JOURNAL OF BACTERIOLOGY, Jan. 1990, p. 260–265
0021-9193/90/010260-06$02.00/0

Vol. 172, No. 1

# Lignin Peroxidase-Negative Mutant of the White-Rot Basidiomycete *Phanerochaete chrysosporium*

K. BOOMINATHAN, S. BALACHANDRA DASS, T. A. RANDALL, R. L. KELLEY, AND C. A. REDDY*
*Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101*

Received 26 June 1989/Accepted 29 September 1989

***Phanerochaete chrysosporium*** **produces two classes of extracellular heme proteins, designated lignin peroxidases and manganese peroxidases, that play a key role in lignin degradation. In this study we isolated and characterized a lignin peroxidase-negative mutant (*lip* mutant) that showed 16% of the ligninolytic activity ($^{14}C$-labeled synthetic lignin $\rightarrow$ $^{14}CO_2$) exhibited by the wild type. The *lip* mutant did not produce detectable levels of lignin peroxidase, whereas the wild type, under identical conditions, produced 96 U of lignin peroxidase per liter. Both the wild type and the mutant produced comparable levels of manganese peroxidase and glucose oxidase, a key $H_2O_2$-generating secondary metabolic enzyme in *P. chrysosporium*. Fast protein liquid chromatographic analysis of the concentrated extracellular fluid of the *lip* mutant confirmed that it produced only heme proteins with manganese peroxidase activity but no detectable lignin peroxidase activity, whereas both lignin peroxidase and manganese peroxidase activities were produced by the wild type. The *lip* mutant appears to be a regulatory mutant that is defective in the production of all the lignin peroxidases.**

*Phanerochaete chrysosporium*, a white-rot basidiomycete, has been extensively studied as a model for fungal lignin degradation (25). Two classes of extracellular heme protein peroxidases, designated lignin peroxidases and manganese peroxidases, and an $H_2O_2$-generating system have been identified to date as the major components of the lignin-degrading enzyme system of this organism (25). Lignin peroxidases are glycosylated heme proteins that catalyze $H_2O_2$-dependent oxidation of a variety of phenolic and nonphenolic lignin model compounds, and that catalyze the oxidative cleavage of β-*O*-4 linkages (the most abundant linkage in lignin polymers), *C*α-*C*β linkages, and other linkages in lignin and lignin substructure model compounds (25). The number of lignin peroxidase isozymes produced by *P. chrysosporium* is reported to vary from 2 to 15, based on the strain, culture conditions, and separation efficiency (24, 30, 41). All the lignin peroxidase isozymes oxidize veratryl alcohol to veratraldehyde but exhibit considerable differences in specific activities (9, 24). Manganese peroxidases constitute a second group of extracellular heme proteins that catalyze the $H_2O_2$-dependent oxidation of Mn(II) to Mn(III), which, in turn, oxidizes various phenolic substrates (25, 44). Also, the manganese peroxidases have been reported to show properties of both an oxidase and a peroxidase (32). Both lignin peroxidases and manganese peroxidases require $H_2O_2$ for activity (15, 40). Several enzymes, including glucose oxidase and glyoxal oxidase, have been reported to contribute to $H_2O_2$ production in lignin-degrading cultures of *P. chrysosporium* (8, 21, 22, 23, 25). Both lignin peroxidases and manganese peroxidases and $H_2O_2$-generating enzymes are produced during secondary metabolism, in response to nitrogen starvation, whereas cultures grown under nitrogen-rich conditions produce no detectable peroxidase activity (12, 17, 25).

In recent years, there has been a relatively rapid expansion of our knowledge of the molecular biology and genetics of the lignin degradation system in *P. chrysosporium*. Three different, but related, cDNAs encoding lignin peroxidases and a cDNA for a manganese peroxidase have been cloned, characterized, and sequenced (7, 33, 42, 45). Several genomic clones of *P. chrysosporium* lignin peroxidases have been isolated and sequenced (1, 3, 18, 37, 38, 43). A number of classes of mutants of *P. chrysosporium* have been characterized (16, 20, 25, 28, 31, 34). Mutants lacking lignin peroxidases, manganese peroxidases, or both could be useful not only for elucidating the relative contribution of each class of these peroxidases to lignin degradation by *P. chrysosporium* and for various biotechnological applications but also for conducting molecular biological analyses of the regulation of production of these enzymes. Information on regulation of production could also be used, eventually, to genetically engineer *P. chrysosporium* strains that are suitable for various biotechnological applications (4, 5, 25, 39). In this report we describe the isolation and characterization of a lignin peroxidase-negative mutant (*lip* mutant) of *P. chrysosporium* which, under ligninolytic culture conditions, produced only manganese peroxidases, but no detectable lignin peroxidases, and was able to degrade lignin to $CO_2$, albeit at a lower level than that degraded by the wild type.

## MATERIALS AND METHODS

**Organism, culture conditions, and media.** *P. chrysosporium* ME446 (ATCC 34571) was maintained through periodic transfer on malt extract agar slants as described previously (20). For conidial inoculum preparations, strains were grown on malt extract agar plates. After 5 days of incubation at 39°C, conidia were washed from plates and filtered through glass wool, and the optical density of the conidia at 650 nm was measured or the number of conidia in suspension was determined with a hemacytometer as described previously (26).

The fungus was grown in shallow liquid cultures (10 ml of medium in 125-ml Erlenmeyer flasks) for lignin peroxidase production. Cultures were routinely grown in defined liquid basal III medium containing 2.4 mM nitrogen (41), except that the dimethyl succinate buffer in the medium was replaced by 20 mM acetate buffer (pH 4.5), which is less expensive and supports production of higher levels of extracellular peroxidases in *P. chrysosporium* (36). The conidial inoculum was added to a final concentration at an optical

* Corresponding author.

density at 650 nm of 0.3 (about $1.6 \times 10^6$ conidia per 10 ml of medium), and the cultures were incubated at 39°C without shaking under 100% oxygen. The flasks were flushed with oxygen at the time of inoculation and again on day 3 of incubation. Agitated cultures (45 ml in 125-ml Erlenmeyer flasks or 750 ml in 2-liter Erlenmeyer flasks) were grown in modified basal III medium (41) as described above. The cultures were agitated at 39°C on a rotary shaker at 200 rpm for the 125-ml flask cultures and at 125 rpm for the 2-liter flask cultures. These cultures were aseptically flushed with oxygen at the time of inoculation and each day thereafter.

**Mutagenesis and isolation of mutants.** Conidia from cultures grown on malt extract agar plates for 7 days were collected and mutagenized as described previously (34). Twenty milliliters of conidial suspension ($2.9 \times 10^6$ conidia per ml) was stirred in water and was irradiated with UV light ($8 \times 10^3$ ergs/cm$^2$ per s) at a distance of 18 cm at 25°C in a petri dish for 2 min. The irradiated conidial suspension (1 to 2% survivors) was diluted and plated onto poly-R medium (11, 14) with the following amendments, per 100 ml: 2 g of sorbose; 10 mg of sodium deoxycholate, and 2 g of agar (Difco Laboratories, Detroit, Mich.). After the medium was autoclaved and cooled to about 60°C, 1.0 ml of poly-R 481 (2 g/100 ml of filter-sterilized stock solution; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added, and the medium was poured into petri dishes. Portions of the irradiated conidial suspension were spread onto these plates and incubated at 39°C. Colonies that were able to decolorize poly-R were surrounded by yellow zones of discoloration. Colonies that failed to decolorize poly-R were isolated from these plates and were repeatedly transferred onto fresh poly-R plates. One such mutant, which was consistently poly-R negative, was isolated from about 4,000 colonies that were screened. A homokaryotic derivative of this mutant, which was isolated as a single basidiospore progeny (13), was designated *lip5* and was used for the experiments described here.

Phenol red medium plates, which were used to detect colonies producing manganese peroxidases, consisted of the basal III medium (41) described above, to which agar (2 g/100 ml; Difco) was added. Filter-sterilized phenol red stock solution (2 g of phenol red in 100 ml of water) was added to the autoclaved and cooled medium to give a final concentration of 20 mg/100 ml.

**Fractionation of peroxidases by FPLC.** The agitated cultures used for lignin peroxidase production were grown as described above. These cultures were pooled on the peak day of activity (usually day 6), and the mycelial pellets were separated by aseptically filtering the cultures through four layers of cheesecloth. The yellow supernatant which contained lignin peroxidase and manganese peroxidase activities was concentrated 20-fold by using a 10-kilodalton cutoff membrane (PM-10; Amicon Div., W. R. Grace & Co., Danvers, Mass.). The preparation was then filtered through 0.4-μm-pore-size filters (Gelman Sciences, Inc., Ann Arbor, Mich.) and dialyzed against 10 mM sodium acetate buffer (pH 6.0) for 24 h with three changes of the buffer. The dialysate was loaded onto a fast protein liquid chromatographic (FPLC) Mono Q anion-exchange column (HR 5/5; Pharmacia, Inc., Piscataway, N. J.) and eluted with a 10 mM to 1 M sodium acetate buffer (pH 6.0) gradient by the procedures described previously (40, 41). The $A_{405}$ of the eluent was monitored. The flow rate was 1.5 ml/min.

**Enzyme and other assays.** The lignin peroxidase activity in portions of the extracellular fluid was determined by measuring the initial rate of oxidation of veratryl alcohol to veratraldehyde, as described previously (40, 41). The cell extracts that were used to assay possible cell-bound lignin peroxidase activity were prepared by washing mycelia with 5 ml of 20 mM sodium acetate buffer (pH 6.0) and centrifuging them at $12,000 \times g$ for 10 min at 4°C. The washed mycelia were suspended in 10 ml of the same buffer, mixed with glass beads (diameter, 0.1 mm) in a 1:1 ratio (glass beads/mycelium [wet weight]), and blended at 4°C (Omni-mixer; Ivan Sorvall, Inc., Norwalk, Conn.) for 15 min. The glass beads and the mycelial debris were removed by centrifugation at $12,000 \times g$ for 15 min at 4°C. Both mycelial washings and cell extracts were assayed separately for lignin peroxidase activity.

Assay mixtures contained the following in a total volume of 1 ml: 450 μl of the culture supernatant, 400 μl of tartaric acid buffer, (125 mM; pH 2.5), 100 μl of veratryl alcohol (20 mM), and 50 μl of 0.4 mM $H_2O_2$. Enzyme activity is expressed as units per liter of the culture fluid.

The glucose oxidase activity in the cell extracts that were prepared as described previously (22) was assayed spectrophotometrically by measuring the peroxidative oxidation of *o*-dianisidine through a horseradish peroxidase-coupled system, using a molar extinction coefficient of 8.3 (22).

The manganese peroxidase activity of the extracellular fluid was measured as phenol red oxidation in the presence and absence of $MnSO_4$ (29). The reaction mixtures contained 0.0025% phenol red, 6.25 mM lactate, 0.025% bovine serum albumin, 2.5 mM $MnSO_4$, and 5 mM 2,2-dimethyl succinate buffer (pH 4.5). The reaction was started by adding 9 μl of 2 mM $H_2O_2$ and run at room temperature. The enzyme activity could be conveniently followed spectrophotometrically by monitoring the change in the $A_{431}$.

Protein was estimated by using a protein assay (Bio-Rad Laboratories, Richmond, Calif.) according to the specifications of the manufacturer. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as the standard.

Ligninolytic activity was monitored as the rate of conversion of 2′-$^{14}$C-labeled synthetic lignin to $^{14}CO_2$, as described previously (10).

## RESULTS

**Ligninolytic activity.** A comparison of the wild type and the *lip5* mutant for their ligninolytic activities showed that the wild type mineralized about 31% of the added $^{14}$C-labeled synthetic lignin in 12 days, whereas the *lip5* mutant showed about 16% of the ligninolytic activity exhibited by the wild type during the same period (Fig. 1).

Decolorization by *P. chrysosporium* of such polymeric dyes as poly-R has been shown to be positively correlated with the onset of secondary metabolism and ligninolytic activity (11, 14). Dye decolorization and the ligninolytic system are known to be repressed in high-nitrogen medium (14). Oxygen induces lignin degradation as well as dye decolorization, and inhibitors of lignin degradation, likewise, inhibit dye decolorization (14). It was therefore of interest that a mutant lacking a detectable ability to decolorize poly-R dye was still capable of low but substantial levels of ligninolytic activity (see below).

**Lignin peroxidase and manganese peroxidase activities.** Lignin peroxidase activity was determined by measuring the initial rate of oxidation of veratryl alcohol to veratraldehyde (40). One unit of activity was defined as 1 μmol of veratraldehyde formed per min. The wild type showed no detectable lignin peroxidase activity on day 4 but showed activities of 62.1, 96.4, 88.0, and 72.9 U on days 5, 6, 7, and 8, respectively. The wild type showed peak lignin peroxidase

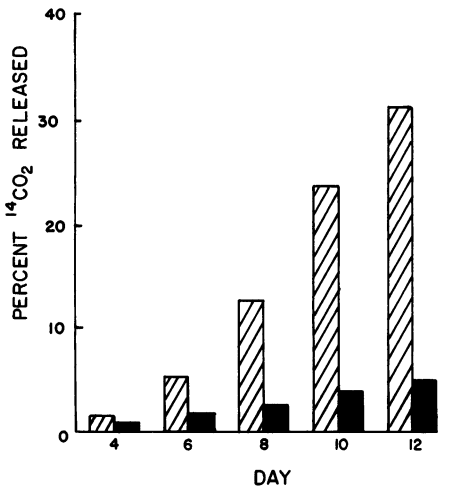


FIG. 1. Ligninolytic activity ($^{14}$C-labeled synthetic lignin → $^{14}CO_2$) of *P. chrysosporium* ME446 (wild type, ▨) and the *lip5* mutant (■). Cultures were grown in the low-nitrogen medium described previously (41).

activity in low-nitrogen medium on day 6 of incubation, in agreement with results of previous investigations (19, 30). Under identical culture conditions, the *lip5* mutant did not exhibit detectable amounts of lignin peroxidase activity on any of the days of incubation. Cell extracts and mycelial washings of the *lip5* mutant also did not exhibit lignin peroxidase activity, suggesting that the mutant does not contain cell-bound or intracellular lignin peroxidase activity. Both the wild type and the *lip5* mutant produced comparable levels of extracellular manganese peroxidases (Table 1). These results are of considerable interest since they document lignin degradation by a *P. chrysosporium* strain that failed to produce detectable levels of lignin peroxidase activity.

FPLC analysis of the extracellular culture fluids of the wild type showed the presence of eight major identifiable heme protein peaks with lignin peroxidase activity and two major heme protein peaks with manganese peroxidase activity. In contrast, in the extracellular culture fluid of the *lip5* mutant, lignin peroxidase peaks were not detectable, but four distinct heme protein peaks with manganese peroxidase activity were seen (Fig. 2). Of these, the elution pattern of the peaks designated MnPc and MnPd matched those of MnP1 and MnP2 of the wild type, respectively, whereas a large protein peak, designated MnPa, and a minor peak, designated MnPb, appeared to be unique to the mutant, as these protein peaks were not detectable in the extracellular fluid of the wild type (Fig. 2 and Table 1).

Both lignin peroxidases and manganese peroxidases are known to decolorize poly-R dye (29). In support of this, our results also showed that partially purified manganese peroxidases of the wild type or the *lip5* mutant decolorized poly-R dye (data not shown). However, when the *lip5* mutant was grown on poly-R plates, it did not show detectable decolorization of the medium after 8 days, in comparison with the wild type. Continued incubation of these plates for 15 days resulted in decolorization of the poly-R dye by colonies of the *lip5* mutant, presumably because of the manganese peroxidase activity. In fact, we have evidence that purified manganese peroxidases decolorize poly-R, although to a lower extent than the lignin peroxidases do.

Manganese peroxidase isozymes are routinely assayed by determining their ability to oxidize phenol red in the presence of $Mn^{2+}$ (29). Therefore, we investigated the ability of the colonies of the *lip5* mutant to decolorize phenol red on plates. The results showed that both the wild type and the *lip5* mutant decolorized phenol red from yellow to red after 2 days of incubation, and the peak activity was detected on day 3, as indicated by the formation of a very intense red discoloration in and around the fungal colony. This observation is in agreement with the fact that *P. chrysosporium* cultures produce manganese peroxidases earlier than lignin peroxidases (30) and is a further indication that in the *lip5* mutant the production of manganese peroxidases is not affected.

TABLE 1. Activities of partially purified $Mn^{2+}$-dependent peroxidases from extracellular culture fluids of *P. chrysosporium* ME446 and the *lip5* mutant[a]

| Strain and enzyme preparation[b] | Addition | Peroxidase activity[c] |
|---|---|---|
| ME446 | | |
| MnP1 | None | 0 |
| | Mn | 1.88 |
| MnP2 | None | 0 |
| | Mn | 1.61 |
| *lip5* | | |
| MnPa | None | 0 |
| | Mn | 1.47 |
| MnPb | None | 0 |
| | Mn | 2.11 |
| MnPc | None | 0 |
| | Mn | 1.69 |
| MnPd | None | 0 |
| | Mn | 1.89 |
| MnPb (boiled) | Mn | 0 |
| No enzyme | Mn | 0 |

[a] The reaction mixture (1 ml) contained 0.0025% phenol red, 6.25 mM lactate, 0.025% bovine serum albumin, 5 mM dimethyl succinate buffer (pH 4.5), and partially purified enzyme (100 μl). Mn was added to the reaction mixture as 2.5 mM $MnSO_4$. The reaction was started by adding 9 μl of 2 mM $H_2O_2$.

[b] The manganese peroxidase (MnP) designations correspond to those used in Fig. 2.

[c] Extracellular fluid from the 6-day-old cultures of the wild type and the *lip5* mutant was fractionated separately on a Mono Q column, and the eluted heme proteins were monitored by measuring the $A_{405}$. Enzyme activity was followed by monitoring the decrease in the $A_{431}$ per minute per milligram of protein.

**Glucose oxidase activity.** Several independent studies have demonstrated the importance of hydrogen peroxide to lignin degradation (8, 10, 21, 23, 35). Glucose oxidase has been shown to be one of the hydrogen peroxide-producing enzymes in ligninolytic cultures of *P. chrysosporium* (8, 21, 35). Glucose oxidase production, similar to lignin peroxidase production, is triggered in response to nitrogen or carbohydrate deprivation; and activities of both enzymes are repressed in media containing high levels of nitrogen or on the addition of exogenous nitrogen sources such as glutamate to lignin-degrading cultures (21). Therefore, it was of interest to investigate whether the *lip5* mutant was able to produce normal levels of glucose oxidase. Our results showed that both the wild type and the *lip5* mutant produce comparable levels of glucose oxidase (Table 2).

## DISCUSSION

The results of this investigation showed that the *lip5* mutant degrades $^{14}$C-labeled synthetic lignin to $^{14}CO_2$ and

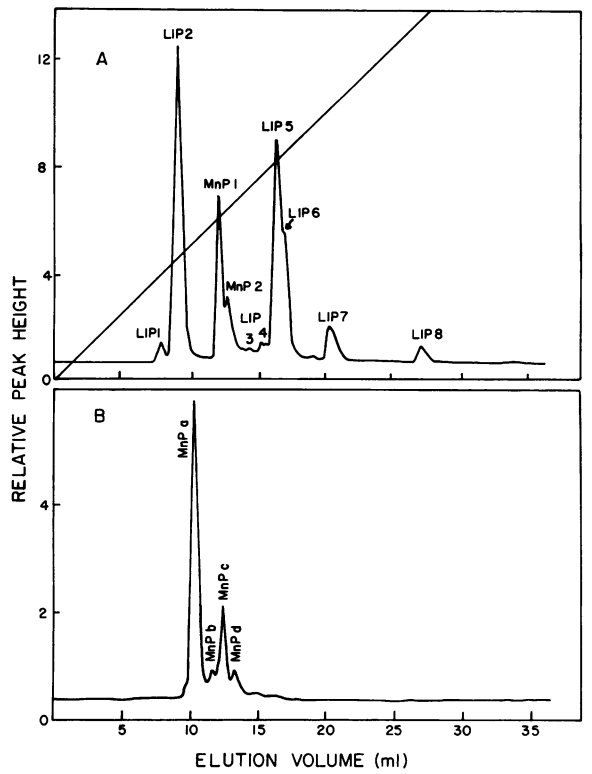


FIG. 2. FPLC profiles of various heme proteins in extracellular culture fluids of the *P. chrysosporium* wild type (A) and the *lip5* mutant (B). Cultures were grown as described in the text. Extracellular fluids of the wild type (850 μg of protein) and the *lip5* mutant (227 μg of protein) were fractionated separately on a Mono Q column, and the elution of the heme proteins was monitored by measuring the $A_{405}$ (indicated here as relative peak height). Abbreviations: LIP, lignin peroxidase; MnP, manganese peroxidase.

lacks lignin peroxidases, but that it elaborates several manganese peroxidases and glucose oxidase at levels comparable to those in the wild type. These data indicate that a strain of *P. chrysosporium* producing manganese peroxidases and no detectable lignin peroxidases is able to degrade lignin to a measurable, although lower, extent. The results are consistent with those of an earlier report by Kirk et al. (27), who reported a lack of good temporal correlation between lignin peroxidase production and lignin degradation. An observation of particular interest was the fact that the ligninolytic activity was detected even before lignin peroxidase production was demonstrable. The observed ligninolytic activity detected by Kirk et al. (27) may be attributable to manganese peroxidases, which are known to be produced earlier than lignin peroxidases (30). Manganese peroxidase activity is usually detected after 65 h of incubation and reaches maximal activity 8 to 10 h later. Lignin peroxidase activity, on the other hand, appears several hours after the appearance of manganese peroxidase activity. In this study we also observed that phenol red oxidation by *lip5* and wild-type colonies reached a peak on day 3 of incubation, indicating manganese peroxidase activity. The results of this investigation indicate that lignin peroxidases are not obligatorily required for the degradation of at least some of the lignin polymer and that a strain of *P. chrysosporium* with manganese peroxidases only, such as the *lip5* mutant, can degrade lignin to a measurable extent. Lignin degradation by other white-rot and brown-rot fungi which do not produce detectable levels of lignin peroxidases has been documented, although at lower levels than degradation by *P. chrysosporium* (2, 6).

TABLE 2. Glucose oxidase activities of *P. chrysosporium* ME446 (wild type) and the *lip5* mutant

| Strain | Glucose oxidase activity on[a]: | | |
|---|---|---|---|
| | Day 4 | Day 5 | Day 6 |
| Wild type | 0.13 | 0.09 | 0.11 |
| *lip5* mutant | 0.10 | 0.11 | 0.08 |

[a] Glucose oxidase activity was assayed spectrophotometrically by measuring the peroxidative oxidation of *o*-dianisidine through a horseradish peroxidase-coupled system (22).

Gold et al. (16) have shown that a phenol oxidaseless mutant (*phe*) of *P. chrysosporium* is defective not only in its ability to degrade lignin and various lignin model compounds but also in its ability to produce fruiting bodies and synthesize veratryl alcohol, a typical secondary metabolite. They concluded that it is a pleiotropic mutant for a set of secondary metabolic characteristics. Liwicki et al. (31) have isolated another class of phenol oxidase mutants which share the following idiophasic traits: increased intracellular cyclic AMP levels, sporulation, extracellular glucan, and veratryl alcohol synthesis. They concluded that mutations that result in the loss of lignin-degrading ability are not necessarily pleiotropic for other idiophasic functions. Kelley et al. (20) have demonstrated that the *gox* mutants of *P. chrysosporium* lack several metabolic activities associated with lignin degradation, such as glucose oxidase and lignin peroxidase production, but unlike the pleiotropic *phe* mutant of Gold et al. (16), *gox* mutants retain some of the major secondary metabolic characteristics such as veratryl alcohol synthesis and conidiation. They concluded that the primary lesion in the *gox* mutants affects the regulation of the onset of a group of secondary metabolic characteristics (20). These results suggest the existence of different levels of secondary metabolism control in *P. chrysosporium*.

The results of this investigation suggest that the *lip5* mutant is yet another class of regulatory mutant in that it is defective only in the production of lignin peroxidases and is not affected in other secondary metabolic processes, such as lignin degradation, manganese peroxidase production, or glucose oxidase production. A regulatory gene specifically involved in controlling the expression of lignin peroxidases is apparently affected in the *lip5* mutant. These results further indicate that manganese peroxidase production is regulated differently, at least in some aspects, from lignin peroxidase production. It appears that specific regulatory controls exist in *P. chrysosporium* for the various enzyme families that are involved in lignin degradation, such as lignin peroxidases and manganese peroxidases, and presumably, other secondary metabolic activities as well.

Studies are in progress to isolate the regulatory gene that complements the *lip* mutation by transforming the *lip5* mutant with a *P. chrysosporium* genomic library and isolating and characterizing transformant colonies that are able to decolorize poly-R. The *lip5* mutant is also being used to elucidate the contribution of lignin peroxidases vis à vis manganese peroxidases to the various degradative activities that are known to be catalyzed by *P. chrysosporium* (4, 5, 25, 39).

## ACKNOWLEDGMENTS

We thank Robert Hausinger for use of the fast protein liquid chromatograph.

This study was supported in part by grant DE-FGO2-85 ER 13369 from the U.S. Department of Energy, Public Health Service grant 1 RO1 GM39032-01 from the National Institutes of Health, and a REF grant from Michigan State University.

## LITERATURE CITED

1. **Asada, Y., Y. Kimura, M. Kuwahara, A. Tsukamoto, K. Koide, A. Oka, and M. Takanami.** 1988. Cloning and sequencing of a ligninase gene from a lignin degrading basidiomycete, *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **29**:469–473.
2. **Bourbonnais, R., and M. G. Paice.** 1988. Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem. J. **255**:445–450.
3. **Brown, A., P. F. G. Sims, U. Raeder, and P. Broda.** 1988. Multiple ligninase related genes from *Phanerochaete chrysosporium*. Gene **73**:77–85.
4. **Bumpus, J. A.** 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55**:154–158.
5. **Bumpus, J. A., and S. D. Aust.** 1988. Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system. BioEssays **6**:166–170.
6. **Buswell, J. A., and E. Odier.** 1987. Lignin biodegradation. Crit. Rev. Biotechnol. **6**:1–60.
7. **de Boer, H. A., Y. Z. Zhang, C. Collins, and C. A. Reddy.** 1987. Analysis of nucleotide sequence of two ligninase cDNAs from a white rot filamentous fungus, *Phanerochaete chrysosporium*. Gene **60**:93–102.
8. **Eriksson, K.-E., B. Patterson, J. Volc, and V. Musilek.** 1986. Formation and partial characterization of glucose-2-oxidase, a $H_2O_2$ producing enzyme in *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **23**:257–262.
9. **Faison, F. D., T. K. Kirk, and R. L. Farrell.** 1986. Role of veratryl alcohol in regulating ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **52**:251–254.
10. **Forney, L. J., C. A. Reddy, M. Tien, and S. D. Aust.** 1982. The involvement of hydroxyl radical derived hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. J. Biol. Chem. **257**:11455–11462.
11. **Glenn, J. K., and M. H. Gold.** 1983. Decolorization of several polymeric dyes by the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **46**:1741–1747.
12. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242**:329–341.
13. **Gold, M. H., and T. M. Cheng.** 1979. Conditions for fruit body formation in the white rot basidiomycete *Phanerochaete chrysosporium*. Arch. Microbiol. **121**:37–41.
14. **Gold, M. H., J. K. Glenn, and M. Alic.** 1988. Use of polymeric dyes in lignin biodegradation assays. Methods Enzymol. **161**: 74–78.
15. **Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn.** 1984. Purification and characterization of an extracellular $H_2O_2$-requiring diarylpropane oxygenase from the white rot basidiomycete *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234**:353–362.
16. **Gold, M. H., M. B. Mayfield, T. M. Cheng, K. Krisnangkura, and M. Shimada.** 1982. A *Phanerochaete chrysosporium* mutant defective in lignin degradation as well as several other secondary metabolic functions. Arch. Microbiol. **132**:115–122.
17. **Gold, M. H., H. Wariishi, and K. Valli.** 1989. Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*, p. 127–136. *In* J. R. Whitaker and P. E. Sonnet (ed.), Biocatalysis in agricultural biotechnology. American Chemical Society, Washington, D.C.
18. **Holzbaur, E. L. F., and M. Tien.** 1988. Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **155**:626–633.
19. **Jager, A., C. Croan, and T. K. Kirk.** 1985. Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **50**:1274–1278.
20. **Kelley, R. L., K. Ramasamy, and C. A. Reddy.** 1986. Characterization of glucose oxidase-negative mutants of lignin degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Microbiol. **144**:254–257.
21. **Kelley, R. L., and C. A. Reddy.** 1986. Identification of glucose oxidase activity as the primary source of hydrogen peroxide production in ligninolytic cultures of *Phanerochaete chrysosporium*. Arch. Microbiol. **144**:248–253.
22. **Kelley, R. L., and C. A. Reddy.** 1986. Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium*. J. Bacteriol. **166**:269–274.
23. **Kersten, P. J., and T. K. Kirk.** 1987. Involvement of a new enzyme, glyoxal oxidase, in extracellular $H_2O_2$ production by *Phanerochaete chrysosporium*. J. Bacteriol. **169**:2195–2202.
24. **Kirk, T. K., S. Croan, M. Tien, K. E. Murtagh, and R. L. Farrell.** 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8**:27–32.
25. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Ann. Rev. Microbiol. **41**: 465–505.
26. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Forenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**: 277–285.
27. **Kirk, T. K., M. Tien, S. Johnsrud, and K.-E. Eriksson.** 1986. Lignin degrading activity of *Phanerochaete chrysosporium* Burds.: comparison of cellulase-negative and other strains. Enzyme Microb. Technol. **8**:75–80.
28. **Kuwahara, M., and Y. Asada.** 1987. Production of ligninases, peroxidases and alcohol oxidase by *Phanerochaete chrysosporium* and its mutant, p. 171–176. *In* E. Odier (ed.), Lignin: enzymic and microbial degradation. I.N.R.A. Publications, Paris.
29. **Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold.** 1984. Separation and characterization of two extracellular $H_2O_2$ dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169**:247–250.
30. **Leisola, M. S. A., B. Kozulic, F. Meussdorffer, and A. Fiechter.** 1987. Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. J. Biol. Chem. **262**:419–424.
31. **Liwicki, R., A. Paterson, M. J. MacDonald, and P. Broada.** 1985. Phenotypic classes of phenoloxidase-negative mutants of the lignin-degrading fungus, *Phanerochaete chrysosporium*. J. Bacteriol. **162**:641–644.
32. **Paszczynski, A., V. B. Huynh, and R. L. Crawford.** 1986. Comparison of ligninase-I and peroxidase M-2 from the white rot fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **244**:750–765.
33. **Pribnow, D., M. B. Mayfield, J. V. Nipper, J. A. Brown, and M. H. Gold.** 1989. Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **264**:5036–5040.
34. **Ramasamy, K., R. L. Kelley, and C. A. Reddy.** 1985. Lack of lignin degradation by glucose oxidase-negative mutants of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **131**:436–441.
35. **Reddy, C. A., and R. L. Kelley.** 1986. The central role of hydrogen peroxide in lignin degradation by *Phanerochaete chrysosporium*, p. 535–542. *In* S. Barry, D. Houghton, C. O'Rear, and G. C. Llewellyn (ed.), Biodeterioration, vol. 6. Commonwealth Agricultural Bureaux, London.
36. **Renganathan, V., K. Miki, and M. H. Gold.** 1985. Multiple molecular forms of diaryl propane oxygenases, an $H_2O_2$ requiring, lignin degrading enzyme from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **241**:304–314.

37. **Schalch, H., G. Gaskell, T. L. Smith, and D. Cullen.** 1989. Molecular cloning and sequences of a lignin peroxidase genes of *Phanerochaete chrysosporium*. Mol. Cell. Biol. **9**:2743–2747.
38. **Smith, T. L., H. Schalch, J. Gaskell, S. Covert, and D. Cullen.** 1988. Nucleotide sequence of a ligninase gene from *Phanerochaete chrysosporium*. Nucleic Acids Res. **16**:1219.
39. **Tien, M.** 1987. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. Crit. Rev. Microbiol. **15**:141–168.
40. **Tien, M., and T. K. Kirk.** 1984. Lignin degrading enzyme from *Phanerochaete chrysosporium*. Purification, characterization and catalytic properties of a unique $H_2O_2$-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81**:2280–2284.
41. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161**:238–249.
42. **Tien, M., and C. P. D. Tu.** 1987. Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*. Nature (London) **326**:520–523.
43. **Walther, I., M. Kalin, J. Reiser, F. Suter, B. Fritsche, M. Saloheimo, M. Leisola, T. Teeri, J. K. C. Knowles, and A. Fiechter.** 1988. Molecular analysis of a *Phanerochaete chrysosporium* lignin peroxidase gene. Gene **70**:127–137.
44. **Wariishi, H., L. Akileswaran, and M. H. Gold.** 1988. Manganese peroxidase from the basidiomycete, *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. Biochemistry **27**:5365–5370.
45. **Zhang, Y.-Z., G. J. Zylstra, R. H. Olsen, and C. A. Reddy.** 1986. Identification of cDNA clones for ligninase from *Phanerochaete chrysosporium* using synthetic oligonucleotide probes. Biochem. Biophys. Res. Commun. **137**:649–656.