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# PHENOLIC DERIVATIVES RELATED TO LIGNIN METABOLISM AS SUBSTRATES FOR *AZOSPIRILLUM* LACCASE ACTIVITY

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**Key Word Index**—*Azospirillum lipoferum*; bacteria; rice; Gramineae; rhizosphere; phenol oxidase; laccase activity; syringic aldehyde, acetosyringone, 2,6-dimethoxy-1,4-benzoquinone.

**Abstract**—Activity of bacterial *Azospirillum lipoferum* laccase on phenolic derivatives was studied by spectrophotometry, HPLC and GC/MS. Phenolic compounds of the syringic type (aldehyde, acid or acetophenone) were transformed into 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ). Comparison has been made with the fungal *Pyricularia oryzae* laccase. Transformation of other phenolic acid derivatives, related to lignin metabolism, have been studied with both bacterial and fungal enzymes, using spectrophotometric analysis.

## INTRODUCTION

Laccases (EC 1.10.3.2.) are phenol oxidases widespread in fungi and higher plants [1, 2], but have been described [3], for the first time, in only one bacterial strain of *Azospirillum lipoferum* (strain 4T), isolated from rice rhizosphere [4]. This coexists in the same rhizosphere with another *A. lipoferum* strain: 4B, genetically very close [5], but which does not show laccase activity. Nevertheless, a spontaneous mutant derived from the latter, *A. lipoferum* 4Bp, shows this activity, suggesting important metabolic modifications.

The characteristic laccase substrates are *o*-diphenols and *p*-diphenols which are oxidized to *o*-quinones and *p*-quinones, respectively [6]. However, fungal laccases are also known to oxidize phenolic compounds that are not diphenolics as, for example, phenolic aldehydes, acids or alcohols in which laccases catalyse the breakdown of the $C_{aryl}$-$C_{alkyl}$ bond [7], that explains its activity in lignin and melanin metabolism. Recently, a comparison between *A. lipoferum* and *Pyricularia oryzae* laccases showed very similar oxidizing characteristics of both enzymes [8] towards simple phenolic substrates. As reported for several fungal laccases, *A. lipoferum* laccase is required for melanin biosynthesis [9], does not oxidize tyrosine [3] and is inhibited by known laccase inhibitors (CTAB and *N*-hydroxyglycine) [8]. In this work, we show by spectrophotometric, HPLC and GC/MS studies that bacterial laccase oxidizes phenolic compounds such as syringic aldehyde, acid or acetosyringone to give the corresponding quinone (2,6-dimethoxy-1,4-benzoquinone). Other phenolic derivatives involved in lignin metabolism were also transformed by the enzyme.

## RESULTS AND DISCUSSION

All the results have been obtained with crude enzymatic extracts from bacteria *A. lipoferum* 4T and 4Bp, and compared with fungal laccase of *Pyricularia oryzae* and, as negative control, crude intracellular extracts from *A. lipoferum* 4B strain. An assay with syringic aldehyde as substrate for the bacterial laccase at the last stage of purification, has given the same results (G. Diamantidis, pers. comm.).

Ethanolic† solutions of syringic derivatives (aldehyde, acid or acetophenone), were treated with these extracts in a MES buffered (pH 6.8) medium, and the reaction followed by HPLC. After 1 hr, syringic aldehyde and acetosyringone were oxidized to syringic acid and another compound that was identified by comparison (UV spectrum, HPLC and GC/MS) with an authentic synthetic sample, as 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (Fig. 1). Syringic acid was also oxidized to the same *p*-quinone but the reaction was less complete: after 24 hr, 68% of the aldehyde had been transformed, but only 35% of syringic acid. Moreover, the disappeared aldehyde seems to have been entirely transformed into the quinone, but only 30% of the syringic acid was transformed in this way. These oxidation reactions are summarized in Fig. 1. The ability of bacterial extracts to modify the UV/

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†With syringaldazine as a substrate we have observed (unpublished results) that addition of solvent (ethanol or methanol) in the enzymatic assay stabilized the activity.

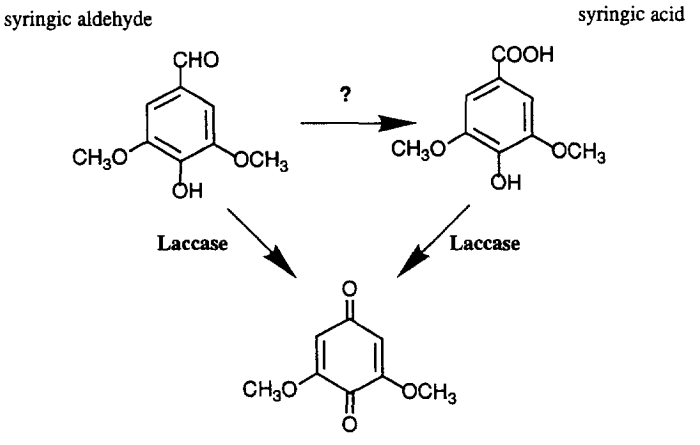
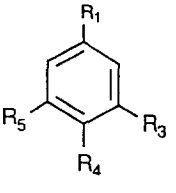



Fig. 1. Transformation of syringic aldehyde and syringic acid by *A. lipoferum* 4Bp and 4T laccases.

visible spectrum of other phenolic compounds involved in lignin metabolism, was also investigated. In the presence of a crude extract from *A. lipoferum* 4B, the laccase minus strain, the spectrum of all compounds tested remain unchanged.

On the contrary, with crude extracts of *A. lipoferum* 4Bp and *P. oryzae* laccase, the spectra of several compounds were modified (Table 1). With caffeic, protocatechuic, and ferulic acids, and ferulic aldehyde, all UV or visible absorbance disappeared after 2 hr of incubation. With caffeic and protocatechuic acids, which show two hydroxyl groups in the *ortho* position, a brown flocculating precipitate was observed in the reaction medium. This indicates a polymerization reaction and/or a tanning process between the reactive *o*-quinonic products and the enzymatic preparations. Because of the absence of any absorption spectrum, HPLC studies have not been conducted on these reaction products. However, the UV spectrum of coniferyl alcohol was modified and an intermediary compound, not studied here, was observed by HPLC ($\lambda_{max}$ 254 nm) after 1 hr of reaction. This intermediary completely disappeared after 24 hr. As expected, veratric substituted compounds (veratric aldehyde and acid and 3,4-dimethoxybenzylic alcohol), that do not have free phenolic groups, were unchanged by both bacterial and fungal laccases. More surprisingly, vanillic acid and aldehyde, which show a guaiacyl ring, like ferulic derivatives, were not oxidized by the bacterial laccase, but were transformed by the fungal one. The response

Table 1. Phenolic compounds transformed (+) or not (−) by *A. lipoferum* 4T or 4Bp enzymatic extracts or *P. oryzae* fungal laccase, as determined by changes in their UV and Visible spectra.




| Substrates | $R_1$ | $R_3$ | $R_4$ | $R_5$ | *A. lipoferum* 4T and 4Bp | *P. oryzae* laccase |
|---|---|---|---|---|---|---|
| Syringic aldehyde | CHO | $OCH_3$ | OH | $OCH_3$ | + | + |
| Syringic acid | COOH | $OCH_3$ | OH | $OCH_3$ | + | + |
| Acetosyringone | $COCH_3$ | $OCH_3$ | OH | $OCH_3$ | + | + |
| Vanillic aldehyde | CHO | $OCH_3$ | OH | H | − | + |
| Vanillic acid | COOH | $OCH_3$ | OH | H | − | + |
| Ferulic aldehyde | CH=CH–CHO | $OCH_3$ | OH | H | + | + |
| Ferulic acid | CH=CH–COOH | $OCH_3$ | OH | H | + | + |
| Coniferylic alcohol | CH=CH–$CH_2OH$ | $OCH_3$ | OH | H | + | + |
| Brotocatechuic acid | COOH | OH | OH | H | + | + |
| Caffeic acid | CH=CH–COOH | OH | OH | H | + | + |
| Veratric aldehyde | CHO | $OCH_3$ | $OCH_3$ | H | − | − |
| Veratric acid | COOH | $OCH_3$ | $OCH_3$ | H | − | − |
| 3,4-Dimethoxybenzylic alcohol | $CH_2OH$ | $OCH_3$ | $OCH_3$ | H | − | − |

to vanillic derivatives would thus appear to distinguish between *A. lipoferum* and *P. oryzae* laccases. However, in the case of several fungal laccases also, vanillic acid gives various results and is less oxidized than ferulic acid, 2,6-dimethoxyphenol, syringic and sinapic acids [10]. From this work it is possible to say that this bacterial laccase has oxidizing abilities similar to fungal laccases. However, unlike most fungal laccases, the *Azospirillum* laccase was not excreted into the medium. The transformation of extracellular substrates by *A. lipoferum* laccase would thus be less efficient than with fungal laccases in lignin catabolism. However, the syringyl type compounds are known to play an important role in rhizosphere signalling phenomena: acetosyringone, for example is an inducer of the *Agrobacterium vir*-genes [11] and, its oxidation product DMBQ *de novo* synthesized during host-parasite interaction, allowed the formation and the fixation of the haustorium of the parasitic weed *Striga* on the roots of grasses [12].

## EXPERIMENTAL

*Chemicals* were obtained from Sigma. Synthetic 2,6-dimethoxy-benzoquinone was a gift of Rhône-Poulenc Agrochimie.

*Strains, cultivation and preparation of cell-free extracts.* Strains of *Azospirillum lipoferum* 4B or 4T, isolated from a rice rhizosphere [4] and *A. lipoferum* 4Bp were cultivated on TY [13] medium. Stationary bacterial cultures were collected, and treated to obtain cell-free extracts as previously described [3].

*Enzymatic assays and HPLC analyses.* For UV spectral studies, chemical compounds were dissolved 0.2% (wt/vol) in EtOH. To 10 $\mu$l of each soln were added 40 $\mu$l of buffer (2-(*N*-morpholino)ethane sulphonic acid, MES) 0.08 M; pH 6) and 10 $\mu$l of the enzyme prepn. A sample without enzyme was used as control. After 3 and 14 hr of incubation (at 21° in the dark), spectral analysis of the reaction mixture was performed. For assays followed by HPLC analysis, 50 $\mu$l of a soln of the phenolic compound (5 mg $ml^{-1}$) in MeOH was added to 300 $\mu$l of MES buffer (pH 6; 0.08 M). 50 $\mu$l of cell-free extracts were added and a 20 $\mu$l sample of the mixture was injected into the column. The HPLC (reversed phase C18 column, 125 × 4 mm, Merck) was used with a photodiode array detector. The solvent was a linear gradient of 0.2% HOAc (100% to 20%)–MeCN (0 to 80%) in 25 min (1 ml $min^{-1}$). An authentic sample of synthetic 2,6-DMBQ was used as a reference.

*GC/MS analysis of DMBQ.* The mixture of syringic aldehyde and DMBQ from the laccase reaction was partially purified on a silica gel (60) column with a hexane–EtOAc gradient. By comparison with a synthetic sample of DMBQ, this mixture was analysed by GC/MS, on a HP MDS 5970 apparatus; column BPX5 SGE, 25 m × 0.22 mm, at first 120° in 1 min then 300°, 8° by 1 min. Retention time (8.7 min) and MS fragments, (*m/z* and abundance) of product DMBQ and authentic synthetic sample were absolutely identical. *m/z* (%): 168 (52)$[M]^+$; 153 (4); 138 (12); 125 (12); 112 (8); 97 (8); 80 (32); 69 (100); 53 (20).

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