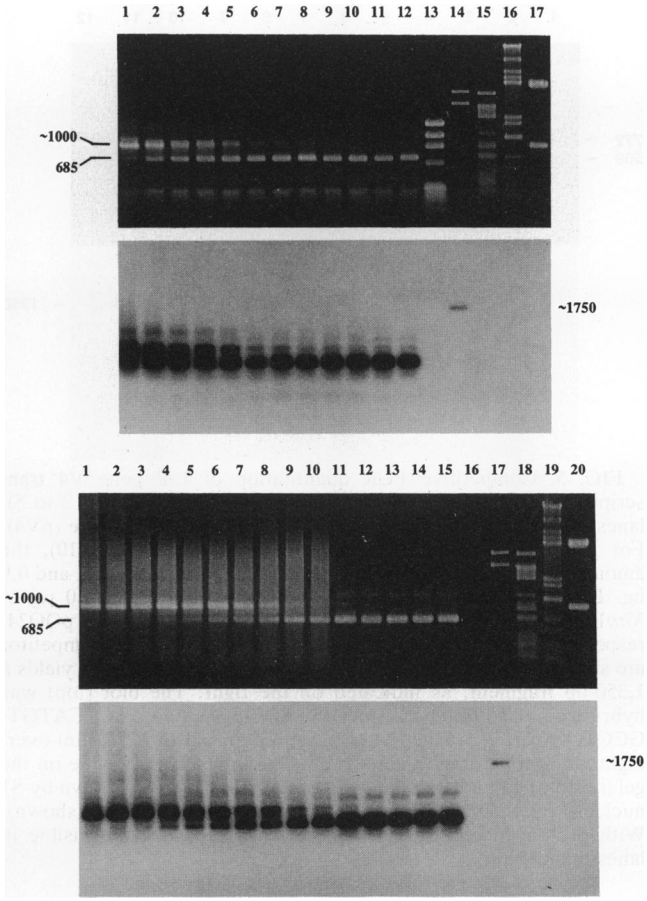

FIG. 4. Competitive PCR quantitation of LiP gene GLG4 transcripts. For RNA derived from N-limited cultures (top), lanes 1 through 11 contain the same amounts of competitive template as listed in the legend to Fig. 3. pGLG4 was used as a template, except in lane 12, which received none. For RNA derived from C-starved cultures (bottom), the amount of pGLG4 template per reaction was increased such that lanes 1 through 14 contained 500, 250, 100, 50, 25, 10, 5, 2.5, 1.0, 0.5, 0.1, 0.05, 0.025, and 0.01 ng of pGLG4. For N-limited reactions, lanes 13 to 17 are as follows: *Hae*III-digested φX174 (lane 13), 0.1 μg of *Not*I-digested pGLG4 (lane 14), 0.6 μg of *Pst*I-*Sph*I-*Xho*I-digested pQQ24 (lane 15), 0.6 μg of *Xho*I-digested pV4 (lane 16), and 0.2 μg of *Xho*I-digested pO282::910 (lane 17). For C-limited reactions, the φX174 standard is excluded and lanes 17 to 20 contain the same plasmid digests to assess probe specificity. The *Not*I-digested pGLG4 yields a fragment estimated to be 1,750 bp, as indicated on the right. Expected sizes of the cDNA and genomic competitor (estimated) are shown on the left in base pairs.

for the O282 gene (Fig. 3). The identity of the ethidium bromide-stained PCR products was confirmed by hybridization with oligonucleotide probes, and the probe specificities were confirmed by including digests of pO282::910 and pQQ24 (Fig. 3, lanes 15 and 16). Except for the GLG5 gene, transcript levels were roughly equivalent in C- and N-limited cultures. In the case of the GLG5 gene, N-limited cDNA levels were estimated to be 25 to 100 pg but no cDNA product was observed in the C-starved cultures. Ten additional PCR cycles failed to amplify the cDNA (data not shown), indicating a complete absence of the transcript in C-starved cultures.

Quantitation of GLG4 in C-limited cultures required a substantial increase in the concentration of competitive template. Estimated cDNA levels were equivalent to 2.5 to 5.0 ng and 25 to 50 pg in C- and N-limited cultures, respectively (Fig. 4). Thus, C-limited cultures show an approximately 1,000-fold increase in GLG4 transcript relative to that in N-starved cultures. Comparison of GLG4 gene PCR results with those of other LiP genes (Fig. 3) must

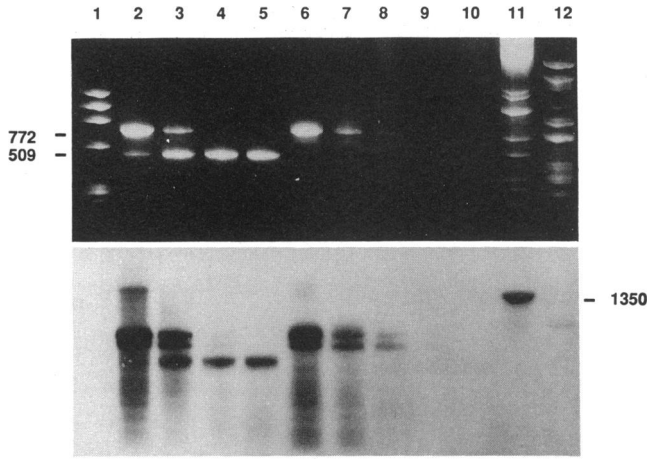

FIG. 5. Competitive PCR quantitation of LiP gene V4 transcripts. For RNA derived from N-limited cultures (lanes 2 to 5), lanes contain 10, 1, 0.1, and 0.0 ng of competitive template (pV4). For RNA derived from C-limited cultures (lanes 6 to 10), the amounts of pV4 template per reaction were 10, 1, 0.1, 0.01, and 0.0 ng. Lane 1, *Hae*III-digested φX174; lanes 11 and 12, 1.0 μg of *Xho*I-digested pV4 and 1.0 μg of *Pst*I-*Sph*I-*Xho*I-digested pQQ24, respectively. Expected sizes of the cDNA and genomic competitor are shown on the left in base pairs. The *Xho*I-digested pV4 yields a 1,350-bp fragment, as indicated on the right. The blot (top) was hybridized at 55°C to a 26-mer (5′-AGGCGGAGAGACTCATGTGCCTCGGC-3′), washed at 60°C, and exposed to XAR film overnight without an amplifying screen. The minor band visible on the gel (bottom) immediately below the genomic band was shown by S1 nuclease treatment to be single-stranded product (data not shown). With prolonged exposures, faint cDNA bands became visible in lanes 9 and 10.

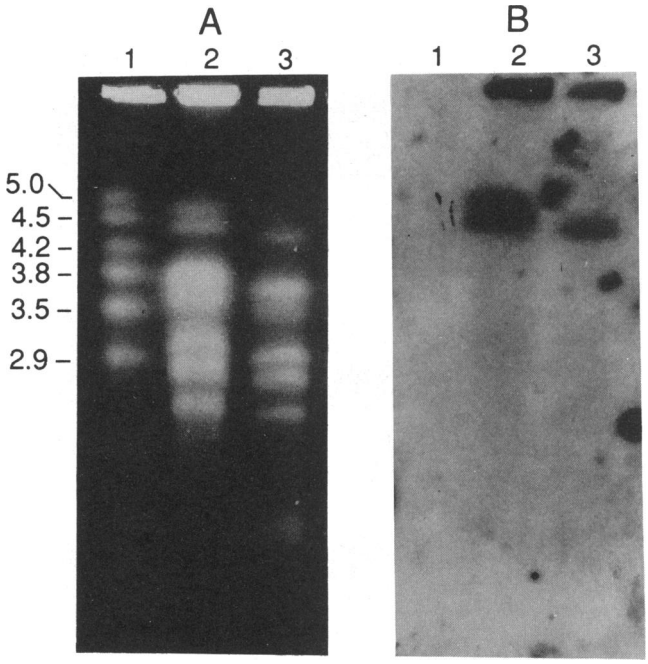

FIG. 6. Chromosomal location of LiP gene GLG4. (A) Ethidium bromide-stained gel; (B) Nytran blot probed with a GLG4 gene fragment. Lane 1, *Aspergillus nidulans* chromosomes; lane 2, dikaryotic parental strain (BKM-1767); 3, homokaryotic derivative of BKM-1767. Estimated sizes of the *A. nidulans* chromosomal standards are shown on the left in megabases.

consider the relative molar concentrations of the LiP genes within competitive templates; pGLG4 is approximately 4.6 kb, whereas cosmids pQQ24 and pO282 are 42 and 50 kb, respectively. When this 10-fold difference is factored into calculations, GLG4 is still the most abundant transcript in C-limited cultures.

Under the conditions described above, transcripts of the V4 gene could not be detected initially. The genomic template, pV4, was readily amplified, indicating that the primers were correct, but the cDNA products from C- and N-limited RNA were not observed even after 45 PCR cycles (data not shown). In further attempts to detect the V4 gene, a second set of primers was synthesized. Downstream and upstream primers were 5′-TTCCGAGTAGGGTCGACGTC-3′ and 5′-GACATCCAGGAGAACCTC-3′, respectively. The predicted sizes of genomic and cDNA products by using these primers were 772 and 509 bp, respectively. The V4 cDNA product was observed only when these primers were used and when the reverse transcription reactions included 0.5 μg of RNA and were extended to 45 min. As a consequence, V4 transcript levels cannot be compared directly with those of the other five LiP genes. However, the data clearly show that the V4 gene is transcribed and that, like the GLG5 gene, N-limited cultures contain relatively more transcript than C-limited cultures (Fig. 5).

Southern hybridization of clamped homogeneous field gels localized the GLG4 gene to the large chromosome bands at approximately 4.4 and 4.8 Mb (Fig. 6, lanes 2). Previous investigations of a linked cellulase gene cluster showed that the bands represent a chromosome-length dimorphism in the dikaryon (5). Consistent with the dimorphic nature of this chromosome, a single hybridization band was observed in the homokaryotic derivative (Fig. 6B, lane 3). The other LiP genes (LiPA, LiPB, GLG5, O282, and V4) were previously assigned to a dimorphic pair at approximately 3.5 and 3.7 Mb (9).

DISCUSSION

Extracellular enzymes of *P. chrysosporium* involved in lignin degradation include GLOX, MnP, and LiP (19). Previous Northern blot experiments showed that LiP and MnP gene transcription occurs during secondary metabolism, particularly under nitrogen limitation (3, 33). In N-limited cultures, MnP expression is regulated by Mn (2). Our results demonstrate that GLOX and MnP transcripts are relatively abundant under C- and N-limited conditions, whereas the expression of certain LiP genes is dramatically altered by medium composition.

The presence of the 1.6-kb GLOX transcript in both C- and N-limited cultures is consistent with a critical role in lignin depolymerization. This peroxide-generating enzyme is produced concurrently with LiP under N-limited conditions, and the activity of GLOX is stimulated by LiP activity (18). Also, peroxide or a peroxide-generating system is required for lignin depolymerization in vitro (14, 37).

MnP transcripts are clearly present in C- and N-limited cultures. The Mn concentration was 30 μM, a level sufficient to activate MnP transcription in N-starved cultures (3). The exact identity of the MnP transcript(s) is not certain. This is because, as with previous Northern analyses of LiP genes (15, 33), the exact number and structural relationships of MnP genes have not yet been established. The sequences of two cDNAs (25, 26) and a corresponding genomic clone (13)

have been reported. The MnP cDNA used as a probe here is identical to MP-1 (25), and on the basis of sequence alignments it would not hybridize to other known clones under the stringencies employed. However, we recently isolated a third MnP cDNA from a BKM-1767 λgt11 library (data not shown), and Datta et al. (6) identified another closely related isozyme in decayed wood. In short, the number and structure of MnP genes remain unclear, but MnP-like transcripts are present in C- and N-limited cultures.

To quantitate closely related LiP transcripts, we devised a PCR approach which offers high levels of sensitivity and specificity. The technique is a modification of the protocol of Gilliland et al. (11), and it is particularly suited to gene families such as the LiPs. This is because a single primer could be used to synthesize the first-strand cDNA. Also, a single cosmid template (pQQ24) was used as a competitor for three of the four genes amplified. One possible disadvantage is the larger size of the competitive templates relative to the cDNA target, i.e., approximately 1,000 versus 680 bp. The longer templates would be expected to be less efficiently amplified, and as a consequence, the original cDNA concentration would be overestimated in each case. However, among the four LiP genes, cDNA and genomic products are highly similar with respect to overall length, intron position, and intron length (Fig. 1). Thus, relative comparisons among the four genes are particularly valid.

Transcriptional regulation of LiP genes GLG4, GLG5, and V4 is dramatically effected by nutrient limitation. Interestingly, their transcripts are almost mutually exclusive; GLG5 and V4 are present in N-limited cultures but undetectable or present in very low levels in C-limited cultures, whereas the GLG4 level is 1,000-fold higher in C-limited cultures than in N-limited cultures. Analysis of concentrated culture filtrates with isoelectric focusing gels showed a major band with a pI of 4.6 in C-limited cultures (data not shown). The absence of GLG5 from C-limited cultures is consistent with the isozyme pattern observed by Glumoff et al. (12) in such cultures. The significance of these patterns of regulation in natural substrates, e.g., wood, remains to be established, although it is clear that lignocellulose is essentially nitrogen limited. In any case, the quantitative PCR system described here may be adapted to identify specific transcripts in such complex substrates.

The relationship between genomic organization and regulation is unclear. Previous investigations have shown the LiPA, LiPB, GLG5, O282, and V4 genes to be located on the same dimorphic chromosome. Three of these genes are closely linked (9). The GLG5 gene is located approximately 15 kb upstream from the LiPA and LiPB genes, which are 750 bp apart. Although LiPA and LiPB transcript levels are comparable in both cultures, GLG5 regulation is entirely different.

We show that the GLG4 gene resides on a large dimorphic chromosome, separate from the other LiP genes. This chromosome was previously shown by Covert (5) to contain a cellulase gene cluster. Subsequent work in this laboratory has shown that the cellulase genes in this cluster are expressed during carbon limitation (unpublished data). Thus, there may be a link between the organization and regulation of these genes involved in lignocellulose degradation.

ACKNOWLEDGMENTS

We thank Ken Hammel and Milena Cotoras for their thoughtful comments.

This work was supported by U.S. Department of Energy grant DE-FG02-87ER13712 to D. Cullen and T. K. Kirk.

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