APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3697-3703 Vol. 62, No. 10
0099-2240/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Expression of *lip* Genes during Growth in Soil and Oxidation of Anthracene by *Phanerochaete chrysosporium*

BILL W. BOGAN,[1,2]† BARRY SCHOENIKE,[2] RICHARD T. LAMAR,[2] AND DAN CULLEN[2]*

Department of Cell and Molecular Biology, University of Wisconsin-Madison, Madison, Wisconsin 53706,[1] and Institute for Microbial and Biochemical Technology, U.S. Department of Agriculture Forest Service Forest Products Laboratory, Madison, Wisconsin 53705[2]

Received 22 April 1996/Accepted 12 August 1996

mRNA extraction from soil and quantitation by competitive reverse transcription-PCR were combined to study the expression of the 10 known lignin peroxidase *(lip)* genes in anthracene-transforming soil cultures of *Phanerochaete chrysosporium.* Levels of extractable *lipA* transcript and protein (LiP H8) were well correlated, although they were separated by a 2-day lag period. The patterns of transcript abundance over time in soil-grown *P. chrysosporium* varied among the nine *lip* mRNAs detected; comparison with *lip* gene expression under different liquid culture conditions suggested an early phase of carbon limitation for the cultures as a whole, which was followed by a transition to nitrogen starvation. Anthracene transformation occurred throughout the 25-day course of the experiment and, therefore, likely involves mechanisms distinct from those involved in oxidation of non-LiP substrate polycyclic aromatic hydrocarbons.

White-rot fungi have been extensively researched in recent years for possible use in the bioremediation of polycyclic aromatic hydrocarbon (PAH)-contaminated soils. To date, most work has centered around one species, *Phanerochaete chrysosporium.* Biochemical data (1, 23, 26, 36, 54), liquid culture studies (1, 6, 24, 25, 46), and bench-scale solid-phase experiments (20, 37, 41, 50) have demonstrated the ability of this species and its extracellular ligninolytic enzymes to degrade numerous PAHs. Field-scale trials of a related species, *Phanerochaete sordida,* have shown promise for remediation of PAH-contaminated sites (10, 31). Degradation of PAHs by *P. chrysosporium* is initiated by free-radical mechanisms; PAHs with ionization potentials at or less than approximately 7.55 eV are substrates for direct one-electron oxidation by lignin peroxidase (LiP) (26), while those higher than this threshold are apparently acted on by radical species formed during manganese peroxidase (MnP)-dependent lipid peroxidation reactions (1, 36).

The biochemistry and molecular genetics of *P. chrysosporium's* ligninolytic system are quite complex. Multiple LiP isozymes are produced in submerged culture, and the genome of *P. chysosporium* contains at least 10 structurally related *lip* genes, which are designated *lipA* through *lipJ* (18). The reason for the multiplicity of *lip* genes is, at present, unclear, although some kinetic and substrate range differences have been observed among LiP isozymes. Kinetic studies of LiP have revealed differences among isozymes with respect to K_m and K_{cat}, values, both for substrates (i.e., veratryl alcohol, 1,4-dimethoxybenzene, and lignin model dimers [14, 22]) and oxidants (H_2O_2 and *tert*-butyl hydroperoxide [14, 22]). Some of these variations are undoubtedly due to differences in the oxidation-reduction potentials of the different isoforms (35), while some may be caused by slight structural variations at the enzyme active site (48). Additionally, it has been proposed that access to the heme group is less restricted in some isozymes, perhaps via differences in flexibility of the surrounding protein (22).

Information regarding enzyme expression in white-rot fungi has mostly been obtained from cultures grown in chemically defined liquid media; extensive data concerning LiP isozyme profiles have been gathered from liquid cultures at the level both of protein (5, 8, 12, 30) and of mRNA transcripts (4, 5, 38, 49). Very little data exist for white-rot fungi grown on solid substrates. MnP has been isolated from *P. chrysosporium* grown on aspen pulp (9), from *Ceriporiopsis subvermispora* cultured on wood chips (51), and from *Bjerkandera adusta*-colonized soil (15). Several LiP and MnP isoforms, as well as the H_2O_2-producing enzyme glyoxal oxidase (GLOX), have recently been recovered from wheat straw-grown *Phlebia radiata* (53). However, quantification of ligninolytic enzymes in solid substrates is frequently plagued by low yield and interference from contaminating substances and enzyme inhibitors (9, 39); this has hampered efforts to investigate the patterns of enzyme expression in complex substrates. Because fungus-colonized soil is the medium most relevant to bioremediation, it is important that methods be developed to address this problem.

Extraction and purification of mRNA from fungus-colonized soils (34) have recently been coupled with competitive reverse transcription-PCR (RT-PCR) (7, 34, 49) to investigate the physiological state of *P. chrysosporium* grown in soil. Initial application of these techniques (34) detected transcripts of two lignin peroxidase genes *(lipA* and *lipC),* as well as two cellobiohydrolases *(cbhl-4* and *cbhl-1)* and mRNA encoding the mitotic spindle protein ß-tubulin, in pentachlorophenol (PCP)-contaminated soil colonized by *P. chrysosporium.* Further soil culture studies (2) correlated the presence of *mnp* mRNAs with active MnP enzyme and with oxidation of two PAH substrates of MnP-dependent lipid peroxidation, fluorene and chrysene. In this paper, we extend these previous studies to encompass the full range of *lip* genes during a bench-scale PAH soil bioremediation experiment. Soil cultures of *P. chrysosporium* transformed anthracene, a LiP substrate PAH, throughout a 25-day time course study. Quantitative RT-PCR analysis was performed on mRNA isolated from these cultures to assess the temporal regulation of *lip* gene expression in soil. The relationship between *lipA* and *glx* transcript abundances

* Corresponding author. Mailing address: USDA Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53705. Phone: (608) 231-9468. Fax: (608) 231-9262. Electronic mail address: dcullen@facstaff.wisc.edu.

† Present address: Intech One-Eighty Corporation, State College, PA 16801.

and extractable LiP H8 (the protein product of *lipA)* and GLOX proteins, as measured by Western blotting (immunoblotting), supported these results.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest commercially available grade. Solvents used in PAH extraction and chromatography were of high-performance liquid chromatography (HPLC) grade. All buffers used in extraction and purification of mRNA were treated with diethylpyrocarbonate according to the method described by Sambrook et al. (45) before use.

Fungi. Stock cultures of *P. chrysosporium* Burdsall BKM-F-1767 (ATCC 24725) were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. These were transferred to yeast extract-malt extract-peptone-glucose slants (33), and the slants were stored at 4°C until use. Spores scraped from three to four such slants were used to inoculate ca. 200 g (dry weight, 60% moisture) of a nutrient-fortified grain-sawdust spawn mixture (L. F. Lambert Spawn Co.. Coatesville, Pa.) in l-quart (ea. 0.9-liter) glass jars with lids containing gas-permeable membranes (11). After 2 to 3 days of growth, this colonized spawn mixture served as an inoculum for soil cultures as described below.

To assess the ability of oligonucleotide primers to amplify both allelic variants of their corresponding *lip* genes, two single-basidiospore isolates of established genotypes were selected (18): SB40 *(lipA2 lipB2 lipC2 lipE2 lipG2 lipH2 lipI2 lipJ2 lipD2 lipF2)* and SB48 *(lipA1 lipB1 lipC1 lipE1 lipG1 [ipH1 lipI1 lipj1 lipD1 lipF1).*

Soil cultures. Sieved Marshan sandy loam (for soil characteristics, see reference 33) was weighed (25 g [dry weight]) into half-pint (0.2-liter) glass jars equipped with gas-permeable membranes. Soil was sterilized by autoclaving (1 h at 121°C) on each of 3 consecutive days. After sterilization, soil was spiked with 400 ppm of anthracene (Aldrich, Milwaukee, Wis.) via addition of a stock solution in 1 ml of methylene chloride. which was then allowed to evaporate for 24 h prior to inoculation. Soil was adjusted to 35% moisture and inoculated with 10% (dry weight basis) of *P. chrysosporium*- colonized Lambert spawn mix. The cultures were maintained at 39°C with periodic watering as necessary.

PAH extraction. Extraction of anthracene and its products from soil involved modifications to Environmental Protection Agency method 3550 (52) as previously described (10, 31). Soil samples (2 to 3 g) were mixed with 6 to 7 g of anhydrous Na_2SO_4 and stored frozen prior to extraction. PAH extraction was done by sonication (2 min) in the presence of 20 ml of acetone-methylene chloride (1:1 [vol/vol]). The soil was allowed to settle for 2 to 3 h, and the supernatant was decanted through glass fiber filters. After a second sonication cycle, the solvent was removed by vacuum filtration. and the soil was rinsed successively with 5 ml of methylene chloride and 5 ml of acetone. Extracts were concentrated under N_2 in a TurboVap ZW700 (Zymark, Hopkinton, Mass.) and redissolved in 5 to 10 ml of acetonitrile for HPLC.

HPLC. All HPLC analyses employed a Vydac 201TP54 (25 by 0.46 cm) C_{18} reverse-phase column (Nest Group, Southhoro, Mass.). The column temperature was maintained at 35°C. The mobile phase gradient for PAH analyses consisted of water-acetonitrile as follows: 0 to 5 min, 60:40; 5 to 30 min, ramped to 0:100; 30 to 35 min, held at 0:100. The flow rate throughout the gradient was 1 ml min^{-1}. UV A_{254} of the column eluate was measured. Anthracene and anthraquinone concentrations were determined by comparison with three-point standard curves *(r* = 0.998) for each compound.

mRNA extraction. Extraction and purification of mRNA from fungal cultures were done as reported by Lamar et al. (34), with modifications (2). Fungus-colonized soil or spawn (10 g) was wrapped in Miracloth (Calbiochem-Novabiochem Corp, La Jolla, Calif.) and snap-frozen in liquid N_2. Frozen samples were ground with a chilled mortar and pestle. The mRNA extraction buffer (4 M guanidinium thiocyanate, 0.1 M Tris base, 1% dithiothreitol, 0.5% Sarkosyl [pH 8.0]) was added in five 2-ml aliquots (seven for spawn-grown cultures) with continuous grinding. The resultant frozen powder was allowed to thaw and was then centrifuged (500 × *g)* for 8 min. The supernatant (approximately 7 ml), following a second centrifugation step, was mixed with 2 volumes of binding buffer (0.1 M Tris base, 0.4 M LiCl, 20 mM EDTA [pH 8.0]). This mixture was centrifuged (8,000 × *g)* for 5 to 10 min. The supernatant was then decanted to a second tube and mixed with 1.5 mg of oligo(dT) Dynabeads (Dynal, Great Neck, N.Y.), which had been previously washed in binding buffer. After a 30-min hybridization on ice, the Dynabeads were isolated in an MPC-1 magnetic concentrator (Dynal) for 5 min and resuspended in 500 μl of washing buffer (10 mM Tris base, 0.15 M LiCl, 1 mM EDTA [pH 8.0]). The remaining steps (three washing cycles, mRNA elution, and Dynabead regeneration) were done in 1.5-ml Eppendorf tubes using the smaller MPC-E-1 magnet (Dynal). mRNA was eluted by heating Dynabeads (65°C) in 200 μl of elution buffer (2 mM EDTA [pH 8.0]). Elution buffer containing mRNA was mixed with 20 μl of 3 M sodium acetate (pH 5.2) and 400 μl of ethanol for storage (–20°C). Regeneration of Dynabeads with 0.1 M NaOH was performed according to the manufacturer's instructions.

Transcript detection and quantitation by RT-PCR. Primer design for quantifying the closely related *lip* transcripts through competitive RT-PCR was guided by several considerations: (i) the primer pairs must selectively amplify both allelic transcripts for each gene, to the exclusion of all other genes; (ii) the amplified regions must encompass one or more introns so that genomic and cDNA products can be size fractionated and their concentrations can be estimated (21); and (iii) for meaningful quantitative comparisons of different transcripts, the amplified regions must be similar in length and, ideally, similar in sequence (40). Satisfying these conditions, *lip*- specific primers were designed to amplify a corresponding region in each *of* the 10 genes (see Fig. 3).

All RT-PCRs were performed in a DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, Conn.). Each reaction mixture contained 3 μl of the final mRNA preparation described above. RT reaction mixtures were as described elsewhere (34, 49) and were primed with oligo(dT) 15-mers. PCR mixtures (100 μl) contained 1.25 U of *Taq* DNA polymerase and 21 pmol of each primer (see Fig. 2). The temperature program for PCR was 94°C (6 min), 54°C (2 min), and 72°C (40 min) for 1 cycle, which was followed by 94°C (1 min), 54°C (2 min), and 72°C (5 min) for 35 cycles and a final 15-min extension at 72°C.

Competitive PCRs were set up as described above, except that known amounts of a genomic DNA (gDNA) competitive template were added as previously described (21, 34, 49). Templates were constructed by cloning gDNA-derived individual *lip* genes and *glx* into plasmid pCRII (Invitrogen, San Diego, Calif.).

The PCR products were analyzed by electrophoresis on 1% SeaKem GTG agarose gels stained with ethidium bromide. The gels were visualized with UV tight and were photographed with a Foto/Analyst Visionary Benchtop Digital Documentation Station (Fotodyne, Hartland, Wis.). The resulting images were digitized, and band intensities were determined with NIH Image software (version 1.58). Equivalence points were determined by plotting ratios of gDNA-to-cDNA band intensities. The point on the resultant linear regression at which this ratio was 1.5 *(lip* genes) or 1.19 *(glx)* was taken as the equivalence point.

LiP-GLOX extraction and Western analysis. LiP was recovered from *P. Chrysosporium* soil cultures by the procedure described by Bollag et al. (3), with minor modifications (2). PAH-contaminated soil cultures for enzyme extraction were set up as described above; at harvest, the entire culture was shaken (15 min at 50 rpm) in 100 ml of 50 μM sodium acetate (pH 6.0). Soil was removed by centrifugation. The resultant supernatant was frozen, thawed, and centrifuged to remove high-molecular-weight polysaccharide slime; aliquots (1.5 ml) of the supernatant were then concentrated ca. 20-fold with Centricon-10 microconcentrators (Amicon, Inc., Beverly, Mass.). The supernatant was then applied to nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) with a Hybri-Dot manifold (Life Technologies, Gaithersburg, Md.). Western blotting was performed with monoclinal antibodies to *P. chrysosporium* LiP H8 and with polyclonal antibodies raised against GLOX.

RESULTS

Anthracene disappearance and transformation. Soil cultures of *P. chrysosporium* were grown in Marshan sandy loam artificially contaminated with anthracene at an initial concentration of 400 ppm. As shown in Fig. 1A, anthracene in these cultures was reduced by ca. 65% in 27 days; this disappearance took place gradually throughout the full duration of the experiment. The primary extractable by-product of anthracene oxidation was 9,10-anthraquinone. Accumulation of 9,10-anthraquinone appeared to level off after approximately 12 days, and in total accounted for roughly 50% of input anthracene (Fig. 1B). Recovery of anthracene from 400-ppm noninoculated control soil remained between 80 and 90% for the duration of the experiment (Fig. 1A), with essentially no anthraquinone formation observed (Fig. 1B).

***lip* and *glx* transcript analysis.** To ensure amplification of both alleles by each primer pair, gDNA was PCR amplified from single basidiospore isolates (Fig. 2). For each gene, both alleles were amplified, including the transposon-containing *lipI2* allele (19). Southern blot experiments with gene-specific probes (Fig. 3) showed no cross-hybridization to nontarget PCR products (data not shown), thus ensuring amplification specificity. The *lip* RT-PCR products (derived from cDNAs isolated from 3-day-old spawn-grown cultures) are shown with their corresponding gDNA PCR products in Fig. 4. Bands of the expected size were observed in all cases, except for *lipF*. No transcripts of this gene *were* detected from spawn-grown cultures, although the primers successfully amplified both the genomic competitive template (Fig. 4) and the gDNA derived from single-basidiospore cultures (Fig. 2).

The temporal variations in titers of nine *lip* mRNAs determined by competitive RT-PCR over the 25-day course of this study are shown in Fig. 5. Transcripts of all nine genes dis-

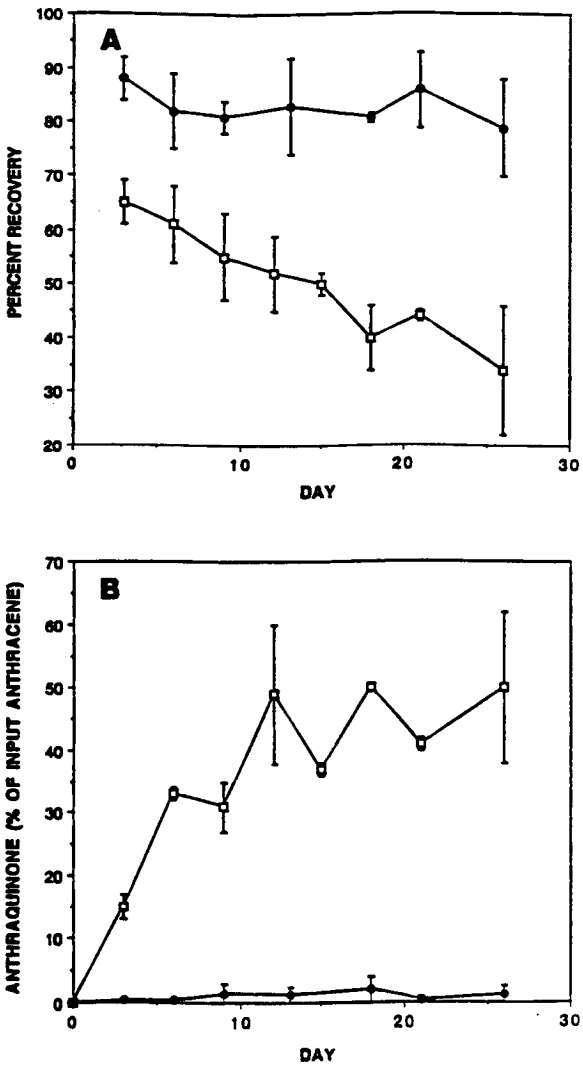

FIG. 1. Disappearance of anthracene (initial concentration, 400 ppm) from soil cultures of *P. chrysosporium* (A) and accumulation of 9,10-anthraquinone (B). Data are means ± 1 standard deviation for two replicate inoculated cultures (□) or two sterile controls (●).

played local maxima during the early phase of fungal growth (ea. days 3 to 6). This peak constituted the maximal mRNA titers attained in some cases (i.e.. *lipA, -B, -D, -H,* and *-I),* whereas others nearly matched *(lipG),* matched (lipE), or exceeded *(lipC* and *lipJ)* these values at later time points.

During this early phase, *lipA* (~8.5 × 10^{-7} pmol per reaction) and *lipD* (~1.0 × 10^{-6} pmol) were the dominant transcripts. Considering the entire time course, *lipA* persisted the longest at high levels and was present at all time points at titers near to or in excess of the maximal values observed for several other *lip* genes (i.e., *lipB, -G, -H,* and *-I).* The highest quantities measured for any of the mRNAs in this study were those of *lipJ,* which was present at up to 6.5 × 10^{-6} pmol per reaction during later time points.

***lipA/glx* transcript correlations with extractable H8-GLOX protein.** Figure 6A shows the relationship between LiP H8 protein, quantitated by Western blotting, and its corresponding transcript, *lipA.* Extractable H8 protein displays an approximately 2-day lag after *lipA* mRNA; however, the quantitative relationship between protein and transcript is quite good, with peaks in mRNA abundance on approximately day 4 (major) and day 11 (minor) corresponding to protein maxima at days 6 to 7 and day 13.

The correlation between *glx* mRNA and GLOX protein (Fig. 6B) was not as good but still showed reasonable agreement. Peaks in mRNA at days 4 to 5 and 11 corresponded to protein maxima. A downward trend in mRNA concentrations from days 6 to 9 corresponded to an overall decrease in protein levels during this time frame, although the magnitudes of the declines did not strictly agree.

DISCUSSION

We report here competitive RT-PCR methods suitable for the quantitative detection of mRNAs corresponding to all known *P. chrysosporium* LiP genes. This methodology was used to systematically quantify *lip* transcripts in anthracene-contaminated soil over a 25-day period.

Unusual patterns of gene expression were observed in the *P.* chysosporium-colonized soil cultures. Early time points were dominated by *lipA* and *lipD.* Transcripts of *lipJ* reached the highest levels observed with any gene but were confined to the latter stages of culture (days 15 to 20). The *lipA* mRNAs maintained fairly high levels (as much as 20% of their peak abundance) for 3 weeks and outnumbered transcripts of several other *lip* genes (i.e., *lipB, -G, -H,* and *-I)* throughout the entire study.

There are few quantitative time course studies of *lip* transcription which are suitable for direct comparison. In nitrogen-limited liquid medium, mRNA corresponding to *lipA* increased rapidly on day 4 and then immediately declined to essentially 0 (4). Single time point analyses in defined liquid media have shown transcripts of *lipD* present at high levels in carbon-limited medium, levels of *lipC* and *lipJ* substantially increased under nitrogen limitation, high *lipA* levels under both conditions, and low *lipB* and *lipI* levels under both (27, 49). Transcripts of *lipE* are abundant in liquid culture, especially under carbon limitation conditions (43, 44).

On the macroenvironmental level, comparison of the transcript patterns observed in anthracene-contaminated soil cultures with those in N- and C-limited defined liquid media thus implies a transition from C limitation (early time points) to conditions of N limitation (later samples). However, *lipC* transcripts, which are essentially absent in C-limited liquid cultures (49), were detected in the early phases of this study, although not at maximal levels. Conversely, *lipD* mRNA, the level of which is approximately 100-fold less under N- versus C-limited liquid culture conditions (27, 49), is not entirely absent from

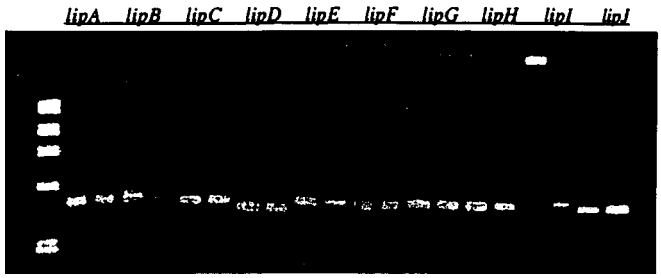

FIG. 2. PCR amplification of allelic variants of *lip* genes from single-basidiospore isolates with primers shown in Fig. 3. The first lane of each pair contains PCR products of strain SB48; SB40 products are the second of each pair. Length dimorphism observed in *lipI* products from the two strains is due to the presence of the *pce1* insertion within the *lipI2* allele (19) in strain SB40. Lane 1, *Hae*III-digested ΦX174 DNA; the molecular sizes of these bands are 1,353, 1,078, 872, 603, 310, and 281 bp.

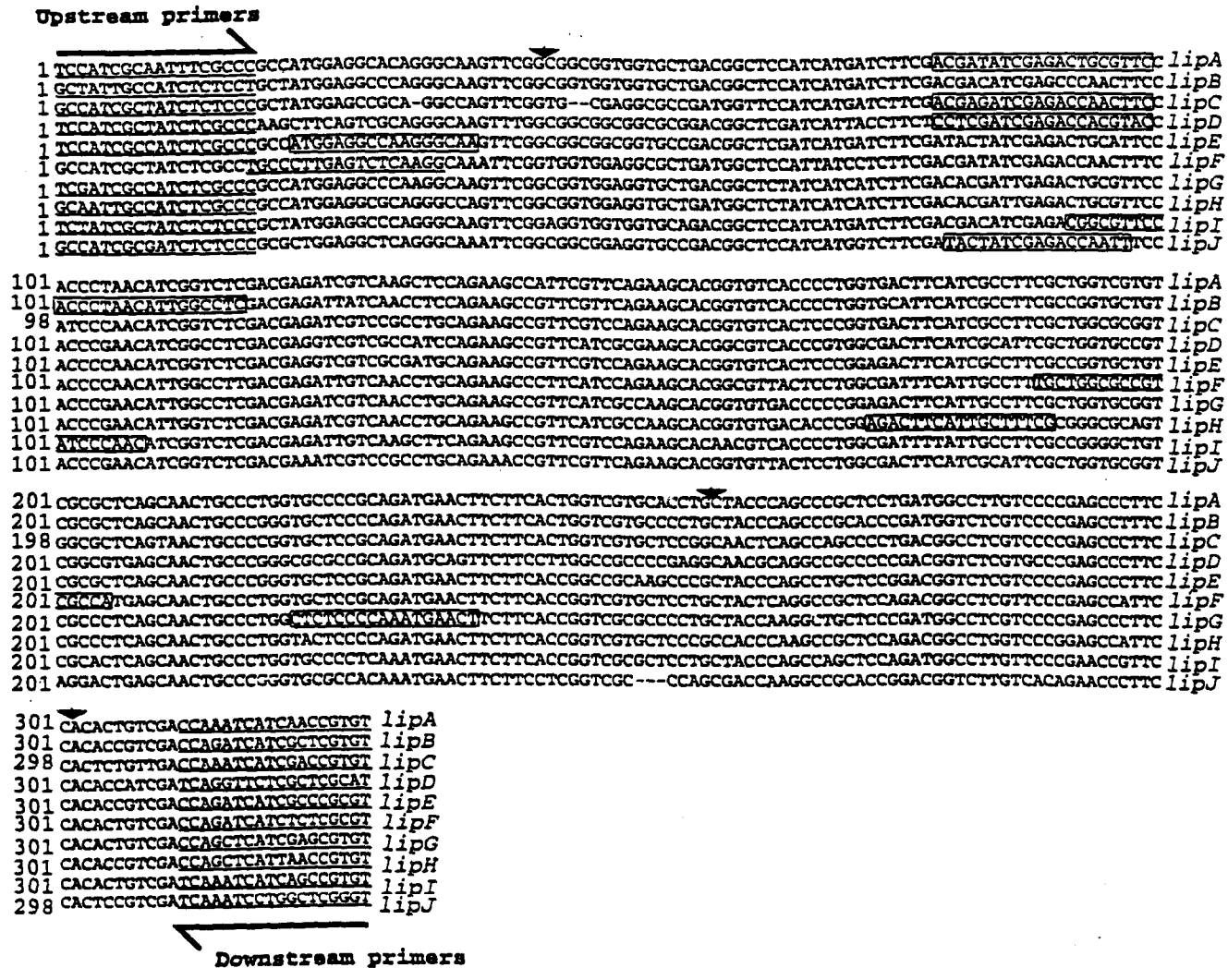

FIG. 3. Partial sequence alignment of the 10 known *P. chrysosporium lip* coding regions. Gene-specific PCR primers and probes are underlined and boxed, respectively. Vertical arrows, positions of introns 3, 4, and 5 of the *lipA* gene.

later time points in the present study. This apparent intermediacy of our present results between extremes of N and C limitation most likely reflects the heterogeneity of the soil-spawn matrix, with hyphae in different microenvironment simultaneously encountering different nutrient limitations.

Transcript abundance patterns in anthracene-amended soil differ somewhat from those in PCP-amended soil cultures (34). Transcripts of *lipD* and *lipE* were not detected in PCP cultures on days 2 and 4, although this may merely reflect slower growth and delayed expression in the presence of PCP. Possibly more significant is the relative abundance of *lipC* versus *lipA* observed in PCP cultures. In contrast to the pattern of *lipC* >> *lipA* found by Lamar et al. (34) in PCP cultures on day 4, the present study found *lipA* >> *lipC* at all early time points. It remains uncertain whether these observed alterations in expression patterns are also due to a growth-mediated difference in nutrient deficiency (as above) or whether the expression patterns are the direct result of the particular organopollutant.

The least prominent transcript of the 10 *lip* genes appears to be that of *lipF*. mRNA from *lipF* was not detected in a 3-day-old spawn-grown *P. chrysosporium* mycelium (Fig. 4); furthermore, its presence in soil mRNA extracts is inconsistent, and levels of mRNA transcript are generally far below those of gDNA contaminant (data not shown). Inasmuch as *lip* mRNA levels of approximately 10^{-10} to 10^{-11} pmol per sample can be detected without gDNA interference, the *lipF* transcript level, even if it is nonzero, must be less than this value. Interestingly, the *lipF* gene resides on a small (2.0-Mbase) chromosome,

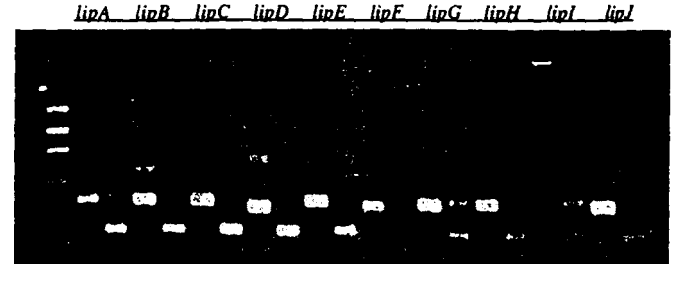

FIG. 4. Products of PCR and RT-PCR amplification of *P. chrysosporium lip* genes from gDNA and mRNA. Within the pair of samples for each gene, the first lane contains the gDNA-derived PCR product; the products in the second lane of each pair are the cDNA-derived products of RT-PCR of mRNAs isolated from spawn-grown cultures. All amplifications used the competitive primers shown in Fig. 3. Lane 1, *Hae*III-digested ΦX174.

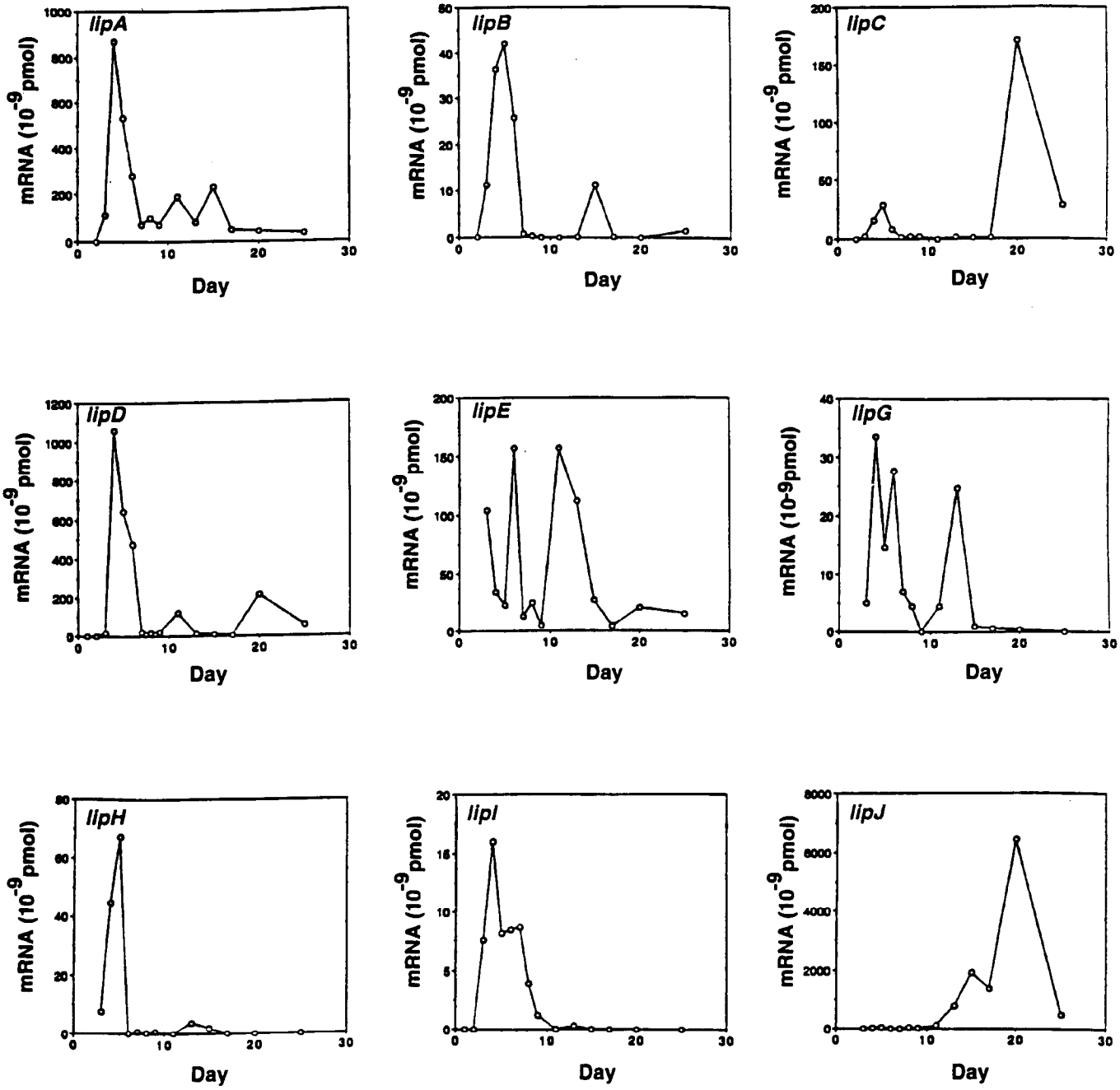

FIG. 5. Time course of *lip* transcripts among mRNA extracted from anthracene-transforming Marshan sandy loam soil cultures of *P. chrysosporium*.

unlinked to other known genes involved in ligninolysis (13, 18). In contrast to *lipF*, many other ligninolytic and cellulolytic genes are tightly linked within the *P. chrysosporium* genome (7, 17, 18, 42). Thus, there may be a relationship between the genomic organization and transcriptional regulation of *lipF*.

Transcript levels of *lipA* and *glx* correlated with extractable LiP H8 and GLOX proteins, as measured by Western blotting (Fig. 6); the relationship observed between *lipA* mRNA titers and levels of LiP H8 protein was particularly good and evidenced the same ca.-2-day lag period seen in previous liquid culture and solid substrate studies. Previous work with liquid culture has shown a correlation between levels of several *lip* transcripts and their corresponding isozymes, measured, respectively, by Northern (RNA) blotting and fast-protein liquid chromatography (4, 5, 38), with a lag (~1 day) frequently observed between peaks in transcript and protein. Liquid culture results with GLOX also showed a temporal correlation between *glx* transcript abundance and enzyme activity (29). Analysis of *mnp* transcripts and MnP activity in soil showed a similar relationship, with maximal enzyme activity delayed 1 to 2 days relative to the peak in *mnp* mRNA (2). These latter findings, coupled with the results presented here, validate competitive RT-PCR methodology by demonstrating definite relationships between mRNA titers measured in solid substrates (i.e., soil) and actual protein and/or activity levels.

Oxidation of anthracene took place throughout the course of this experiment. This is in direct contrast with oxidation of fluorene (2, 20) and depletion of chrysene (2), both of which

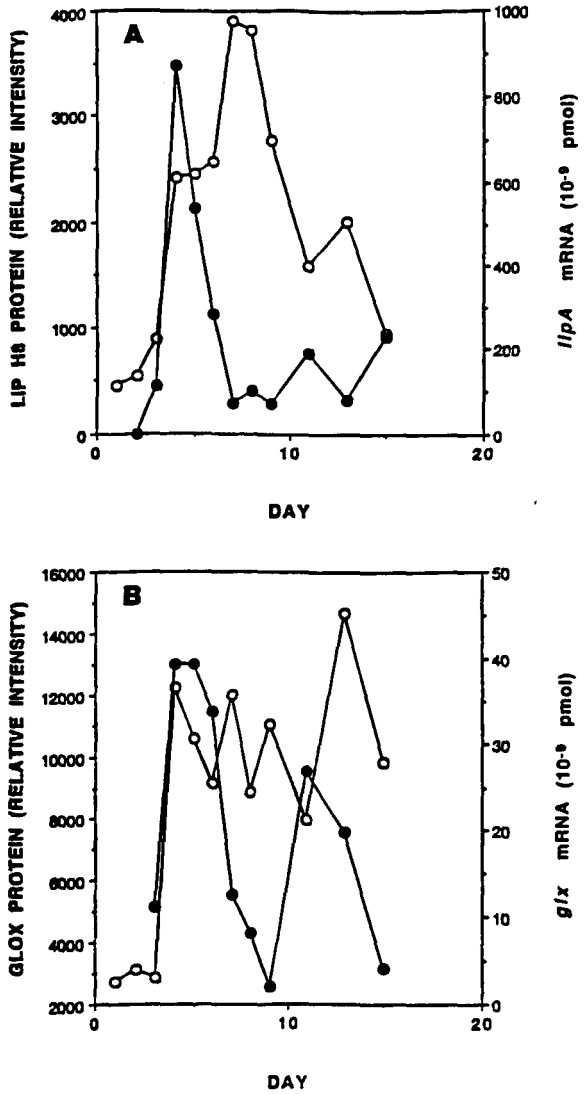

FIG. 6. Correlation between levels of transcript and protein for *lipA* (LiP H8) (A) and *glx* (GLOX) (B). ●, transcripts (*lipA* and *glx*); ○, protein (LiP H8 and GLOX).

were restricted to the first 6 to 7 days of fungal growth. This difference most likely reflects the different pathways by which high- and low-ionization potential PAHs are oxidized. The results with fluorene and chrysene were hypothesized (2) to be due to the depletion of some component of the MnP-based lipid peroxidation system (1, 36), which is believed to play a major role in the oxidation of non-LiP substrate PAHs. Anthracene, on the other hand, is an LiP substrate (26), and its transformation would be expected to require only the presence of LiP (transcripts for which are present throughout) and hydrogen peroxide.

Monitoring the physiological state of microbes in situ provides a useful tool to help ensure bioremediation performance. RT-PCR-based approaches have been applied to *Pseudomonas putida* in bioreactors (47) and during remediation of naphthalene-contaminated soils (16). Methods which employ this type of mRNA analysis have distinct advantages over other approaches used with white-rot fungi, such as quantitation of ergosterol (11) or PCR-based quantitation of fungal DNA (28). First, tailoring of PCR primers allows a species specificity not possible with ergosterol measurements. Second, and more importantly, mRNA quantitation data are superior to both of the methods described above in that they provide insight into the physiological status of the fungus, rather than merely quantifying biomass, and thus result in a more informative monitoring of the remediation process. In summary, the RT-PCR methodology detailed in this paper allows for monitoring expression of all 10 members of the *lip* gene family during growth of *P. chrysosporium* in a soil-spawn system.

The methods described here are readily adaptable to a variety of genes and substrates. For example, we have recently identified the same nine *lip* transcripts quantified herein in nonsterile soil collected from a hazardous waste site at Brookhaven, Miss. (31, 32), following inoculation with *P. chrysosporium*-colonized spawn. No transcripts were detected from noninoculated soil. Together with similar methods for measuring *mnp* gene expression (2), they allow for a more accurate assessment of the physiological state of *P. chrysosporium* during soil remediation efforts. Extension of these methods to other substrates (i.e., fungus-colonized wood chips during biological pulping) is currently under way in our laboratory.

ACKNOWLEDGMENTS

We thank J. Gaskell and A. Vanden Wymeienberg for *lipE*, *lipF*, and *glx* competitive templates and P. Stewart and T. K. Kirk for helpful criticism of the manuscript.

B.W.B. was supported by a predoctoral fellowship from the Howard Hughes Medical Institute. The research herein was funded in part by Department of Energy grant DE-FG02-87ER13712 (to D. C.) and U.S. Environmental Protection Agency cooperative agreement DW12931791-01-8 (to R.T.L.).

REFERENCES

1. **Bogan, B. W., and R. T. Lamar.** 1995. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **61:**2631–2635.
2. **Bogan, B. W., B. Schoenike, R. T. Lamar, and D. Cullen.** 1996. Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **62:**2381–2386.
3. **Bollag, J.-M., C.-M. Chen, J. M. Sarkar, and M. J. Loll.** 1987. Extraction and purification of a peroxidase from soil. Soil Biol. Biochem. **19:**61–67.
4. **Boominathan, K., T. M. D'Souza, P. S. Naidu, C. Dosoretz, and C. A. Reddy.** 1993. Temporal expression of the major lignin peroxidase genes of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**3946–3950.
5. **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.
6. **Bumpus, J. A.** 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55:**154–158.
7. **Covert, S. F., A. Vanden Wymelenberg, and D. Cullen.** 1992. Structure, organization and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**2168–2175.
8. **Dass, S. B., and C. A. Reddy.** 1990. Characterization of extracellular peroxidases produced by acetate-buffered cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **69:**221–224.
9. **Datta, A., A. Bettermann, and T. K. Kirk.** 1991. Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl. Environ. Microbiol. **57:**1453–1460.
10. **Davis, M. W., J. A. Glaser, J. W. Evans, and R. T. Lamar.** 1993. Field evaluation of the lignin-degrading fungus *Phanerochaete sordida* to treat creosote-contaminated soil. Environ. Sci. Technol. **27:**2572–2576.
11. **Davis, M. W., and R. T. Lamar.** 1992. Evaluation of methods to extract ergosterol for quantitation of fungal biomass. Soil Biol. Biochem. **24:**189–198.
12. **Dosoretz, C. G., and H. E. Grethlein.** 1991. Physiological aspects of the regulation of extracellular enzymes of *Phanerochaete chrysosporium*. Appl. Biochem. Biotechnol. **28/29:**253–265.
13. **D'Souza, T. M., S. B. Dass, A. Rasooly, and C. A. Reddy.** 1993. Electrophoretic karyotyping of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Mol. Microbiol. **8:**803–807.
14. **Farrell, R. L., K. E. Murtaugh, M. Tien, M. D. Mozuch, and T. K. Kirk.** 1989. Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **11:**322–328.

15. **Field, J. A., H. Feiken, A. Hage, and M. J. J. Kotterman.** 1995. Application of a white-rot fungus to biodegrade benzo(*a*)pyrene in soil. p. 165–171. *In* R. E. Hinchee, J. Fredrickson, and B. C. Alleman (ed.), Bioaugmentation for site remediation. Battelle Press, Columbus, Ohio.
16. **Fleming, J. T., J. Sanseverino, and G. S. Sayler.** 1993. Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. **27:**1068–1074.
17. **Gaskell, J., E. Dieperink, and D. Cullen.** 1991. Genomic organization of lignin peroxidase genes of *Phanerochaete chrysosporium*. Nucleic Acids Res. **19:**599–603.
18. **Gaskell, J., P. Stewart, P. J. Kersten, S. F. Covert, J. Reiser, and D. Cullen.** 1994. Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. Bio/Technology **12:**1372–1375.
19. **Gaskell, J., A. Vanden Wymelenberg, and D. Cullen.** 1995. Structure, inheritance and transcriptional effects of *pce1*, and insertional element found within *Phanerochaete chrysosporium* lignin peroxidase gene *lipI*. Proc. Natl. Acad. Sci. USA **92:**7465–7469.
20. **George, E. J., and R. D. Neufeld.** 1989. Degradation of fluorene in soil by fungus *Phanerochaete chrysosporium*. Biotechnol. Bioeng. **33:**1306–1310.
21. **Gilliland, G., S. Perrin, and H. F. Bunn.** 1990. Competitive PCR for quantitation of mRNA, p. 60–69. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
22. **Glumoff, T., P. J. Harvey, S. Molinari, M. Goble, G. Frank, J. M. Palmer, J. D. G. Smit, and M. S. A. Leisola.** 1990. Lignin peroxidase from *Phanerochaete chrysosporium*: molecular and kinetic characterization of isozymes. Eur. J. Biochem. **187:**515–520.
23. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo(*a*)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. Veratryl alcohol and stability of ligninase. J. Biol. Chem. **261:**6900–6903.
24. **Hammel, K. E., W. Z. Gai, B. Green, and M. A. Moen.** 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**1832–1838.
25. **Hammel, K. E., B. Green, and W. Z. Gai.** 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA **88:**10605–10608.
26. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **261:**16948–16952.
27. **Holzbaur, E., and M. Tien.** 1988. Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **155:**626–633.
28. **Johnston, C. G., and S. D. Aust.** 1994. Detection of *Phanerochaete chrysosporium* in soil by PCR and restriction enzyme analysis. Appl. Environ. Microbiol. **60:**2350–2354.
29. **Kersten, P. J., and D. Cullen.** 1993. Cloning and characterization of a cDNA encoding glyoxal oxidase, a H_2O_2-producing enzyme from the lignin-degrading fungus *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. USA **90:**7411–7413.
30. **Kirk, T. K., S. Croan, M. Tien, K. E. Murtaugh, and R. L. Farrell.** 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8:**27–32.
31. **Lamar, R. T., M. W. Davis, D. M. Dietrich, and J. A. Glaser.** 1994. Treatment of a pentachlorophenol- and creosote-contaminated soil using the lignin-degrading fungus *Phanerochaete sordida*: a field demonstration. Soil Biol. Biochem. **26:**1603–1611.
32. **Lamar, R. T., J. W. Evans, and J. A. Glaser.** 1993. Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degrading fungi. Environ. Sci. Technol. **27:**2566–2571.
33. **Lamar, R. T., M. J. Larsen, and T. K. Kirk.** 1990. Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp. Appl. Environ. Microbiol. **56:**3519–3526.
34. **Lamar, R. T., B. Schoenike, A. Vanden Wymelenberg, P. Stewart, D. M. Dietrich, and D. Cullen.** 1995. Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. Appl. Environ. Microbiol. **61:**2122–2126.
35. **Millis, C. D., D. Cai, M. T. Stankovich, and M. Tien.** 1989. Oxidation-reduction potentials and ionization states of extracellular peroxidases from the lignin-degrading fungus *Phanerochaete chrysosporium*. Biochemistry **28:**8484–8489.
36. **Moen, M. A., and K. E. Hammel.** 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60:**1956–1961.
37. **Morgan, P., S. A. Lee, S. T. Lewis, A. N. Sheppard, and R. J. Watkinson.** 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. **25:**279–287.
38. **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.
39. **Orth, A., D. Royse, and M. Tien.** 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl. Environ. Microbiol. **59:**4017–4023.
40. **Pannetier, C., S. Delassus, S. Darche, C. Saucier, and P. Kourilsky.** 1993. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. Nucleic Acids Res. **21:**577–583.
41. **Qiu, X., and M. J. McFarland.** 1991. Bound residue formation in PAH contaminated soil composting using *Phanerochaete chrysosporium*. Hazard. Waste Hazard. Mater. **8:**115–126.
42. **Raeder, U., W. Thompson, and P. Broda.** 1989. RFLP-based genetic map of *Phanerochaete chrysosporium* ME446: lignin peroxidase genes occur in clusters. Mol. Microbiol. **3:**911–918.
43. **Reiser, J., I. S. Walther, C. Fraefel, and A. Fiechter.** 1993. Methods to investigate the expression of lignin peroxidase genes by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**2897–2903.
44. **Ritch, T. G., and M. H. Gold.** 1992. Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chrysosporium*. Gene **118:**73–80.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Plainview, N.Y.
46. **Sanglard, D., M. S. A. Leisola, and A. Fiechter.** 1986. Role of extracellular ligninases in biodegradation of benzo(*a*)pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209–212.
47. **Selvaratnam, S., B. A. Schoedel, B. L. McFarland, and C. F. Kulpa.** 1995. Application of reverse transcriptase PCR for monitoring expression of the catabolic *dmpN* gene in a phenol-degrading sequencing batch reactor. Appl. Environ. Microbiol. **61:**3981–3985.
48. **Sinclair, R., B. Copeland, I. Yamazaki, and L. Powers.** 1995. X-ray absorption spectroscopy comparison of the active site structures of *Phanerochaete chrysosporium* lignin peroxidase isoenzymes H2, H3, H4, H5, H8 and H10. Biochemistry **34:**13176–13182.
49. **Stewart, P., P. Kersten, A. Vanden Wymelenberg, J. Gaskell, and D. Cullen.** 1992. Lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation and identification of a second dimorphic chromosome. J. Bacteriol. **174:**5036–5042.
50. **Stroo, H. F., M. A. Jurek, J. A. Bumpus, M. F. Torpy, and S. D. Aust.** 1989. Bioremediation of wood preserving wastes using the white rot fungus *Phanerochaete chrysosporium*. Proc. Am. Wood-Preserv. Assoc. **85:**236–243.
51. **Urzua, U., L. F. Larrondo, S. Lobos, J. Larrain, and R. Vicupa.** 1995. Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. FEBS Lett. **371:**132–136.
52. **U.S. Environmental Protection Agency.** 1986. Test methods for evaluating solid waste, SW-846. Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency, Washington, D.C.
53. **Vares, T., M. Kalsi, and A. Hatakka.** 1995. Lignin peroxidases, manganese peroxidases, and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation of wheat straw. Appl. Environ. Microbiol. **61:**3515–3520.
54. **Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak.** 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl. Environ. Microbiol. **60:**459–466.