# Transcript patterns of *Phanerochaete chrysosporium* genes in organopollutant-contaminated soils and in wood.

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We describe here recent methods for quantitative assessment of specific *P. chrysosporium* mRNAs in organopollutant contaminated soils and in Aspen wood chips. Magnetic capture techniques were used to rapidly purify poly(A)-RNA, and quantitative RT-PCR protocols were developed for all known lignin peroxidase (*lip*) and cellobiohydrolase (*cbh1*) genes. The methodology is extremely sensitive and highly specific. Results indicated that gene transcript patterns varied dramatically in response to substrate. In general, transcript patterns in defined media were unreliable predictors of expression in soil or wood. No apparent relationship was observed between genomic organization and transcript patterns. Results provide a framework for future investigations and help guide strain improvement programs.

## Introduction.

Lignocellulose and organopollutant degradation by *P. chrysosporium* involves complex families of structurally related genes (for review see: Gold & Alic, 1993; Broda *et al.,* 1996; Cullen, 1997; Kirk & Cullen, 1998)). Multiple lignin peroxidase (LiP) isozymes are encoded by, a minimum of 10 genes which have been designated *1ipA* through *1ipJ* (Gaskell *et a1.*, 1994). Transcriptional regulation of *lipA, lipC, lipD,* and *lipE* have been studied in defined media (Holzbaur & Tien, 1988; James *et al.,* 1992; Ritch & Gold, 1992; Stewart *et al.,* 1992; Reiser *et al.,* 1993). *lipD,* and to a lesser extent, *lipE* were shown to be preferentially expressed under carbon limitation, whereas *lipC* transcripts were relatively more abundant under nitrogen limitation. Single basidiospore cultures show >97% cosegregation of *lipA, lipB, lipC, lipE, lipG, lipH, lipI,* and *lipJ,* and cosmid analysis has generated a detailed map of the cluster; *lipD* and *lipF* are located on chromosomes separate from each other and all other known peroxidases.

Differential regulation in submerged culture has also been demonstrated for six structurally related genes encoding cellobiohydrolase 1 (CBH1). Designated *cbh*1-1 through *cbh*1-6, all except *cbh1-1* feature the catalytic and binding domains typical of microbial cellulases. *Cbh1-1* lacks the cellulose binding domain (CBD) and in submerged culture it's transcript levels are 1000-fold less than *cbh1-4* (Covert *et al.,* 1992b; Vanden Wymelenberg *et al.,* 1993). Genetic analysis, cosmid mapping and pulsed field gel electrophoresis show *cbh1-1, cbh1-2* and *cbh1-3* to be clustered within 30 kb, whereas *cbh1-4* and *cbh1-5* are distantly linked on a separate chromosome. *Cbhl-6* appears to be unlinked to all known *cbh's* and *lip's* (Covert *et al.,* 1992a; Gaskell *et al.,* 1994).

The precise roles and interactions of individual genes in lignocellulose and organopollutant degradation are unclear. We describe here recent efforts to assess the transcript levels of specific genes in soil and in wood.

## Materials and methods.

Figure 1 illustrates competitive RT-PCR of *P. chrysosporium cbhl-5* transcripts from 4-week-old aspen wood chip cultures (Akhtar, 1997). Approximately ten grams of *P. chrysosporium-colonized* sample are wrapped in Miracloth (Calbiochem Corporation, La Jolla, CA), snap frozen in liquid nitrogen, and ground in a mortar and pestle precooled with dry ice. Extraction buffer (4M guanidinium thiocyanate, 0.1 M Trizma base, 1% dithiothreitol, and 0.5% Sarkosyl, pH 8.0) is added while grinding and the slurry then centrifuged at 2,000 x g for 8 min. The supernatant is centrifuged a second time and the final supernatant mixed with binding buffer (100 mM Trizma base, 400 mM LiCl, and 20 mM EDTA, pH 8.0). Oligo-(dT)25 Dynabeads (Dynal Inc., Great Neck, NY), are added and the mixture hybridized for 30 min on ice. Bound poly (A)-RNA is isolated by magnetic concentration with a Dynal Magnetic Particle Concentrator and then repeatedly washed. Poly (A)-RNA is eluted at 65°C for 2 min in 2 mM EDTA, pH 8.0. Dissociated Dynabeads can be recovered by magnetic concentration and regeneration according to the manufacturer's recommendations. RNA is precipitated with two volumes of ethanol and stored at 20°. Single stranded cDNA pools are typically synthesized using an oligo (dT)15 primer, although gene-specific 'downstream' primers can be used to optimize sensitivity and reduce non-specific PCR amplifications. Competitive PCR was performed as described (Gilliland *et al.,* 1990; Stewart *et al.,* 1992). In the example shown, replicate PCR reactions containing *cbh1 -5*-specific primers are prepared and known concentrations of a competitive template is added as a serial dilution to each reaction tube. Generally, the competitive template is an intron-containing genomic clone in plasmid pCRII (Invitrogen Inc., San Diego CA). However, any vector, cosmid, or λ subclone of *cbh1-5* would be adequate provided that the entire amplification target is included and that the region contains at least one intron. A variety of thermocycling conditions may be used, although excessive cycling should be avoided (Pannetier *et al.,* 1993).

Following amplification, the target cDNA and competitive template are size fractionated on agarose gels. The initial concentration of *cbh1-5* cDNA is determined by estimating the dilution point at which target cDNA and competitive template are equivalent. Typically, 10- or 5-fold serial dilutions are initially used to approximate cDNA concentrations. When necessary, increased resolution is achieved using many smaller dilutions, e.g. 1- or 2-fold. The equivalence point is attained by scanning the ethidium bromide stained gels and quantifying band intensity using imaging software such as NIH Image. At very low transcript levels, reverse transcriptase inhibition of PCR amplifications may underestimate certain cDNAs and appropriate controls may be needed (Chandler *et al.,* 1998). To confirm the identity of PCR products, agarose gels may be blotted to nylon membranes and probed with $^{32}$P-labeled oligonucleotides specific for cDNA targets (Stewart *et al.,* 1992)

## Results

Several *Phanerochaete chrysosporium* transcripts were quantified in soil and wood. Transcripts of all 10 LiP genes as well as 3 structurally-related manganese peroxidase genes were assayed in anthracene-contaminated soli (Bogart *et al.,* 1996a; Bogart *et al.,* 1996b). In aspen wood chips (Akhtar, 1997), transcripts corresponding to six CBHI genes and a cellobiose dehydrogenase gene (*cdh*) (Li *et al.,* 1996; Li *et al.,* 1997) have been quantified (Vallim *et al.,* 1998).

In anthracene-contaminated soil, patterns of *lip* transcript levels were markedly different from those previously observed in submerged cultures. Transcripts of one gene, *LipF*, were absent from *P. chrysosporium* colonized soil. *LipA, lipB, lipD, lipH,* and *lipI* transcripts peaked within the first week of growth on anthracene amended soil but declined by day 10. In contrast, *lipJ* transcripts appeared only after 10 days incubation and, by day 20, they were the

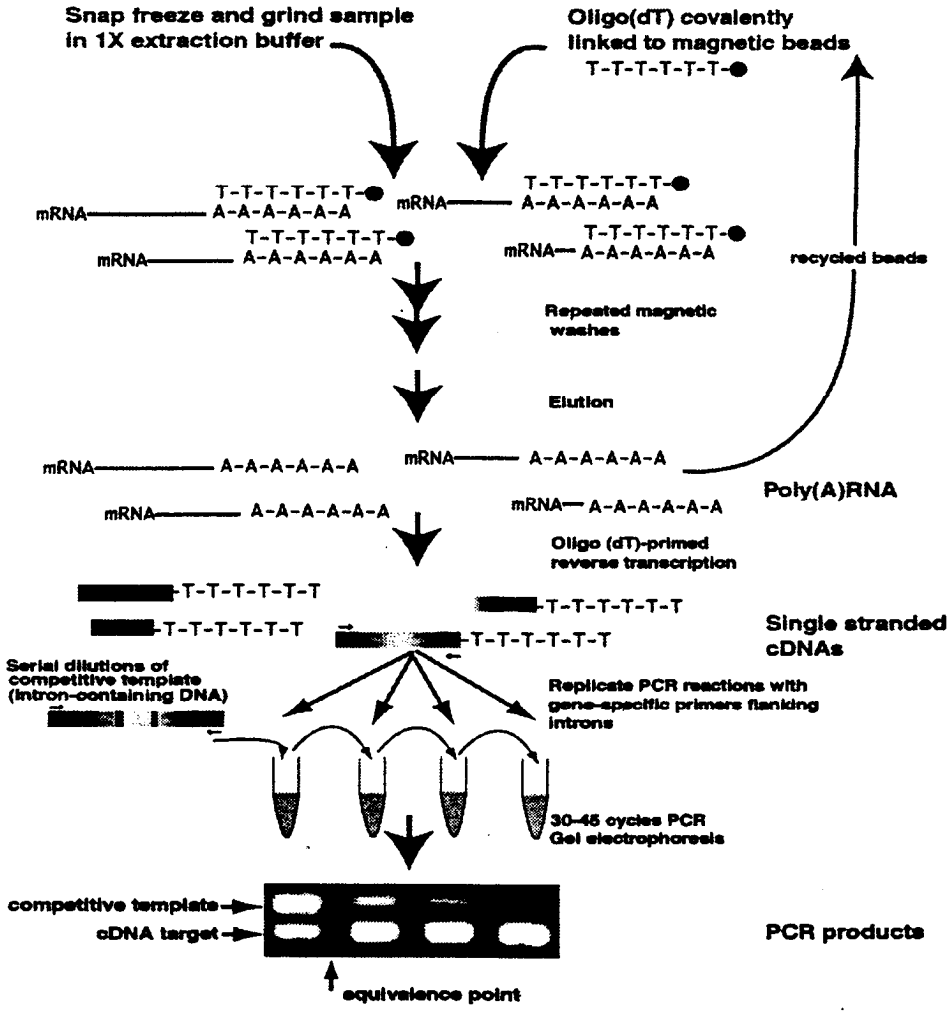


Figure 1. General protocol for mRNA purification and cDNA quantified by competitive RT-PCR. The ethidium bromide stained gel shows cbh1-5 amplification products.

dominant transcript. Transcripts of *lipA* far exceeded *lipC* in anthracene-contaminated soils (Bogan *et al.,* 1996a), while the reverse (i.e. *lipC>>lipA)* was observed in pentachlorophenol contaminated soils (Lamar *et al.,* 1995). Following these observations, the ability of particular organopollutants to influence gene expression in soil is being examined by suppression subtractive hybridization experiments (unpublished).

With minor modifications, the published magnetic capture/RT-PCR techniques have been adapted to *cbhI* and *cdh* transcript analysis in wood chips. Transcript patterns were dramatically different from previous studies of submerged cultures. After 4 weeks of incubation, *cbhl-5* transcript levels were highest while *cbhl-4* transcripts were barely detectable in Aspen chips. In contrast to these findings, *cbh1-4* was the dominant transcript in submerged cultures (Vanden Wymelenberg *et al.*, 1993). Interestingly, transcripts of the CBD-deficient CBHI gene, *cbh1-1*, were consistently detected at relatively high levels in wood as were transcripts of *cdh*. The results strongly support roles for the *cbh1-1* and *cdh* genes in the degradation of native cellulose.

## Discussion.

Quantitative transcript analysis features the sensitivity and specificity of DNA amplifications for identification purposes. However, the approach also provides a measure of fungal biomass and a glimpse of physiological processes *in situ*. The competitive RT-PCR technique is particularly suited for differentiating genes within complex gene families as exemplified by the peroxidases and cellobiohydrolases of white-rot fungi. The absence of certain peroxidase transcripts in *P. chrysosporium* colonized soil provides strong evidence against a significant role for these particular genes in anthracene degradation. Similarly, *cbh1-2* and *cbhl-6* could not be detected in Aspen wood chips and are, therefore, unlikely to have an important role in biopulping. Thus, magnetic capture and quantitative RT-PCR can clarify the role of specific genes in a variety of complex processes such as organopollutant degradation, biomechanical pulping and lignin degradation.

## References.


Akhtar, M., (1997). Method for enhancing biopulping efficacy. U.S. Patent, Wisconsin Alumni Research Foundation.

Bogan, B., Schoenike, B., Lamar, R. & Cullen, D. (1996a) Expression of *Zip* genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **62,** 3697-3703.

Bogan, B., Schoenike, B., Lamar, R & Cullen, D. (1996b) Expression of *mnp* genes during bioremediation of PAH-contaminated soil with *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **62,** 2381-2386.

Broda, P., Birch, P., Brooks, P. & Sims, P. (1996). Lignocellulose degradation by *Phanerochaete chrysosporium*: gene families and gene expression for a complex process. *Molecular Microbiology* **19,** 923-932.

Chandler, D., Wagnon, C.A. & Bolton, H. (1998) Reverse transcriptase (RT) inhibition of PCR are low concentrations of template and its *implications* for quantitative RT-PCR. *Applied and Environmental Microbiology* **64,** 669-677.

Covert, S., Bolduc, J. & Cullen, D. (1992a) Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium. Current Genetics* **22,** 407-413.

Covert, S., Vanden Wymelenberg, A. & Cullen, D. (1992b) Structure, organization and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **58,** 2168-2175.

Cullen, D. (1997) Recent advances on the molecular genetics of ligninolytic fungi. *Journal of Biotechnology* **53,** 273-289.

Gaskell, J., Stewart, P. & Kerken, P., Covert, S., Reiser, J. & Cullen, D. (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.

Gilliland, G. Perrin, S., Blanchard, K. & Bunn, HF. (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proceedings of the National Academy of Sciences USA* **87,** 2725-2729.

Gold, M.H., Cheng, T.M. & Alic, M.(1983) Formation, fusion and regeneration of protoplasts from wild type and auxotrophic mutant strains of the white rot basidiomycete *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **46,** 260-263.

Holxbaur, E. & Tien, M.(1988) *Structure* and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium. Biochemical and Biophysical Research Communications* **155,** 626-633.

James, C.M., Felipe, M.S.S., Sims, P.F.G. & Broda, P. (1992) Expression of a single lignin peroxidase-encoding gene in *Phanerochaete chrysosporium* strain ME446. *Gene* **114,** 217-222.

Kirk, T.K. & Cullen D. (1998). Enzymology and molecular genetics of wood degradation by white-rot fungi. In: *Environmentally Friendly Technologies for the Pulp and Paper Industry.* (Eds, Young, R.A. & Akhtar, M.) John Wiley & Sons, New York, pp 273-308.

Lamar, R.T., Schoenike, B. Vanden Wymelenberg, A, Stewart, P., Dietrich, D.M. & Cullen, D. (1995) Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. *Applied and Environmental Microbiology* **61,** 2122-2126.

Li, B., Nagalla, S. & Renganathan, V. (1996) Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **62,** 1329-1335.

Li, B., Nagalla, S. & Renganathan, V. (1997) Cellobiose dehydrogenase from *Phanerochaete chrysosporium* is encoded by two allelic variants. *Applied and Environmental Microbiology* **63,** 796-799.

Pannetier, C., Delassus, S., Darche. S., Saucier, C. & Kourilsky, P. (1993) Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. *Nucleic* Acid *Research* **21,** 577-583.

Reiser, J., Walther, I., Fraefel, C. & Fiechter. A. (1993) Methods to investigate the expression of lignin *peroxidase* genes by the white-rot fungus *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **59,** 2897-2903.

Ritch, T.G. & Gold, M.H. (1992) Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chiysosporium. Gene* **118:** 73-80.

Stewart, P., Kersten, P., Vanden Wymelenberg, A., Gaskell, J. & Cullen, D. (1992). The lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation, and the identification of a second dimorphic chromosome. *Journal of Bacteriology* **174,** 5036-5042.

Vallim, M., Janse, B., Gaskell, J., Pizzirani-Kleiner, A. & Cullen, D. (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts *in* wood. *Applied and Environmental Microbiology* **64,** (In Press).

Vanden Wymelenberg, A., Covert, S. & Cullen, D. (1993) Identification of the gene encoding the major cellobiohydrolase of the white-rot fungus *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **59,** 3492-3494.

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Publications

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