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Nitrogen Regulation of Lignin Peroxidase Gene Transcription

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Western blot (immunoblot) analysis with a polyclonal antibody to lignin peroxidase (LiP) isozyme H8 from the white rot basidiomycete *Phanerochaete chrysosporium* demonstrates that LiP protein is detectable in the extracellular media of 5- and 6-day-old nitrogen-limited, but not nitrogen-sufficient, cultures. Northern (RNA) blot analysis demonstrates that *lip* mRNA is detectable from 5- and 6-day old cells grown in nitrogen-limited, but not nitrogen-sufficient, cultures. These results indicate that LiP expression is regulated at the level of gene transcription by nutrient nitrogen. Since lignin degradation by *P. chrysosporium* is derepressed by nitrogen starvation, it appears that lignin degradation and LiP expression are coordinately regulated in this organism. These results contradict a recent report which concluded that LiP protein expression is not regulated by nutrient nitrogen (C. G. Johnston and S. D. Aust, Biochem. Biophys. Res. Commun. 200:108–112, 1994).

The white rot basidiomycete *Phanerochaete chrysosporium* degrades lignin (6, 10, 17) and a variety of aromatic pollutants (5, 11, 13, 28) during the secondary metabolic (idiophasic) stage of growth, the onset of which is triggered by depletion of nutrient nitrogen. Two isozyme families of secreted heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), and an H_2O_2-generating system constitute the known major components of this organism's extracellular lignin degradative system (10, 17). Genes encoding various isozymes of LiP and MnP have been cloned and characterized (8). *lip*, *mnp*, and glyoxal oxidase (*glox*) mRNAs have been detected from cultures of *P. chrysosporium* only after the nitrogen in the medium has been depleted and the organism has entered idiophase (2, 15, 27). Furthermore, *mnp* mRNA is detectable from nitrogen-deficient but not from nitrogen-sufficient medium (23). These results have been taken as evidence that LiP and MnP are regulated by nitrogen at the level of gene transcription (8). There also is evidence that various LiP and MnP isozymes may be differentially regulated by nitrogen and carbon (21, 26). Finally, there are several reports of the isolation of *P. chrysosporium* mutants that are nitrogen deregulated for LiP, MnP, and GLOX production and lignin degradation (1, 19, 20). In direct contrast to this previous work, a recent report suggests that nutrient nitrogen regulates LiP expression at the level of heme insertion into the apoprotein rather than at the level of gene transcription (12). Therefore, we decided to reexamine this question by using Northern (RNA) blot and Western blot (immunoblot) analysis.

P. chrysosporium was maintained on slants as described previously (9). The organism was grown at 37°C from a conidial inoculum in 20-ml stationary cultures in 250-ml Erlenmeyer flasks, as described previously (3). Cultures were incubated under air for 3 days and then purged with 100% O_2. The medium was as previously described (3, 18), with 2% glucose as the carbon source, 1.2 mM ammonium tartrate (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen), and 20 mM sodium 2,2-dimethyl succinate (pH 4.5) as the buffer. The media contained 0, 30, or 180 μM $MnSO_4$.

A Western blot of the extracellular media from 5- and 6-day-old nitrogen-deficient and nitrogen-sufficient cultures is shown in Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as previously described (3). The blot was probed with a polyclonal antibody to LiP isozyme H8 from *P. chrysosporium* OGC101 (24) as previously described (3). Whereas LiP protein was detected in the extracellular media of both 5- and 6-day-old nitrogen-deficient cultures at all Mn concentrations, no LiP protein was detectable in the extracellular medium of nitrogen-sufficient cultures.

To determine if *lip* genes were transcribed under conditions of nitrogen sufficiency, Northern blots of total RNA from 5- and 6-day-old cultures were probed with the cDNA corresponding to LiP isozyme H8 (L18) (24). Cells were filtered through Miracloth (Calbiochem), rinsed twice with cold, distilled water, and quick-frozen in liquid nitrogen. The frozen cells were disrupted and the RNA was isolated by homogenization in the presence of TRI reagent (Molecular Research Center, Inc.) as described previously (4). After spectrophotometric quantitation, the RNAs (20 μg per lane) were denatured in the presence of 2.2 M formaldehyde and 50% formamide for 15 min at 68°C and electrophoresed in a denaturing (0.6 M formaldehyde–1% agarose) gel. The RNA was transferred to Magna NT (MSI) membranes and hybridized at 42°C with a ^{32}P-labeled probe as previously described (2). The L18 cDNA (24) was used as a template for random-primed synthesis of [α-^{32}P]dCTP-labeled (Dupont-New England Nuclear) probes, using a Multiprime DNA Labeling Kit (Amersham). RNA blots were washed and exposed to Kodak XAR-5 X-ray film. Figure 2 demonstrates that whereas *lip* mRNA was detected in nitrogen-deficient cultures at all Mn concentrations, no *lip* transcript was detectable in any nitrogen-sufficient culture.

The results presented here indicate that *P. chrysosporium lip* is regulated by nitrogen at the mRNA level. These results are in agreement with previous work indicating that gene transcription of *lip* isozyme H8 from strain BKM-F-1767 is regulated by nitrogen at the level of gene transcription (26, 27). These results also are in agreement with a large body of evidence indicating that lignin degradation by *P. chrysosporium* (6, 8, 10, 16, 18), although not necessarily by other white rot

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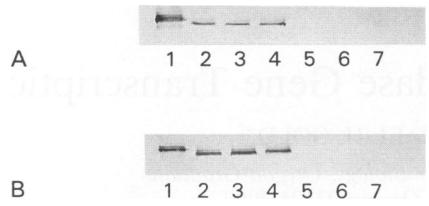

FIG. 1. Immunoblot analysis of extracellular LiP from nitrogen-limited and nitrogen-sufficient cultures. Samples of extracellular medium from 5-day-old (A) and 6-day-old (B) cultures were subjected to SDS-PAGE, electrophoretic transfer, and immunodetection as described in the text. Lane 1, purified LiP isozyme H8; lanes 2 to 4, cultures contained 1.2 mM NH_4 tartrate and 0, 30, and 180 μM $MnSO_4$, respectively; lanes 5 to 7, cultures contained 12 mM NH_4 tartrate and 0, 30, and 180 μM $MnSO_4$, respectively.

fungi (6, 14, 17, 22), is regulated by nitrogen limitation. *mnp* mRNA from *P. chrysosporium* also is detected only under conditions of nitrogen depletion (2, 23), and several other components of this organism's ligninolytic system appear to be coordinately regulated with LiP and MnP production during secondary metabolism. These include GLOX production (15) and synthesis of the secondary metabolite veratryl (3,4-dimethoxybenzyl) alcohol (25). It has been suggested that activator protein 2 (AP-2) sequences present in the promoter regions of many *lip* genes, as well as the *mnp1* gene, may be involved in the regulation of gene transcription by nitrogen deficiency (7).

The results presented here are in contrast to a recent report which concluded that *lip* isozyme H8 mRNA is produced in nitrogen-sufficient as well as nitrogen-deficient cultures of *P. chrysosporium* (12). In that study, a small amount of *lip* mRNA was detected from nitrogen-sufficient cultures and a large amount was detected from nitrogen-deficient cultures by reverse transcription and PCR. However, dot blot analysis indicated substantially more *lip* mRNA from nitrogen-sufficient cultures compared with nitrogen-deficient cultures. Contamination of the mRNA used on the dot blots with genomic DNA is a possible explanation for this discrepancy. The authors of that study also claim that although no LiP activity was detectable in the extracellular medium of nitrogen-sufficient cultures, fast protein liquid chromatography (FPLC) elution profiles indicated the presence of LiP apoprotein. However, no attempt was made, by using antibodies, for example, to verify that the FPLC peaks corresponded to LiP. Furthermore, it is highly unlikely that LiP apoprotein would elute on FPLC at the same position as the holoenzyme, as claimed in that study. The results presented in Fig. 1 indicate that no LiP protein is present in the extracellular medium of nitrogen-sufficient cultures of *P. chrysosporium*, strongly contradicting the assertions in reference 12.

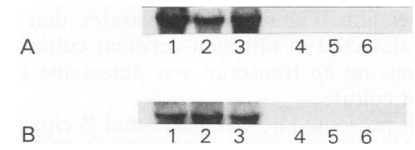

FIG. 2. Detection of *lip* mRNA from nitrogen-limited and nitrogen-sufficient cultures. Both 5-day-old (A) and 6-day-old (B) cultures were harvested, and total RNA was extracted, electrophoresed, transferred to a membrane, and probed as described in the text. Lanes 1 to 3, cultures contained 1.2 mM NH_4 tartrate and 0, 30, and 180 μM $MnSO_4$, respectively; lanes 4 to 6, cultures contained 12 mM NH_4 tartrate and 0, 30, and 180 μM $MnSO_4$, respectively.

The results shown in Fig. 1 and 2 further demonstrate that the appearance of *lip* mRNA and protein is independent of the concentration of Mn in the medium. We showed previously that *mnp* gene transcription and protein synthesis are dependent on the presence of Mn in the culture medium, with maximum *mnp* gene expression occurring at 180 μM Mn (2, 3). Our earlier results also indicated that Mn had little effect on LiP activity and no effect on veratryl alcohol production (3). Our results here confirm that in 2,2-dimethylsuccinate buffer, Mn concentrations ranging from 0 to 180 μM have no apparent effect on either *lip* gene transcription or protein synthesis. We are continuing our studies on the regulation of *lip* and *mnp* gene transcription by nitrogen and other factors.

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