Applied and Environmental Microbiology, Jan. 1995, p. 341–345 Vol. 61, No. 1
0099-2240/95/$04.00+0
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Regulation of Manganese Peroxidase Gene Transcription by Hydrogen Peroxide, Chemical Stress, and Molecular Oxygen

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Received 23 May 1994/Accepted 28 October 1994

The expression of manganese peroxidase (MnP) in nitrogen-limited cultures of the lignin-degrading fungus *Phanerochaete chrysosporium* is regulated at the level of gene transcription by H_2O_2 and various chemicals, including ethanol, sodium arsenite, and 2,4-dichlorophenol, as well as by Mn(II) and heat shock. Northern (RNA) blot analysis demonstrates that the addition of 1.0 mM H_2O_2 to 5-day-old cultures grown in the absence of Mn results in the appearance of *mnp* mRNA within 15 min. Higher levels of *mnp* mRNA are obtained with simultaneous induction by Mn and H_2O_2 than with H_2O_2 alone. Although neither MnP activity nor associated protein is detectable in H_2O_2-induced cultures grown in the absence of Mn, simultaneous induction with Mn and H_2O_2 results in a 1.6-fold increase in MnP activity compared with the MnP activity resulting from Mn induction alone. In the presence of Mn, purging of low-nitrogen cultures with 100% O_2, in contrast to incubation under air, results in an increase in the accumulation of *mnp* mRNA and a 13-fold increase in MnP activity on day 5. However, in contrast to the effects of H_2O_2 and heat shock, O_2 purging of Mn-deficient cultures results in negligible accumulation of *mnp* mRNA.

The white rot basidiomycete *Phanerochaete chrysosporium* degrades lignin (8, 21, 27) and a variety of aromatic pollutants (7, 22, 24, 45) during the secondary metabolic (idiophasic) stage of growth. Two isozyme families of secreted 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 (21, 27). Elucidation of the catalytic mechanisms of these enzymes (21, 27, 46, 48); the cloning, characterization, and expression of the genes encoding various isozymes (18, 20); and the recent determinations of the crystal structures of LiP and MnP (13, 36, 42) have increased our understanding of the biochemistry and genetics of this unique extracellular oxidative system.

In the presence of manganous ion [Mn(II)], MnP catalyzes the H_2O_2-dependent oxidation of a variety of phenolic lignin model compounds (21, 44, 48) and the in vitro depolymerization of lignin (47). The enzyme catalyzes the oxidation of Mn(II) to Mn(III). Mn(III), complexed with an organic acid such as oxalate secreted by *P. chrysosporium*, oxidizes the terminal phenolic substrate (14, 21, 44, 48). MnP occurs as a series of isozymes encoded by a family of genes, and the sequences of two *mnp* cDNAs (33, 37) and *mnp* genomic clones (*mnp1* and *mnp2*) (16, 31) have been reported.

Previously, we reported that MnP is regulated by nutrient nitrogen at the level of gene transcription (37) and that in *P. chrysosporium* and other white rot fungi, MnP activity is dependent on the presence of Mn(II) in the culture medium (2, 4, 35, 38). We also demonstrated that the addition of Mn to *P. chrysosporium* cultures grown in the absence of Mn induces *mnp* gene transcription (3, 4) and that the expression of MnP in nitrogen-limited cultures is regulated by heat shock at the level of gene transcription (5). Treatment with H_2O_2 or a variety of other chemicals can induce the heat shock response in both prokaryotes and eukaryotes (9, 11, 32). Since lignin degradation is an oxidative process and *P. chrysosporium* produces H_2O_2 as part of its extracellular lignin-degrading system, we decided to examine the effects of oxidative and chemical stresses on *mnp* gene expression and MnP activity.

MATERIALS AND METHODS

Culture conditions. *P. chrysosporium* OGC101 was maintained on slants as described previously (19). The organism was grown at 37°C from a conidial inoculum in 20-ml stationary-phase cultures in 250-ml Erlenmeyer flasks, as described previously (4). Cultures were incubated under air for 2 days, after which they were purged daily with 100% O_2 for 10 min, unless otherwise noted. The medium was as described previously (4, 28), 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. As indicated, $MnSO_4$ was added to a final concentration of 180 μM or cultures were heat shocked by transfer to a 45°C water bath. The effect of H_2O_2 on cell viability was measured by inoculating mycelial plugs onto agar plates containing growth medium (19) supplemented with various concentrations of H_2O_2 and comparing the degree of radial growth.

Chemicals. H_2O_2, sodium arsenite, and cycloheximide were obtained from Sigma, and peracetic acid and 2,4-dichlorophenol were obtained from Aldrich Chemical Co. H_2O_2 concentration was measured by the horseradish peroxidase method (10).

RNA preparation and Northern (RNA) blot hybridization. Cells were filtered through Miracloth (Calbiochem), rinsed twice with cold distilled water, quick-frozen in liquid nitrogen, and stored at −80°C. 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 (5). 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 membranes (Micron Separations, Inc.) and hybridized at 42°C with ^{32}P-labeled probes as previously described (3). The *mnp1* cDNA (37) was used as a template for randomly primed synthesis of [α-^{32}P]dCTP-labeled (Dupont-New England Nuclear) probes with a Multiprime DNA Labeling Kit (Amersham). RNA blots were washed and exposed to Kodak XAR-5 X-ray film.

MnP enzyme assays and protein analysis. Aliquots (50 to 100 μl) of the extracellular medium were added to the reaction mixture (1 ml) consisting of 50 mM malonate (pH 4.5) and 0.5 mM $MnSO_4$. Reactions were initiated by the addition of H_2O_2 to a final concentration of 0.1 mM. Oxidation of Mn(II) to Mn(III) was measured by the increase in A_{270} (48). Intracellular protein prep-

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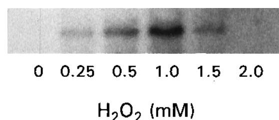

FIG. 1. H_2O_2 induction of *mnp* mRNA. H_2O_2 was added to 5-day-old nitrogen-limited, Mn-deficient cultures at the indicated final concentrations. The cells were harvested after 1 h, and total RNA was extracted, electrophoresed, transferred to a membrane, and probed as described in Materials and Methods.

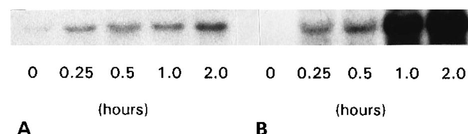

FIG. 3. Time course for the appearance of *mnp* mRNA following H_2O_2 induction in the presence and absence of Mn. H_2O_2 (1.0 mM) was added to 5-day-old, nitrogen-limited, Mn-deficient cultures with (B) or without (A) concurrent induction with Mn. The cells were harvested at the indicated times following the addition of H_2O_2, and total RNA was extracted and probed as described in Materials and Methods.

arations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot (immunoblot) analysis were carried out as previously described (4).

RESULTS

H_2O_2 induction of *mnp* mRNA. H_2O_2 was added to 5-day-old, nitrogen-limited, Mn-deficient cultures of *P. chrysosporium* to final concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 mM. The cells were harvested after 1 h. Accumulation of *mnp* mRNA was detected from cell cultures exposed to H_2O_2 concentrations of from 0.25 to 1.5 mM, with maximum accumulation at 1.0 mM. No *mnp* mRNA was detected in the absence of H_2O_2 or with H_2O_2 concentrations above 1.5 mM (Fig. 1). The concentration of endogenous H_2O_2 in the extracellular medium of 5-day-old, nitrogen-limited cultures was determined to be less than 4 μM. Over a 24-h period, radial growth of *P. chrysosporium* on plates containing 0.5 mM H_2O_2 was inhibited by less than 3% compared with growth on plates lacking added H_2O_2. Plates containing 1.0 mM H_2O_2 exhibited less than 13% inhibition of growth over the same period, whereas 26% inhibition was observed for cultures with 2.0 mM H_2O_2.

No *mnp* mRNA was detectable following H_2O_2 induction of nitrogen-sufficient cultures (data not shown). The growth stage dependence of H_2O_2 induction of *mnp* mRNA in nitrogen-deficient cultures is shown by the results in Fig. 2. Three- to seven-day-old, Mn-deficient cultures were treated with 1.0 mM H_2O_2, with or without concurrent Mn induction, and the cells were harvested after 1 h. H_2O_2 induction of *mnp* mRNA in the absence of Mn was first detectable on day 4, was maximal on days 5 and 6, and declined on day 7. *mnp* mRNA induction in cells treated concurrently with Mn and H_2O_2 was maximal on day 5 and declined on subsequent days. On days 4, 5, and 6, more *mnp* mRNA was detected from cultures that were simultaneously induced with Mn and H_2O_2 than from those induced with H_2O_2 alone.

A time course for H_2O_2 induction of *mnp* mRNA in 5-day-old, nitrogen-limited, Mn-deficient cultures is shown in Fig. 3A. *mnp* mRNA was detected in cells harvested 15 min after induction with 1.0 mM H_2O_2 and continued to accumulate for at least 2 h. Again, accumulation of *mnp* mRNA appeared to be greater at all time points following concurrent Mn and H_2O_2 induction than accumulation after induction with H_2O_2 alone. (Fig. 3B).

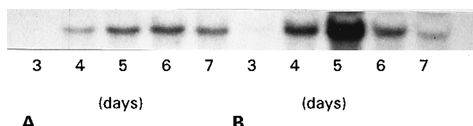

FIG. 2. Growth-stage-specific induction of *mnp* mRNA by H_2O_2 in the presence or absence of Mn. Nitrogen-limited, Mn-deficient cultures were grown for the indicated number of days, after which 1.0 mM H_2O_2 was added with (B) or without (A) simultaneous induction with Mn. The cells were harvested after 1 h, and total RNA was extracted and probed as described in Materials and Methods.

MnP activity and the production of MnP protein by cells under H_2O_2 induction in the presence and absence of Mn were examined. Triplicate 5-day-old, Mn-deficient, nitrogen-limited cultures of *P. chrysosporium* were induced with 180 μM Mn, 0.5 mM H_2O_2, or both, and MnP activity in the extracellular medium was assayed at various intervals over the following 48 h. MnP activity was not detected in the extracellular medium of cells treated with H_2O_2 alone. However, the level of MnP activity in the extracellular medium of cells treated with both Mn and H_2O_2 was higher at all time points than the level of MnP activity from cultures treated with Mn alone (Fig. 4A). At 35 h after induction, MnP activity was increased 1.6-fold by Mn plus 0.5 mM H_2O_2 over that by Mn alone. Western blot analysis was performed on extracellular (Fig. 4B) and intracellular (data not shown) proteins from cells grown in Mn-deficient, nitrogen-limited medium for 5 days prior to an additional 4-h incubation with or without 1.0 mM H_2O_2 in the presence or absence of 180 μM Mn. MnP protein was detected only from cultures incubated in the presence of Mn.

Other chemical stresses. Five-day-old, Mn-deficient, nitro-

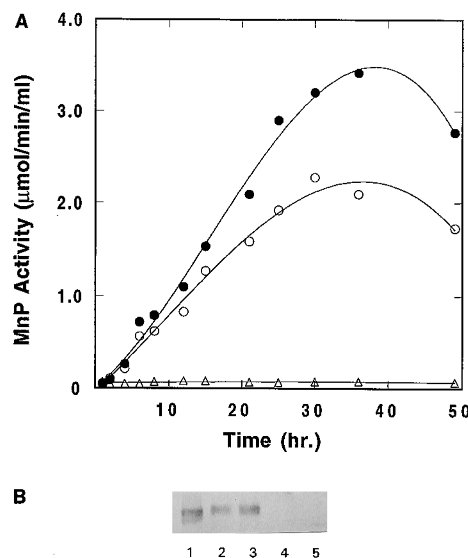

FIG. 4. MnP activity and immunoblot analysis of extracellular MnP following induction by Mn and/or H_2O_2. (A) Activities of Mn-deficient nitrogen-limited cultures grown for 5 days prior to the addition of H_2O_2 alone (0.5 mM) (Δ), Mn alone (○), or H_2O_2 plus Mn (●). MnP activity in the extracellular medium was assayed at the indicated times as described in the text. (B) Western blot of the extracellular media from cultures prepared as described for panel A, with the addition of an untreated control (lane 5). After 4 h, samples of extracellular medium were subjected to sodium dodecyl sulfate-polyacryamide gel electrophoresis, electrophoretic transfer, and immunodetection as described previously (4). The lanes were loaded with purified MnP isozyme 1 alone (lane 1) or with medium from cultures with Mn (lane 2), H_2O_2 (1.0 mM) plus Mn (lane 3), or H_2O_2 alone (lane 4) or with the untreated control (lane 5).

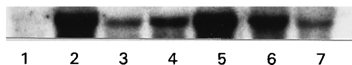

FIG. 5. Induction of *mnp* gene transcription by various chemical stresses. Five-day-old, Mn-deficient, nitrogen-limited cultures were incubated with chemicals at the final concentrations indicated in parentheses: lane 1, untreated control; lane 2, H_2O_2 (1.0 mM); lane 3, peracetic acid (1.0 mM); lane 4, ethanol (10%, vol/vol); lane 5, sodium arsenite (1.0 mM); lane 6, 2,4-dichlorophenol in *N,N*-dimethylformamide (0.5 mM); and lane 7, *N,N*-dimethylformamide (1.65%, vol/vol). The cells were harvested after 1 h, and total RNA was extracted and probed as described in Materials and Methods.

gen-limited cultures of *P. chrysosporium* were incubated, for 1 h prior to harvesting, with each of the following chemicals at the final concentration indicated in parentheses: peracetic acid (1.0 mM), ethanol (10%, vol/vol), sodium arsenite (1.0 mM), 2,4-dichlorophenol (0.5 mM) dissolved in *N,N*-dimethylformamide (1.65%, vol/vol), and *N,N*-dimethylformamide alone (1.65%, vol/vol). The addition of each of the above-mentioned compounds resulted in the accumulation of detectable *mnp* mRNA (Fig. 5). Other organic peroxides such as *t*-butyl hydroperoxide and cumene hydroperoxide at concentrations of 1.0 mM did not induce *mnp* mRNA (data not shown). Cycloheximide added to a final concentration of 50 μg/ml did not prevent the induction of *mnp* mRNA by either heat shock or H_2O_2. However, at that concentration, cycloheximide alone appeared to induce *mnp* gene transcription, resulting in several bands that probed with the *mnp1* cDNA but that were larger than the normally induced *mnp* transcript (data not shown).

Effect of O_2 purging. The effect of O_2 purging on MnP activity is shown in Fig. 6A. Cultures were grown in low-nitrogen medium containing 180 μM $MnSO_4$. Daily purging with 100% O_2 for 10 min on days 3 through 6 resulted in an eightfold increase in MnP activity on day 5 and at least a

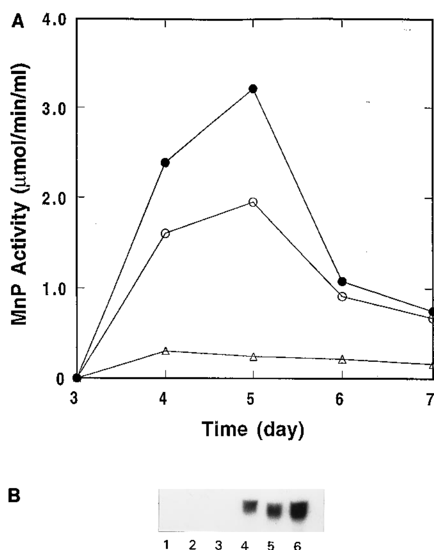

FIG. 6. Effects of O_2 purging on MnP activity and *mnp* gene transcription. (A) Activities of nitrogen-limited cultures that were supplemented with 180 μM Mn and grown under air for 2 days, after which they were purged daily for 10 min with 100% O_2 (○), purged daily for 2 h with 100% O_2 (●), or not purged (Δ). MnP activity in the extracellular medium was assayed on days 3 through 7 as described in the text. (B) Northern blot of nitrogen-limited, Mn-deficient cultures (lanes 1 to 3) and cultures supplemented with 180 μM Mn (lanes 4 to 6), grown under air for 2 days, and then purged for 10 min with 100% O_2 on days 3 and 4 (lanes 2 and 5), purged for 2 h with O_2 on days 3 and 4 (lanes 3 and 6), or not purged with O_2 (lanes 1 and 4). The cells were harvested on day 5. Total RNA was extracted and probed as described in Materials and Methods.

fourfold increase at all time points compared with activities under an air atmosphere. Daily purging with O_2 for 2 h resulted in a 13-fold activity increase on day 5 compared with activities under air alone.

The effects of O_2 purging on *mnp* gene transcription in the presence and absence of Mn are shown in Fig. 6B. Considerably more *mnp* mRNA was detected from 5-day-old Mn-supplemented cells that were purged with O_2 for 2 h each on days 3 and 4 than from Mn-sufficient cultures incubated under air or purged with O_2 for 10 min only on days 3 and 4 (compare Fig. 6B, lane 6 with lanes 4 and 5). Under the conditions used in this study, O_2 purging did not result in detectable *mnp* mRNA under Mn-deficient conditions (Fig. 6B, lanes 1 to 3). However, prolonged exposure of the probed Northern blot resulted in a faint signal from Mn-deficient cultures that were purged with O_2 for 2 h on days 3 and 4 (data not shown).

DISCUSSION

The lignin degradative system of *P. chrysosporium* is expressed during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting of nutrient nitrogen (8, 18, 27). Likewise, LiP and MnP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth (27) and Northern blot analysis has demonstrated that expression of LiP and MnP is controlled at the level of gene transcription by nutrient nitrogen (29, 37, 43). There also is evidence that various isozymes of LiP and MnP may be differentially regulated by carbon and nitrogen (23, 34, 40).

We have been examining additional specific inducers of MnP expression and have shown that MnP is regulated by Mn(II), the substrate for the enzyme, at the level of gene transcription (3, 4). Both the *mnp1* and *mnp2* promoters contain multiple putative consensus metal response elements (16, 18, 31) identical to *cis*-acting sequences that are responsible for heavy metal induction of mammalian metallothionein genes (12). The *mnp1* and *mnp2* promoters also contain putative heat shock elements (16, 18, 31), and we recently reported on parameters affecting heat shock induction of *mnp* gene transcription and suggested that the heat shock elements are physiologically functional (5). Neither metal response elements nor heat shock elements have been found in the promoter regions of sequenced *lip* genes.

In addition to thermal stress, a wide variety of chemical agents are known to induce heat shock proteins (30, 32, 41). These factors include heavy metals, various organic compounds, and oxidants (32, 39). Since lignin degradation is an oxidative process during which the fungus generates H_2O_2 as the cosubstrate for LiP and MnP, we examined the induction of *mnp* gene transcription by H_2O_2 and other chemical agents, as well as the effect of incubation under 100% O_2.

Northern blot analysis indicates that maximum accumulation of *mnp* mRNA occurs with H_2O_2 added to a final concentration of 1.0 mM (Fig. 1). No *mnp* mRNA is detectable when the cells are induced with 2.0 mM H_2O_2. Our results indicate that 1.0 mM exogenous H_2O_2 has only minimal effects on the growth of *P. chrysosporium* cultures. As with Mn induction (3, 4) and heat shock induction (5) of *mnp* gene transcription, H_2O_2 induction of *mnp* gene transcription occurs only under conditions of nitrogen limitation. Thus, *P. chrysosporium* appears to employ a hierarchy of regulatory strategies for controlling MnP production.

The growth-stage-specific H_2O_2 induction (Fig. 2) and the time course for H_2O_2 induction of *mnp* gene transcription (Fig. 3) are similar to parameters observed for heat shock induction

of *mnp* mRNA (5), suggesting that heat shock and H_2O_2 may be acting via the same or related mechanisms. In contrast, the additive effects of H_2O_2 and Mn induction (Fig. 2 and 3) or heat shock and Mn induction (5) suggest that Mn induces *mnp* gene transcription via a different mechanism.

No MnP activity or protein is detectable after H_2O_2 induction in the absence of Mn (Fig. 4). However, in the presence of Mn, more MnP activity is obtained after simultaneous induction with Mn and H_2O_2 than after induction with Mn alone (Fig. 4A). A similar effect is seen with heat shock induction of MnP (5).

Many other compounds are known to induce heat-shock-like responses in a variety of systems (32). In this study, peracetic acid, ethanol, sodium arsenite, 2,4-dichlorophenol, and *N,N*-dimethylformamide were all found to induce *mnp* gene transcription (Fig. 5). Ethanol and arsenite are known to induce the synthesis of heat shock proteins in bacteria and fungi (25, 32). As with H_2O_2 and heat shock, no MnP protein was obtained with these inducers in the absence of Mn.

It is not yet clear why induction with heat shock or chemical stress does not result in production of MnP protein. The elevated activity levels obtained with simultaneous induction by Mn and either heat shock (5) or H_2O_2 (Fig. 4A) suggest that the transcript induced by these agents is translatable in the presence of Mn and that the presence of H_2O_2 does not interfere with translation of the *mnp* mRNA. The *mnp* genes are not typical of genes transcribed during heat shock in that they contain six or seven small introns and lack a long 5′ untranslated leader sequence (16, 31). Although Northern blot analysis shows no obvious differences between the *mnp* mRNA induced by either heat shock or H_2O_2 and that induced by Mn, small differences in transcript size, resulting from a variable transcription start site or incomplete processing, would not necessarily be detectable.

It is possible that Mn has a role in mRNA stability. However, our recent results indicate that only the region 5′ to the *mnp1* translation initiation codon is required for conferring Mn inducibility on a heterologous reporter gene (15). It also is possible that Mn has an additional role in posttranscriptional processing of MnP. For example, Mn may be required for correct processing or translation of the *mnp* message synthesized under heat shock or chemical or oxidative stress.

H_2O_2 recently was shown to activate the heat shock transcription factor in fibroblasts, and this reaction was inhibited by an iron chelator, suggesting that the H_2O_2 effect may be mediated via the generation of hydroxyl radicals by the Fenton reaction (6). Alternatively, H_2O_2 may act indirectly, perhaps by causing membrane damage or the denaturation of preexisting proteins which, in turn, may result in a heat shock response (1, 11, 17).

An intracellular peroxidase in *Neurospora crassa* also is known to be induced by heat shock and H_2O_2. Treatment with H_2O_2 leads to thermotolerance in this organism, and it has been suggested that the induced peroxidase protects the cell from active oxygen species, including H_2O_2, that accumulate during heat shock and other stress (25). It is not clear why *mnp* gene transcription is induced by H_2O_2. However, *P. chrysosporium* produces H_2O_2 as part of its extracellular lignin-degrading system, and induction of *mnp* by H_2O_2 or other oxidants produced during lignin degradation may be a factor in regulating the peroxide levels in this system. We are investigating the *mnp* promoter elements that are responsible for H_2O_2 and/or heat shock induction of *mnp* gene transcription.

Lignin degradation is an oxidative process, and a high-O_2 atmosphere enhances ligninolytic activity by *P. chrysosporium* (26, 28). We therefore examined the effects of O_2 on MnP activity and *mnp* gene transcription. As seen from the results in Fig. 6A, purging with 100% O_2 for 10 min on days 3 and 4 resulted in a large increase in MnP activity compared with MnP activity in cultures growth under air. Increasing the duration of O_2 purging to 2 h resulted in a further increase in MnP activity. In the absence of Mn, O_2 purging resulted in only a negligible amount of *mnp* mRNA. However, in the presence of Mn, 2 h of O_2 purging on days 3 and 4 resulted in substantially more *mnp* mRNA on day 5 than was obtained from cells grown under air or purged for only 10 min (Fig. 6B). This observation suggests that although the effect of O_2 is, at least in part, at the level of *mnp* gene transcription, it appears to be distinct from the mechanism of H_2O_2 induction. It is possible that O_2 is positively affecting Mn induction of *mnp* gene transcription. This is the first observation of an O_2 effect on *mnp* gene transcription, and it may explain, at least in part, the positive effect of O_2 on lignin degradation (26, 28). We are continuing to investigate the effects of O_2 and other oxidants on MnP activity and gene transcription.

ACKNOWLEDGMENTS

This work was supported by grants MCB 9405978 and MCB-9207997 from the National Science Foundation and FG 06292 ER-20093 from the U.S. Department of Energy, Division of Energy Biosciences.

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