Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 436-441

LACK OF LIGNIN DEGRADATION BY GLUCOSE OXIDASE-NEGATIVE MUTANTS OF *Phanerochaete chrysosporium* +

K. Ramasamy*, R. L. Kelley and C. A. Reddy**

Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan 48824-1101

Received July 18, 1985

Glucose oxidase-negative (*gox*$^-$) mutants of *Phanerochaete chrysosporium* were isolated after exposing conidia to UV irradiation. The *gox*$^-$ mutants exhibited little or no ability to degrade lignin (2-(^{14}C)-synthetic lignin to $^{14}CO_2$); however, they retained other secondary metabolic features such as the ability to conidiate and produce veratryl alchol, suggesting that they are not pleiotropic for secondary metabolism. Lignin degradation activity was restored in *gox*$^+$ revertants. These results, in support of earlier evidence, indicate that glucose oxidase activity plays an important role in lignin degradation by *P. chrysosporium*.

The physiology, biochemistry and genetics of lignin degradation by *Phanerochaete chrysosporium*, a white-rot basidiomycete, are areas of intensive study worldwide (1,2). Recent studies indicate that hydrogen peroxide (H_2O_2) plays a critical role in lignin degradation by this organism. For example, H_2O_2 production and H_2O_2-derived hydroxyl radical (.OH) production, similar to lignin degradation, are idiophasic events triggered in response to nitrogen or carbohydrate starvation (3,4,5,6). A correlation between ligninolytic activity and H_2O_2 production has been observed in cultures grown with different growth substrates (4). H_2O_2-producting periplasmic microbodies are observed in lignin-degrading cultures but not in non-ligninolytic cultures (6). Extracellular lignin-degrading enzymes (7-10) and a lignin demethylase (11) from *P. chrysosporium* have been shown to have an obligate requirement for H_2O_2 for activity. Glucose oxidase (E.C.1.1.3.4.) activity has been shown to

+Journal article no. 11688 from the Michigan Agricultural Experiment Station.
*Present address: Dept. of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbafore, Tamil Nadu, India.
**To whom correspondence should be addressed.

0006-291X/85 $1.50

be the predominant physiological source of H_2O_2 in ligninolytic cultures of P. chrysosporium (4,5). We have described here the isolation of glucose oxidase-negative (gox$^-$) mutants of P. chrysosporium which are deficient in lignin degradation and gox$^+$ revertants which have regained their ability to degrade lignin. (This work was presented in part at the 85th Annual Meeting of the American Society for Microbiology, 3-7 March 1985, Las Vegas, Nevada.)

MATERIALS AND METHODS

Organism and culture conditions. P. chrysosporium (ATCC 34541) was maintained through periodic transfer on malt extract agar (MEA) slants as previously described (12) except that 10mM Na 2,2'-dimethylsuccinate replaced the o-phthalate buffer. Composition and preparation of the low N medium used in these studies was previously described (12). Unless described otherwise 0.5 cm mycelial disc from 7 d-old GOX plates (see below) served as the inoculum and cultures were incubated in air without agitation at 39°C.

Mutagenesis and Isolation of Mutants and Revertants. Conidia from cultures grown on MEA plates for 7 d were collected as previously described (12). Twenty ml of conidial suspension (2.9 x 10^6 conidia/ml) stirred in water were irradiated with UV light (8 x 10^3 ergs/cm^2/sec) at a distance of 18 cm at 25°C in a Petri plate for 2 min. The irradiated conidial suspension (1-2% survivors) was diluted and plated on GOX medium containing per 100 ml: malt extract (0.3g), Saponin (0.08g) and Bacto agar (2.0g). Filter-sterilized ortho-anisidine (in 95% ethanol) was added to this medium (2.5mM final concn.) prior to plating. Gox$^+$ colonies produce a brownish violet discoloration of the medium in 5-8 days, whereas Gox$^-$ colonies show no discoloration. This mutation procedure yielded one mutant colony for every 30 colonies observed, whereas the spontaneous mutation rate under identical conditions was 3.4 x 10^{-3}. A total of 40 putative gox$^-$ mutants which lacked glucose oxidase activity based on the spectrophotometric assay described below, were isolated and data on four of the mutants are provided here.

To produce gox$^+$ revertants, conidia from glucose oxidase-negative strain gox-10 were mutagenized and plated on Gox medium as described above. One to two gox$^+$ revertants were isolated for every 100 colonies screened. Each of the revertants were shown to be gox$^+$ based on the spectrophotometric assay described below. Two of these revertants (R_1 and R_2) were selected for further characterization.

Preparation of cell extracts and assay of glucose oxidase activity. Cultures grown in low N medium (3 x 50ml) for 6 days were harvested by centrifugation (12,000 x g for 10 min at 4°C), washed once with 20ml of 10mM 2,2'-Na-dimethylsuccinate buffer (DMS; pH 4.5), and resuspended in 20ml of the same buffer. Preparation of cell extracts and the glucose oxidase assay were done as previously described (5).

Polyacrylamide gel electrophoresis of cell extracts, employed to demonstrate glucose oxidase activity band, was performed using a 8% native gel with a 5% stacking gel (14). Samples (50 µl) were placed on the gel in 0.05 M Tris-HCl buffer (pH 6.7) containing 55% w/v glycerol and subjected to electrophoresis at 30mA for 3.5 hr. Glucose oxidase bands in the gel were stained by a diaminobenzidine/horseradish peroxidase-staining solution previously described (15) for 4 hours at 25°C. This staining procedure is based on the peroxidatic oxidation of DAB; hence, glucose oxidase which generates H_2O_2 upon oxidation of the substrate will appear brown. Protein bands in parallel gels were visualized by the silver staining method described by Morrissey (16).

Other Assays. Assays used for ligninolytic activity (3), and veratryl alcohol production (17) were performed as previously described.

RESULTS AND DISCUSSION

Characterization of gox⁻ mutants

All the gox⁻ mutants exhibited either negligible glucose oxidase activity or no activity compared to that of the wild-type (Table 1). The loss of glucose oxidase activity in the mutants was positively correlated with a dramatic decrease in their ability to degrade 2-^{14}C-synthetic lignin to $^{14}CO_2$. Furthermore, none of the glucose oxidase-negative mutants were able to decolorize poly R-481 which is known to serve as a substrate for the lignin degradation system of P. chrysosporium (data not shown). Cell yields (mg dry weight/ml) of the gox⁻ mutants in malt extract medium, were 87 to 100% of the values obtained for the wild type indicating that the loss of glucose oxidase and ligninolytic activities in the mutants is not due to their poor growth relative to the wild type.

Table 1. Selected characteristics of wild type and glucose oxidase-negative (gox⁻) mutants of P. chrysosporium[a]

Strain	Glucose Oxidase (U/mg)	Lignin degradation (%)	Veratryl alcohol production[b] (nmol/ml culture)	Conidiation[c] (conidia/ml)
Wildtype (WT)	1.73 (100%)	41.0	285	2.0×10^7
Gox-1	0.07 (4.0%)	3.1	86	1.2×10^7
Gox-4	0.01 (0.6%)	1.3	71	2.1×10^7
Gox-6	0.0 (0.0%)	1.1	86	2.5×10^5
Gox-10	0.01 (0.6%)	3.0	71	2.8×10^7
Heat Killed WT	0.0 (0.0%)	1.1	0.0	--------

[a]The Low N medium used for growth and other culture conditions were described in the text. Values are means of three replicates. Lignin degradation values represent the percent 2'-(^{14}C)-synthetic lignin converted to $^{14}CO_2$ after 15 d incubation in low N medium.

[b]For determining veratryl alcohol production, cultures were grown in low N medium under 100% O_2 for 7 days and veratryl alcohol produced was quantified as previously described (17).

[c]Conidial numbers were determined as described in materials and methods.

(S.D. were ± 5% except for conidiation which was ± 15%.)

The above results, in support of the cumulative evidence obtained previously (4,5), suggested that glucose oxidase activity is important for lignin degradation. However, the possibility that these are pleiotropic mutants and the loss of ligninolytic activity is just one of a set of various secondary metabolic activities lost by the organism, as previously demonstrated for the phenol oxidase mutants of P. chrysosporium (18) could not be ruled out. Therefore, we determined the ability of these mutants to synthesize veratryl alcohol, a typical secondary metabolite in P. chrysosporium, and to conidiate (Table 1). The results showed that gox$^-$ mutants, which have $\leq$4% of the glucose oxidase activity of the wild type, have retained 24.9-30.2% of the wild type's ability to synthesize veratryl alcohol. Conidiation by the gox$^-$ mutants was comparable to that of the wild type, except Gox-6 which exhibited much lower lovel of conidiation compared to the wild type. These results show significant levels of uncoupling between glucose oxidase activity and two of the widely accepted secondary metabolic activities in P. chrysosporium suggesting that the gox$^-$ mutants are not truly pleiotropic for secondary metabolism. Furthermore, we have recently isolated a ligninase deficient mutant of P. chrysosporium which had 8% and 74%, respectively, of the ligninase and glucose oxidase activities observed with the wild type, indicating an uncoupling between glucose oxidase and ligninase activities both of which are known to be idiophasic events.

Characteristics of gox$^+$ Revertants

Considering the close correlation between glucose oxidase activity and ligninolytic activity, it was of interest to obtain revertants of gox$^-$ mutants and to determine if ligninolytic activity had been restored to these revertants. The gox$^+$ revertants R1 and R2, respectively, had 152% and 139%, of the glucose oxidase activity exhibited by the wild type and the corresponding ligninolytic activities by the same revertants were 114% and 117%, respectively, compared to that observed with the wild type (results not shown). The time course of lignin degradation was comparable between the wild type and the gox$^+$ revertant R2 (Fig. 1). Similar results were obtained with strain R1. Native polyacrylamide gel electrophoresis of the extracts of wild

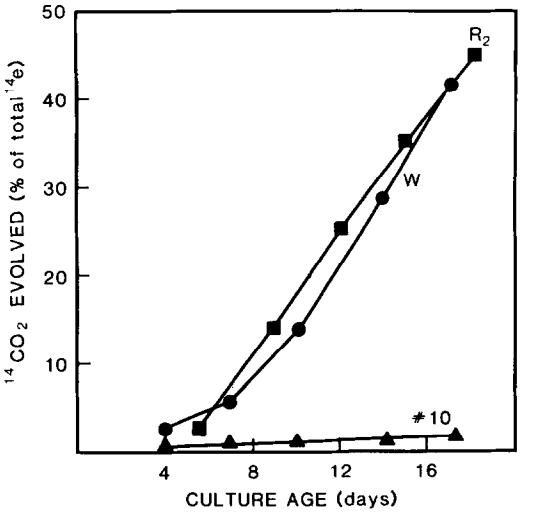

Figure 1. Time course of lignin degradation (2-(^{14}C)-synthetic lignin → $^{14}CO_2$) by wild type (WT), gox$^-$ mutant (#10) and gox$^+$ revertant (R2). Procedures employed for determining the ligninolytic activity were similar to those previously described (3).

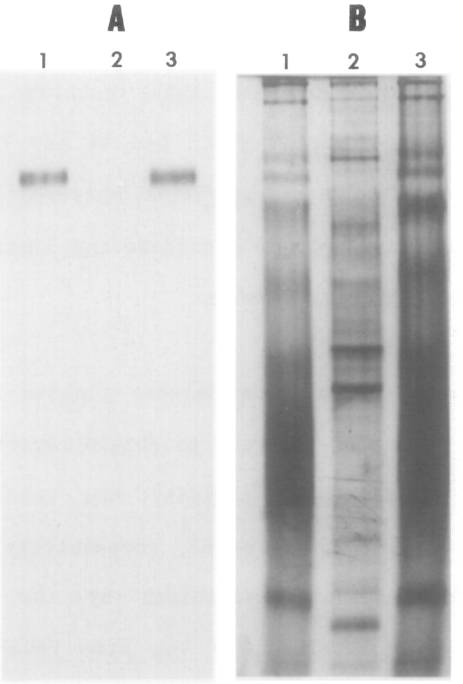

Figure 2. Polyacrylamide gel electrophoresis of cell extracts from 6 day old cultures of the wild type, gox$^-$ mutant (#10) and gox$^+$ revertant (R_2). Gels in panel A were stained for glucose oxidase activity utilizing the diaminobenzidine/horseradish peroxidase staining procedure and those in panel B were stained for protein by the silver staining procedure (see methods). In each panel, lanes 1, 2 and 3 represent extracts of the wildtype, gox$^-$ mutant and gox$^+$ revertant, respectively.

type, gox⁻ mutant and gox⁺ revertant, followed by staining of the gels for glucose oxidase activity (see methods) showed the presence of a single, glucose oxidase activity band in extracts of the wild type and the gox⁺ revertant (Figure 2, Section A, lanes 1 and 3, respectively) but not in extracts of the gox⁻ mutant (lane 2, Section A). Parallel gels stained for protein (Figure 2, Section B) showed protein bands corresponding to glucose oxidase activity bands in extracts of the wild type and the gox⁺ revertant (lanes 1 and 3) but not in extracts of the gox⁻ mutant (lane 2).

The results of this study, in support of earlier evidence, show that glucose oxidase activity plays an important role in lignin degradation by P. chrysosporium as evidenced by the lack of ligninolytic activity in gox⁻ mutants and restoration of this activity in the gox⁺ revertants.

REFERENCES

1. Reddy, C. A. (1984) in Klug, M. J. and Reddy, C. A. (eds.), Current Perspectives in Microbial Ecology, pp. 558-571, American Society for Microbiology, Washington, D.C.
2. Kirk, T. K. (1984) In D. T. Gibson (ed.), Biochemistry of Microbial Degradation, pp. 399-437, Marcel Dekker, New York.
3. Forney, L. J., Reddy, C. A., Tien, M. and Aust, S. D. (1982) J. Biol. Chem. **257**:1455-1462.
4. Reddy, C. A., Forney, L. J. and Kelley, R. L. (1983) In Higuchi, T., Chang, H. M. and T. K. Kirk (eds.), Recent Advances in Lignin Biodegradation Research, pp. 153-163, Uni Publishers, Tokyo.
5. Reddy, C. A. and Kelley, R. L. (1985) Proc. VIth International Biodeterioration Symposium (in press).
6. Forney, L. J., Reddy, C. A., and Pankratz, H. S. (1982) Appl. Environ. Microbiol. **44**:732-736.
7. Tien, M. and Kirk, T. K., (1984) Proc. Natl. Acad. Sci. USA **81**:2280-2284.
8. Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984) Arch. Biochem. Biophys. **234**:353-362.
9. Kersten, P. J., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) J. Biol. Chem. **260**:2609-2612.
10. Kuwahara, M., Glenn, J. K., Morgan, M. A. and Gold, M. H. (1984) FEBS Lett. **169**:247-250.
11. Frick, T. D. and Crawford, R. L. (1983) in Higuchi, T., Chang, H. M. and Kirk, T. K. (eds.) Advances in Lignin Biodegradation Research. pp. 143-152, Uni Publishers, Tokyo.
12. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Arch. Microbiol. **117**:277-285.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. **193**:265-275.
14. Melachouris, N. (1968) J. Dairy Sci. **52**:456-459.
15. Cohen, H. J. (1973) Anal. Biochem. **53**:208-222.
16. Morrissey, J. K. (1981) Anal. Biochem. **117**:307-310.
17. Shimada, M., Nakatsubo, F., Kirk, T. K. and Higuchi, T. (1981) Arch. Microbiol. **134**:299-302.
18. Gold, M. H., Mayfield, M. B., Cheng, T. M., Krisnangkura, K., Shimada, M., Enoki, A. and Glenn, J. K. (1982) Arch. Microbiol. **132**:115-122.