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Manganese Regulates Expression of Manganese Peroxidase by *Phanerochaete chrysosporium*

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The appearance of manganese peroxidase (MnP) activity in nitrogen-limited cultures of *Phanerochaete chrysosporium* is dependent on the presence of manganese. Cultures grown in the absence of Mn developed normally and produced normal levels of the secondary metabolite veratryl alcohol but produced no MnP activity. Immunoblot analysis indicated that appearance of MnP protein in the extracellular medium was also dependent on the presence of Mn. Intracellular MnP protein was detectable only in cells grown in the presence of Mn. MnP mRNA was detected by Northern (RNA) blot analysis only in cells grown in the presence of Mn. If Mn was added to 4-day-old nitrogen-limited Mn-deficient cultures, extracellular MnP activity appeared after 6 h and reached a maximum after 18 h. Both actinomycin D and cycloheximide inhibited the induction of MnP activity by Mn. These results indicate that Mn, the substrate of the enzyme, is involved in the transcriptional regulation of the MnP gene.

Lignin, the second most abundant natural polymer, is a complex, optically inactive phenylpropanoid matrix that constitutes 20 to 30% of woody plants (6, 36). White rot basidiomycetes are primarily responsible for the initiation of the decomposition of lignin in wood (4, 16, 20). The white rot basidiomycete *Phanerochaete chrysosporium* degrades lignin during the secondary metabolic phase of growth, which is triggered by limiting cultures for nutrient nitrogen (4, 20). Under ligninolytic conditions, *P. chrysosporium* secretes two extracellular heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), which, along with an H_2O_2-generating system, are apparently the major components of its lignin degradation system (4, 16, 20). The structure and mechanism of LiP have been studied extensively (16, 20, 28, 42), and cDNA (7, 44) and genomic sequences (2, 41, 46) encoding several LiP isozymes have been reported.

MnP was discovered in our laboratory (22) and has been purified and characterized (12, 13, 16, 22, 29, 31, 47). This peroxidase is an H_2O_2-dependent heme glycoprotein of M_r ~46,000 with an iron protoporphyrin IX prosthetic group. Like LiP, MnP exists as a series of isozymes (26). Nucleotide sequences of cDNAs encoding two MnP isozymes have recently been reported (32, 33). MnP catalyzes the Mn(II)-dependent oxidation of a variety of phenolic lignin model compounds (12, 13, 16, 31, 49). Most importantly, MnP preferentially oxidizes Mn(II) to Mn(III), and the latter is chelated by organic acids (13, 16). The Mn(III)-organic acid complex produced is primarily responsible for the oxidation of the organic substrates (12, 13, 31, 47, 48). Herein, we demonstrate that the production of MnP by cultures of *P. chrysosporium* is regulated by Mn as well as by nutrient nitrogen (33). Furthermore, inhibitor studies and Northern (RNA) blot analysis suggest that this regulation occurs at the level of gene transcription.

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MATERIALS AND METHODS

Culture conditions. *P. chrysosporium* OGC101 (1) was maintained on slants as previously described (14). The organism was grown at 38°C from a conidial inoculum in 20-ml stationary cultures in 250-ml Erlenmeyer flasks as described previously (9). Cultures were incubated under air for 2 days, after which they were purged daily with 100% O_2. Under these conditions, lignin degradation is optimal (16, 20). Unless otherwise indicated, the medium was as previously described (21), with 2% glucose as the sole carbon source and 1.2 mM ammonium tartrate (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen) and 20 mM sodium-2,2-dimethylsuccinate (pH 4.5) as the buffer. As indicated, various concentrations of $MnSO_4$ were added to a modified trace elements solution (21). The 10× basal medium contained the following per liter of glass-distilled water: KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; thiamine, 0.01 g; and mineral solution, 10.0 ml. Minerals (per liter of glass-distilled water) consisted of the following: nitrilotriacetate, 1.5 g; $MgSO_4 \cdot 7H_2O$, 3.0 g; NaCl, 1.0 g; $FeSO_4 \cdot 7H_2O$, 100 mg; $CoSO_4$, 100 mg; $CaCl_2 \cdot 2H_2O$, 100 mg; $ZnSO_4$, 100 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $AlK(SO_4)_2$, 10 mg; H_3BO_3, 10 mg; and $NaMo \cdot O_4$, 10 mg. $MnSO_4 \cdot H_2O$ was added to the indicated concentrations. Mycelium weights were determined after collection and drying on tared filter paper disks.

Enzyme assays. MnP activity was measured by using the diammonium 2,2′-azino-bis(3-ethyl-6-benzothiazolinesulfonate) (ABTS) assay as previously described (13). Assay mixtures (1 ml) contained 20 mM sodium succinate (pH 4.5), 50 mM sodium lactate, 200 μM $MnSO_4$, gelatin (3 mg), ABTS (80 μg), and 100 μM H_2O_2. Reactions were initiated by the addition of 10 μl of culture medium and monitored at 415 nm (13). LiP activity was measured by using the veratryl alcohol oxidation assay as previously described (15, 43). Veratryl alcohol in the extracellular medium was separated by high-performance liquid chromatography using a C-18 reverse-phase column and monitored at 310 nm.

Disc electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as previously described (17, 23). Electrophoretic transfer to

nitrocellulose was as described previously (45), using a Bio-Rad transfer apparatus. Rabbit polyclonal antibody was prepared against purified MnP isozyme I (33). Immunodetection was performed according to a modification of a previous procedure (3, 24). Nitrocellulose transfers were first incubated in TTBS (10 mM Tris chloride [pH 8.0] containing 0.05% Tween 20 and 150 mM NaCl) and 3% gelatin. Transfers were incubated with primary antibody (25 μl of rabbit anti-MnP in 50 ml of TTBS) and washed three times with TTBS. Transfers were then incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase in TTBS. After three further washes, alkaline phosphatase was detected by using the 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium assay (24). Color development was stopped by rinsing the transfer in distilled water. All incubations and washes were carried out at room temperature with gentle shaking. Intracellular protein extracts were prepared by grinding frozen mycelia with a mortar and pestle in liquid N_2 and extracting with 20 mM sodium succinate (pH 4.5).

RNA preparation and Northern blot hybridizations. Cultures were filtered through Miracloth, washed twice with distilled water, quick-frozen in liquid N_2, and stored at −80°C until used. Frozen mycelia (~2 g) were ground to a powder with a mortar and pestle under liquid N_2 and homogenized with a Polytron (Brinkmann Instruments, Inc.), using three 10-s bursts in 10 ml of TSE (10 mM Tris chloride [pH 7.5] containing 1 mM sodium dodecyl sulfate and 5 mM EDTA) and 0.2 mg of proteinase K (Sigma Chemical Co.) per ml. The mixture was incubated at 45°C for 1 h and then extracted with an equal volume of water-saturated phenol. The organic phase was back-extracted with 0.5 volumes of TSE, and the two aqueous phases were reextracted with an equal volume of phenol. The resultant aqueous phase was extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol and then dissolved in water. RNA was precipitated with 2 M LiCl, dissolved in water, and stored at −80°C.

MnP isoenzyme I cDNA (33) was used as a template for random-primed synthesis of ^{32}P-labeled probes (10). Random hexanucleotide primers and [α-^{32}P]dCTP (800 Ci/mmol) were obtained from Pharmacia LKB Biotechnology Inc. and from Dupont, NEN Research Products, respectively. RNA was electrophoresed in 1.0% agarose gels containing 0.7 M formaldehyde, transferred to Biotace RP membranes, and hybridized at 42°C as described elsewhere (R. M. Fourney, J. Miyakoshi, R. S. Day III, and M. C. Paterson, Focus **10**:5, 1988).

RESULTS

A time course for MnP activity in the extracellular medium of nitrogen-limited cultures of *P. chrysosporium* is shown in Fig. 1. Cultures grown in the presence or absence of 180 μM Mn(II) were assayed for enzyme activity from day 1 through day 8. Cultures grown in the absence of Mn had no detectable MnP activity through day 8, whereas MnP activity was detectable by day 4 in cultures grown in the presence of 180 μM Mn(II) and reached a maximum on day 5, after which MnP activity steadily decreased. Under these conditions, cultures grown in the presence of Mn had approximately two times more LiP activity than cultures grown in the absence of this metal. However, the appearance of LiP activity was not absolutely dependent on Mn (data not shown). Mn(II) could not be replaced by 180 μM Fe(III), Ni,

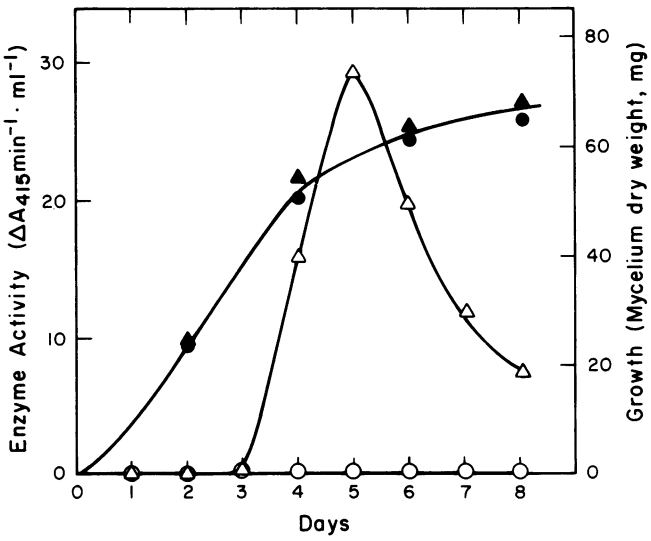

FIG. 1. Effect of Mn supplementation on the appearance of extracellular MnP activity. Nitrogen-limited cultures were grown from a conidial inoculation as described in the text. Extracellular MnP activity from duplicate cultures grown in the presence (△) or absence (○) of 180 μM Mn was assayed as described in the text. Mycelia from triplicate cultures grown in the presence (▲) or absence (●) of Mn were collected by filtration, dried, and weighed.

Mo, Zn, Co, or Mg as an inducer of MnP. Figure 1 also shows the growth curve for cultures grown in the presence and absence of Mn. Cultures entered stationary phase on approximately day 3, and MnP activity in the extracellular medium reached a maximum on day 5. These results indicate that the addition of excess Mn did not have a significant effect on growth. The extracellular medium of cultures grown in either the presence or absence of Mn contained approximately 350 μM veratryl alcohol on day 6. There was also no difference in the linear growth rates (1.54 mm/h) as measured in growth tubes (35) when *P. chrysosporium* was grown on solid, nitrogen-sufficient medium in the presence or absence of Mn.

The appearance of MnP in the extracellular medium as detected by immunoblot analysis correlated with enzyme activity (Fig. 2). In cultures grown in the presence of Mn, MnP protein was detected starting on day 4, persisting through day 6. MnP protein was not detectable in the extracellular medium of cultures grown in the absence of Mn. To determine whether the absence of MnP in the extracellular medium of Mn-deficient cultures was the result of defective secretion, the presence of intracellular MnP was determined. Immunoblot analysis of intracellular MnP from 5-day-old cells demonstrated MnP protein in cells grown in the presence of Mn but not in its absence (data not shown).

The effect of Mn concentration on MnP induction is shown in Fig. 3. *P. chrysosporium* was grown for 4 days in the absence of Mn, after which the indicated concentrations of Mn were added to the media, and the cultures were then purged with 100% O_2 and reincubated. No MnP activity was detected before the addition of Mn(II). MnP activity first appeared 6 h after the addition of Mn and reached a maximum 18 to 24 h after the addition. Furthermore, the extent of induction of MnP activity correlated with the Mn(II) concentration up to ~180 μM Mn. Above this concentration, the effect of induction leveled off (data not shown). Addition of Mn to the cell-free extracellular medium

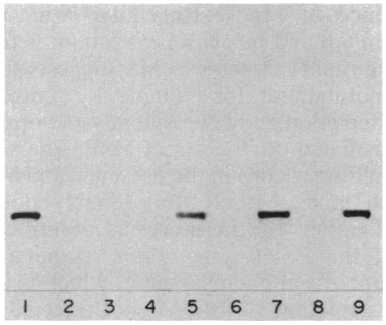

FIG. 2. Immunoblot analysis of extracellular MnP protein. Samples of the extracellular medium from Mn-deficient and complete cultures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretic transfer, and immunodetection as described in the text. Samples were as follows: MnP standard (lane 1); day 3, Mn deficient (lane 2); day 3, Mn complete (lane 3); day 4, Mn deficient (lane 4); day 4, Mn complete (lane 5); day 5, Mn deficient (lane 6); day 5, Mn complete (lane 7); day 6, Mn deficient (lane 8); day 6, Mn complete (lane 9).

from cultures grown for 5 days in the absence of Mn did not result in detectable MnP activity.

Figure 4 shows an immunoblot analysis of the effect of various Mn concentrations on MnP induction. Nitrogen-limited cultures of *P. chrysosporium* were grown for 4 days in the absence of Mn. Mn was then added to the indicated final concentrations, and the cultures were incubated for an additional 18 h. The intensity of the protein bands detected by Western immunoblot analysis correlated with the amount of Mn added to the cultures and reached a maximum at 180 μM Mn.

The effects of the inhibitors cycloheximide and actinomycin D on the induction of MnP by Mn are shown in Fig. 5. When either of these inhibitors was added with Mn to 4-day-old cultures grown in the absence of Mn, subsequent appearance of MnP activity was inhibited at least 85%. In

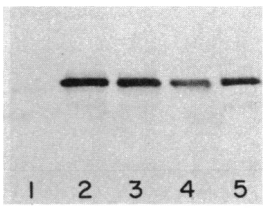

FIG. 4. Effect of Mn concentration on MnP protein induction. Various concentrations of Mn were added to 4-day-old Mn-deficient cultures. Samples were removed and subjected to immunodetection as described in the text. Final Mn concentrations in the cultures were as follows: 0 μM (lane 1), 90 μM (lane 2), 180 μM (lane 3), 360 μM (lane 4). Lane 5, MnP standard.

vitro MnP activity was not significantly inhibited by these compounds. The inhibiting effect of the RNA synthesis inhibitor actinomycin D suggests that Mn regulates MnP gene transcription, either directly or indirectly. Because of the inhibition of the appearance of MnP activity by actinomycin D, we determined the effect of Mn on the levels of MnP mRNA. No MnP mRNA was detectable in nitrogen-limited cultures grown in the absence of Mn (Fig. 6). In cultures grown in the presence of 180 μM Mn, MnP mRNA was detected on day 4 and reached a maximum on day 5. This result is in agreement with the appearance of enzyme activity and MnP protein in the presence or absence of 180 μM Mn.

DISCUSSION

MnP is a heme-containing enzyme isolated from the extracellular medium of ligninolytic cultures of the white rot basidiomycete *P. chrysosporium* (12, 13, 22, 31, 47). MnP requires H_2O_2 as a cosubstrate and catalyzes the Mn(II)-dependent oxidation of a variety of simple phenols and phenolic lignin substructures (12, 13, 31, 47, 49). The enzyme oxidizes Mn(II) to Mn(III). The generated Mn(III) is chelated by an organic acid, and the complex diffuses from

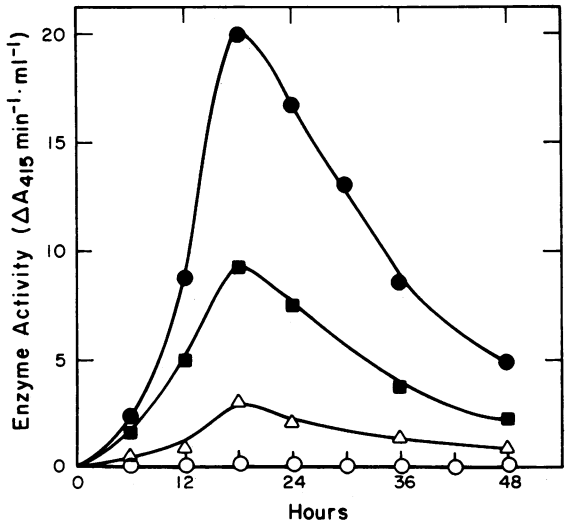

FIG. 3. Effects of various concentrations of Mn on the appearance of extracellular MnP activity. Mn-deficient cultures were grown for 4 days, after which Mn was added to a final concentration of 0 μM (○), 10 μM (△), 50 μM (■), or 180 μM (●). At the indicated time intervals after the addition of Mn, the extracellular MnP activity was assayed as described in the text.

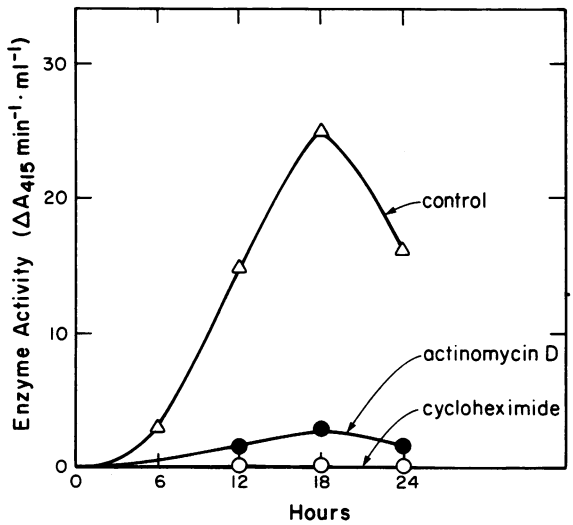

FIG. 5. Effects of actinomycin D and cycloheximide on the induction of MnP activity. Mn-deficient cultures were grown for 4 days, after which Mn (180 μM) was added alone (△), simultaneously with actinomycin D (50 μg/ml) (●), or with cycloheximide (50 μg/ml) (○). Extracellular enzyme activity was assayed at the indicated intervals after the additions, as described in the text.

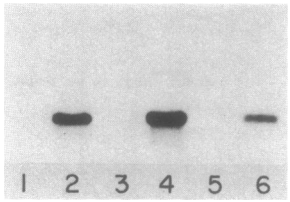

FIG. 6. Northern blot analysis of *P. chrysosporium* RNA probed with ^{32}P-labeled MnP cDNA. RNA from Mn-deficient and complete cultures was isolated, separated by electrophoresis, and probed as described in the text. Samples were as follows: day 4, Mn deficient (lane 1); day 4, Mn complete (lane 2); day 5, Mn deficient (lane 3); day 5, Mn complete (lane 4); day 6, Mn deficient (lane 5); day 6, Mn complete (lane 6).

the enzyme to oxidize the organic substrate (12, 13, 31, 47). Thus, Mn(III)/Mn(II) acts as a redox couple which presumably can diffuse into the wood and oxidize polymeric lignin. Although both Mn(II) and phenols are able to reduce the oxidized enzyme intermediate, MnP compound I, to MnP compound II, only Mn(II) is capable of efficiently reducing MnP compound II to the native resting enzyme (47, 48). Thus, MnP cannot complete its catalytic cycle in the absence of Mn(II). This appears to explain the absolute dependence of MnP catalytic activity on Mn(II).

Lignin degradation by *P. chrysosporium* is a secondary metabolic process that is triggered by nitrogen limitation (4, 20); LiP and MnP activities appear in the extracellular medium only during the secondary metabolic phase of growth (4, 16, 20, 22, 44). Northern blot analysis has demonstrated that the expression of LiP (44) and MnP (33) is controlled at the level of gene transcription by available nutrient nitrogen.

Previous work indicated that the rate of lignin degradation by *P. chrysosporium* and *Lentinus edodes* (20, 25, 34) and the production of lignin peroxidase by *P. chrysosporium* (19) are influenced by the concentration of Mn and other trace elements in the culture medium. This finding encouraged us to examine the effects of Mn on the production of MnP by *P. chrysosporium*.

Comparisons of *P. chrysosporium* linear growth rates and mycelial biomass production (Fig. 1) in Mn-deficient and complete cultures indicate that Mn has no significant effect on growth. In contrast, accumulation of MnP activity in the extracellular medium of nitrogen-limited cultures is absolutely dependent on the presence of Mn (Fig. 1). Addition of Mn to the cell-free extracellular medium of cultures grown in the absence of Mn does not restore MnP activity, indicating that the role of Mn is not as an activator of the enzyme. This was tested directly by using protein immunoblotting (Western blotting). Antiserum detects extracellular MnP protein only in Mn-supplemented cultures, indicating that no active or inactive extracellular MnP protein is present in the absence of Mn (Fig. 2). The possibility that the antiserum used in these experiments does not recognize precursors of MnP or an apoprotein can be ruled out because the primary translation product synthesized in vitro is recognized (unpublished results).

Enzyme and immunoblot assays of intracellular extracts prepared from nitrogen-limited cultures demonstrate that MnP is detectable only in extracts of cells grown in the presence of Mn. This finding further suggests that Mn regulates the synthesis of the MnP protein rather than its secretion.

When Mn is added to cultures previously grown for 4 days in the absence of Mn, extracellular MnP accumulation ensues within 6 h and reaches a maximum within 18 h (Fig. 3 and 4). The rapid response to Mn suggests that this effect is probably not indirect, for example, by controlling the rate of nitrogen depletion and consequently the onset of secondary metabolism and production of MnP. The similar growth curves for cultures grown in the presence and absence of Mn (Fig. 1) also indicate that Mn probably does not affect nitrogen utilization. Furthermore, as described above, the synthesis of the *P. chrysosporium* secondary metabolite veratryl alcohol from phenylalanine, which is also triggered by nitrogen depletion (20, 40), is not affected by Mn. The concentration of veratryl alcohol in the extracellular medium of Mn-deficient cultures was the same as in complete cultures (350 μM). The decrease in enzyme activity observed after the optimal activity is reached may be due to a decrease in transcription (Fig. 6), a decrease in RNA or protein stability, or inactivation of the enzyme.

The rapid positive response to the addition of Mn to 4-day-old cultures suggests that the metal affects transcriptional or translational processes during the synthesis of MnP protein. The effects of the inhibitors actinomycin D and cycloheximide suggest that Mn is specifically involved in the transcriptional control of the MnP genes (Fig. 5). The translational inhibitor cycloheximide would prevent the appearance of MnP regardless of whether Mn affects transcription or translation (Fig. 5). In contrast, the inhibitory effect of the RNA synthesis inhibitor actinomycin D (Fig. 5) strongly suggests that Mn is involved in the transcriptional control of MnP synthesis. Since preexisting MnP mRNA would probably be translated in the presence of actinomycin D, it is unlikely that Mn exerts its influence solely by stabilizing mRNA or by affecting the rate of MnP mRNA translation. When Mn and actinomycin D are added simultaneously to 4-day-old cultures, MnP accumulation is strongly inhibited (Fig. 5), suggesting that the Mn induction effect is at the level of transcription. Northern blot analysis confirms that Mn exerts its influence at the level of transcription. MnP mRNA is detectable in 4-day-old Mn-sufficient cultures but not in Mn-deficient cultures (Fig. 6). All of these results indicate that Mn, the substrate of the enzyme, is also regulating transcription of the MnP gene.

The regulation of secondary metabolic processes by metal ions has been recognized for several decades (50, 51). Mn is required for the synthesis of such secondary metabolites as patulin (37–39), malformin (51), and bacitracin (52). In the best-studied system, *Penicillium urticae*, Scott et al. (37–39) have shown that the synthesis of the patulin biosynthetic enzyme *m*-hydroxybenzyl alcohol dehydrogenase is regulated by Mn at the level of transcription.

Clarification of the details of Mn regulation of MnP synthesis will require further study. The metal may complex to a specific DNA-binding protein, as demonstrated for the induction of yeast metallothionein by Cu (5, 11). This is an attractive mechanism because of the high degree of specificity involved. Metal ion activation of gene transcription has recently been reviewed (30). Examples include the Hg-MerR activation of Hg resistance genes of *Escherichia coli* (30), molybdenum regulation of nitrogenase in *Azotobacter vinelandii* (18), and the Fe-regulating system in *E. coli* (8). However, other, less specific mechanisms for Mn induction are also possible. The activation of an idiophase-specific RNA polymerase has been proposed for the regulation of patulin biosynthesis (38). Alternatively, Mn levels might regulate a secondary messenger such as cyclic AMP. For example, Mn-specific adenyl cyclases such as have been

found in yeast cells (27) could be regulating cyclic AMP levels. However, the latter mechanisms seem less likely because they would be expected to result in global responses affecting a variety of secondary metabolic functions such as the synthesis of veratryl alcohol. We are undertaking additional experiments to elucidate the site of Mn control of MnP gene transcription.

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