

Manganese Peroxidase mRNA and Enzyme Activity Levels during Bioremediation of Polycyclic Aromatic Hydrocarbon-Contaminated Soil with *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, USDA Forest Service Forest Products Laboratory, Madison, Wisconsin 53705²

Received 7 February 1996/Accepted 29 April 1996

mRNA extraction from soil and quantitation by competitive reverse transcription-PCR were combined to study the expression of three manganese peroxidase (MnP) genes during removal of polycyclic aromatic hydrocarbons from cultures of *Phanerochaete chrysosporium* grown in presterilized soil. Periods of high *mnp* transcript levels and extractable MnP enzyme activity were temporally correlated, although separated by a short (1- to 2-day) lag period. This time frame also coincided with maximal rates of fluorene oxidation and chrysene disappearance in soil cultures, supporting the hypothesis that high ionization potential polycyclic aromatic hydrocarbons are oxidized in soil via MnP-dependent mechanisms. The patterns of transcript abundance over time in soil-grown *P. chrysosporium* were similar for all three of the *mnp* mRNAs studied, indicating that transcription of this gene family may be coordinately regulated under these growth conditions.

The white rot basidiomycete *Phanerochaete chrysosporium* has been extensively studied for possible use in the bioremediation of contaminated soils. For example, biochemical data (1, 19, 22, 30, 47), liquid-culture studies (1, 5, 20, 21, 40), and bench scale solid-phase experiments (15, 31, 37, 43) have demonstrated the ability of *P. chrysosporium* and its extracellular enzymes to degrade numerous polycyclic aromatic hydrocarbons (PAHs), such as those found in creosotes and coal tars. Similar results have been obtained in field trials (9, 26) of a related species, *P. sordida*. *P. chrysosporium* initiates PAH degradation by free-radical mechanisms: PAHs with ionization potentials at or below a cutoff of approximately 7.55 eV are substrates for direct one-electron oxidation by lignin peroxidase (LiP) (22), whereas those with ionization potentials above this threshold are apparently acted upon by radical species formed during manganese peroxidase (MnP)-dependent lipid peroxidation reactions (1, 2, 30).

The biochemistry and molecular genetics underlying the ligninolytic systems of *P. chrysosporium* are quite complex. Multiple LiP isozymes are produced, and the genome of *P. chrysosporium* contains at least 10 structurally related genes encoding LiP proteins. These genes have been designated *lipA* through *lipJ* (14). Similarly, several MnP isozymes (designated H3, H4, and H5) are produced in submerged culture, and three *mnp* genes from *P. chrysosporium* have been characterized. The designations used herein for the three *mnp* genes are as follows: *mnp-1* is the H4-encoding *MP-1* gene of Pease et al. (35), and *mnp-3* is *MP-2*, which encodes the H3 isoform (32). Our *mnp-2* corresponds to the gene isolated from strain ME446 by Godfrey et al. (18), who referred to it as *mnp1*; the protein product of this gene is unknown. The reason for the multiplicity of *lip* and *mnp* genes is unclear, although some substrate range and kinetic differences have been observed among the different LiP (17) and MnP (36) isozymes.

Information concerning patterns of enzyme expression in white rot fungi has been confined almost entirely to chemically defined liquid media; extensive data concerning LiP and MnP isozyme profiles have been gathered from liquid cultures at the levels of both protein (4, 7, 11, 25) and mRNA transcripts (4, 42). Very little data exist at either level for white rot fungi grown on solid substrates. MnP has been recovered from *P. chrysosporium* grown on aspen pulp (8), *Ceriporiopsis subvermispora* cultured on wood chips (44), and *Bjerkandera adusta*-colonized soil (12). A recent report (46) detailed the purification of several LiP and MnP isoforms, as well as the H₂O₂-producing enzyme glyoxal oxidase, from wheat straw-grown *Phlebia radiata*. Purification of proteins from solid substrates, however, has frequently been impeded by low yield and interference from contaminating substances; this has hampered efforts to investigate the patterns of enzyme expression in complex substrates. Inasmuch as one such substrate, fungus-colonized soil, is the medium most relevant to bioremediation, it is important to circumvent this problem.

Recent advances in competitive reverse transcription (RT)-coupled PCR analysis of mRNAs from *P. chrysosporium* (6, 28, 42) and in extraction and purification of mRNA from fungus-colonized soils (28) can be combined to allow investigation of the physiological state of fungi during growth in soil. Initial application of these techniques to pentachlorophenol-contaminated soil colonized by *P. chrysosporium* (28) detected transcripts corresponding to two LiPs (*lipA* and *lipC*), two cellobiohydrolases (*cbh1-4* and *cbh1-1*), and the mitotic spindle protein β -tubulin (*tub*). In this study, we examined the expression of *mnp* genes during a bench scale PAH soil bioremediation experiment. *P. chrysosporium* cultured in soil caused transformation of fluorene and disappearance of chrysene, both substrates for MnP-dependent lipid peroxidation (1, 2), during the early phase of fungal colonization. Transcripts from three *mnp* genes were present during this time. Quantitative RT-PCR analyses were performed on these and older soil cultures to study the temporal regulation of *mnp* gene expression in soil. Potential housekeeping mRNAs were investigated to ensure that mRNA extraction efficiency was uniform in all sam-

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

TABLE 1. Primers and probes used for competitive PCR of cDNA products, cloning of full-length gDNA copies for template construction, and Southern blot hybridization for primer specificity experiments

Gene	Primer used	5' Primer	3' Primer	Probe
<i>mnp-1</i>	Competitive PCR	CAG ACG GTA CCC GCG TCA CC	AGT GGG AGC GGC GAC ATC AC	CCA GAG CCT AGG TCC TC
	Full-length gDNA	GCA ATG GCC TTC GGT TCT CT	TTA GGC AGG GCC ATC GAA CT	
<i>mnp-2</i>	Competitive PCR	CCG ACG GCA CCC GCG TCA GC	CGA GCG GGA GCG GCG ACG CC	TCG CTC CCA GGG CCC CA
	Full-length gDNA	CAG ATG GCC TTC AAG TCC CT	TTA TGC GGG ACC GTT GAA CT	
<i>mnp-3</i>	Competitive PCR	CCG ACG GTA CCA AGG TCA AC	AGC GGC AGC GGC GAC GCG AC	GCA AAG CAA GGG ACC GA
	Full-length gDNA	GCA CTC AAG CCA GCG CAA TG	TGT CCG GCG CGT CAG ACT TA	
<i>tub</i>	Competitive PCR	AGG TCG TCT CAG ACG AAC ACG	GAT CAG GAG AGT ACC CAT GCC	
	Full-length gDNA	AAT CGA CGT GCT GAC GT	CGT GCA GTG ATG ACA TG	
<i>gpd</i>	Competitive PCR	CGT ATC GTC CTC CGT AAT GC	ACT CGT TGT CGT ACC AGG AG	
	Full-length gDNA	ATG CCG GTC AAA GCA GGA AT	TTA GAG GGC ACC GTC GAC CT	

ples. Lastly, this method of analysis was validated by demonstrating a temporal correlation between *mnp* transcript abundance and extractable MnP activity.

MATERIALS AND METHODS

Chemicals. [9-¹⁴C]fluorene (11.3 mCi/mmol, >98% radiochemical purity) was purchased from Sigma (St. Louis, Mo.). All of the other chemicals used were of the highest commercially available grade. Solvents employed for PAH extraction and chromatography were high-performance liquid chromatography (HPLC) grade. The buffers used for mRNA extraction and purification were treated with diethyl pyrocarbonate as described by Sambrook et al. (39) before use.

Fungi. Stock cultures of *P. chrysosporium* Burds. 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 (27) and stored at 4°C until use. Spores were scraped from three or four such slants and used to inoculate 200 g (dry weight; 60% moisture) of a nutrient-fortified grain-sawdust mixture (spawn mixture), obtained from L. F. Lambert Spawn Co. (Coatesville, Pa.), in 1-quart (0.946-liter) glass jars with lids containing gas-permeable membranes as previously described (9). Following 2 to 3 days of growth, the colonized spawn mixture was used as the inoculum for soil cultures as described below.

Soil cultures. Sieved Marshan sandy loam (27) was weighed (25-g dry weight) into half-pint (0.2365-liter) glass jars equipped with gas-permeable membranes. Soil was sterilized by autoclaving (1 h at 121°C) on each of 3 consecutive days. Following sterilization, soil was spiked with a PAH (fluorene or chrysene) to the desired concentration; PAHs were added as a stock solution in approximately 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 the *P. chrysosporium*-colonized Lambert spawn mixture. Cultures were maintained at 39°C, and the moisture content was maintained by periodic watering as necessary. Cultures which were to receive [¹⁴C]fluorene contained 3 g (dry weight) of sterilized Marshan soil (spiked, watered, and inoculated in the same manner as larger cultures) and were incubated in capless 20-ml borosilicate glass scintillation vials inside half-pint (0.2365-liter) jars. [¹⁴C]fluorene (50,000 dpm) and unlabeled fluorene (500 ppm) were added (in 50 μl of CH₂Cl₂) on day 2 or 8. Following 4 weeks of incubation, PAHs were extracted from entire cultures as described below for analysis.

PAH extraction. Extraction of PAHs from soil involved a modified version of Environmental Protection Agency method 3550 (45) as previously described (9, 26). Soil (2 to 3 g) was mixed with 6 to 7 g of anhydrous Na₂SO₄ and stored frozen prior to extraction. Samples were extracted by sonication (2 min) in the presence of 20 ml of acetone-methylene chloride (1:1, vol/vol). Soil was allowed to settle for 2 to 3 h, and the supernatant was decanted through glass fiber filters. Following a second sonication cycle, 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 N₂ in a TurboVap ZW700 (Zymark, Hopkinton, Mass.) and redissolved in 5 to 10 ml of acetonitrile for HPLC.

Ergosterol extraction. Total neutral-extractable ergosterol was extracted as described by Davis and Lamar (10). Five-gram soil samples were extracted by overnight shaking with 25 ml of methanol-hexane (4:1). Following vacuum filtration and rinsing of soil (5 ml of methanol, 5 ml of hexane), extracts were saponified by addition of 2.5 g of KOH and shaken overnight. After addition of 5 ml of H₂O, tubes were vigorously shaken; following clarification of the aqueous layer, the hexane phase was removed. Three additional 5-ml hexane extractions were performed. Extracts were evaporated to dryness under N₂ and redissolved in 0.5 ml of methanol for quantitation by HPLC.

HPLC. All HPLC analyses employed a Vydac 201TP54 (25 by 0.46 cm) C₁₈ reverse-phase column (Nest Group, Southboro, Mass.). The column temperature was maintained at 35°C for PAH separation and 45°C for ergosterol quantitation.

The HPLC 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 absorbance of the column eluent was measured at 254 nm. PAH concentrations were determined by comparison with four-point standard curves ($r \geq 0.998$) for each compound. [¹⁴C] profiles were generated by passing column eluent through a Flo-One radiochromatography detector (Packard Instrument Co., Downers Grove, Ill.) operated in TR-LSC mode with Flo-Scint V (5-ml min⁻¹ flow rate) scintillation cocktail (Packard). The counts per minute thus obtained were converted to disintegrations per minute by applying a quench curve in accordance with the manufacturer's specifications.

Ergosterol was eluted with methanol (0.75 ml min⁻¹) and monitored at 282 nm. Soil-extracted ergosterol coeluted (retention time, 15 min) with an authentic standard and was quantitated by using a three-point standard curve (10 to 200 μg ml⁻¹, $r = 0.999$).

mRNA extraction. Magnetic capture techniques were used for rapid purification of poly(A) RNA. Extraction and purification of mRNA from fungal soil cultures were done as previously reported (28) and modified as necessary for scale up. Colonized soil or spawn (10 g) from cultures was wrapped in Miracloth (Calbiochem-Novabiochem Corp., La Jolla, Calif.) and snap-frozen in liquid N₂.

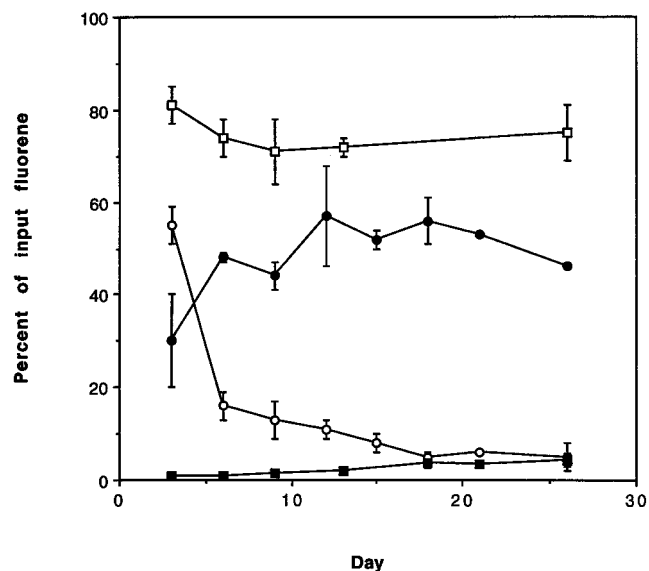


FIG. 1. Disappearance of fluorene at 500 ppm (open symbols) and conversion to 9-fluorenone (filled symbols) in control soil cultures (□ and ■) and those inoculated (○ and ●) with *P. chrysosporium*. Reported values correspond to averages of two replicate cultures; error bars represent one standard deviation.

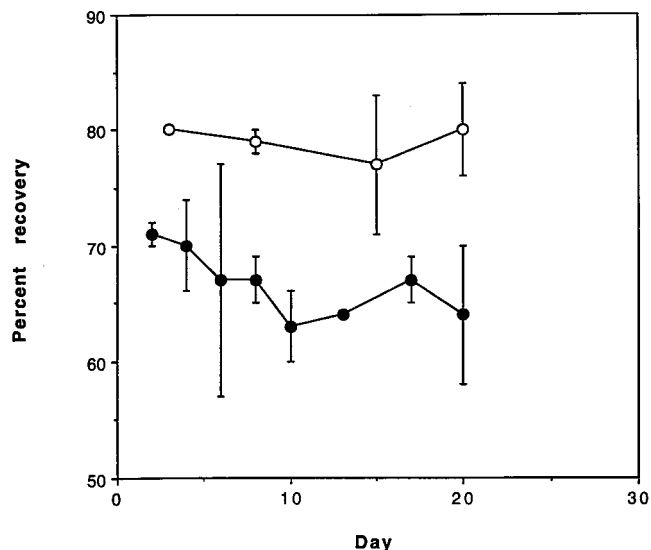


FIG. 2. Disappearance of chrysene (120 ppm) from control soil cultures (○) and those inoculated (●) with *P. chrysosporium*. Values are averages of two replicate cultures; error bars represent one standard deviation.

Frozen samples were ground with a mortar and pestle, which were kept chilled in dry ice. The mRNA extraction buffer (4 M guanidinium thiocyanate, 0.1 M Trizma base, 1% dithiothreitol, 0.5% Sarkosyl [pH 8.0]) was added in five 2-ml aliquots (seven for spawn-grown cultures) with grinding. The resultant frozen powder was allowed to thaw, and the resultant slurry was centrifuged in a swinging-bucket rotor (500 × g) for 8 min. Following a second centrifugation step, the supernatant (≈7 ml) was mixed with 2 volumes of binding buffer (0.1 M Trizma 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 into a second tube and mixed with 1.5 mg of oligo(dT)₂₅ Dynabeads (DynaL, Great Neck, N.Y.), which had been previously washed in binding buffer. Following 30-min of hybridization on ice, Dynabeads were isolated with an MPC-1 magnetic concentrator (DynaL) for 5 min, the supernatant was removed, and the Dynabeads were resuspended in 500 μl of washing buffer (10 mM Trizma 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 by using the smaller MPC-E-1 magnet (DynaL). Elution of mRNA was done by heating Dynabeads (65°C) in 200 μl of 2 mM EDTA (pH 8.0). The mRNA-containing elution buffer was mixed with 20 μl of 3 M sodium acetate (pH 5.2) and 400 μl of ethanol and stored at -20°C. This procedure yielded sufficient mRNA for approximately 200 RT-PCRs in approximately 1/10 of the time of the previously reported (28) protocol. Regeneration of Dynabeads with 0.1 M NaOH was performed in accordance with the manufacturer's instructions; RT-PCR of material "eluted" from Dynabeads immediately after regeneration showed that no mRNA carryover occurred (data not shown).

Transcript detection and quantitation by RT-PCR. All RT-PCRs were performed with a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, Conn.). Each reaction mixture contained 3 μl of the final mRNA preparation described above; this represented mRNA recovered from a ca. 0.05-g soil sample. RT reactions were prepared as previously described (28, 42) and were primed with oligo(dT) 15-mers. PCRs (100 μl) contained 1.25 U of *Taq* DNA polymerase and 21 pmol of each primer (see Table 1). The PCR temperature program was 94°C for 6 min, 54°C for 2 min, and 72°C for 40 min for 1 cycle, followed by 94°C for 1 min, 54°C for 2 min, 72°C for 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 (16, 28, 42). To prepare templates, gDNA was PCR amplified with primers encompassing the translational start and stop codons (Table 1). These full-length products were directly subcloned into plasmid pCRII (Invitrogen, San Diego, Calif.). All *mnp* competitive templates thus contained full-length genomic *mnp* sequences. Southern blot analysis with gene-specific oligonucleotide probes (Table 1) ensured the specificity of each competitive PCR primer pair for its target *mnp* sequence; no cross-hybridization of any probes with nontarget PCR products was observed (data not shown).

Transcripts of mRNAs corresponding to β-tubulin (*tub*) (38) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) were examined as potential housekeeping genes. Competitive templates for *tub* and *gpd* sequences were constructed in the same manner as those for the *mnp* genes; primers used for cloning and competitive PCR amplification of *tub* and *gpd* sequences are given in Table 1.

PCR products were analyzed by electrophoresis on 1% SeaKem GTG agarose

gels stained with ethidium bromide. Gels were visualized with UV light and photographed with a Foto/Analyst Visionary Benchtop Digital Documentation Station (Fotodyne, Hartland, Wis.). The resulting images were digitized, and band intensities were determined with National Institutes of Health 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 1.3 (*mnp* genes), 1.68 (*tub*), or 1.19 (*gpd*) was taken as the equivalence point.

MnP extraction and assay. MnP activity was recovered from *P. chrysosporium* soil cultures by the procedure of Bollag et al. (3), with minor modifications. 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 polysaccharides; 10-ml aliquots were then dialyzed against 5 mM sodium acetate (pH 6.0) prior to assaying for MnP. Vanillyl acetone was used as the enzyme substrate (34).

RESULTS

PAH disappearance and transformation. Soil cultures of *P. chrysosporium* were grown in Marshan sandy loam artificially contaminated with fluorene at an initial concentration of 500 ppm. The fluorene concentration in these cultures decreased rapidly to approximately 30 ppm in 6 days, followed by a more gradual rate of disappearance (Fig. 1). Fluorene levels in *P. chrysosporium*-inoculated soil declined to roughly 10 ppm by day 27. The primary extractable by-product of fluorene oxidation was the ketone 9-fluorenone. Conversion to 9-fluorenone accounted for ca. 50% of the input fluorene by day 12, after which time 9-fluorenone concentrations leveled off (Fig. 1). Accumulation of 9-hydroxyfluorene (2) was insignificant (data not shown). Recovery of fluorene in 500-ppm noninoculated controls remained at or slightly above 80% for the duration of the experiment.

Similar results were obtained from experiments examining chrysene disappearance from soil cultures. In cultures containing chrysene at an initial concentration of 120 ppm, disappearance took place primarily during the first 10 days of fungal growth, followed by a decline in the apparent rate of depletion (Fig. 2).

To determine whether the decrease in observed PAH trans-

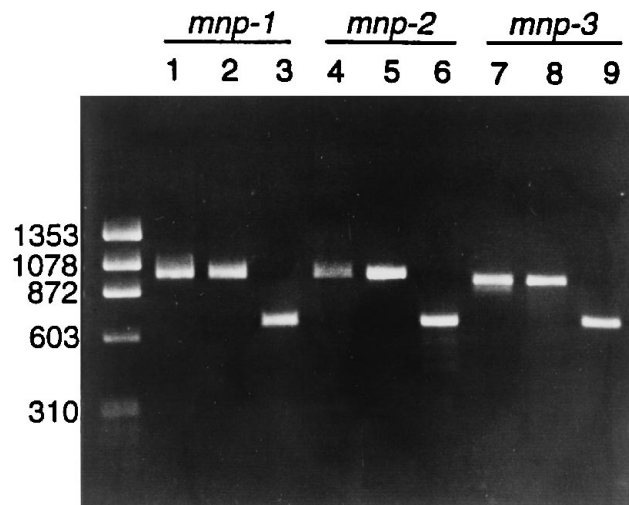


FIG. 3. Products of PCR and RT-PCR of *mnp* genes and transcripts with competitive PCR primers (Table 1). Lanes 1, 4, and 7, products derived by PCR amplification of *P. chrysosporium* genomic DNA; 2, 5, and 8, corresponding treatment of *mnp* competitive templates. Samples in lanes 3, 6, and 9 were generated by RT-PCR of poly(A) RNA extracted from a *P. chrysosporium*-colonized Lambert spawn mixture. The leftmost lane contained *Hae*III-digested ϕ X174 molecular size markers, and sizes of bands (base pairs) are on the left.

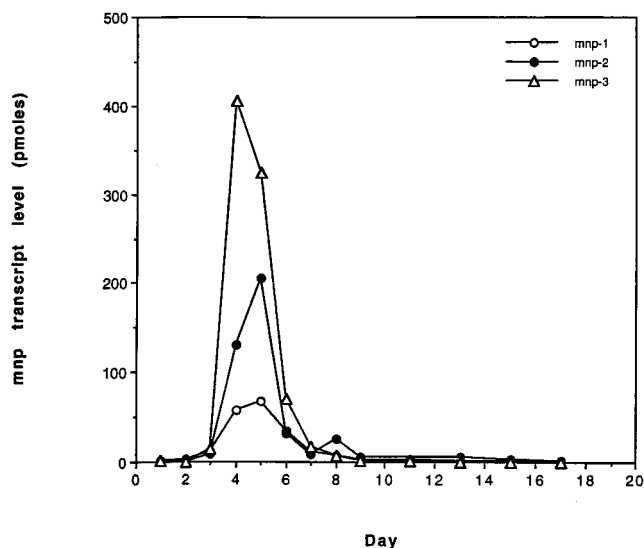


FIG. 4. Temporal variation in abundances of *mnp* mRNAs extracted from *P. chrysosporium* soil cultures.

formation rates observed at later time points was due to the physiological state of the fungus or to potential decreases in the bioavailability of the residual PAH, a 4-week experiment was conducted in which [¹⁴C]fluorene was used to spike cultures at different time points and extracted at the conclusion of the experiment. [¹⁴C]fluorene added on day 2 was extensively converted to 9-fluorenone (79% ± 4% of the recovered ¹⁴C) and 9-hydroxyfluorene (17% ± 5%). In contrast, when spiking was performed on day 8, all of the recovered ¹⁴C remained as unchanged fluorene. Total recovery in both cases was low (47% ± 4% and 47% ± 2%, respectively). Combustion of soil spiked on day 2 with trapping and counting of ¹⁴CO₂ revealed that ca. 80% of the input ¹⁴C remained in the soil at the conclusion of the experiment (data not shown).

***mnp* transcript analysis.** PCR primers were developed (Table 1) which were capable of gene-specific amplification of *mnp-1*, *mnp-2*, and *mnp-3*. The results of PCR of gDNA and RT-PCR of spawn-grown *P. chrysosporium* mRNA are presented in Fig. 3. Gene-specific probes (Table 1) were used in Southern blotting experiments with gDNA-derived PCR products to ensure the specificity of each primer pair for its assigned gene; nonspecific amplification products were not detected (data not shown). mRNA purified from spawn-grown cultures yielded cDNA-derived products of the lengths (671 to 674 bp) expected for each of the three genes (Fig. 3).

Figure 4 shows the temporal variations in the levels of *mnp-1*, *mnp-2*, and *mnp-3* mRNAs in *P. chrysosporium*-colonized soil cultures. All three transcripts appeared on day 1 or 2, increased to peak abundance on day 6, and then dropped off. The observed decrease during the later phase was rapid from approximately day 7 to day 9 and was more gradual through day 15. To assess the reproducibility of the entire mRNA extraction-quantitation procedure (extraction, RT-PCR, and image analysis), 16 individual datum points were replicated. All 16 pairs were within an order of magnitude, with an average standard deviation of 57%. To ensure that the observed variability in *mnp* transcript levels was not due to fluctuations in mRNA extractability, the mRNAs corresponding to the *P. chrysosporium* β-tubulin gene (*tub*) (38) and the reportedly (29) constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) were investigated as potential house-

keeping RNAs. The relationships between *tub* and *gpd* mRNA levels with extractable ergosterol levels are shown in Fig. 5. Levels of both transcripts peaked early (days 4 to 5), declined rapidly and evidenced no strict connection with the ergosterol content of the cultures.

***mnp* transcript correlation with enzyme activity.** MnP activity extracted from PAH-contaminated *P. chrysosporium* soil cultures during the 2.5-week duration of the experiment is shown in Fig. 6. Peak levels of ca. 3,000 total MnP U (approximately 70 U/g of soil) were observed on days 5 to 7. Figure 6 also shows the cumulative levels of the three *mnp* transcripts, quantified by competitive RT-PCR, in these same soil cultures. Maximal enzyme activity occurred 1 to 2 days after the highest abundance of *mnp* transcripts; enzyme activity displayed a slower decay following this peak than did mRNA levels.

DISCUSSION

PAHs with ionization potential values of greater than 7.55 eV are not substrates for LiP (22). The initial oxidative steps in their degradation are believed to occur by MnP-dependent lipid peroxidation (1, 2, 30). Depletion of two such compounds, fluorene (assessed by both disappearance of fluorene and accumulation of 9-fluorenone) and chrysene (disappearance), from *P. chrysosporium*-colonized soil occurred primarily during a time in which transcripts of the three known *mnp* genes were present at their maximal levels. This result, coupled with the extractability of high titers of MnP activity from these soil cultures during the same period, lends credence to the hypothesis that MnP-dependent reactions play a role in soil bioremediation by these fungi.

The rates of decrease in the concentrations of fluorene and chrysene from soil cultures appeared to decline after roughly 7 to 10 days of fungal growth, despite the presence of live biomass well beyond this point (Fig. 5). Similar results have been reported by George and Neufeld (15); removal of fluorene at 75 ppm from *P. chrysosporium* soil cultures essentially ceased after 6 to 8 days, with 15 to 25 ppm remaining. The nutrient supplements employed by those investigators were quite different from ours (a glucose-mineral salts-based liquid supplement versus a spawn mixture); direct comparison of these

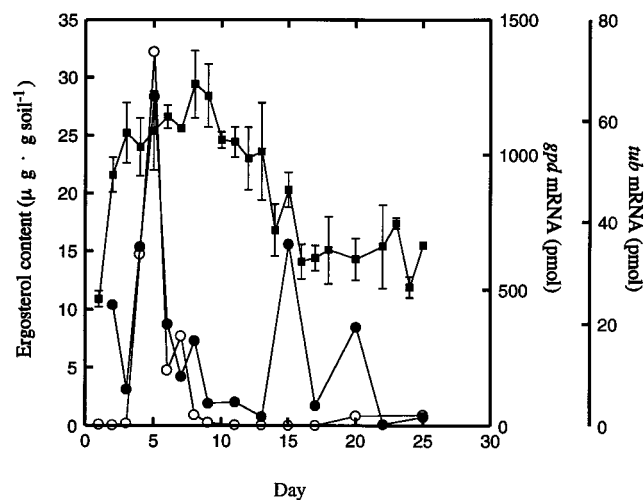


FIG. 5. Comparison of biomass level (measured as ergosterol content) (■) and levels of two potential housekeeping mRNAs. Abundances of transcripts encoding β-tubulin (*tub*) (●) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) (○) are depicted.

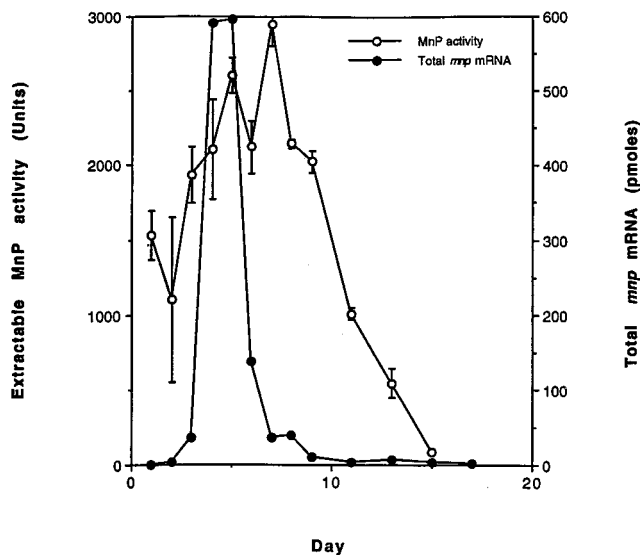


FIG. 6. Correlation between extractable MnP activity and total RT-PCR-quantitated *mnp* mRNA (sum of *mnp-1*, *mnp-2*, and *mnp-3*) for *P. chrysosporium* soil cultures.

results may therefore be inappropriate. Experiments in which [^{14}C]fluorene was used to spike soil on day 2 or 8 of growth revealed a definite temporal variability in the fluorene-transforming capacity of fungal cultures. MnP enzyme and *mnp* transcript levels, although not maximal, did persist beyond day 8; roughly 30% of the total MnP activity recovered from soil cultures (Fig. 6) was observed between days 8 and 15. This indicates that other changes in the physiological state of the fungus may have been involved. For example, if MnP-dependent lipid peroxidation was responsible for the transformation of fluorene and chrysene, declines in the level(s) of other components of the system (i.e., unsaturated lipids or lipid hydroperoxides) may have caused the observed decreases in rates of PAH oxidation. Recent liquid culture work (24) with *Panus tigrinus* has indicated a decline in lipid peroxidation (measured as thiobarbituric acid-reactive products and/or lipid hydroperoxides) with culture age. However, the magnitude of this effect does not (at least in *P. tigrinus*) appear to be great enough to cause cessation of MnP-dependent lipid peroxidation. For example, levels of lipid hydroperoxides in *P. tigrinus* cultures declined only ca. 30% (as a percentage of the total extractable lipid) between the exponential phase and the late, "dying-off" phase (24).

The work described here extends the mRNA extraction and competitive RT-PCR techniques previously applied (28) to analysis of *lip* and *cbh* transcripts in *P. chrysosporium*-colonized soil to encompass three closely related *mnp* genes. Quantitative PCR can be prone to error, particularly when unrelated sequences and/or sequences of various lengths are compared (33). In this study, primers were selected which amplified the same region within the three *mnp* genes. Thus, our competitive PCR methodology is especially well suited for comparative analysis of the *mnp* family.

Transcripts of all three of the *mnp* genes examined were detected in mRNA isolated from *P. chrysosporium* grown on a commercial lignocellulosic substrate (spawn mixture), as well as PAH-transforming soil cultures of this fungus. Quantitation of three *mnp* mRNAs during a 2.5-week time course of fungal colonization and growth revealed coordinate regulation of the gene family under these culture conditions. This presents some

contrast with the regulation of these genes under liquid culture conditions, in which *mnp-1* and *mnp-3* are known to be differentially controlled by the Mn^{2+} concentration in the medium (36). The degree of error present in the mRNA extraction-quantitation procedure does not permit absolute statements concerning the relative abundances of all transcripts at all time points. However, the magnitudes of many of the fluctuations observed in this work (i.e., in the cases of individual transcripts over time) are far greater than the level of error and must be regarded as real. The observed fluctuations in *mnp* mRNA abundance were most likely not artifacts of differential extractability. The observed abundances of several *lip* mRNAs were much less variable throughout the course of the experiment, whereas several others were present at their highest levels during periods of lowest *mnp* transcript recovery (2a).

Total *mnp* mRNA peaked 1 to 2 days in advance of maximal levels of enzyme activity. This may reflect an actual delay between transcription and translation. Alternatively, the enzyme extraction method used here may recover only the extracellular enzyme; that fraction of the translated enzyme which has not yet been secreted may be unaccounted for, thus causing a net underestimate of the total activity produced. The decline in MnP activity was much slower than that of the transcript level, indicating that the MnP enzyme is relatively stable under these conditions. Enzyme activity on days 1 to 3 was higher than expected from the low levels of *mnp* RNA. This may reflect protein present in the colonized inoculum; the absence of high transcript levels on days 1 and 2 may indicate that the biomass in the inoculum has reached a period analogous to the later portion of the soil time course.

The observation that *mnp* mRNA levels correlate with MnP enzyme production, and with the disappearance of high ionization potential PAHs during *P. chrysosporium*-based soil remediation, suggests that extraction of mRNA and RT-PCR analysis will provide a useful tool for monitoring the physiological state of the fungus to help ensure bioremediation performance. This approach has been applied to bacterial systems, such as the expressions of *dmpN* during phenol degradation by *Pseudomonas putida* in bioreactors (41) and *nahA* by the same species during mineralization of naphthalene in contaminated soils (13). The advantages of methods employing mRNA determination over other approaches, e.g., quantitation of ergosterol (10) or PCR-based quantitation of fungal DNA (23), center around two factors. First, tailoring of PCR primers allows a species specificity not possible with ergosterol measurements. Second, and more important, mRNA quantitation data provide insight into the physiological status of the organism, rather than merely revealing its presence, and thus allows more informative monitoring of the remediation process.

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