Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1242–1246, February 1993
Biochemistry

Stimulation of Mn peroxidase activity: A possible role for oxalate in lignin biodegradation

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Communicated by T. Kent Kirk, October 29, 1992 (received for review August 24, 1992)

ABSTRACT **Oxalate is produced by numerous wood-degrading fungi. Our studies here show that the white-rot fungus *Phanerochaete chrysosporium* produces extracellular oxalate under conditions that induce synthesis of the ligninolytic system. Little or no oxalate was detected in cultures grown under high nutrient nitrogen or carbon. This extracellular oxalate was identified and quantitated by HPLC. Its identity was further substantiated by its decomposition by the enzyme oxalate oxidase. The oxalate content of the extracellular fluid (peaking at 60 μM) paralleled the extracellular activity of the lignin-degrading enzyme, Mn peroxidase. Significantly, we demonstrated that oxalate, at physiological concentrations, substantially stimulated Mn peroxidase-catalyzed phenol red oxidation, presumably by its ability to chelate Mn. Stopped flow studies also indicate that oxalate accelerates the turnover of Mn peroxidase. Furthermore, we discovered that oxalate can support Mn peroxidase-catalyzed oxidations in the absence of exogenous H_2O_2 and in the presence of dioxygen. These results allow us to propose an important role for oxalate, a ubiquitous compound produced by wood-destroying fungi, in lignin biodegradation.**

Results of the present study indicate a role for oxalate in lignin biodegradation by white-rot fungi. Lignin is an aromatic polymer composed of phenylpropanoid subunits. It makes up over 20% of the biomass renewable carbon. Its biodegradation is brought about predominantly by fungi (1). *Phanerochaete chrysosporium* is a wood-destroying fungus of the white-rot type that has been extensively characterized for its lignin-degrading activity. Under conditions that trigger the synthesis of the ligninolytic system (2, 3), it secretes a large number of hemoprotein peroxidases (EC 1.11.1.7), which catalyze the depolymerization of lignin (4–6). These peroxidases can be divided into two isozyme families based on the catalytic activities, the lignin peroxidases (4, 5) and the Mn peroxidases (7, 8). The lignin peroxidases catalyze the oxidation of a large number of nonphenolic substrates resulting in carbon–carbon bond cleavage of its aromatic substrates (9, 10). The Mn peroxidases catalyze the oxidation of divalent Mn to trivalent Mn (11). Trivalent Mn is a diffusible oxidant capable of oxidizing a large variety of phenolic substrates.

Of significance to the present study are the Mn peroxidases. The enzymes exhibit an absolute requirement for the transition metal Mn as a reducing substrate (12). The rate of catalysis is dependent on the presence of Mn chelators (7, 12, 13). Not surprisingly, the reactivity of transition metals is highly dependent on their chelation states. Past studies have shown that chelators affect not only the reaction of divalent Mn with Mn peroxidase (12) but also the reaction of trivalent Mn with its organic substrates (13). Due to the importance of chelators to Mn peroxidase action and due to the relative importance of this enzyme in lignin biodegradation (6), we investigated whether *P. chrysosporium* produces chelators that would stimulate Mn peroxidase. In accord with a previous report (14), we found that oxalate is produced by *P. chrysosporium* and that at physiological concentrations it is able to stimulate Mn peroxidase activity. These results are of widespread significance because oxalate is a ubiquitous compound found in wood-destroying fungi (14–19).

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MATERIALS AND METHODS

Materials. Veratryl alcohol (3,4-dimethoxybenzyl alcohol) and phenol red were purchased from Aldrich; the former was purified by vacuum distillation. Oxalic acid and oxalate oxidase were obtained from Sigma. All other chemicals were reagent grade and were used without further purification. The H_2O_2 concentration was determined spectrophotometrically at 240 nm using an extinction coefficient of 39.6 $M^{-1} \cdot cm^{-1}$.

Growth of *P. chrysosporium*. *P. chrysosporium* strain BKM-F-1767 (ATCC 24725) was grown in 10-ml stationary cultures in 125-ml Erlenmeyer flasks (20). Agitated cultures were not characterized in the present study. The cultures were flushed with 100% water-saturated dioxygen on day 2 of growth. Cultures were grown in a nitrogen-limited culture medium containing 1.0% glucose and 1.1 mM ammonium tartrate (20). The extracellular fluid was collected daily from 10 replicate cultures from day 0 to day 7 of growth. The mycelium was separated from the extracellular fluid by passage through cheesecloth. After removal of the mycelium, the pooled extracellular fluid was frozen immediately for later analysis.

Purification of Mn Peroxidase Isozymes. An overproducing strain of *P. chrysosporium*, PSBL-1 (21, 22), was used for production of Mn peroxidases. Extracellular fluid from day 5 cultures was harvested and separated by using an FPLC Mono Q column (23). Peaks containing isozymes H3 (pI 4.9), H4 (pI 4.5), and H5 (pI 4.2) were combined and dialyzed against 50 mM sodium succinate at pH 4.5. These isozymes were further purified by the procedure of Glenn and Gold (24). The enzymes were subjected to chromatography on an 18 cm × 3 cm (≈100-ml volume) blue agarose column (Cibracon blue 3GA type 3000-L; Sigma) and eluted with 400 ml of a 0–0.4 M NaCl gradient in 50 mM sodium succinate at pH 4.5. Isozyme H4 had been purified to homogeneity at this step and was used in the study here.

Enzyme Activity Assays. One unit is defined as 1 μmol of product formed per min. Lignin peroxidase activity was measured by monitoring oxidation of veratryl alcohol at 310 nm (20). Mn peroxidase activity in the extracellular fluid was determined by phenol red oxidation (25). Phenol red oxidation was used in studies characterizing the effect of oxalate on Mn peroxidase activity; lactate and gelatin were omitted from these incubations. Lactate and gelatin have been reported to stimulate Mn peroxidase activity (24).

HPLC Analyses of Organic Acids. The extracellular fluid collected from cultures on days 0–7 was analyzed by HPLC with a Supelcogel C-610H organic acid column (Supelco) at

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room temperature. The mobile phase was 0.1% H_3PO_4 at a flow rate of 0.4 ml/min. Samples were diluted with 2 vol of 0.1% H_3PO_4 and filtered before injection onto the organic acid column. Organic acids were monitored at 210 nm. Oxalate was identified tentatively by its retention time as compared to an oxalate standard. To substantiate its identity, aliquots of the extracellular fluid (usually 300 μl) were incubated with 0.025 unit of oxalate oxidase at room temperature overnight and then analyzed by HPLC. To determine oxalate content, peaks were integrated with CHROMATOCHART (Interactive Microware, State College, PA) and quantified by using external standard calibration.

Stopped-Flow Experiments. The stopped-flow apparatus used in the present study was as described by Johnson (26). One syringe was filled with 1.5 μM Mn peroxidase and 200 μM $MnSO_4$ in 20 mM sodium succinate at pH 4.5. The other syringe contained 100 μM H_2O_2 and various concentrations of oxalic acid. The multiple turnovers of Mn peroxidase were monitored at 406 nm after mixing from the two syringes. Each kinetic trace was the average of three runs.

RESULTS

Identification of Oxalate. Upon nitrogen limitation, *P. chrysosporium* produces a large number of oxidative enzymes involved in lignin biodegradation (4–6). Along with these enzymes, the fungus produces organic acids as evidenced by the drop in pH and the strong buffering capacity of the extracellular fluid of fungal cultures. The organic acids of the extracellular fluid were analyzed by HPLC without further concentration or treatment. As shown in the upper chromatogram of Fig. 1, a peak with the same retention time as oxalate at 11 min was observed.

Because coelution of different organic acids might occur, the identity of oxalate was further confirmed in incubations with oxalate oxidase. In the presence of O_2, oxalate oxidase oxidizes oxalate to generate H_2O_2 and CO_2. Incubations of the extracellular fluid with oxalate oxidase resulted in the loss of the 11-min peak, thus confirming its identity as oxalate (Fig. 1, lower chromatogram). The validity of this method was further confirmed by the identification of H_2O_2 as a product. The generation of H_2O_2 was monitored by coupling the reaction with lignin peroxidase. Lignin peroxidase catalyzes the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2. As expected, the oxidation of veratryl alcohol was observed when the extracellular fluid was added to the oxalate oxidase assay mixture. The amount of veratraldehyde produced was equivalent to the oxalate present in the samples (data not shown). No veratraldehyde formation was found if oxalate oxidase or lignin peroxidase was omitted.

Quantitation of Oxalate. Oxalate was quantitated by its 210-nm absorbance after separation by HPLC. Extracellular fluid was collected from day 1 to day 7 and acidified. Acidification results in solubilization of calcium oxalate crystals. The oxalate concentration as well as the lignin and Mn peroxidase activities of day 1 to day 7 cultures are shown in Fig. 2. Only a small amount of oxalate was found in the extracellular fluid during primary growth (day 1 and 2). However, on day 3 of growth, when the extracellular Mn peroxidase first appeared and actually peaked, the concentration of oxalate increased to 60 μM. Thereafter, both the Mn peroxidase activity and the oxalate concentration decreased to a low level. The extracellular lignin peroxidase activity appeared after the Mn peroxidase and oxalate first appeared and exhibited maximal activity on day 5. No oxalate was detected in cultures grown with high nutrient nitrogen and carbon (data not shown).

Effect of Oxalate on Phenol Red Oxidation. The appearance of oxalate in the extracellular fluid of *P. chrysosporium*

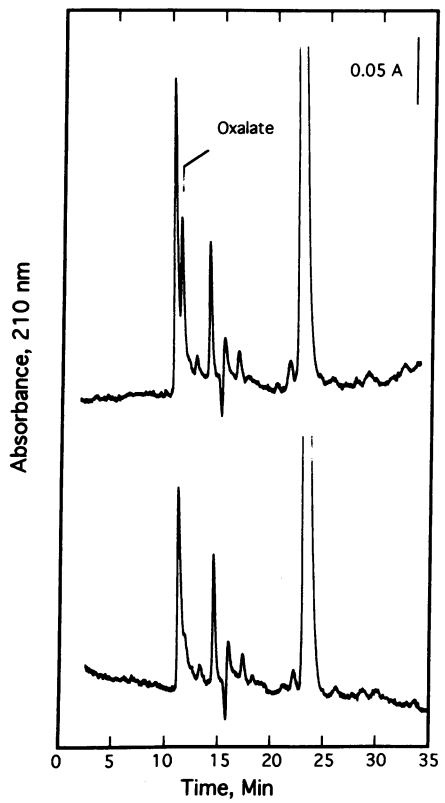

FIG. 1. Identification of oxalate in the extracellular fluid of *P. chrysosporium*. The organic acids of the extracellular fluid from nitrogen-limited cultures were analyzed by HPLC as described in *Materials and Methods*. The extracellular fluid was analyzed for oxalate before (upper chromatogram) and after (lower chromatogram) treatment with oxalate oxidase. The oxalate peak is indicated. The bar in the upper right corner indicates an absorbance of 0.05 unit.

correlated with the production of Mn peroxidase and lignin peroxidase. This correlation suggested a role for oxalate in the oxidative reactions catalyzed by these lignin-degrading enzymes. Mn peroxidase activity is dependent on Mn ions and is known to be enhanced by Mn chelators such as lactate. Due to the known Mn-chelating ability of organic acids, we investigated the effect of oxalate on Mn peroxidase activity. Mn peroxidase activity was measured by phenol red oxidation with sodium succinate as a buffer. Succinate is unable to form a stable complex with Mn^{3+} and is not able to stimulate Mn peroxidase activity (12). As shown in Fig. 3, oxalate stimulated Mn peroxidase-catalyzed phenol red oxidation in a concentration-dependent manner up to 500 μM. At concentrations above 500 μM oxalate, the rate of phenol red oxidation gradually decreased. Aside from stimulating the rate of phenol red oxidation, oxalate was also able to increase the net amount of phenol red oxidized. This result suggests that oxalate might stabilize Mn^{3+} from spontaneous decomposition.

Enzyme-Monitored Turnover Experiments. The stimulation of Mn peroxidase activity by oxalate, observed in the phenol red assay, was also observed in enzyme-monitored turnover experiments. The reaction of H_2O_2 with resting Mn peroxidase results in oxidation of the enzyme by two electrons forming compound I (12). The enzyme then returns to the resting state by oxidizing two molecules of divalent Mn to trivalent Mn. In incubations containing H_2O_2 and excess divalent Mn, the enzyme undergoes multiple turnovers until the H_2O_2 is depleted, at which time it returns to the resting state. During steady state, the predominant form of the enzyme is the one-electron oxidized enzyme intermediate, compound II, because the reaction of compound II with

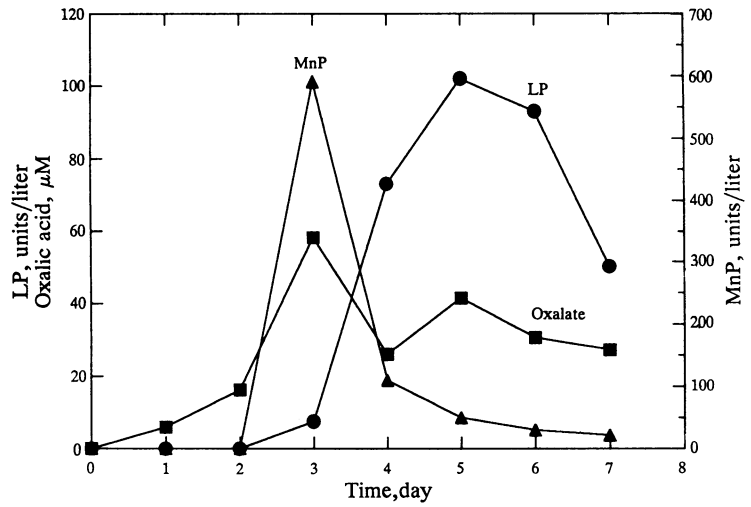

FIG. 2. Extracellular lignin peroxidase (LP) activity, Mn peroxidase (MnP) activity, and oxalate content as a function of culture age. The lignin peroxidase activity (●; left axis) was measured by veratryl alcohol oxidation, and Mn peroxidase activity (▲; right axis) was assayed by phenol red oxidation. Oxalate content (■; left axis) was measured by quantifying the oxalate peak eluted from an organic acid column as described in Fig. 1.

Mn^{2+} is rate-limited. The rate at which it returns to the resting enzyme is reflective of the rate of catalysis. Due to the spectral changes associated with compound I and II formation, enzyme turnover can be monitored by measuring absorbance changes in the Soret region; 406 nm is the Soret maximum of native Mn peroxidase. Compounds I and II, however, exhibit a decreased extinction coefficient at 406 nm. As shown in Fig. 4, increasing the oxalate concentration resulted in an increase in the rate of enzyme turnover. These results are similar to those obtained with the phenol red assay; the acceleration in rate reached a maximum at a concentration of 500 μM oxalate.

Oxalate-Supported Mn Peroxidase-Catalyzed Phenol Red Oxidation. Mn peroxidase-catalyzed reactions exhibit an absolute dependence on peroxide. We found that in addition to stimulating Mn peroxidase activity, presumably through its ability to chelate Mn, oxalate was also able to support Mn peroxidase activity in the absence of added peroxide (Fig. 5). In incubations containing Mn peroxidase, Mn^{2+}, and oxalate, phenol red was oxidized after a long lag period. If any one of the components was omitted, no phenol red oxidation occurred. The lag period could be eliminated by the addition of low levels of H_2O_2 (Fig. 5). No oxidized phenol red was formed when the reactions were carried out under anaerobic conditions, indicating that oxygen is required for this oxalate-supported oxidative activity (Fig. 5).

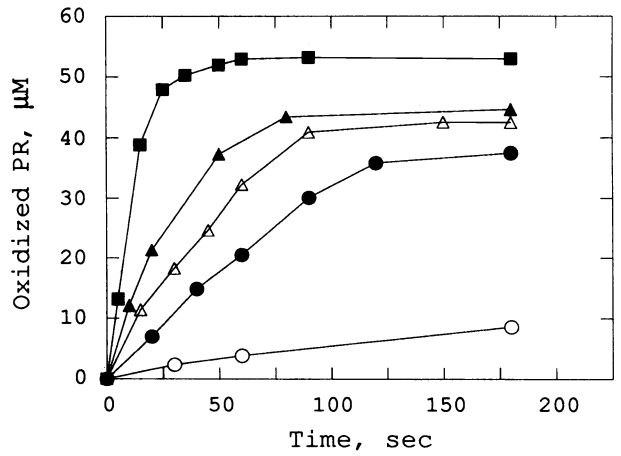

FIG. 3. Effect of oxalate on Mn peroxidase-catalyzed phenol red (PR) oxidation. The assay mixture contained 0.1 mM $MnSO_4$, 0.28 mM phenol red, 0.025 μM Mn peroxidase, and 50 μM H_2O_2 in 50 mM sodium succinate at pH 4.5 and 0 (○), 25 (●), 50 (△), 100 (▲), or 500 (■) μM oxalic acid.

DISCUSSION

Oxalate has been found in many wood-destroying fungi (14–19). The ubiquity of oxalate in these fungi would suggest that it is involved in wood decay. Indeed, a number of roles have been proposed. Oxalate has been proposed to function as an extracellular buffering agent, allowing the organism to control the pH of its environment (15). Although this is a simplistic role, it is by no means trivial due to the importance of extracellular enzymes in fungal metabolism and the need to maximize their activity. Oxalate has also been proposed to serve as a reducing agent of iron in brown-rot fungi. Schmidt *et al.* (16) proposed that the reduction of iron by oxalate results in eventual hydroxyl radical formation via the Fenton reaction; the hydroxyl radical then purportedly oxidizes cellulose.

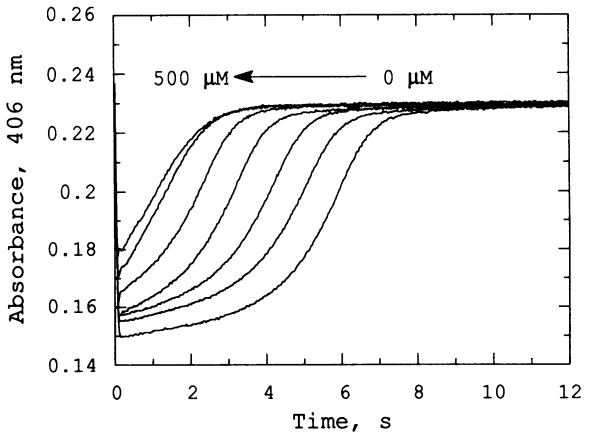

FIG. 4. Effect of oxalate on the turnover rate of Mn peroxidase. Turnover of Mn peroxidase was monitored by the change in absorbance at 406 nm. One syringe contained Mn peroxidase (0.75 μM after mixing) and $MnSO_4$ (100 μM after mixing) in 20 mM sodium succinate at pH 4.5 (after mixing). The other syringe contained H_2O_2 (50 μM after mixing) and various concentrations of oxalic acid (0, 25, 50, 100, 200, 400, or 500 μM after mixing).

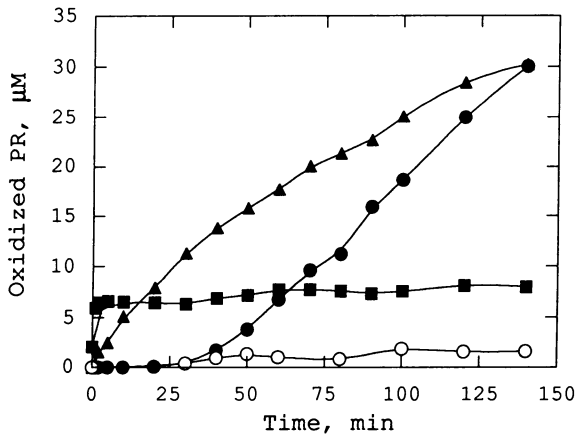

FIG. 5. Oxalate-supported phenol red (PR) oxidation by Mn peroxidase. The rate of phenol red oxidation was monitored in reaction mixtures containing 5 mM sodium oxalate (●), 5 μM H_2O_2 (■), or 5 mM sodium oxalate and 5 μM H_2O_2 (▲). All assay mixtures contained 0.1 mM $MnSO_4$, 0.28 mM phenol red, and 0.05 μM Mn peroxidase in 10 mM sodium succinate at pH 4.5. The reaction mixture containing 5 mM sodium oxalate was also examined under anaerobic conditions (○), which was maintained by purging with argon.

Another role proposed for oxalate is that of a calcium-sequestering agent (17). In necrotrophic plant pathogenic fungi, the chelation of calcium by oxalate and the resultant crystal formation effectively removes calcium from the middle lamellae (17). This in turn would result in an increase in pore size, which would then permit more effective penetration of lignocellulosic enzymes.

Although a number of roles have been proposed for oxalate in wood decay, its role in fungal metabolism has not been fully established. Most of the work on oxalate in wood decay has been with brown-rot fungi. Oxalate has also been found in white-rot fungi (14, 18, 19). Difficulty in detecting oxalate in liquid cultures has been attributed, in part, to the presence of an enzyme, oxalate decarboxylase, which rapidly metabolizes oxalate in white-rot fungi (27); oxalate decarboxylase has yet to be reported in *P. chrysosporium*. It is only recently that oxalate has been considered as an agent involved in lignin degradation in white-rot fungi. Popp *et al.* (14) were the first to suggest such a role. These workers demonstrated that oxalate is capable of mediating the oxidation of Mn^{2+} to Mn^{3+} via lignin peroxidase and veratryl alcohol. This was proposed to occur through a series of reactions in which oxalate is first oxidized by the veratryl alcohol radical, producing the oxalate radical. The oxalate radical then reduces molecular oxygen, producing superoxide. Superoxide, in turn, is the agent that oxidizes Mn^{2+} to Mn^{3+}. This series of reactions, in effect, converts the lignin peroxidases into Mn peroxidases.

Our work here does not negate any of the proposed roles for oxalate, but it does suggest an additional role for it in lignin biodegradation via stimulation of Mn peroxidase activity. Glenn *et al.* (11) were the first to demonstrate the significance of a chelator in Mn peroxidase-catalyzed oxidations. They demonstrated that 50 mM lactate stimulates Mn peroxidase-catalyzed oxidation of phenol red. We have shown in the present study that oxalate stimulates Mn peroxidase-catalyzed oxidation of phenol red. This stimulation is concentration-dependent and is observed at concentrations of oxalate found in the extracellular fluid of *P. chrysosporium*.

Lactate has long been known to stabilize the higher valence state of manganese (Mn^{3+}). Gold and coworkers (11) suggested that in addition to stabilizing trivalent Mn, lactate stimulated enzyme activity by increasing the rate of product release. Their transient-state kinetic studies suggested that the rate of Mn^{3+} release from the enzyme was stimulated by lactate (over that of succinate). Although a similar mechanism of enhancement may be operative with oxalate, our data do not permit us to comment on where oxalate has its effect on Mn peroxidase turnover. In enzyme-monitored turnover studies, we demonstrated that oxalate greatly increased the rate of Mn peroxidase turnover relative to succinate. The finding that oxalate is able to exhibit its effect on Mn peroxidase at micromolar concentrations—concentrations found in the extracellular fluid—is consistent with proposing that this is its physiological role. The stimulation that Glenn *et al.* (11) demonstrated with lactate was observed at 50 mM. Although we have found lactate in the extracellular fluid (unpublished results), its concentration is also micromolar and it is not able to stimulate Mn peroxidase activity at this concentration.

We have also found that oxalate can support Mn peroxidase activity in the absence of H_2O_2. This activity is dependent on the presence of molecular oxygen and a lag period is observed. A lag period would suggest the formation of an essential precursor. Due to the dependence of molecular oxygen in this reaction, we suspect that this precursor is H_2O_2, possibly formed from the autoxidation of oxalate. This is supported by the finding that a low equivalent concentration of added H_2O_2 can eliminate the lag period resulting in the immediate oxidation of phenol red.

In conclusion, we have shown that (*i*) oxalate is produced extracellularly by ligninolytic cultures of *P. chrysosporium* and that this production coincides with extracellular Mn peroxidase activity; (*ii*) oxalate, a known chelator of Mn, stimulates Mn peroxidase activity as demonstrated by phenol red oxidation and enzyme-monitored turnover experiments; (*iii*) the stimulation of Mn peroxidase activity occurs at micromolar concentrations of oxalate, concentrations found physiologically; and (*iv*) oxalate is able to support Mn peroxidase-catalyzed reactions in the absence of H_2O_2. On the basis of these collective results, we propose that oxalate plays a key role in lignin biodegradation by *P. chrysosporium* through its stimulation of Mn peroxidase activity.

This work was supported in part by U.S. Department of Energy Grant DE-FG02-87ER13690 and Public Health Service Grant 1-P42ES04922-01. M.T. is the recipient of National Science Foundation Presidential Young Investigator Award DCB-8657853.

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