APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1993, p. 3946–3950
0099-2240/93/113946-05$02.00/0

Vol. 59, No. 11

Temporal Expression of the Major Lignin Peroxidase Genes of *Phanerochaete chrysosporium*

K. BOOMINATHAN,[1]† T. M. D'SOUZA,[1] P. S. NAIDU,[1] C. DOSORETZ,[2] AND C. A. REDDY[1,2]*

Department of Microbiology[1] and NSF Center for Microbial Ecology,[2] Michigan State University, East Lansing, Michigan 48824-1101

Received 26 March 1993/Accepted 10 August 1993

DNA probes specific for the genes encoding major lignin peroxidase (LIP) isozymes H2, H8, and H10 of *Phanerochaete chrysosporium* were constructed. These probes were used to study the temporal expression of the three *lip* genes in defined low-nitrogen medium. H2 gene transcripts were produced at high levels on days 4, 5, and 7 and at low levels on day 6, while the H8 gene transcripts peaked on day 4 and were produced in substantially lower amounts thereafter. H10 transcripts, on the other hand, peaked on day 4, dropped precipitously on day 5, and were barely detectable on days 6 and 7. There was no precise correlation between *lip* transcript and isozyme levels.

During secondary metabolism, the lignin-degrading white rot basidiomycete *Phanerochaete chrysosporium* produces two families of catalytically distinct, extracellular, glycosylated, heme peroxidases, referred to as lignin peroxidases (LIPs) and manganese-dependent peroxidases, which play key roles in lignin degradation (3, 6, 12). The LIP enzyme family consists of a number of closely related isozymes with molecular masses ranging from 38 to 43 kDa. The cross-reactivities of polyclonal antibodies raised against different LIP isozymes and the results of analyses of peptides obtained after V8 protease digestion indicated that the LIP isozymes, although closely related to each other, can be divided into at least three subfamilies (9, 13, 18). Several *lip* cDNAs and genes have been cloned and sequenced (18). Structural analyses of the *lip* cDNAs and genes revealed that the different LIP isozymes are encoded by distinct genes, that the different *lip* genes exhibit high levels of nucleotide homology to each other, and that there are at least three subfamilies of *lip* genes within the *lip* gene family of *P. chrysosporium* (18).

A number of cultural parameters, including the levels of carbon, nitrogen, and Mn(II) in the medium, are known to effect marked changes in the amounts and types of LIP isozymes produced by *P. chrysosporium* (3, 4, 9, 11, 12). However, previously there has been no information concerning the temporal patterns of expression of the individual *lip* genes because of a lack of gene-specific probes. This study was initiated to construct probes that are specific to the major *lip* genes encoding LIP isozymes H2, H8, and H10 and to use these probes to investigate the temporal patterns of expression of these genes in *P. chrysosporium*.

Culture conditions. Liquid cultures of *P. chrysosporium* BKM-F 1767 were grown in acetate-buffered low-N (2.4 mM N) liquid medium (45-ml portions in 125-ml Erlenmeyer flasks) as described by Dass and Reddy (7). The cultures were incubated on a rotary shaker (200 rpm) at 39°C and were flushed with 100% oxygen at the time of inoculation and every day thereafter.

Specificity of the *lip* gene probes. Previously described *lip* gene sequences (15, 17, 21) were aligned by using GENEPRO (Riverside Scientific Enterprises, Seattle, Wash.) to identify DNA sequences that have a high probability of being specific to a given *lip* gene. The *lip* gene probes (size range, 195 to 298 bp) constructed in this way exhibited levels of nucleotide homology of 48 to 52% to each other and included the 5′ coding and noncoding regions of *lip* genes *GLG1* (EMBL accession no. X15599 [17]), *GLG2* (21), and *GLG3* (15), which encode the major LIP isozymes H2, H10, and H8, respectively (Fig. 1). These isozymes were purified, their N-terminal sequences were determined (7), and the isozymes were matched to the corresponding *lip* genes and cDNAs cloned and sequenced previously (15, 17, 21). The *GLG1*-specific probe contained no intron, while the probes specific for *GLG2* and *GLG3* contained 52- and 36-bp introns, respectively.

Southern blots of the restriction digests of *lip* genomic clones pUGLG1 through pUGLG6 (21), each of which contained the coding region for a different *lip* gene, were hybridized to the newly constructed probes for *GLG1*, *GLG2*, and *GLG3*. Our results showed that each of the three probes was specific for a *lip* gene, and no cross-hybridization was observed (Fig. 2). Recently, Brooks et al. (5) described a polymerase chain reaction procedure for constructing probes specific for *lip* genes *LIG1* and *LIG5* of *P. chrysosporium* ME-446.

Temporal production of *lip* gene transcripts. Total RNA was extracted as previously described (10) by using day 2 through day 7 cultures grown in low-N medium because most of the previous studies (3) showed that LIP production in this medium peaks around day 6. RNA was quantified by determining the A_{260}; 25 μg was loaded into each lane of the gel, and Northern (RNA) blots were prepared as described by Sambrook et al. (19). The blots were hybridized (4) with different *lip* gene probes and were densitometrically analyzed by using an AMBIS image analyzer (Ambis Systems, Inc., San Diego, Calif.) to determine the relative amounts of the transcripts produced.

Previous studies showed that in cultures grown under nitrogen-limited conditions, LIP activity first appears in the extracellular culture fluid on day 3 or 4 and reaches a peak around day 6 (4, 8). The results of this study (Fig. 3) showed that the level of H2-encoding *GLG1* transcripts peaked on day 4, steeply declined by day 6, and peaked again on day 7 (Fig. 3A). The level of H10-encoding *GLG2* transcripts, however, peaked on day 4, declined dramatically on day 5,

* Corresponding author.
† Present address: Novo Nordisk Biotech, Inc., Davis, CA 95616.

***GLG1*-specific probe (226 bp)**

GTACACCGTTCCCTGGTTTCAACGCCAAGCCCGCCTGCTATTGGATGTCCTCAACGTTCT
ACCTCAATCGACCCGTTATGGCAGACCAAGTTCGCCCTGCGCCATCTGCACTTGTCTGCG
CCTCGACCTCAGGAACGGCGGGTATATAAGTGCTCGCGACGAGCCTCGGAAACTCCAGCA
CCTCCCAGTGTTCTACTTTCTACAGCTCACCGTCCGGTCTCAGCAG

***GLG2*-specific probe (298 bp)**

CGCGCCGGCCGGCTTGTCCGACTGCGGCAGCGCGTTGGCGACAATGCTTGTCTCAGACGA
AACTCGTCATAGATATGGACCAGAGTATATAAGCAGCCTATGCACGATCGGTGTCTTCAG
GTCATTCTCAGCCTCTTCAGACTCTCCAACGGTTGCCTTTGGACAGACATGGCCTTCAAG
AAGCTCCTTGCTGTTCTTACCGCCGCTCTCTCCCTCCGTGCTGCTCAGGGTGCGTCCCGG
ATGGCTACCGACGCATTGCACAACTAACAGCTACGCGATAGGTGCGGCCGTTGAGAAG

***GLG3*-specific probe (195 bp)**

GTCGACCACGCCTAGGGTATAAAAGGGCGACAGGACCACCGCAGTCCCTCAGACATCCAG
TCTCTTCAGTCCCACTCAGCACCAGCAACACAGCGGACATGGCCTTCAAGCAGCTCTTCG
CAGCTATCTCTCTCGCTCTCTCGCTCTCGGCTGCGAACGGTACGCCCATCGCAGTTATCG
TTGACCAGTGGACTG

FIG. 1. Nucleotide sequences of *lip*-specific probes. Probes specific for *lip* genes *GLG1*, *GLG2*, and *GLG3* (which encode LIP isozymes H2, H10, and H8, respectively) are *Hin*dIII-*Sst*I fragments from the 5′ regions of the respective genes which were subcloned in M13 vectors and sequenced as described previously (15, 17, 21).

and was almost zero thereafter (Fig. 3B). The level of H8-encoding *GLG3* transcripts peaked on day 4, declined to a substantially lower level on day 5, and remained essentially at that level through day 7 (Fig. 3C). Several independent lines of evidence support the conclusion that the H2-, H8-, and H10-encoding *lip* genes each have a distinct temporal transcription pattern, as shown in this study. Similar results were obtained consistently in at least three separate experiments. In independent experiments, RNA blots similar to those in Fig. 3 were probed with rRNA genes from *Neurospora crassa*, and comparable amounts of transcripts were observed in each of the lanes (data not shown), indicating that the variation in the transcription patterns of the *lip* genes described above was not due to anomalies in RNA loading rates. Additional evidence in support of the idea that the observed differences in *lip* transcription patterns in Fig. 3 were not due to differences in RNA loading rates is the fact that when the same RNA blot was hybridized to three different probes (Fig. 3), three distinct hybridization patterns were obtained. For example, the RNA contained in lanes 4 was the same for Fig. 3A, B, and C, yet the transcription patterns of the three *lip* genes were different. Similarly, the same amount of RNA was contained in lanes 6 of Fig. 3A, B, and C; however, the hybridization patterns obtained for the RNA with the three *lip* gene probes were very different. Marked temporal variations in *lip* gene expression were also observed by Black (1) for two *lip* genes of another white rot fungus, *Trametes versicolor*. Furthermore, our transcription data for H8-encoding *GLG3* are in agreement with the results of Moukha et al. (14), who reported that the maximum

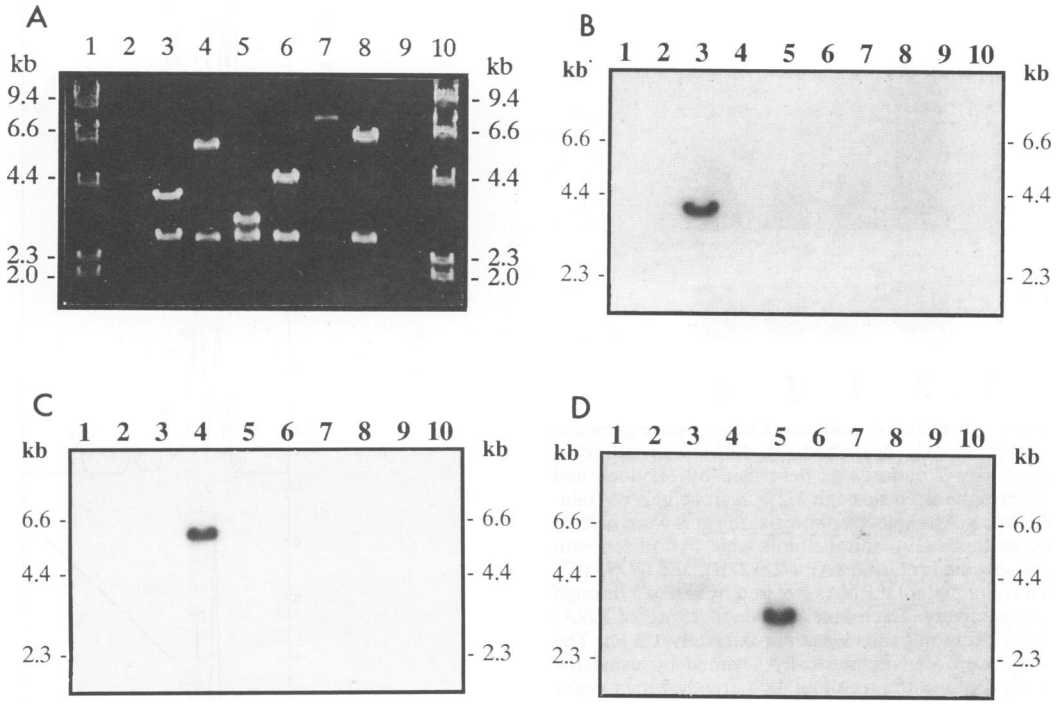

FIG. 2. Specificity of the *lip* probes. Southern hybridization analyses were performed by using probes specific for *lip* genes *GLG1*, *GLG2*, and *GLG3*. The *lip* genomic clones pUGLG1, pUGLG2, pUGLG4, pUGLG5, and pUGLG6, each of which contains a different *lip* gene (15–17, 21), were digested with *Bam*HI, while pUGLG3, which contains the coding region for the H8 gene, was digested with *Bam*HI and *Hin*dIII. The digests were electrophoresed on a 1% agarose gel (A) and Southern blotted by using standard methods (19). The same blot was successively hybridized with ^{32}P-labeled probes specific for *GLG1* (B), *GLG2* (C), and *GLG3* (D) at 42°C for 12 h and washed under high-stringency conditions as described by Sambrook et al. (19). Lanes 1 and 10 contained size markers (*Hin*dIII-digested lambda DNA), while lanes 3 through 8 contained DNAs from pUGLG1, pUGLG2, pUGLG3, pUGLG4, pUGLG5, and pUGLG6, respectively. No DNA was added to lanes 2 and 9.

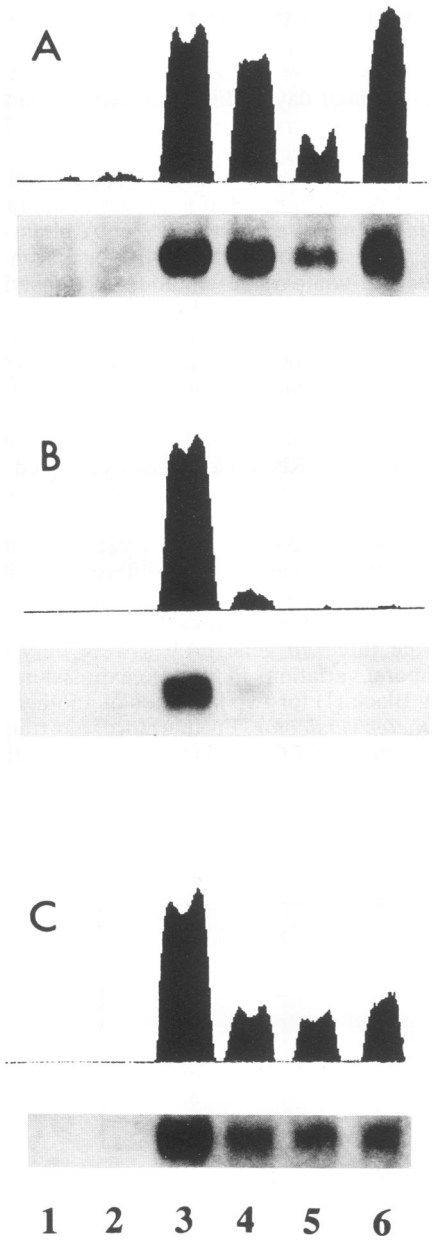

FIG. 3. Northern hybridization analyses. Cultures were grown in low-N medium as previously described (7). RNA was extracted from day 2 through day 7 cultures as described by Haylock and Broda (10) and electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde. After electrophoresis, the gels were blotted by using standard methods (19), and the blots were hybridized with ^{32}P-labeled probes specific for *GLG1* (A), *GLG2* (B), and *GLG3* (C). Lanes 1 through 6 contained total RNAs isolated from day 2 through day 7 cultures, respectively. Each lane contained 25 μg of RNA. The size of the hybridization bands was approximately 1.3 kb. The hybridization bands were densitometrically scanned by using the AMBIS image analyzer, and the resulting data are shown as peaks above the bands.

transcript levels for the H8-encoding gene occurred on day 4 and that these transcript levels decreased dramatically on days 5 and 6.

LIP isozyme levels. Twentyfold-concentrated extracellular

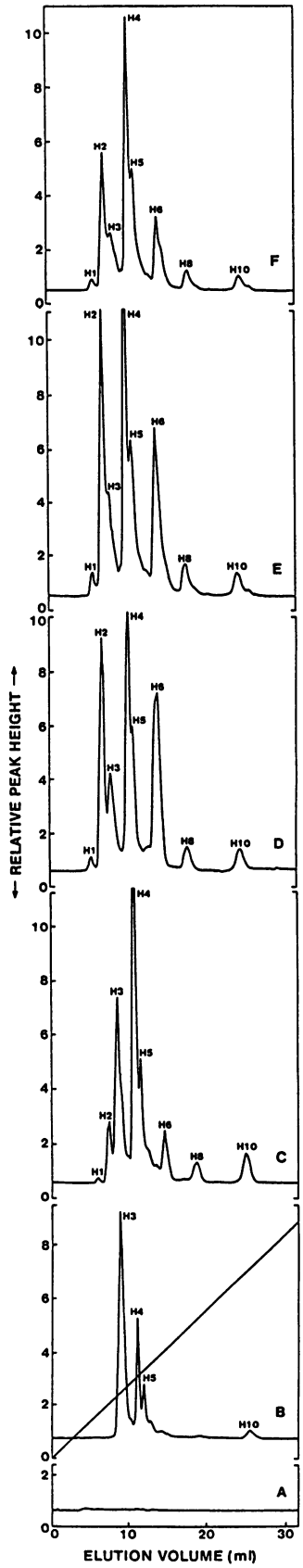

FIG. 4. FPLC profiles of heme proteins in the extracellular fluid of *P. chrysosporium* cultures grown in low-N medium. Equal amounts of protein (480 μg) from 20-fold-concentrated and dialyzed samples of extracellular fluid from day 2 through day 7 cultures (see the legend to Fig. 3) were analyzed for heme proteins by using FPLC as previously described (2, 4). LIP isozymes are represented by heme protein peaks H1, H2, H6, H8, and H10, while peaks H3, H4, and H5 represent manganese-dependent peroxidase isozymes. Heme protein profiles A through F are profiles of day 2 through day 7 cultures, respectively. LIP activity was measured by determining the initial oxidation of veratryl alcohol to veratraldehyde (20).

fluids from day 2 through day 7 cultures (see above) were subjected to fast protein liquid chromatography (FPLC) as described by Dass and Reddy (7). A positive correlation was observed between the patterns of transcript production and LIP isozyme H2 production, although there was a noticeable lag (approximately 1 day) between the appearance of H2 transcripts and the appearance of H2 isozyme (Fig. 3A and 4). However, there was no precise temporal correlation between the production of H8 and H10 transcripts and the production of the corresponding isozymes. The observed differences may have been due to variation in the stability of the different *lip* mRNA species, the relative sensitivities of the blotting procedures and the FPLC procedure, and perhaps other factors that are not clear at this time. It should also be mentioned that the isozyme levels were determined by using 20-fold-concentrated extracellular culture fluid, and this may explain why certain LIP isozymes were observed but the corresponding transcripts were not. Recently, Moukha et al. (14) also described the lack of precise correlation between the time of appearance of LIP H8 transcripts and the time of appearance of the LIP H8 isozyme; the appearance of LIP H8 was delayed with respect to the appearance of the H8 transcript. Furthermore, there was some lack of correlation between the appearance of peroxidase activity as detected by H_2O_2-dependent benzidine oxidation and the time of appearance of H8 transcripts.

The transcription pattern of the H2-encoding gene described above is consistent with the idea that the H2 isozyme is important among the LIP isozymes of *P. chrysosporium* since it is produced in substantial amounts compared with the other isozymes, regardless of the culture conditions employed (7, 11, 20). Holzbaur and Tien (11) reported the production of the H2 isozyme under nitrogen-limiting conditions as well as carbon-limiting conditions; this is in contrast to the H8 isozyme, which is produced only under nitrogen-limiting conditions. Moreover, the H2 isozyme is produced in substantial amounts under shaken or static conditions and in cultures growing with acetate or dimethyl succinate buffers, which are the two most commonly used buffers in *P. chrysosporium* culture media (7, 20). Recently, Brooks et al. (5) also reported that transcripts of the *LIG5* gene of strain ME-446, which is supposedly equivalent to the *GLG1* gene of strain BKM-F 1767, are expressed in media containing various levels of nitrogen.

Currently, an important limitation in the study of the regulation of expression of individual *lip* genes of *P. chrysosporium* is the lack of probes which are specific enough to discriminate between different *lip* transcripts. The availability of such probes, as described in this paper and in the study of Brooks et al. (5), should facilitate further studies to understand the regulation of *lip* gene expression in response to variations in C, N, O, and Mn(II) levels in culture media. Gene-specific probes should also be useful in studies of the regulation of *lip* gene expression when *P. chrysosporium* is grown on different species of softwood and hardwood.

This work was supported in part by the Michigan Agricultural Experiment Station and by grant DE-FG02-85ER from the U.S. Department of Energy.

REFERENCES

1. **Black, A. K.** 1993. Structure and molecular regulation of the lignin peroxidase genes from the white-rot fungus *Trametes versicolor*. Ph.D. dissertation. Michigan State University, East Lansing.
2. **Boominathan, K., S. B. Dass, T. A. Randall, R. L. Kelley, and C. A. Reddy.** 1990. Lignin peroxidase-negative mutant of the white rot basidiomycete *Phanerochaete chrysosporium*. J. Bacteriol. **172:**260–265.
3. **Boominathan, K., and C. A. Reddy.** 1992. Fungal degradation of lignin: biotechnological applications, p. 763–822. *In* D. K. Arora, R. P. Elander, and K. G. Mukerji (ed.), Handbook of applied mycology, vol. 4. Fungal biotechnology. Marcel Dekker, New York.
4. **Boominathan, K., and C. A. Reddy.** 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. USA **89:**5586–5590.
5. **Brooks, P., P. Sims, and P. Broda.** 1993. Isozyme specific polymerase chain reaction analysis of differential gene expression: a general method applied to lignin peroxidase genes of *Phanerochaete chrysosporium*. Bio/Technology **11:**830–834.
6. **Buswell, J. A., and E. Odier.** 1987. Lignin biodegradation. Crit. Rev. Biotechnol. **6:**1–60.
7. **Dass, S. B., and C. A. Reddy.** 1990. Characterization of extracellular peroxidases produced by acetate-buffered cultures of *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **69:**221–224.
8. **Dosoretz, C. G., S. B. Dass, C. A. Reddy, and H. E. Grethlein.** 1990. Protease-mediated degradation of lignin peroxidases in liquid cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **56:**3429–3434.
9. **Farrell, R. L., K. E. Murtagh, M. Tien, M. D. Mozuch, and T. K. Kirk.** 1989. Physical and enzymatic properties of lignin peroxidase isozymes from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **11:**322–328.
10. **Haylock, R., and P. Broda.** 1988. Preparation and characterization of mRNA from ligninolytic fungi. Methods Enzymol. **161:**221–227.
11. **Holzbaur, E. L. F., and M. Tien.** 1988. Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **155:**626–633.
12. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
13. **Leisola, M. S. A., B. Kozulic, F. Meussdorffer, and A. Fiechter.** 1987. Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. J. Biol. Chem. **262:**419–424.
14. **Moukha, S. M., H. A. B. Wösten, E.-J. Mylius, M. Asther, and J. G. H. Wessels.** 1993. Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. J. Bacteriol. **175:**3672–3678.
15. **Naidu, P. S., and C. A. Reddy.** 1990. Nucleotide sequence of a new lignin peroxidase gene *GLG3* from the white-rot fungus, *Phanerochaete chrysosporium*. Nucleic Acids Res. **18:**7173. (Corrigendum, Nucleic Acids Res. **20:**4124.)
16. **Naidu, P. S., Y. Z. Zhang, and C. A. Reddy.** 1990. Characterization of a new lignin peroxidase gene (*GLG6*) from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **173:** 994–1000.
17. **Naidu, P. S., Y. Z. Zhang, and C. A. Reddy.** 1989. Analysis of nucleotide sequence of a lignin peroxidase gene from a white-rot basidiomycete, *Phanerochaete chrysosporium*, abstr. H70, p. 181. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.

18. **Reddy, C. A.** 1993. An overview of the recent advances on the physiology and molecular biology of lignin peroxidases of *Phanerochaete chrysosporium*. J. Biotechnol. **30:**91–107.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161:**238–249.
21. **Zhang, Y. Z., C. A. Reddy, and A. Rasooly.** 1991. Cloning of several lignin peroxidase (LIP)-encoding genes: sequence analysis of the *LIP6* gene from the white-rot basidiomycete *Phanerochaete chrysosporium*. Gene **97:**191–198.