Genomic organization of lignin peroxidase genes of
Phanerochaete chrysosporium

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ABSTRACT

Three lignin peroxidase (LiP) genes from the
basidiomycete Phanerochaete chrysosporium were
cloned on a single 30 kb cosmld insert. One gene,
GLG5, is the genomic equivalent of a previously
reported cDNA clone, CLG5. The other two LiP genes
are transcriptionally convergent and map to a position
approximately 15 kb downstream of GLG5. The
translational stop codons of these genes are separated
by 1.3 kb. Analysis of homokaryons established allelic
relationships to previously described LiP clones. Using
clamped homogeneous electrical field electrophoresis
(CHEF), seven chromosomal bands were resolved from
P. chrysosporium
genomic DNA. On CHEF gel
Southern blots, the LiP gene family was localized to a
single, dimorphic chromosome.

INTRODUCTION

Lignin depolymerization is catalyzed by extracellular peroxidases
of white-rot basidiomycetes such as Phanerochaete chrysosporium
(1). In submerged culture, production of multiple lignin
peroxidase (LiP) isozymes is derepressed under carbon, nitrogen,
or sulfur limitation (2-4). The roles of the individual isozymes
in lignin degradation and their genetic regulation are unknown.

The lignin peroxidases of P. chrysosporium are encoded by
a family of structurally related genes (5-12), but their total
number is unclear. To date, clones from P. chrysosporium strain
BKM-1767 include the cDNA encoding isozyme H8 (5), the
corresponding genomic clone (9), cDNA's designated CLG4 and
CLG5 (6), genomic clone GLG2 which is nearly identical to
University), genomic clones V4 and O282 (11), genomic and
cDNA clones designated ML4 (10), and most recently, genomic
clonelPOB which is closely linked to H8 (=LPOA). A large
family of structurally similar genes has also been identified in
P. chrysosporium strain ME446 (8). An allelic relationship
between H8 and ML4 has been proposed (10) but direct
experimental evidence is lacking. The genomic organization of
LiP genes is also poorly understood. This is in part due to the
lack of a well-defined recombination system in P. chrysosporium(13).

Wild-type P. chrysosporium strains are heterokaryotic, and
following meiosis haploid recombinants can be recovered as
homokaryotic basidiospores (13—15). Recombinants vary
substantially with respect to numerous phenotypic traits, including
pigmentation, sporulation, and lignin peroxidase activity (16,
unpublished). Analyses of single basidiospore cultures have been
used to exclude allelic relationships among several LiP clones
(11) and to generate a genetic map using restriction fragment
length polymorphisms (RFLP's)(16). The latter study involved
strain ME446 and utilized a 33-mer oligonucleotide which
hybridized to eight different genomic LiP clones under stringent
conditions.

We report here the genomic organization of three very closely
linked lignin peroxidase genes. In order to investigate the
chromosomal organization of the entire LiP gene family, P.
chrysosporium chromosomes were resolved using pulse field
electrophoresis and the LiP genes were localized to a single,
dimorphic chromosome.

MATERIALS AND METHODS

DNA cloning and characterization
A 6,000-member library of P. chrysosporium strain BKM-1767
(ATCC242725) was prepared in cosmid pWE15 (17). Clones were
maintained at -90°C in 96-well microtitre plates (18). The
library was probed with a nick-translated genomic clone encoding
LiP isozyme H8 (11) at low stringency (35% formamide, 37°Q.
Standard Southern and colony hybridization techniques were used
throughout (19-21).

Cosmid restriction sites were mapped directly as described by
Wahl et al. (17) and by analyses of fragments subcloned into
Bluescript II vectors (Stratagene, La Jolla, CA). Double stranded
templates were sequenced by the dideoxy chain termination
method (22) using universal primers or synthetic oligonucleotides
synthesized by the University of Wisconsin Biotechnology Center.
Nucleotide and amino acid alignments were generated by the
method of Wilbur and Lipman (23) using DNASTAR Inc.
software (Madison, WI).

To ascertain allelic relationships, BstHI site polymorphisms
(Fig.1) were exploited by Southern hybridization of
homokaryons. Strain BKM-1767 was fruiting as described (14)
and single, germinating basidiospores were isolated using a dissecting microscope. DNA was extracted from parent and single basidiospore cultures (24), digested with BssHI, size fractionated in 0.8% agarose, blotted to Nytran (S&S, Keene, NH), and UV crosslinked (UV Stratalinker 1800, Stratagene Inc.). BssHI digests of cosmids pQQ24 (Fig. 1, top map) and of a plasmid containing the LPOB/LPOA genes (Fig.1, bottom) were included as size standards.

The blot was probed twice; first with a nick-translated 1081 bp BglII/KpnI fragment derived from pQQ24 and then with a 28-mer oligonucleotide (5'-AAACCTTTTGATTCGCG-ATTTTATTACCGGATT-3'). The fragment included 516 bp LiPA coding region plus 565 bp 3' untranslated intergenic region (Fig. 1). Under the hybridization stringencies employed, this probe would differentiate LiPA (2353 bp) from its' allele LPOA (2573 bp), and it would also hybridize to LiPB (1475 bp) and its' allele LPOB (1593 bp). The 28-mer hybridizes to a position 600 bp 3' of LiPA's translational stop codon. This region of intergenic LiPB-LiPA shows no homology to LPOB-LPOA (see below). The 28-mer was synthesized by the University of Wisconsin Biotechnology Center and further purified by acrylamide gel electrophoresis and reverse phase chromatography (21).

Chromosomal location of LiP genes
Clamped homogeneous electric field (CHEF) electrophoresis was performed on a BIO RAD CHEF II apparatus as described by Chu et al (25). Plug preparations, run conditions, and blotting were similar to Brody and Carbon (26) with the following modifications. To prepare protoplasts, 1 x 10^8 conidiospores were germinated in modified Vogel's medium, washed by centrifugations (< 1,000xg). The final pellet was resuspended in plugs and stored as described (26). Protoplasts were equilibrated to 37°C, mixed with an equal volume of Beckman LMP agarose (1.4%) in GMB, and then poured into a 1x9x20 mm plug mold. Protoplasts were lysed in plugs and stored as described (26). Gels were prepared using 0.8% SeaKem GTG (FMC) agarose in 0.5 X TAE. Final gel dimensions were 14 X 13 X 1 cm. Plugs were trimmed to approximately 1 x 9 x 10 mm and then sealed into wells (1.5 x 10 x 10 mm) with 0.8% SeaKem GTG. Run conditions were as described (26) except pulsing intervals and their duration were: 50 min/80 hr; 45 min/13.5 hr; 37 min/80 hr. Gels were stained with ethidium bromide, destained, blotted to Nytran, and UV crosslinked.

Nick-translated probes included cosmids pQQ24 (Figure 1), pO282 (11), pFFF62, which contains LiP gene V4 (11), and a plasmid containing the LPOA (=H8) gene (9,12). Specific activities of probes exceeded 1 x 10^8 dpm/μg. Hybridizations were at 50°C in 50% formamide, 7% SDS, 0.125 M Na2HPO4, 1 mM EDTA. Final washes were at 60°C in 1% SDS, 0.025 M Na2HPO4. A 33-mer (M33) that hybridizes to at least 8 LiP clones of P. chrysosporium strain ME446 (8,27) was also used. It was synthesized, purified, end-labeled, separated from unincorporated label, and hybridized as described above except that 1 x 10^7 cpm/ml hybridization buffer were used. Final washes were at 60°C in 1× SSC, 0.1% SDS.

RESULTS
Isolation and characterization of closely-linked LiP genes
When the cosmid library was probed under low stringency with the gene encoding LiP isozyme H8 (=LPOA), approximately 30 clones hybridized with varying intensities. Subsequent restriction digests and Southern analyses of these cosmids showed that many contained previously described genes (5,6,10) and that several contained more than one H8-homologous region. One clone, designated pQQ24, gave a particularly strong signal even after extensive washing and was found to contain 3 intact LiP-like regions. This cosmid was mapped in detail, subcloned, and

Figure 1. Partial restriction map of all LiP gene clusters. Top map shows approximately 8 kb from cosmid pQQ24 which contains 3 intact LiP-like regions. The distance between GLG5 and LiPB is approximately 14 kb. The allelic homologue is shown in the bottom map (12). No restriction sites have been mapped 5' to LPOB. LPOA is identical to H8 (9). Abbreviations: B, BssHII; K, KpnI; R, EcoRI.
approximately 8 kb were sequenced (Figure 1). Southern blots of genomic DNA digested with ClaI plus NolI showed the 26 kb LiP-containing region to be contiguous (data not shown).

Excluding the intron sequences, the nucleotide sequence of GLG5 (EMBL accession No. X54256) is identical to a previously published cDNA clone, CLG5 (6). GLG5 is remarkably similar to another genomic clone, GLG2 (Y. Zhang, 1987, Ph.D. Thesis, Michigan State University). The nucleotide and amino acid alignments of these two clones are 97.7% and 100% identical, respectively. All nucleotide mismatches are located in the third positions of codons or within introns. Both GLG2 and GLG5 contain nine introns as opposed to eight found in other LiP clones (7–12). GLG2 and GLG5 may be allelic forms of the same gene.

Two transcriptionally convergent LiP-like clones, LiPA and LiPB, are located approximately 14 kb downstream of GLG5 (Figure 1). The nucleotide sequence of the entire region was determined (EMBL accession No. X54256). This region is strikingly similar in structure to a recently reported λEMBL3 subclone which contains two lignin peroxidase genes designated LPOA and LPOB (Figure 1)(12). The overall nucleotide sequence identity between the LPO and the LiP clones, including the intergenic region, is 93%. The translational stop codons of the LiP and LPO genes are separated by 1360 bp and 1322 bp, respectively. Within this region the nucleotide identity is 87%. Intron numbers and their relative positions are conserved between the homologous LPO and LiP genes.

Sequence comparisons reveal LiPA to be identical to ML-4 (10) and LPOA identical to LiPH8 (9,10). The nucleotide sequence identity between the LiPA and LPOA coding regions is 99%. Their deduced amino acid sequences are identical except for a single amino acid change in the signal sequence. In contrast, LiPB and LPOB nucleotide and amino acid sequences are 96.5% and >99% identical, respectively. The single amino acid difference is at position 298 with a Leu residue in LiPB and a His in LPOB.

The close structural similarities between the LPO and LiP clones suggested an allelic relationship. Segregation of BssHII bands is consistent with allelism (Figure 2A). Bands specific to LiPB (2353 bp), LiPA (1475 bp), LPOB (2573 bp) and LPOA (1593 bp) are evident in the dikaryon (lane 4) and then segregate into the homokaryotic cultures (lanes 5 and 6). Higher molecular weight bands visible in lanes 4–6 represent related LiP genes such as O282 (11). Segregation of allelic variants was confirmed.
by probing the blot with the 28-mer (Fig. 2, panel B). The cosmid standard (lane 2), dikaryon (lane 4), and only one of the homokaryons (lane 6) hybridize.

CHEF gel analysis

Under the CHEF conditions described above, at least seven chromosome bands were resolved from P. chrysosporium DNA (Figure 3). At least two of these are doublets, as judged by the relative intensity of ethidium bromide staining. Based upon comparisons with Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Aspergillus nidulans, the P. chrysosporium bands are approximately 2.0, 2.8, 3.2, 3.6, 3.8, 4.4, 4.8 mb.

The band pattern is similar for strain ME446 (data not shown), but differs substantially among homokaryons because of meiotic recombination and chromosome polymorphisms. For example, basidiospore culture No. 2 (Fig. 5, lane 3) has bands at approximately 4.3, 3.7, 3.5, 3.0, 2.8, and 2.0 mb. Assuming doublets at 2.8, 3.0, and 3.7 mb the total genome size is approximately 29 mb, somewhat lower than the previous estimate of 36.5 mb for the haploid genome (27).

Six different LiP clones from BKM-1767 hybridized to electrophoretic bands at 3.7 and 3.5 mb (Figure 4). The clones were H8 (=LPOA), O282, V4 (pFFF62), and the three genes residing on pQQ24 (Fig. 1). In addition, the 33-mer (M33) which hybridizes to 8 of the 11 LiP clones of ME446, hybridizes to the same two bands. These results could be interpreted as interchromosomal duplications/translocations of LiP genes. Alternatively, homologous chromosomes in the dikaryon might be dimorphic with respect to electrophoretic mobility. The latter explanation is supported by the presence of single bands in homokaryotic derivatives (Figure 5). This, in turn, is entirely consistent with the identification of specific alleles in these cultures (Fig. 3). Subsequent probing of the same CHEF gel with genes encoding glyoxal oxidase and a cellobiohydrolase have identified other dimorphic chromosomes (P. Kersten and S. Covert, unpublished data). Chromosome length polymorphisms have been observed in Plasmodium falciparum (28), S. cerevisiae (29), Candida albicans (30), Ustilago maydis (31), and Coprinus cinerus (M. Zolan, personal communication). The latter two fungi belong to the same taxonomic class as P. chrysosporium, the Basidiomycetes.

No evidence for a second LiP cluster was observed using the six LiP clones as probes. In addition, the 33-mer that hybridizes to two RFLP-defined clusters in ME446 (27), hybridized to the 3.7 and 3.5 mb bands of BKM-1767 (Figure 4) and ME446 (data not shown).

DISCUSSION

The cosmid and CHEF analyses described here clearly demonstrate clustering of LiP genes at both kilobase and megabase levels. The clustering of LiP genes is consistent with the RFLP finding that large variations in LiP activity among haploid recombinants do not correlate with the distribution of LiP alleles (16). Recombination between closely linked structural genes is expected to be relatively low and the cluster would segregate as a unit. (Accurate estimates of recombination frequencies would require complete mapping of all LiP genes on the chromosome.) Variation among strains is undoubtedly due to complex interactions involving many genes, linked and unlinked. Raeder et al. (16) detected one locus unlinked to LiP genes that exerted a significant effect on LiP activity.

Other structural genes probably involved in lignin depolymerization include those encoding Mn-dependent peroxidases (32,33), glyoxal oxidase (34), and perhaps additional LiP genes. It is unlikely that unidentified LiP genes are present outside the dimorphic chromosome, unless their nucleotide sequences are substantially different from the six clones and 33-mer used to probe CHEF gels in this study.

The CHEF separation described here will allow rapid and convenient chromosome mapping of a variety of markers in wild type and genetically transformed (35) strains. For example, we have mapped the genes encoding glyoxal oxidase and several cellulase genes to chromosomes other than those carrying the LiP cluster (P. Kersten and S. Covert, unpublished). Some of the cellulase genes are very closely linked; i.e. within 1 kb (Covert and Cullen, submitted).

The mechanism(s) giving rise to chromosome length polymorphisms may also be related to variations in LiP activity. In P. falicurum, polymorphisms are caused by homologous recombination in distal regions of chromosomes, and it has been suggested that recombination in these dynamic regions may be related to antigenic variation (28). Extensive mapping of the chromosome homologues will be required to determine if a similar mechanism is operative in P. chrysosporium. These investigations will be greatly facilitated by the preparation of chromosome-specific genomic libraries from haploid recombinants, now possible using the CHEF separation we describe together with other recent techniques (e.g., 36,37).

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