

Lignocellulose Affects Mn^{2+} Regulation of Peroxidase Transcript Levels in Solid-State Cultures of *Pleurotus ostreatus*

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The effect of Mn^{2+} amendment on peroxidase gene expression was studied during *Pleurotus ostreatus* growth on cotton stalks. Four peroxidase-encoding genes were expressed differentially and in a manner different from that observed in defined media. Mn^{2+} affects *mnp3* expression even 2 h after its addition to the cultures, suggesting a direct effect of the metal ion on expression.

Pleurotus species belong to the group of white rot fungi that are responsible for the degradation of lignin (16, 20). *Pleurotus ostreatus* produces three types of oxidative extracellular enzymes: manganese-dependent peroxidases (MnPs) (16a), versatile peroxidases (VPs) (26), and laccases (25). VPs have been found in fungi from the genera *Pleurotus* and *Bjerkandera* and are suggested to be hybrids of MnP and lignin peroxidase (8, 21). The importance of Mn^{2+} ions, which are naturally present in lignocellulosic substrates, for the lignin degradation process by *Pleurotus* species has been demonstrated (6, 9, 17, 18). In recent years, different peroxidases have been purified from *Pleurotus* species, and their genes have been sequenced and analyzed. These include *mnp1* (2), *mnp2* (11), and *mnp3* (14) from *P. ostreatus* as well as *vpl* (corresponding to the *P. ostreatus mnp4* gene analyzed here) and *vps1* from *Pleurotus eryngii*, which show high identity with the *P. ostreatus mnp2* gene (7, 24). Ruiz-Dueñas et al. (23) studied the regulation of *P. eryngii mnp1* transcript levels in peptone-containing liquid medium and stated that no *vpl* transcript could be detected when Mn^{2+} (25 μ M) was present in the medium. Previously, we described the regulation of *P. ostreatus* peroxidase activity and *mnp* gene expression by Mn^{2+} as well as the lignin mineralization rate in peptone medium (PM) under solid-state fermentation (SSF) conditions (9). The reduction in VP gene (*mnp1*, *mnp2*, and *mnp4*) transcript levels and the increase in *mnp3* (which encodes an MnP) transcript level were colinear with the changes observed in the enzyme activity profiles. Bogan et al. (3) analyzed gene expression during *Phanerochaete chrysosporium* bioremediation of organopollutants in soil. Janse et al. (15) analyzed the expression of the *P. chrysosporium* lignin peroxidase-, MnP-, and glyoxal oxidase-encoding genes in wood. In both studies, the observed transcription patterns were different from those found in defined media.

Since the environmental conditions that prevail in defined liquid or solid cultures are different from those in the natural substrate, extrapolation of results obtained during studies performed on cultures growing in defined media to those occur-

ring under natural growth conditions should be performed with caution. As information concerning extracellular peroxidases of white rot fungi accumulates, we believe that it is timely to advance such analyses to conditions which are more similar to the natural lignocellulosic substrate. Such analyses would not only provide information relevant to the natural niche but would also provide a basis for comparing results obtained while analyzing the more amenable liquid culture medium system to the natural growth substrate.

The study of gene expression during SSF in natural substrates is challenging due to the presence of plant polyphenols and polysaccharides, which are known to inhibit PCR and reverse transcription (RT)-PCR (12). In the present study, we report on the comparative abundance of *P. ostreatus* MnP and VP gene transcripts during fungal SSF on a natural lignocellulosic substrate. *P. ostreatus* (pregrown on poor medium as described in reference 9) was used to inoculate sterile cotton stalks (3 g in polypropylene cups). In order to determine the expression levels of the *mnp* genes, we used a relative RT-PCR approach with oligonucleotide primer sets synthesized on the basis of nonconserved sequences of the different peroxidases, as previously described (9). As total RNA could not be used for RT-PCR (due to RT inhibitors present in the natural extract), we used mRNA (isolated with a PolyA-Tract kit; Promega). Quantitative calibration of mRNA was performed using β -tubulin transcript abundance analyses as described previously (9). Briefly, the degrees of abundance of the RT-PCR products obtained from different transcripts and treatments were compared in a reaction that was terminated well within the linear phase of product accumulation. Preliminary results (data not shown) indicate that, under the reaction conditions chosen, 30 cycles of amplification were appropriate. RT-PCR amplifications were performed with RNA samples from different culture conditions in order to compare the relative abundances of *mnp1*, *mnp2*, *mnp3*, *mnp4*, and β -tubulin gene transcripts. Although the natural substrate contains 5 ppm of manganese, our Mn^{2+} amendment (73 ppm) to the cotton stalks affected the expression of the different genes (Fig. 1). Transcripts of *mnp1*, *mnp2*, *mnp3*, and *mnp4* were detected. The most significant effect of Mn^{2+} amendment was on *mnp3* transcript abundance on day 7. The transcript levels of *mnp2* increased on day 13, while *mnp1* transcript levels were appar-

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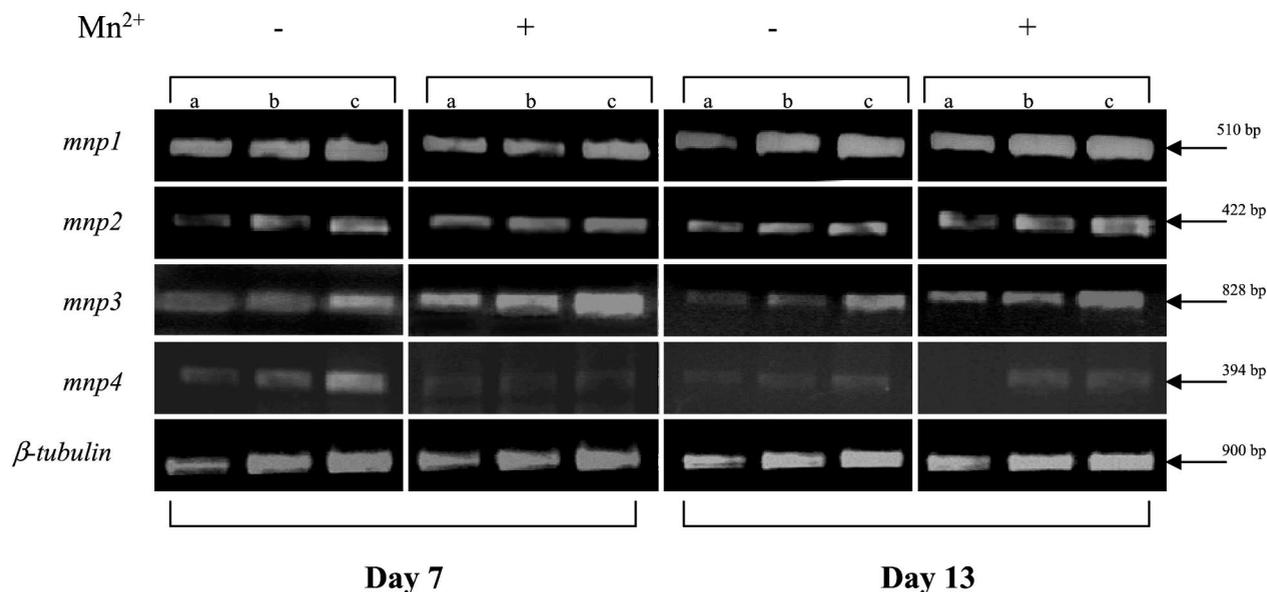


FIG. 1. The effect of Mn^{2+} amendment (73 ppm) on peroxidase gene expression of *P. ostreatus* grown under SSF conditions on cotton stalks. Gene expression was determined by relative RT-PCR utilizing specific primer sets for each gene. β -Tubulin gene expression was used as an internal control. Amplicons with increasing amounts (1-, 1.3-, and 1.5-fold amounts of RNA in panels a, b, and c, respectively) of mRNA template per gram of substrate, established on the basis of the linear range of the β -tubulin amplicon produced (see reference 9), are shown.

ently not affected during the experiment. The *mnp4* transcripts were almost undetectable in all the cultures analyzed, especially in the presence of Mn^{2+} , which corresponds to its preferential expression in liquid cultures, and inhibition by Mn^{2+} as described for *P. eryngii* (23). These results indicate a trend of expression different from that reported when the fungus was cultured under defined conditions using PM and perlite as solid supports. Under these conditions, Mn^{2+} amendment increased MnP-encoding gene transcript levels, while the levels of the VP-encoding gene transcripts declined (9). These differences could be explained by additional factors that affect gene expression, such as nutrient nitrogen (22) or natural inducers that are present in the cotton stalks. Such compounds have been reported to increase laccase activity and lignin degradation by *P. ostreatus* (1) and MnP activity in PM (R. Cohen et al., unpublished results).

Mn^{2+} amendment enhanced lignin degradation during *P. ostreatus* growth on cotton stalks (18). This effect was demonstrated by measuring the [^{14}C]lignin mineralization rate, as well as lignin content, under the same culture conditions used in the present study. Under these conditions cellulose degradation was not affected. Thus, an increase in preferential lignin degradation was achieved and in vitro digestibility of organic matter was enhanced. It was suggested that this may be the result of an increase in the activity of lignin-degrading enzymes (18). In the present study we aim to support this hypothesis by the analysis of changes in peroxidase gene transcript levels.

The regulation of *mnp* and *vp* genes over short periods in *P. chrysosporium* and *P. eryngii*, respectively, has been studied (4, 23). However, induction during SSF on a natural substrate has not been demonstrated in white rot fungi. To determine the kinetics of the alterations in *mnp* transcript levels following Mn^{2+} amendment, we conducted a series of short-term (0 to 8 h) Mn^{2+} induction experiments. Mn^{2+} (23 ppm) was added

to 7-day-old nonamended cultures. The Mn^{2+} was added in the presence of 1% Tween 20 to enable the uptake of Mn^{2+} by the fungus, which produces a hydrophobic surface. Samples were collected after 2, 4, and 8 h. Addition of Tween 20 alone did not affect gene expression (Fig. 2B). A significant and positive effect of Mn^{2+} on *mnp3* expression was detected after 2 h, while the expression levels of *mnp2* were only slightly affected and *mnp1* transcript levels were apparently unchanged. The expression of *mnp4* was hardly detected during the course of this experiment (data not shown).

The same trend was obtained after 4 and 8 h (data not

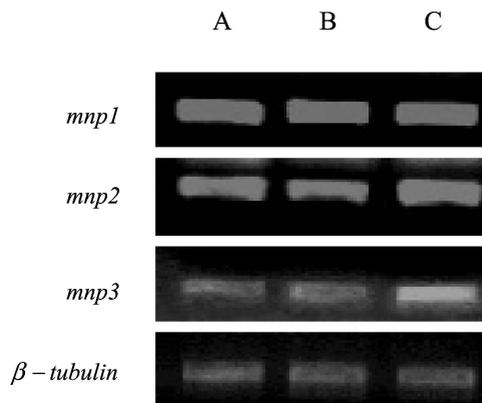


FIG. 2. *P. ostreatus* *mnp* gene expression, 2 h after addition of Mn^{2+} (23 ppm in 1% Tween 20) to a 7-day-old culture grown on cotton stalks. Gene expression was determined using relative RT-PCR and specific primer sets for each gene as explained in the Fig. 1 legend. Since the same pattern of linear range was observed, only one RNA concentration is presented. β -Tubulin gene expression was used as an internal control. (A) Nonamended control; (B) 1% Tween 20 control; (C) Mn^{2+} amendment (23 ppm).

shown). In *P. chrysosporium*, detectable *mnp* mRNA was found within 20 to 40 min of Mn²⁺ amendment of Mn²⁺-deficient nitrogen-limited cultures. It was suggested that the Mn²⁺ may be directly involved in activating gene expression (4).

Different studies have shown that MnP regulation occurs at the level of gene transcription by means of nutrient nitrogen, manganese, heat shock, and other factors (4, 5, 10, 19, 23). These data and those presented here suggest the presence of regulatory elements in the promoter regions of these genes. Since *mnp3* was sequenced without its promoter region (14), we isolated this region (1,000 nucleotides [nt]) (using a GenomeWalker kit; Clontech) and analyzed it in comparison with the other *mnp* genes. Interestingly, this region contained only one metal response element. We therefore continued the sequence analysis further upstream (approximately 2,500 nt sequenced on both strands; GenBank accession number AF435445). Most of the regulatory elements of the *mnp3* promoter were located between 1,000 and 2,400 nt upstream of the ATG, whereas in the other genes the regulatory elements were reported in the first 1,000 nt. We identified seven putative metal response elements in *mnp3*. These findings provide a basis for future functional dissection of the significant and positive effect of Mn²⁺ on this gene's expression. The presence of nitrogen regulatory elements in the promoter region of *mnp3* and in the other genes could explain the differences in gene expression between cotton stalks and medium containing peptone. The advances in use of the *P. ostreatus* DNA-mediated transformation system (13) should prove useful in future alteration of the regulation of peroxidase genes in this fungus.

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