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Quantitation of Fungal mRNAs in Complex Substrates by Reverse Transcription PCR and Its Application to *Phanerochaete chrysosporium*-Colonized Soil

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Thorough analysis of fungi in complex substrates has been hampered by inadequate experimental tools for assessing physiological activity and estimating biomass. We report a method for the quantitative assessment of specific fungal mRNAs in soil. The method was applied to complex gene families of *Phanerochaete chrysosporium,* a white-rot fungus widely used in studies of organopollutant degradation. Among the genes implicated in pollutant degradation, two closely related lignin peroxidase transcripts were detected in soil. The pattern of lignin peroxidase gene expression was unexpected; certain transcripts abundant in defined cultures were not detected in soil cultures. Transcripts encoding cellobiohydrolases and ß-tubulin were also detected. The method will aid in defining the roles of specific genes in complex biological processes such as organopollutant degradation, developing strategies for strain improvement, and identifying specific fungi in environmental samples.

The physiological activities of fungi in complex substrates are poorly understood. Relative to defined culture media, purification and characterization of enzymes from fungus-colonized plant material and soil are severely complicated by interfering substances and low yields. Fungal biomass in complex substrates is often estimated by ergosterol and chitin measurements (for examples, see references 7 and 45), but these techniques lack sensitivity and specificity. Recently, increased specificity has been attained by PCR amplification of the internal transcribed spacer region of nuclear rRNA genes (21, 33).

Phaneroehaete chrysosporium, a ligninolytic basidiomycetes, has been extensively studied for its ability to degrade organopollutants in defined media (17), soil cultures (26, 28, 29), and large-scale field trials (26, 27). Free radical-based peroxidase mechanisms have been demonstrated both in ligninolysis (25) and in the degradation of anthracene (16), 2,4-dichlorophenol (42), 2,4-dinitrotoluene (41), 2,7-dichlorodibenzo- *p* -dioxin (43), 2,4,5 -trichlorophenol (22), dibenzo- *p* -dioxin (18, 23), and various dyes (34).

Lignocellulose and organopollutant degradation by *P. chrssosporium* involves complex families of structurally related genes (for reviews, see references 2, 6, and 15). Multiple lignin peroxidase (LiP) isozymes are encoded by a minimum of 10 genes which have been designated *lipA* through *lipJ* ***(12).*** The LiP isozymes differ with respect to their physical properties and substrate specificities (14, 24, 30, 34). On the basis of experimentally determined pIs and N-terminal amino acid sequences, the major isozymes H2, H7, H8 (14), and H10 (9) are encoded by *lipD, lipE, lipA,* and *lipC,* respectively. The transcriptional regulation of *lipA, lipC, lipD,* and *lipE* has been studied in detail (19, 20, 35, 37, 39). Gene *lipD* and, to a lesser extent, gene *lipE* are preferentially expressed under conditions of carbon limitation, whereas *lipC* transcripts are relatively more abundant under conditions of nitrogen limitation. In contrast, *lipA* transcript levels remain approximately equal under conditions of carbon or nitrogen deficiency. Differential regulation within the cellobiohydrolase (CBH) gene family has also been demonstrated. The major CBH isozyme in submerged culture is encoded by *cbhl-4,* and transcript levels exceed those of a closely related gene, *cbhl-1,* by greater than 1,000-fold (5, 44). The precise roles and interactions of individual genes in lignocellulose and organopollutant degradation are unclear, and their expression in fungus-colonized soil has not been investigated.

We report here on the development of generally applicable techniques for the quantitative assessment of specific mRNAs in soil. The approach is demonstrated with *P. chrysosporiun* cultured on pentachlorophenol (PCP)-contaminated soil. The transcript patterns observed are unlike those found in defined media. The results have important implications in defining the roles of specific genes in organopollutant degradation and in the development of strain improvement strategies.

MATERIALS AND METHODS

Soil cultures. PCP-amended soil cultures were prepared and analyzed as described previously (28. 29), with minor modifications. as follows. Marshan sandy loam was sterilized by fumigating it with 98% methyl bromide-2% chloropicrin (Bromo Gas; Great Lakes Chemical Corporation, West Lafayette, Ind.). This fumigation procedure was previously used to sterilize soil in laboratory soil cultures (28) and in field trials (26). PCP dissolved in acetone was added to the sterile soil to a final concentration of 50 μg g^{-1}. Soil moisture was adjusted to 39% with sterile deionized water. Cultures were set up in 125-ml Erlenmeyer flasks fitted with air exchange ports. Each flask contained 30 g of soil. Cultures were inucuiated with 5.25 g of Lambert spawn (L. F. Lambert Spawn Company, Coatsville, Pa.) colonized with *P. chrysosporium* BKM- 1767. Control cultures contained 5.25 g of sterile spawn. The cultures were incubated at 39°C. For PCP analysis, three inoculated and three control cultures were harvested at 0,2,4, and 6 days. PCP was extracted and analyzed by gas chromatography-electron *capture* detection (26).

RNA purification. PoIy(A) RNA was extracted with oligo$(dT)_{25}$ Dynabeads (Dynal Inc., Great Neck. NY.). with moditifications made to the manufacturer's recommendations. Ten grams of spawn inoculum or inoculated soil culture was wrapped in Miracloth (Calbiochem-Novabiochem Corporation. La Jolla. Cal if.),

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TABLE 1. Primers for PCR amplification of *P. chrysosporium* genes

Gene[a]	5′ Primer[b]	3′ Primer[b]	Size (bp)[c]	
			gDNA	cDNA
β-Tubulin	AGGTCGTCTCAGACGAACAC	GGCATGGGTACTCTCCTGATC	640	495
cbh1-1	TCCTTTTGTTGGGCGTCGTC	ACCTCCAACACGGGCTTTTG	817	766
cbh1-4	AAGGTCGTCCTCGACTCGAA	CTCCAAGCCCTTCACCGTCG	795	741
lipA	GCAGCTATCTCTCTCGCTCT	TCCCAGTTCTTCGTCGAG	1,012	683
lipC	GCTGTTCTTACCGCCGCTCT	TCCCAGTTCTTCGTCGAG	999	677
lipD	CAGCCCTCTCCGTCGCCCTG	TCCCAGTTCTTCGTCGAG	999	685
lipE	TCGCCGCGATCACCGTCGCC	TCGCAGTTCTTCGTCGAG	1,006	685

[a] *P. chrysosporium* genes encoding β-tubulin, cellobiohydrolases (*cbh1-1*, *cbh1-4*), and lignin peroxidases (*lipA*, *lipC*, *lipD*, *lipE*).
[b] Primer design is based on published sequences or database accession numbers: β-tubulin (36), *cbh1-1* (5), *cbh1-4* (44), *lipA* (38, 40), *lipC* (11), *lipD* (8, 9) (GenBank accession number X15599), *lipE* (35, 37).
[c] gDNA, size predicted for PCR amplification of genomic sequence; cDNA, size predicted for PCR amplification of cDNA sequence.

snap frozen in liquid nitrogen, and ground in a mortar and pestle precooled with dry ice. While grinding, 2-ml aliquots of extraction buffer (4 M guanidinium thiocyanate, 0.1 M Trizma base. 1% dithiothreitol. 0.5% Sarkosyl, [pH8.0]) were added sequentially until a total of 10 ml was added. The slurry was centrifuged at 500 x g for 8 min. The supernatant was centrifuged a second time at 500 x g for 8 min. and 400-μl aliquots of the final supernatant were mixed with 800 μl of binding buffer (100 mM Trizma base. 400 mM LiCl, 20 mM EDTA [pH 8.0]) in Eppendorf tubes. The tubes were centrifuged at 8,000 x g for 2 min. and the supernatant was added to 1.5 mg of Dynabeads which were previously washed in binding buffer. The mixture was hybridized for 5 min at mom temperature. Bound poly(A) RNA was isolated by magnetic concentration and was washed three times with 10 mM Trizma base-O.l5 M LiCl-l mM EDTA (PH 8.0). Poly(A) RNA was eluted at 65°C for 2 min in 20 μl of 2 mM EDTA (PH 8.0). Dissociated Dynabeads were recovered by magnetic concentration and were regenerated according to the manufacturer's recommendations. RNA was precipitated with 2 volumes of ethanol and was stored at -20°C. Each 10-g soil sample yielded sufficient RNA for approximately 240 reverse transcription PCR (RT-PCR) reactions.

RT-PCR detection and quantitation of transcripts. RT reactions were as described previously (39), except that oligo(dT)$_{15}$ instead of a *lip-specific* downstream primer served as the primer. PCRs were in 100-μl volumes with 5 U of *Taq* DNA Polymerase and 21 pmol of each primer (Table 1). The larger genomic PCR products corresponding to each cDNA were obtained from parallel reactions with <1 ng of genomic DNA as template. Reactions were subjected to an initial cycle of denaturation (6 min. 94°C), annealing (2 min, 54°C), and prolonged extension (40 min. 72°C): this was followed by 35 cycles of denaturation (1 min. 94°C). annealing (2 min, 54°C). and extensition (5 min, 72°C). A final 15-min extension at 72°C was also included,

Competitive RT-PCR techniques (13, 39) were used to quantitate *lipA* and *lipC* transcripts in day 4 soil cultures and to quantitate ß- tubulin transcripts in day 2 and 4 cultures. Genomic clones of *lipA*, *lipC*, and ß- tubulin in plasmid pCRII (Invitrogen Inc., San Diego. Calif.) served as competitive templates. The clones were generated from genomic DNA by PCR and contained full-length sequences from translational start to stop codons. To confirm the identities of the *lip* PCR products, agarose gels were blotted onto nylon membranes and were probed with ^{32}*P*- labeled oligonuclcotides specific for each gene (39).

RESULTS

Detection of specific transcripts in ***P. chrysosporium-colo***nized soil entailed direct RNA isolation and then RT-PCR amplification. Poly(A) RNA was affinity purified by disrupting soil cultures in the presence of oligo(dT) covalently attached to magnetic beads. Following repeated washes, RNA was eluted from the magnetic particles. Oligo(dT) was then used to prime RT, and the resulting first-strand cDNA was subjected to PCR amplifications with gene-specific primers (Table 1).

Poly(A) RNA derived from inoculum and soil cultures was a suitable template for RT-PCR amplification of several genes (Fig. 1). Initial experiments focused on the LiP genes most highly expressed in defined media ***(lipA, lipD, lipE) (19, 35,37, 39), two*** CBH1 genes ***(cbhl-1, cbhl-4)*** (5, 44), and the gene encoding ß- tubulin (36). Ail cDNAs were detected (Fig. 1A) in the fungal spawn inoculum, which is a pure culture of ***P. chrysosporium*** grown on a nutrient-fortified grain-sawdust mixture (27). Of the three ***lip*** transcripts initially sought in soil, only ***lipA*** was detected. The ***lipA*** cDNA product was clearly observed in 4.-day-old soil samples (Fig. 1C, lane 10), and a less intense band was visible in cultures harvested on day 2 (Fig. lB). cDNAs specific for *cbhl-1. cbhl-4*, and ß- tubulin were amplified from 4-day-old soil cultures and from the spawn

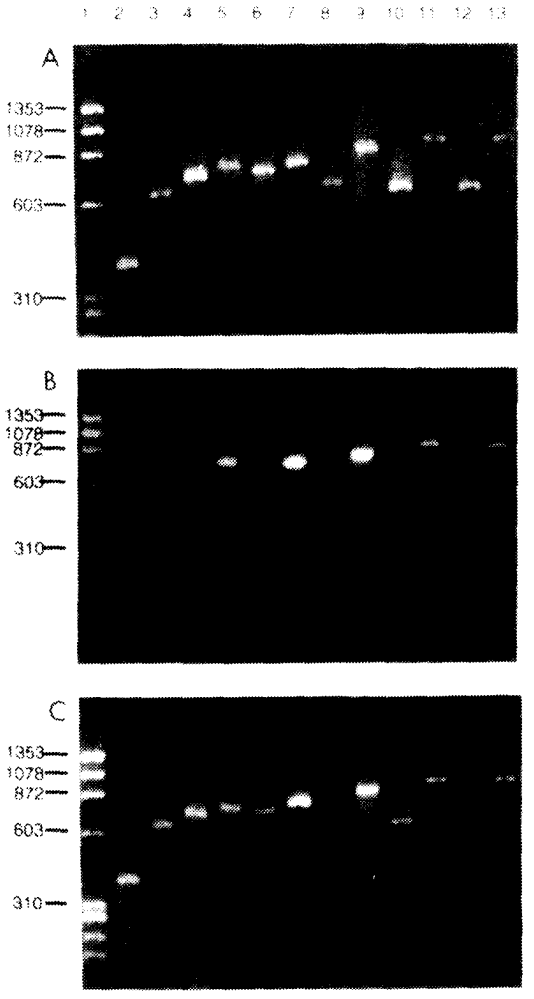

FIG. 1. PCR amplifications of genes from spawn inoculum (A), day 2 soil cultures (B), and day 4 soil cultures (C). The six genes analyzed are arranged in pairs; the first lane of each pair contains the RT-PCR-generated cDNA product and the second lane contains the larger PCR-generated genomic equivalent. Sample order is *Hae*III-digested ϕX174 (lane 1), β-tubulin (lanes 2 and 3), *cbh1-4* (lanes 4 and 5), *cbh1-1* (lanes 6 and 7), *lipD* (lanes 8 and 9), *lipA* (lanes 10 and 11), and *lipE* (lanes 12 and 13). Molecular mass markers (*in base pairs*) are indicated on the left margin.

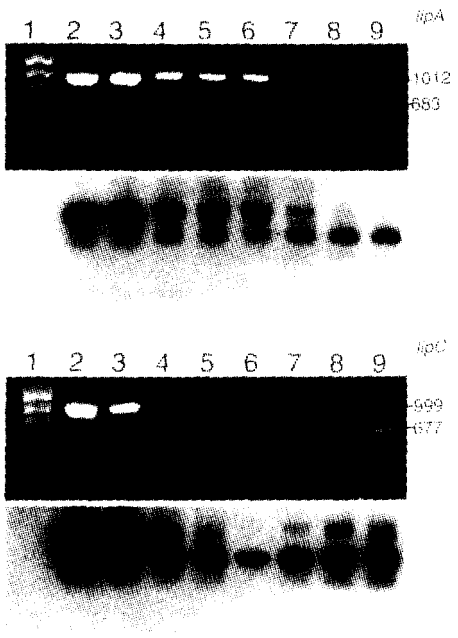

FIG. 2. Competitive RT-PCR quantitation of mRNA of *lipA* and *lipC*. Lanes 2 through 8 were loaded with PCR mixtures that received decreasing amounts of competitive templates: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng. The competitive templates were full-length genomic clones of *lipA* and *lipC*. No competitive template was added to the eighth reaction (lane 9). Lane 1, *Hae*III-digested ϕX174 as a molecular size marker. The sizes of the cDNA and genomic PCR products are indicated on the right margin in base pairs. Southern blots probed with gene-specific oligonucleotides (39) are shown immediately below the corresponding ethidium bromide-stained gels.

inoculum. Low levels of the ß- tubulin cDNA were also detected in day 2 soil samples (Fig. lB, lane 2).

Relative transcript levels of several genes were estimated by competitive PCR techniques (13, 39). Briefly, the process involved the addition of competitive templates, in the form of genomic DNA, to the PCR mixtures. The competitors were added in a series of dilutions of known concentration, and introns within the competitive templates allowed the target cDNA and genomic DNA to be size fractionated on agarose gels. The initial concentration of specific cDNA was estimated by the dilution point at which target cDNA and competitive

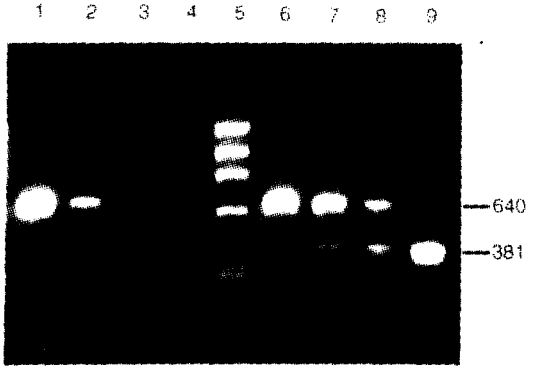

FIG. 3. Competitive RT-PCR of β-tubulin mRNA derived from day 2 (lanes 1 to 4) and day 4 (lanes 6 to 9) soil cultures. Competitive template, in the form of a genomic clone, was added to the PCR mixtures. Lanes 1 to 4, 10^{-3}, 10^{-6}, 10^{-7}, and 10^{-8} ng, respectively; lanes 6 to 9, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} ng, respectively; lane 5, *Hae*III-digested ϕX174 as a molecular size marker. The sizes of the genomic DNA and cDNA products are indicated on the right margin in base pairs.

template were equivalent. In the cases of ***lipA*** and ***lipC,*** the equivalence points on ethidium bromide-stained gels were approximately 10^{-6} to 10^{-7} ng (Fig. 2, lanes 6 and 7) and 10^{-4} to 10^{-5} ng (Fig. 2, lanes 4 and 5), respectively. Thus, ***lipC*** transcript levels were at least 10-fold greater than ***lipA*** transcript levels in 4-day-old soil cultures. To confirm the identities of the ethidium bromide-stained bands, the gels were blotted and probed with gene-specific probes (Fig. 2). Equivalence points based on the autoradiogram confirmed the results obtained on the stained gels.

Transcript levels of ß -tubulin, an essential gene involved in microtubule formation but not directly involved in lignocellulose degradation, were also measured (Fig. 3). PCR products corresponding to genomic and cDNA templates were observed at 640 and 381 bp, respectively. As expected, relative levels increased substantially from day 2 to day 4.

DISCUSSION

Investigations of fungi in complex substrates have been limited. Recently, PCR amplification of specific DNA sequences has proven to be useful for the identification of fungi in plant material and soil (for examples, see references 21 and 33). The method described here also permits strain identification, but it substantially extends these techniques by assessing the physiological activities of fungi in soil. *P. chrysosporium,* with its families of closely related isozymes, exemplifies the difficulties in soil analysis and provides a demonstration of our method.

Transcript levels of *P. chyrosporium* LiP genes were measured in soil cultures and were shown to differ markedly from those in defined media. Under an array of defined culture conditions, *lipA, lipD,* and *lipE* have been shown to be highly expressed (19, 20, 35, 37, 39). Transcript levels of *lipC* are extremely low in carbon-limited cultures and are almost equal to the transcript levels of *lipA* in nitrogen-limited cultures (39). In soil, the number of *lipC* transcripts substantially exceeded the number of *lipA* mRNAs. in carbon-limited cultures, the numbers of transcripts of *lipD* and, to a lesser extent, *lipE* increase relative to the numbers in nitrogen-limited cultures (19, 35, 39). Neither *lipD* or *lipE* transcripts were detected when most PCP degradation occurs (days 2 to 4), suggesting that they have no significant role in the process. Similar stabilities and turnover rates would be expected among structurally related *lip* mRNAs, but this has not been established experimentally. It is therefore formally possible that *lipD* and *lipE* are expressed, perhaps at low levels. Also, by analogy with defined media (3, 39), *lip* transcription patterns are likely to be dramatically altered by soil composition and culture age.

The role(s) of *lipA* and *lipC* in PCP degradation remains to be established. A temporal association of transcripts with PCP degradation does not prove direct involvement of specific isozymes, In defined media, *lip* transcript levels are directly related to specific isozymes (4, 31), but the many difficulties associated with extracting and purifying peroxidase isozymes preclude such investigations in soil. The precise role of transcriptionally active genes in organopollutant and lignin degradation will require additional investigations, perhaps involving the disruption of specific genes (1). In general, then, the techniques described here identify potentially important genes in complex processes such as organopollutant and lignin degradation. The absence of a transcript provides strong evidence against any significant role for a given gene.

The methodology is applicable to any intron-containing gene for which the nucieotide sequence is known. In addition to LiPs, cDNAs encoding CBHS and ß- tubulin were amplified, Transcript detection in a spawn inoculum, a mixture of sawdust

and grains, strongly supports the applicability of the approach to other important substrates such as molded grains and woody materials. (By using nearly identical methods, ß-tubulin transcripts have been detected in Aspen wood chips colonized by *P. chysosporium* [data not shown]). Transcription of ***cbhl-1*** is noteworthy because expression is exceedingly low in submerged culture (5, 44) and because the gene lacks the cellulose binding domain common to other CBHS (5). The transcript levels of ***cbhl-1*** relative to those of other CBH genes remain to be established. It would be of considerable interest to assess the transcript levels of all known ***cbh*** and ***lip*** genes in field trials with fumigated (26) and nonfumigated (27) soils contaminated with PCP.

Unambiguous identification and biomass estimation of fungi in solid substrates have been long-standing problems. Direct estimates are complicated by difficulties in isolating hyphal fragments. Glucosamine, ergosterol, laccase, and other indirect measurements lack sensitivity and specificity (32). Selective plating techniques can be highly specific (10), but the quantitative recovery of hyphae and spores is not possible. In contrast, PCR amplification of genomic DNA such as the internal transcribed spacer region of the 18S rRNA gene can be extremely specific and sensitive (21, 33).

Quantitative analysis of mRNA features all of the attributes of DNA amplification while simultaneously providing an assessment of physiological processes within the substrate. For example, the detection of ***lip*** and ***cbhl*** transcripts strongly suggests an active lignocellulose degradation system. Increased expression of ß-tubulin (Fig. 3), an essential gene involved in spindle formation, indicates mitotic division. When needed, a more comprehensive assessment of *P. chrysosporium* physiological activity could be attained by extending the methodology to additional transcripts. For instance, basic carbon catabolism might be reflected by expression of *gpd* (GenBank accession number M81754) or *facA* (GenBank accession number X56211), amino acid synthesis by *trpC* (Gen Bank accession number X56047), and DNA synthesis by ***his4.1*** (GenBank accession number Z15134).

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