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Organization and Differential Regulation of a Cluster of Lignin Peroxidase Genes of *Phanerochaete chrysosporium*

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The lignin peroxidases of *Phanerochaete chrysosporium* are encoded by a minimum of 10 closely related genes. Physical and genetic mapping of a cluster of eight *lip* genes revealed six genes occurring in pairs and transcriptionally convergent, suggesting that portions of the *lip* family arose by gene duplication events. The completed sequence of *lipG* and *lipJ*, together with previously published sequences, allowed phylogenetic and intron/exon classifications, indicating two main branches within the *lip* family. Competitive reverse transcription-PCR was used to assess *lip* transcript levels in both carbon- and nitrogen-limited media. Transcript patterns showed differential regulation of *lip* genes in response to medium composition. No apparent correlation was observed between genomic organization and transcript levels. Both constitutive and upregulated transcripts, structurally unrelated to peroxidases, were identified within the *lip* cluster.

Lignin is second only to cellulose as the most abundant form of carbon, and its mineralization is a pivotal step in the carbon cycle. The white-rot basidiomycete *Phanerochaete chrysosporium* has become the model system for studying the physiology and genetics of lignin degradation (for reviews, see references 1, 12, and 31). Under nutrient limitation in defined media, *P. chrysosporium* secretes multiple isozymes of lignin peroxidase (LiP). In vitro depolymerization of lignin by LiP has been demonstrated previously (22, 23), although the role and interaction of individual isozymes remain uncertain.

The LiPs are encoded by a family of 10 structurally related genes, designated A to J (16). In 1992, four *lip* subfamilies were proposed based on the intron/exon structure of the five known *P. chrysosporium lip* sequences (21, 44). Segregation of restriction fragment length polymorphisms and allele-specific markers (16, 41, 42) demonstrated linkage of *lipA*, *lipB*, *lipC*, *lipE*, *lipG*, *lipH*, *lipI*, and *lipJ*. Southern blots of pulsed field gels supported the observed genetic linkage and localized *lipD* and *lipF* to chromosomes separate from each other and from the eight linked genes (13, 15, 16, 46). Mapping of cosmid (15) and λ clones (26) established precise distances and transcriptional orientation for *lipA*, *lipB*, and *lipC*, but the genomic organization of *lipE*, *lipG*, *lipH*, *lipI*, and *lipJ* has not been described.

When *P. chrysosporium* is grown on defined media containing limiting amounts of carbon or nitrogen (35, 36, 38, 47), *lip* genes are upregulated (3, 6, 7, 25, 27, 37, 49, 51). Quantitative transcript analyses has been limited to a subset of *lip* genes, and results have often been contradictory, perhaps due to differences in methodology. Nevertheless, as Northern blots first demonstrated for *lipA* and *lipD* (25), it is now firmly established that certain *lip* genes are differentially regulated in response to medium composition. To distinguish closely related transcripts, quantitative reverse transcription-PCR techniques were developed (8, 9, 46), and it was shown that *lipI*, *lipC*, and *lipJ* transcript levels are also differentially regulated under carbon versus nitrogen limitation (46). Nuclease protection assays showed *lipE* transcripts to be upregulated in C-limited media compared to N-limited media (43). Broda and coworkers found *lipD* transcripts dominating under all growth conditions examined (8, 9, 27).

To elucidate relationships between *lip* gene structure, organization, and transcriptional regulation, we have sequenced two *lip* genes, mapped the *lip* gene cluster, and systematically assessed relative transcript levels for all 10 *lip* genes under standard conditions of nitrogen and carbon starvation. In addition, we have identified constitutive and upregulated transcripts within the *lip* cluster.

MATERIALS AND METHODS

Fungal strains and culture conditions. *P. chrysosporium* BKM-F-1767 was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis., and used throughout the study. Standard B3 salts media with limiting carbon or nitrogen were grown statically at 39°C as previously described (10, 32) and harvested on days 4 and 5, respectively. Mycelia were harvested by filtration through Miracloth (Calbiochem, La Jolla, Calif.), immediately immersed in liquid nitrogen, and stored at −90°C. LiP activities as measured by veratryl alcohol oxidation (48) were 7.3 and 12.8 nmol min^{-1} ml^{-1} in C-limited and N-limited cultures, respectively. Mycelia were also harvested from log-phase B3 cultures containing nonlimiting levels of carbon or nitrogen and lacked extracellular peroxidase activity.

DNA sequencing and analysis. Nucleotide sequences were determined by using the ABI prism dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, Calif.) with an ABI373 DNA sequencer. Nucleotide and amino acid sequence similarity searches used the BLAST method (2) on the National Center for Biotechnology Information databases. Nucleotide and amino acid sequences were analyzed and phylogenetic trees constructed by using DNASTAR software (DNASTAR, Madison, Wis.).

Genomic organization of *lip* genes. Cosmid clones containing different *lip* genes were identified from a pWE15-based cosmid library (15) by using *lip*-specific probes. Preliminary restriction maps of the cosmids were constructed, and *lip* intergenic regions were PCR amplified by using the GeneAmp XL PCR kit (Perkin-Elmer, Foster City, Calif.) according to manufacturer's recommendations. Specifically, each PCR mixture (100 μl) contained 20 to 50 ng of cosmid template, 1× XL buffer, 0.8 to 1.0 mM magnesium acetate, 40 pmol of each primer, 10 mM (each) deoxynucleoside triphosphate, 5% dimethyl sulfoxide, and 2 to 4 U of rT^{th} polymerase. Cycling conditions after hot start were 94°C, 1 min, 1 cycle, followed by 16 cycles of 94°C for 30 s and 68°C for 10 min. These conditions were repeated for an additional 12 cycles, with an autoextension of 15 s/cycle. This was followed by a final extension at 72°C for 10 min. The primer pairs for intergenic regions were as follows: 5′-ATGGCGTCGGAAACCTGG GAACTT-3′ and 5′-TGAGGAGCATGTCCCGAGGTAGAG-3′ for *lipA* and *lipE*, 5′-CGCCATCGCTATCTCTCCCGC-3′ and 5′-AACACGAGCGATGAT CTGGTCG-3′ for *lipB* and *lipC*, and 5′-GAGCGTCGGAGCGCGAGAACC-3′ and 5′-CTTTACCAGCCGATTACAGAGATG-3′ for *lipG* and *lipH*. PCR prod-

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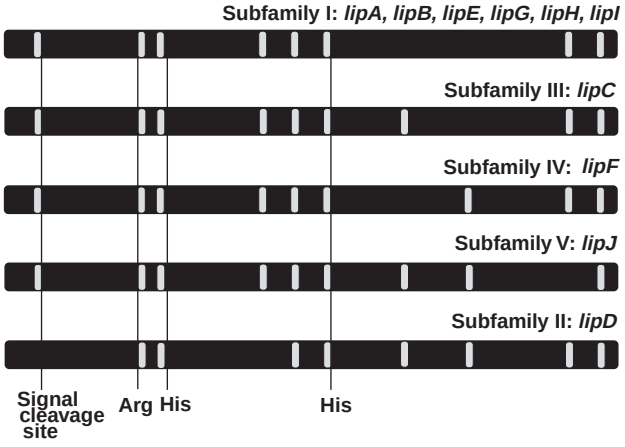

FIG. 1. Classification of *lip* genes by intron/exon structure. Introns and exons are represented by white and black bars, respectively. The positions of conserved amino acid residues coordinating the heme (49) the relative positions of signal cleavage sites and are indicated.

ucts were electroeluted, subcloned into pCRTOPO-XL, and transformed into *Escherichia coli* TOP10 (Invitrogen, Carlsbad, California) following the manufacturer's recommendations.

RNA isolation. Total RNA from *P. chrysosporium* was extracted from frozen mycelium and pelleted in CsCl (45, 50). Poly(A) RNA was extracted from total RNA by using a magnetic capture technique involving oligo(dT)25 Dynabeads (Dynal, Great Neck, N.Y.), following the manufacturer's recommendations.

Competitive RT-PCR of *lip* genes. To quantify *lip* transcripts, a competitive reverse transcription-PCR (RT-PCR) protocol was adapted from Gilliland et al. (19) as previously described (4, 46). Specifically, each RT reaction contained 2 μl of poly(A) RNA and was primed with 15 pmol of oligo(dT) 15-mers. Competitive PCRs (100 μl) contained 1.25 U of *Taq* DNA polymerase, 21 pmol of each primer, and competitive template added as 10-fold serial dilutions. Full-length *lip* genomic clones served as competitive templates, and *lip*-specific primers were as described previously (4). Reactions were cycled for 94°C (6 min), 54°C (2 min), and 72°C (40 min) for 1 cycle, followed by 94°C (1 min), 54°C (2 min), and 72°C (5 min) for 35 cycles and a final 72°C extension (15 min). Experiments quantifying *lipA*, *lipD*, *lipI*, and *lipJ* transcripts with various amounts of poly(A) template in RT-PCRs showed no evidence of reverse transcriptase inhibition (11).

PCR products were electrophoresed, and ethidium bromide-stained gel images were acquired by using Photoshop 3.0 (Adobe, San Jose, Calif.). National Institutes of Health (NIH) Image 1.61 software was used for image analysis and assigning equivalence points. The image was labeled by using Illustrator 7.0 (Adobe).

Transcript analyses of *lip* intergenic regions. The intergenic regions between *lipE* and *lipA*, *lipB* and *lipC*, and *lipG* and *lipH* were XL-PCR amplified to aid in identifying areas of transcriptional activity. Fragments were size fractionated on 0.8% agarose gels, transferred to Nytran membranes (Schleicher and Schuell, Keene, N.H.), and probed with ^{32}P-labeled cDNA prepared from C-limited, N-limited, and log-phase B3 media. Total cDNA was prepared from oligo(dT)-primed poly(A) RNA by using the Smart cDNA library kit (Clontech, Palo Alto, Calif.) and labeled by nick translation. Blots were hybridized and washed at high stringency and exposed to XAR film (Kodak) for 1 to 3 days. Film was scanned in Adobe Photoshop 3.0, and the image was labeled with Adobe Illustrator 7.0.

Nucleotide sequence accession number. Nucleotide sequences for *lipJ* and *lipG* were assigned GenBank accession no. AF140062 and AF140063, respectively. Noncoding regions between *lipI* and *lipG* and between *lipH* and *lipJ* were assigned no. AF140064 and AF140065, respectively.

RESULTS

Structure and phylogeny of *lip* genes. The complete nucleotide sequences of *lipG* and *lipJ* were determined, and all members of the *lip* family were then classified by intron/exon structure as proposed by Ritch and Gold (44). Five distinct subfamilies emerged (Fig. 1). The *lipG* intron positions were identical to *lipA*, *lipB*, *lipE*, *lipH*, and *lipI*. The number of introns varied within the *lip* family; *lipD* and the members of the *lipG* subfamily contained eight introns, and the others, *lipC*, *lipF*, and *lipJ*, contained nine. The positions of five introns were invariant among all *lip* genes. Two introns were missing in *lipD* but conserved among all other *lip* genes—one intron adjacent to the signal sequence and the other intron immediately preceding intron no. 4. All introns, except the second to the last, are conserved in *lipJ*. Intron-exon junctions of *P. chrysosporium's lip* family conform to those of other filamentous fungi, specifically, PuPy (usually GT) at the 5′ end and AG at the 3′ end (21).

Cladistic analysis based on deduced amino acid sequences established two main branches within the LiP family (Fig. 2). The branch consisting of LiPD and LiPJ was the most divergent from all other LiPs, correlating with their unique intron structures. The second branch indicated that LiPA, LiPB, LiPE, LiPG, LiPH, and LiPI were the most recent members of the LiP family to have emerged. This result is further supported by the conserved intron/exon structures of the respective genes in this branch. The more distantly related LiPC and LiPF appear to have diverged much earlier from this branch.

Genomic organization of *lip* genes. A detailed physical map of the genomic organization of the eight lignin peroxidase-encoding genes was constructed (Fig. 3). Four genes, *lipA*, *lipB*, *lipC*, and *lipE*, resided within a 35-kb region. The remaining genes, *lipG*, *lipH*, *lipI*, and *lipJ*, lie within a 15-kb region. Six genes were paired and transcriptionally convergent (*lipA* and *lipB*, *lipG* and *lipI*, and *lipH* and *lipJ*). The regions that separated paired genes were 1.3 kb or less but lacked significant nucleotide similarity to one another or to any database sequences. Of the eight genes, *lipE* and *lipC* appear to be the only unpaired members and flank the *lipA* and *lipB* pair by >10 kb.

Competitive RT-PCR indicates *lip* genes are differentially regulated. Transcript levels of all *lip* genes in chemically de-

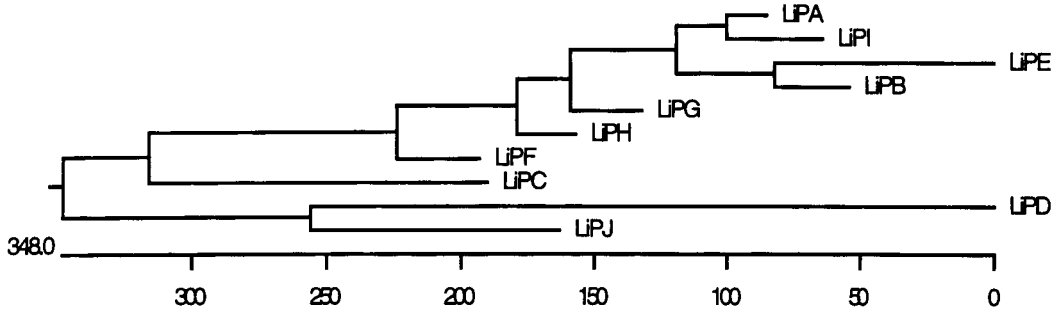

FIG. 2. Cladistic analysis of deduced amino acid sequences. The underlying scale shows the number of substitutions between sequences. Analysis was done with the Jotun Hein (24) algorithm on DNASTAR software.

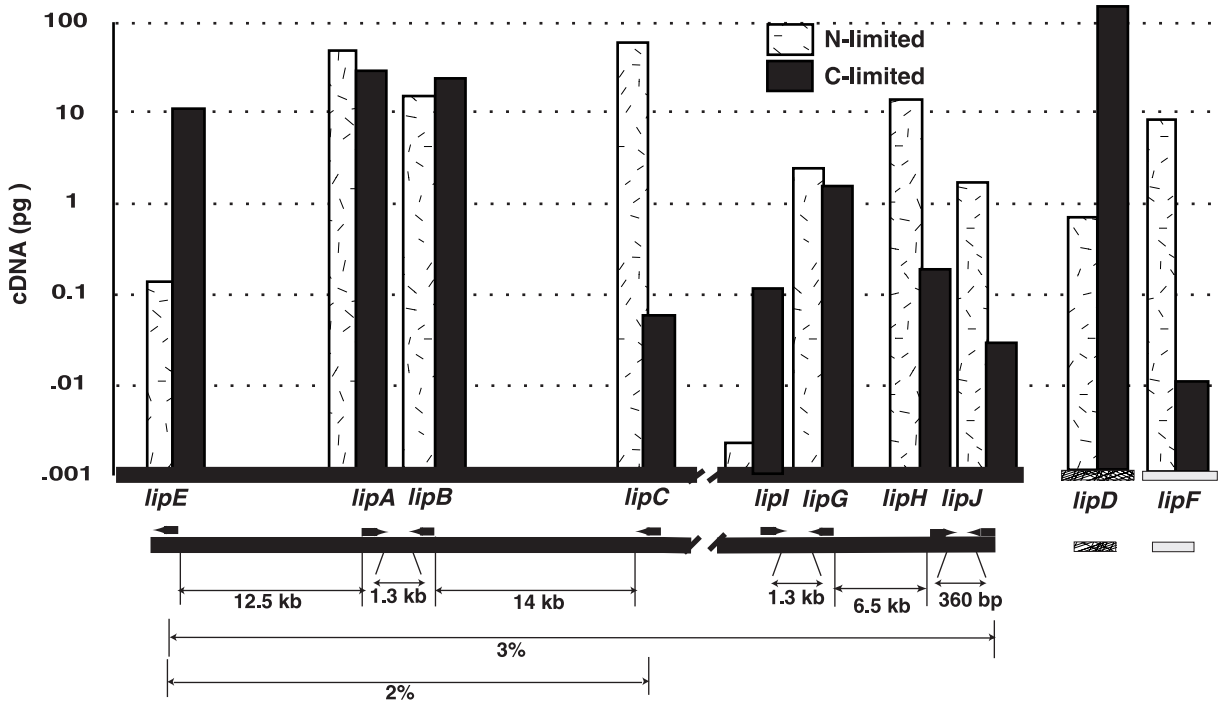

FIG. 3. Transcript levels and genomic organization of the *lip* genes. Genomic organization is shown under the graph. Thickened arrows indicate transcriptional orientation. Physical distances are in kilobases, and genetic distances are in percent recombination. Two unlinked genes, *lipD* and *lipF*, are detached to the right. Transcript levels were determined by competitive RT-PCR and are given in picograms of cDNA.

fined media were quantified by competitive RT-PCR (Fig. 4) and analyzed with respect to their genomic organization (Fig. 3). The paired genes *lipA* and *lipB* maintained similar transcript levels when limited for either carbon or nitrogen, suggesting coordinate regulation. However, transcript patterns of other *lip* genes, paired or otherwise, changed depending upon the limiting nutrient, indicating that most *lip* genes were differentially regulated. *lipC* had the highest transcript level of all *lip* genes under nitrogen limitation but one of the lowest transcript levels under carbon limitation. The dominant transcript under carbon limitation, *lipD*, had only moderate transcript levels under nitrogen-limiting conditions.

Previous studies indicated *lipE* transcripts dominating in *P. chrysosporium* cultures under carbon limitation (43, 44). Our data show that *lipA*, *lipB*, and *lipD* transcript levels exceed those of *lipE* in carbon-limited media. Broda and coworkers, working with the closely related strain ME446, reported *lipD* to be the only member of the *lip* family to be highly transcribed in carbon- or nitrogen-limited cultures (8, 9, 27). In strain BKM, we found all *lip* genes to be transcribed under both culture conditions. However, *lipD* was the most abundant transcript in carbon-limited cultures.

Transcript analyses in *lip* intergenic regions. PCR-amplified intergenic regions were blotted and probed with labeled cDNA isolated from cultures grown under either ligninolytic or nonligninolytic conditions (Fig. 5). In the region between *lipB* and *lipC*, a substantial signal was observed in all media tested. In contrast, transcriptional activity between *lipE* and *lipA* was observed under carbon and nitrogen limitation but not under nonligninolytic B3 medium. Transcripts arising between *lipG* and *lipH* were barely detectable and only under nitrogen limitation (Fig. 5, 5.3-kb signal).

DISCUSSION

The lignocellulosic component of plant cells is comprised of lignin, cellulose, and hemicellulose. To attack these complex polymers, *P. chrysosporium* produces an array of enzyme families, including lignin peroxidases, manganese peroxidases, and cellulases. Why *P. chrysosporium* maintains multiple isozymes to catalyze presumably similar reactions remains unclear. Some substrate and kinetic differences between LiP isozymes have been observed previously (14, 20) and may indicate specific roles for individual LiPs during lignin depolymerization. Alternatively, it is possible that the majority of LiPs are redundant, having arisen through various chromosomal rearrangements such as duplications, translocations, or unequal crossover events during meiosis. The report of an insertion element that transcriptionally inactivates *lipI2* indicates that not all alleles are necessary for efficient lignin depolymerization (17). In addition, there is growing evidence that redundant genes are maintained if they are not deleterious to the organism (18, 39).

The evolutionary origins of the *lip* family remain unclear despite various attempts to categorize them by intron/exon structure or deduced amino acid similarity. The full sequences of *lipG* and *lipJ*, presented here, allow for a comprehensive analysis of all known members of the *lip* family for the first time. Ritch and Gold first proposed dividing the *lip* genes into four subfamilies based on intron/exon structure (44). A fifth subfamily, consisting solely of *lipJ*, is now evident (Fig. 1). Interestingly, only subfamily I (consisting of *lipA*, *lipB*, *lipE*, *lipH*, *lipI*, and now *lipG*) has more than one member, suggesting that these genes are the most recent members of the *lip* family to have emerged. Cladistic analysis of deduced LiP amino acid sequences (Fig. 2) further supports the high degree of similarity and recent emergence of this subfamily. These six

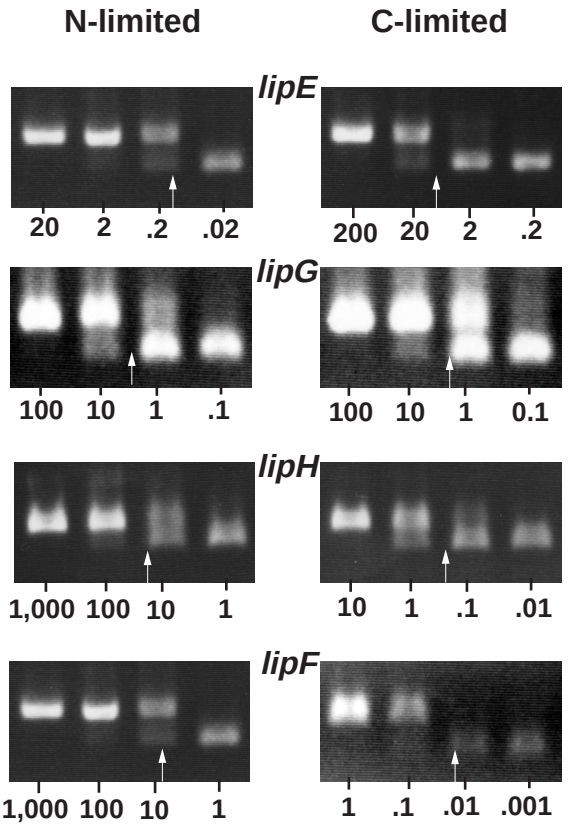

FIG. 4. Competitive PCRs comparing four *lip* transcripts in samples harvested from nitrogen- or carbon-limited conditions. Transcript levels were estimated by determining the equivalence points (arrows) with competitive templates (46). The amount of plasmid competitive template added to each PCR is indicated below the gels and expressed in picograms. Ethidium bromide-stained gels were photographed with a Foto/Analyst Visionary system (Fotodyne, Hartland, Wis.) and scanned with a Microtek ScanMaker III and Adobe Photoshop 3.0.

genes are tightly linked to *lipC* and *lipJ* (Fig. 3) but are more similar to the unlinked *lipF*. Thus, clustering of the *lip* genes does not appear to require sequence conservation.

The genomic organization of the *lip* cluster displays a striking pattern; six genes occurred in pairs and were transcriptionally convergent (Fig. 3). This unusual organization may indicate that these pairs arose by duplication events of an ancestral pair. The intergenic distance between paired *lip* genes is conserved at 1.3 kb except for the pair *lipH* and *lipJ*, which is separated by only 364 bp. These intergenic regions lack significant nucleotide similarity to one another and to database sequences.

It has been proposed that selective advantages may drive some gene families to cluster (30, 34). Such advantages include a greater likelihood for the entire family to be horizontally transferred or the simplicity of regulating a coordinately expressed cluster versus individual regulation of separated genes. Portions of the fungal secondary metabolic pathways of penicillin and cephalosporin may have been horizontally transferred from prokaryotes (40, 52), and coordinate expression of fungal gene clusters has been well documented (for a review, see reference 30). Information related to *lip* genomic organization in other white-rot fungi is limited, but two *lip* genes and a manganese peroxidase-encoding gene from *Trametes versicolor* are tandemly arranged within a 10-kb region (29), tentatively supporting a selective advantage in *lip* clustering. In *P. chrysosporium* two unlinked genes, *lipD* and *lipF*, were abundantly transcribed (Fig. 3), indicating that linkage is not essential for function.

Models for the origin and function of *lip* gene clustering must also take into account the presence of seemingly unrelated genes in the region (Fig. 5). For example, the signal consistently observed between *lipB* and *lipC* has been tentatively attributed to a gene with very high sequence similarity to *Saccharomyces cerevisiae* elongation factor G (BLASTX P = 2.7^{-136}). Upregulated transcripts were also observed between *lipA* and *lipE* and between *lipG* and *lipH*. Although the precise genes have not yet been identified, Southern blots and sequence analysis exclude peroxidase sequences in these regions (data not shown).

To investigate the relationship between genomic organization and regulation, competitive RT-PCR was used to quantify transcript levels in defined media under carbon or nitrogen limitation (Fig. 4). Surprisingly, no correlation between *lip* genomic organization and transcript levels was apparent (Fig. 3). Only the *lipA* and *lipB* pair displays similar transcript patterns under both culture conditions, suggesting coordinate regulation. A clear pattern is not evident for the remaining *lip* genes, either in pairs or for general clusters. Furthermore, transcript levels do not correlate with intron/exon structure or with amino acid sequence comparisons. For example, transcript levels within intron subfamily I vary over a 1,000-fold range (carbon-limited levels of *lipA* versus *lipJ*), and LiPs that are over 90% similar at the amino acid level may vary over 10,000-fold (e.g., nitrogen-limited levels of *lipA* versus *lipI* transcripts). This observation may support the theory that individual *lip* genes are regulated for specific biological roles.

Recently, *lip* transcript levels from *P. chrysosporium* colonized wood chips and organopollutant-contaminated soil have been measured (4, 28, 33). The complexity of these substrates restricts observations concerning *lip* regulation, but the overall patterns reported are significantly different from those seen in defined media. This may reflect the occurrence of multiple layers of regulation in these substrates. As in defined media, transcript levels from solid substrates do not correlate with intron subfamilies, genomic organization, or amino acid sequence similarity (data not shown).

Although RT-PCR is an accurate method for quantifying transcript levels, it is not intended to gauge levels of active protein. It is conceivable that some *lip* transcripts are relatively unstable or are subject to other forms of posttranscriptional regulation that would not be detected by RT-PCR. However, competitive RT-PCR remains the most accurate method for assessing LiP transcripts, and in general, transcript levels have been shown to correlate well with enzyme activity (4, 5). The

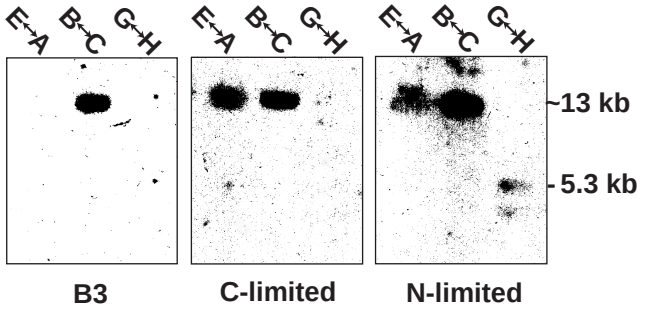

FIG. 5. Southern blots of XL-PCR-amplified intergenic regions probed with ^{32}P-labeled cDNA from log-phase B3, C-limited B3, and N-limited B3 media.

precise relationship between *lip* genes and specific isozymes is unclear except for *lipA* and *lipD*, which encode isozymes H8 and H2, respectively.

Differential regulation of the lignin peroxidases supports specific biological roles for individual isozymes. However, the possibility that some *lip* genes are redundant but have accumulated mutations altering their expression and physical properties cannot be excluded. The repeated pattern of genomic organization indicates that the *lip* family probably arose via a series of duplication events. Detailed physical examination of the regions surrounding the *lip* cluster may indicate if these areas are the result of duplications. Further analysis is also needed to identify regulatory sequences which must play a critical role orchestrating the expression of *lip* genes.

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