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 lipA through lipJ (Gaskell et al., 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 cbh1-1 through cbh1-6, 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. Cbh1-6 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 32P-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 soil (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 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).

Figure 1. General protocol for mRNA purification and cDNA quantified by competitive RT-PCR. The ethidium bromide stained gel shows cbh1-5 amplification products.
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, cbhl-4 was the dominant transcript in submerged cultures (Vanden Wymelenberg et al., 1993). Interestingly, transcripts of the CBD-deficient CBHI gene, cbhI-1, were consistently detected at relatively high levels in wood as were transcripts of cdh. The results strongly support roles for the cbhI-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, cbhI-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.**


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