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Comparative Study of Substrates and Inhibitors of *Azospirillum lipoferum* and *Pyricularia oryzae* Laccases

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***Azospirillum lipoferum* and *Pyricularia oryzae* laccases were compared, using several substrates and inhibitors. Sixteen phenolic or nonphenolic compounds were found to be substrates of both fungal and bacterial laccases. In the presence of different phenol oxidase inhibitors, *P. oryzae* and *A. lipoferum* laccase activities had similar properties.**

In animals, plants, fungi, and eubacteria, the main phenol oxidases (PO) are catechol oxidases (tyrosinases) and laccases. In the presence of molecular oxygen, laccases typically oxidize *p*- and *o*-diphenols, whereas catechol oxidases oxidize monophenols and *o*-diphenols (19), and the quinonic products may be polymerized into large molecules, such as melanins (4). Laccases have been detected in many fungi and higher plants (19, 26), but so far only in one bacterium: *Azospirillum lipoferum* (13). Laccases have been implicated in the lignification of plant tissues (3) and in the phytopathogenicity of several fungi (4); these oxidizing enzymes have also been associated with browning in food and have been used for the removal of natural phenols or xenobiotics (5, 7, 22). The list of substrates oxidized by laccases has increased significantly in recent years: methoxy- or amino-monophenols and several nonphenolic compounds such as aromatic diamines, 2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-naphthol, hydroxyindoles, and syringaldazine are laccase substrates (8, 19, 26). Moreover, in the presence of ABTS, laccases are able to oxidize several compounds which are not laccase substrates on their own (6).

Unlike *A. lipoferum* 4B, the nonmotile *A. lipoferum* 4T and *A. lipoferum* 4Bp, a spontaneous nonmotile form derived from *A. lipoferum* 4B, all exhibit a laccase activity (2, 13). *A. lipoferum* 4B and 4T were isolated from the same rice rhizosphere (25) and are very close genetically (12, 15). As determined by Tn*5* mutagenesis in *A. lipoferum* 4T, laccase activity is required for melanin production (11). *Azospirillum* spp. colonize the rhizospheres of several plants and are used as agricultural inoculants (10). Both laccase activity and melanization, as O_2 or toxic phenol scavengers, could contribute to the adaptation of *A. lipoferum* to rhizospheric life (5, 16, 24).

Fungal and plant laccases have been extensively described by the several substrates and inhibitors active on them (19, 28), but few data on bacterial laccase activity have been reported (13). In this work, commercial fungal laccase and catechol oxidase, purified from *Pyricularia oryzae* and *Agaricus bisporus*, respectively, have been used as laccase or catechol oxidase references. By using several substrates and PO inhibitors, we showed that bacterial and fungal laccase activities are very similar.

Strains, enzyme preparation, and chemicals. Acellular extracts of *A. lipoferum* 4B and 4Bp were prepared as previously described (13) and stored at −20°C. All chemicals were purchased from Sigma (St. Louis, Mo.). *N*-Hydroxyglycine was a gift from E. G. E. Jahgen (17).

Substrates of bacterial laccase activity. Five classes of chemical compounds were investigated as substrates for laccase: (I) L-tyrosine and several substituted monophenols such as *p*-coumaric and *o*-hydroxyphenylacetic or salicylic acids; (II) *o*-diphenols (catechol, pyrogallol, guaiacol, and protocatechic, gallic, and caffeic acids), L-3,4-dihydroxyphenylalanine, and *o*-aminophenol, which could be oxidized by both laccase and catechol oxidase; (III) *p*-diphenol and *p*-substituted aromatic compounds as typical *p*-phenol oxidase (*p*-PO) substrates such as hydroquinone, *p*-cresol, *p*-aminophenol, and *p*-phenylenediamine; (IV) *m*-diphenols such as resorcinol, orcinol, 4-hexylresorcinol, and 5-pentadecylresorcinol; and (V) other laccase substrates such as syringaldazine, 1-naphthol, ABTS, and 4- and 5-hydroxyindoles. All of these chemicals compounds were dissolved 0.2% (wt/vol) in deionized water or absolute ethanol. To 10 μl of each chemical solution were added 400 μl of buffer [2-(*N*-morpholino)ethanesulfonic acid [MES], 0.08 M; pH 6] and 10 μl of the enzyme preparation of *A. lipoferum* 4B, *A. lipoferum* 4Bp, or *P. oryzae*. A sample without enzyme was used as a control. After 3 and 14 h of incubation (at 21°C in the dark), spectral analysis of the reaction mixture was performed (200 to 700 nm; 200 nm/min; Uvikon 930, Kontron, Zurich, Switzerland). When the spectrum obtained with *A. lipoferum* 4Bp extract was (i) different from the control, (ii) different from the spectrum with *A. lipoferum* 4B extract, or (iii) similar to the spectrum with *P. oryzae* laccase, compounds were considered substrates of *A. lipoferum* 4Bp laccase.

The range of substrates used by *A. lipoferum* laccase was similar to that used by *P. oryzae* laccase. As diagnostic substrates of the plant and fungal laccases, *o*-diphenols, *o*-aminophenol (class II), *p*-diphenol, and *p*-aminophenol (class III) were oxidized by *A. lipoferum* laccase. Because L-tyrosine and several monophenols (class I) were not oxidized by *A. lipoferum* 4Bp extract, *A. lipoferum* 4Bp had no *o*-hydroxylation activity, which is characteristic of catechol oxidase (20). *m*-Diphenols (class IV) were not oxidized by most plant or fungal catechol oxidases and laccases or by *Azospirillum* laccase. As noted above, the range of accepted characteristic laccase substrates has recently been expanded (19, 26). Additional compounds such as syringaldazine, *p*-phenylenediamine, 1-naphthol, and 4- and 5-hydroxyindoles were also found to be oxidized by *A. lipoferum* extract (class V). Thus, for all diagnostic substrates, *A. lipoferum* laccase was similar to *P. oryzae* laccase. However, the one difference between the two enzymes,

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TABLE 1. Comparison of the effectiveness of various PO inhibitors on *A. bisporus* catechol oxidase and on *P. oryzae* and *A. lipoferum* laccases

Substrate	Enzyme prepn	Inhibitor concn needed for 50% inhibition of PO activity				
		NaN_3 (μM)	*N*-Hydroxyglycine (μM)	SHAM (μM)	Tropolone (μM)	Kojic acid (mM)
o-Aminophenol	*A. bisporus* catechol oxidase	ND[a]	NI[b]	0.2	0.7	0.2
	P. oryzae laccase	30	800	NI	NI	20
	A. lipoferum 4Bp extract	2	NI	NI	NI	20
p-Aminophenol	*P. oryzae* laccase	20	200	NI	NI	0.3
	A. lipoferum 4Bp extract	3	500	NI	NI	0.4
Syringaldazine	*P. oryzae* laccase	5	50	80	NI	2
	A. lipoferum 4Bp extract	2	150	80	NI	2

[a] ND, not determined.
[b] NI, not inhibited; i.e., in the presence of PO inhibitors, PO activities remained equal or superior to 80% of PO activities in the absence of these inhibitors.

namely, the ability to oxidize *p*-cresol, varied from one laccase to another (20). Several chemicals which are not laccase substrates on their own are affected by fungal or bacterial laccase activity in the presence of a coupling reagent such as ABTS (6, 13, 23). Thus, almost all compounds which were oxidized by plant and fungal laccases were also oxidized by *A. lipoferum* laccase.

Effects of PO inhibitors on bacterial laccase activity. Sensitivity to several specific inhibitors is the second characteristic of PO that we investigated. Because *o*-aminophenol, *p*-aminophenol, and syringaldazine were substrates of *o*-diphenol oxidase (*o*-PO) or *p*-PO, and because they were rapidly oxidized into a product with absorbance in the visible spectrum, they are convenient for measuring the inhibition kinetics of PO (14, 27). Ten microliters of each enzyme preparation was incubated for 10 min at 37°C in 200 μl of buffer (0.08 M MES, pH 6) with or without inhibitor; kojic acid (9), salicylhydroxamic acid (SHAM) (1), tropolone (18), *N*-hydroxyglycine (21), or NaN_3 (20). Fifty microliters of substrate solution (1 mM; in 70% [vol/vol] ethanol) was added, and the PO activities were analyzed at 37°C with a microplate reader ($THERMO_{max}$; B. Braun ScienceTec, Les Ulis, France) at 540 (syringaldazine) or 450 (*o*- and *p*-aminophenols) nm. For all enzyme preparations, the V_{max} without inhibitor was adjusted to 2.5, 10, and 50 10^{-3} DO/min with *p*-aminophenol, *o*-aminophenol, or syringaldazine as substrate, respectively. Concentrations of each inhibitor were tested over the ranges 0.8 to 800 μM (NaN_3 and *N*-hydroxyglycine), 0.4 to 80 μM (tropolone), 0.4 to 512 μM (SHAM), and 0.4 to 51.2 mM (kojic acid). The results are the means of three replicates and are expressed as a percentage of the activity observed in the absence of inhibitor (standard deviation, <6%).

Catechol oxidases and laccases, as well as *A. lipoferum* laccase, were affected by metal chelators such as EDTA and Na azide and, more specifically, by copper chelators such as diethyldithiocarbamate (13, 20) (Table 1). These characteristics suggest that *A. lipoferum* laccase is similar to fungal and plant laccases, as well as catechol oxidases, in that copper is necessary for these enzyme activities (20, 26). However, *A. lipoferum* laccase activity was affected by two *p*-PO inhibitors (28), hexadecyltrimethylammonium bromide (13) and *N*-hydroxyglycine (Table 1). *o*-PO activity of *A. bisporus* catechol oxidase was inhibited by low concentrations of SHAM (7 μM) and tropolone (8 μM), while *o*-PO and *p*-PO activities of both *A. lipoferum* and *P. oryzae* laccases remained unaffected by these inhibitor concentrations. With syringaldazine as substrate, both fungal and bacterial laccase activities were only affected by high concentrations (80 μM) of SHAM (Table 1). All PO enzymes were inhibited by kojic acid, but *A. bisporus o*-PO activity was 100 times more sensitive than *P. oryzae* and *A. lipoferum o*-PO activities. SHAM, tropolone, and *N*-hydroxyglycine can thus be used to distinguish between catechol oxidase and laccase activities.

The comparison of *A. lipoferum* and *P. oryzae* laccase activities showed that their substrates ranges and sensitivities to several inhibitors are similar. These features suggest a similar oxidizing ability among plant, fungal, and bacterial laccases.

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REFERENCES

1. **Allan, A. C., and J. R. Walker.** 1988. The selective inhibition of catechol oxidases by salicylhydroxamic acid. Phytochemistry **27:**3075–3076.
2. **Bally, R., D. Thomas-Bauzon, T. Heulin, J. Balandreau, C. Richard, and J. De Ley.** 1983. Determination of the most frequent N_2 fixing bacteria in the rice rhizosphere. Can. J. Microbiol. **29:**881–887.
3. **Bao, W., D. M. O'Malley, R. Whetten, and R. R. Sederoff.** 1993. A laccase associated with lignification in loblolly pine xylem. Science **260:**672–674.
4. **Bell, A. A., and M. H. Wheeler.** 1986. Biosynthesis and functions of fungal melanins. Annu. Rev. Phytopathol. **24:**411–451.
5. **Bollag, J. M., K. L. Shuttleworth, and D. H. Anderson.** 1988. Laccase-mediated detoxification of phenolic compounds. Appl. Environ. Microbiol. **54:**3086–3091.
6. **Bourbonnais, R., and G. G. Paice.** 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′-azino-di(3-ethylbenzothiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. **36:** 823–827.
7. **Brenna, O., and E. Bianchi.** 1994. Immobilised laccase for phenolic removal in Mustand wine. Biotechnol. Lett. **16:**35–40.
8. **Cai, W., R. Martin, B. Lemaure, J. L. Leuba, and V. Pétiard.** 1993. Hydroxyindoles: a new class of laccase substrates. Plant Physiol. Biochem. **31:**441–445.
9. **Chen, J. S., C. Wei, and M. R. Marshall.** 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. J. Agric. Food Chem. **39:**1897–1901.
10. **Fages, J.** 1994. *Azospirillum* inoculants and field experiments, p. 87–109. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Inc., Boca Raton, Fla.
11. **Faure, D., M. L. Bouillant, and R. Bally.** 1994. Isolation of *Azospirillum lipoferum* 4T Tn*5* mutants affected in melanization and laccase activity. Appl. Environ. Microbiol. **60:**3413–3415.
12. **Givaudan, A., and R. Bally.** 1991. Similarity between large plasmids of *Azospirillum lipoferum*. FEMS Microbiol. Lett. **78:**245–252.
13. **Givaudan, A., A. Effosse, D. Faure, P. Potier, M. L. Bouillant, and R. Bally.** 1993. Polyphenoloxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. FEMS Microbiol. Lett. **108:**205–210.
14. **Harkin, J. M., and J. R. Obst.** 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. Experientia **29:**381–387.
15. **Haurat, J., D. Faure, R. Bally, and P. Normand.** 1994. Molecular relationship of an atypical *Azospirillum* strain 4T to other *Azospirillum* species. Res. Microbiol. **145:**633–640.
16. **Hynes, M. F., K. Brucksch, and U. Priefer.** 1988. Melanin production encoded by a cryptic plasmid in a *Rhizobium leguminosarum* strain. Arch.

Microbiol. **150:**326–332.
17. **Jahngen, E. G. E., and E. F. Rossomando.** 1982. The synthesis of hadacidin: sodium cyanoborohydride reduction of a-oximinoic acids. Synth. Commun. **12:**601–606.
18. **Kahn, V., and A. Andrawis.** 1985. Inhibition of mushroom tyrosinase by tropolone. Phytochemistry **24:**905–908.
19. **Mayer, A. M.** 1987. Polyphenol oxidases in plants—recent progress. Phytochemistry **26:**11–20.
20. **Mayer, A. M., and E. Harel.** 1979. Polyphenol oxidases in plants. Phytochemistry **18:**193–215.
21. **Murao, S., Y. Hinode, E. Matsumura, A. Numata, K. Kawai, H. Ohishi, H. Jin, H. Oyama, and T. Shin.** 1992. A novel laccase inhibitor, N-hydroxyglycine, produced by *Penicillium citrinum* YH-31. Biosci. Biotechnol. Biochem. **56:**987–988.
22. **Sariaslani, F. S.** 1989. Microbial enzymes for oxidation of organic molecules. Crit. Rev. Biotechnol. **9:**171–257.
23. **Shin, T., S. Murao, and E. Matsumura.** 1987. A chromogenic oxidative coupling reaction of laccase: application for laccase and angiotensin I converting enzyme assay. Anal. Biochem. **166:**380–388.
24. **Shivprasad, S., and W. J. Page.** 1989. Catechol formation and melanization by Na^+-dependent *Azotobacter chroococcum*: a protective mechanism for aeroadaptation? Appl. Environ. Microbiol. **55:**1811–1817.
25. **Thomas-Bauzon, D., P. Weinhard, P. Villecourt, and J. Balandreau.** 1982. The spermosphere model—its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. Can. J. Microbiol. **28:**922–928.
26. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
27. **Toussaint, O., and K. Lerch.** 1987. Catalytic oxidation of 2-aminophenol and ortho hydroxylation of aromatic amines by tyrosinase. Biochemistry **26:**8567–8571.
28. **Walker, J. R. L., and R. F. McCallion.** 1980. The selective inhibition of *ortho*- and *para*-diphenol oxidases. Phytochemistry **19:**373–377.