ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 3, pp. 327–333. © Pleiades Publishing, Ltd., 2010.
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EXPERIMENTAL ARTICLES

Phenol Oxidase Activity in Bacteria of the Genus *Azospirillum*

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Received July 6, 2009

Abstract—Phenol oxidase activity was detected for the first time in a number of strains belonging to various *Azospirillum* species. Both extracellular and intracellular activities of laccase, Mn-peroxidase, lignin peroxidase, and tyrosinase were observed. Extracellular enzymes were found to have higher activity. Significant differences in phenol oxidase activities were observed between species and strains.

Key words: Azospirillum, phenol oxidase activity, laccase, Mn-peroxidase, lignin peroxidase, tyrosinase.

DOI: 10.1134/S0026261710030082

Bacteria of the genus *Azospirillum* are among the most intensely studied associative plant partners. These bacteria stimulate plant growth and development and are able to colonize both root and stem surfaces and internal tissues without formation of specific structures [1].

Laccases, peroxidases, and tyrosinases belong to the large group of phenol-oxidizing enzymes that are widespread in nature. Numerous studies concentrated on detection and description of the enzymes in eukaryotes (fungi and plants). However, recent experimental data demonstrated the presence of phenol oxidases (mainly laccases) in prokaryotes as well. Laccaselike activity was detected in melanin-synthesizing *Sinorhizobium melioti* strains [2]. Laccase CotA isolated from *Bacillus subtilis* endospore envelope was described [3–5]. Laccase activity of cloned multicopper oxidase from *Bacillus halodurans* was revealed [6]. Laccases were isolated from *Sinorhizobium melioti* [7] and *Pseudomonas putida* [8]. Laccaselike and tyrosinase activities were observed in the marine bacterium MMB-1 [9]. The presence of tyrosinase was reported in *Rhizobium, Azotobacter*, and *Streptomyces* [10–14]. Tyrosinase of *Rhyzobium melioti* GR4 was characterized [15]. In practically all cases, tyrosinase activity was associated with pigment formation.

As for phenol oxidase activity of *Azospirillum*, few facts are found in the literature concerning the mutant strain *Azospirillum lipoferum* 4T characterized by immotility and brown pigmentation [16–18]. There is no data on the enzymatic activity in other azospirilla species. We therefore consider it of utmost importance to find out whether the pigmented and immotile mutant *A. lipoferum* 4T is the only strain capable of laccase production or if other strains of different *Azospirillum* species, motile and nonpigmented, also possess laccase activity. Moreover, it is reasonable to suppose that, along with laccase, the whole complex of phenol oxidizing enzymes is produced by bacteria since all of the enzymes contribute to the substrate degradation.

The aim of the present work was to reveal and study intercellular and extracellular phenol oxidase activity in a number of *Azospirillum* strains of various species.

MATERIALS AND METHODS

Bacteria and culturing conditions. The following *Azospirillum* strains were used in this work: *A. brasilense* Sp245 (J. Dobereiner, Brazil), Sp7 (Institute of Microbiology, Moscow, Russia), Sp107 (J. Dobereiner, Brazil), and Cd (Y. Bashan, Mexico); *A. lipoferum* 43 (Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov, Russia) and 59b (VKM B-1519); *A. irakense* KA-3 and KBC-1 (C. Elmerich, France); and *A. amazonense* 14 (J. Dobereiner, Brazil).

Bacteria were grown on a number of agarized media, i.e., a synthetic medium containing the following (g/l): KH_2PO_4, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.026; $MgSO_4$, 0.2; KNO_3, 0.05; and glucose, 1.44 (pH 6.8) [19], a synthetic medium of the same composition supplemented with a water extract of wheat root sprouts, 10% potato medium, and malt agar (4° Balling). Azospirilla were cultured at 37°C.

Qualitative detection of oxidase activity in the process of bacterial growth. The presence of oxidase activity was judged by the ability of a growing culture to oxidize 2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulfonate), (ABTS, Sigma, United States), 2,6-dimethoxyphenol (DMOP, Acros, United States), and syringaldazine (Acros, United States) in petri dishes after 12, 24, and 36 h of growth. The surface of growing bacterial colonies was treated with 0.2 mM ABTS in 50 mM Na–

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tartrate buffer (pH 4.5) [20], 0.2% DMOP solution in 50 mM Na-tartrate buffer (pH 4.5) [21], and 0.02% syringaldazine in 50 mM Tris–HCl buffer (pH 7.5) [22]. Laccase activity was determined by the appearance of green coloration of bacteria and the medium in the first case, brown coloration in the second, and pink in the third. To reveal the activity of Mn-peroxidase, 0.2% *ortho*-dianisidine (Acros, United States) in 50 mM Na–tartrate buffer (pH 4.5) was used with the addition of 0.1 mM H_2O_2 and 0.2 mM Mn^{2+}. Activity was manifested by the appearance of oxidation products of greenish-brown color [23]. Tyrosinase was detected with 2 mM L-3-(3,4-dihydroxyphenylalanine), (L-DOPA, Acros, United States) in 50 mM Tris–HCl buffer (pH 7.5) by the appearance of dark brown coloration of bacteria and the medium [24]. Lignin peroxidase was detected with 0.01% phenol red in 20 mM Na–succinate buffer (pH 4.5) with the addition of 0.1 mM H_2O_2 by the appearance of pink coloration [25].

Enzyme isolation conditions. Bacterial biomass was washed off the agar surface with 20 mM Na–K phosphate buffer (pH 6.0); then the cells were washed several times and resuspended in a minimal volume (2 ml) of the same buffer. In order to obtain the enzyme fraction from bacterial cell surface, we used the modified "shaving" method [26] initially proposed by Eshdat et al. [27]. The modification was as follows: cellular suspension was passed several times through a syringe with a 0.8 × 38 needle according to the technique of Eshdat et al.; however, the obtained protein fraction was not treated with 2-nitro-5-thiocyanobenzoic acid in order to retain its enzymatic activity. The suspension was then centrifuged at 12000 *g* for 15 min, and the supernatant was separated and filtered.

To obtain intracellular enzymes, after isolation of surface proteins, the cell pellet was disintegrated with 5-min ultrasonic treatment using the same buffer and centrifuged at 12000 *g* for 15 min; the supernatant was collected and filtered.

Quantitative determination of enzymatic activity. Solutions of the relevant phenolic substrates were added to the intercellular and extracellular bacterial extracts of interest and left at 4°C for 3, 24, or 48 h. After 3 h, the absorbance spectrum of each sample was recorded, samples were left for another 21 h, the procedure was repeated, and samples were left for another day to finally record spectra at 48 h.

Enzyme activity was determined at 18°C using a Specord M 40 spectrophotometer (Carl Zeiss, Germany). Laccase activity was determined by the rate of oxidation of 0.2 mM ABTS in 50 mM Na–tartrate buffer (pH 4.5). ABTS oxidation to a stable cation radical was measured by the increase in absorbance at 436 nm (ε_{436} 29300 M^{-1} cm^{-1}) [20].

Mn-peroxidase activity was determined by the rate of oxidation of 0.2 mM ABTS in 50 mM Na–tartrate buffer (pH 4.5) supplemented with 0.1 mM H_2O_2 and 0.2 mM Mn^{2+}. ABTS oxidation to a stable cation radical was measured by the increase in absorbance at 436 nm (ε_{436} 29300 M^{-1} cm^{-1}) [23].

Tyrosinase activity was determined by the rate of oxidation of 2 mM L-DOPA in 50 mM Tris–HCl buffer (pH 7.5). L-DOPA oxidation to DOPA quinone was measured by the increase in absorbance at 475 nm (ε_{475} 3700 M^{-1} cm^{-1}) [24].

Lignin peroxidase activity was determined using 0.01% phenol red in 20 mM Na–succinate buffer (pH 4.5) supplemented with 0.1 mM H_2O_2, 25 mM lactate, and 0.1% ovalbumin. Phenol red oxidation was measured by the increase in absorbance at 610 nm [25].

In all cases, the reaction time was 3, 24, and 48 h. The enzyme amount that catalyzed transformation of 1 μmol substrate or formation of 1 μmol product in 1 h per 1 mg protein was taken as an activity unit.

Protein determination. Protein concentration was determined according to the Bradford procedure [28].

RESULTS AND DISCUSSION

Phenol oxidase activity of *Azospirillum* bacteria during cultivation. Using qualitative methods, we determined the ability of chosen azospirilla strains to produce a complex of extracellular phenol oxidases, that is, laccases, Mn-peroxidases, lignin peroxidases, and tyrosinases.

Preliminary experiments demonstrated that agarized potato medium was optimal for growth of all strains under study and for obtaining the most intense qualitative phenol oxidase reactions. Bacteria grown on malt agar, synthetic, and wheat root extract media exhibited significantly poorer enzymatic activity. It was also noted that enzyme activity was much higher in cells grown on agarized media rather than in submerged culture. Phenol oxidase activity increased with culture growth and biomass accumulation. The growth maximum was detected after 36 h of cultivation and correlated with the maximum oxidase activity. Therefore, for further studies we chose 36-h bacterial cultures grown on agarized potato medium.

All strains under study exhibited phenol oxidase activity that was revealed visually within several hours upon treatment with the relevant reagents by accumulation of colored products of phenolic substrate oxidation. Coloration intensity and differences in the colored area and rate of coloration were distinctly seen. However, various azospirilla strains decomposed phenolic compounds with different rates exhibiting various affinities to substrates. DMP was most efficiently decomposed by *A. brasilense* strains Sp245 and Sp7 and *A. irakense* strains KA-3 and KBC-1. This was evidenced by the appearance of colored (dark-brown) oxidation products in contrast to pale-creamy control cultures against the yellowish and transparent background of the culturing medium (Fig. 1). The tyrosinase substrate L-DOPA was decomposed by all strains, but the largest amount of colored products was

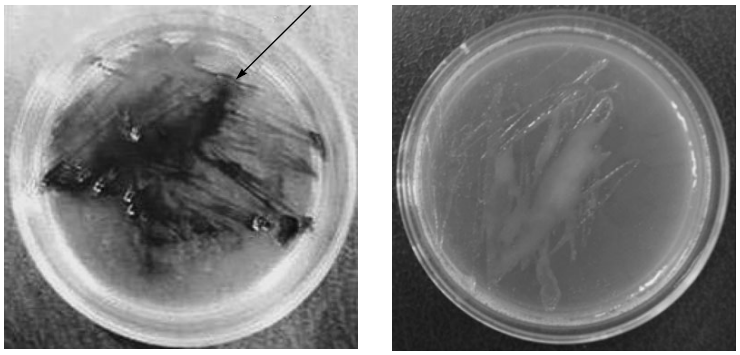

Fig. 1. Qualitative color reaction with dimethoxyphenol to test laccase activity in *A. brasilense* Sp245 grown on solid medium. The arrow indicates colored *A. brasilense* Sp245 colonies.

observed in the cultures of *A. lipoferum* 43 and 59b. *A. brasilense* strains Sp245 and Sp7 demonstrated a positive reaction with syringaldazine, with the appearance of pale pink coloration in plates with the bacteria cultures. Other strains did not exhibit activity toward this substrate. Under the given conditions, ABTS was only oxidized by *A. lipoferum* 43 and *A. irakense* KA-3 and KBC-1 exhibiting bright green coloration. *A. brasilense* Sp245 and Sp7 and *A. lipoferum* 43 and 59b could not decompose ortho-dianisidine. Other strains demonstrated activity toward the substrate. It is important to mention that a positive reaction by Mn-peroxidase toward ortho-dianisidine was only revealed on the bacterial surface and not in the culture medium. On the contrary, a reaction toward tyrosinase and laccase substrates was observed by coloration of both bacterial surface and the culturing medium, which evidences that enzymes were excreted beyond the bacterial surface.

A qualitative color test for lignin peroxidase with phenol red yielded positive results in *A. irakense* KA-3 and KBC-1, *A. lipoferum* 43, and *A. brasilense* Sp7 as well as in *A. amazonense* 14. The medium was colored bright pink to violet. The most intense coloration was observed for *A. lipoferum* strain 43; it developed already 15 min after the reagent was applied. After 30 min of incubation, coloration started to develop in the cultures of *A. irakense* KA-3 and KBC-1. Although it developed slower, it was as intense as in the case of *A. lipoferum* 43. *A. brasilense* Sp7 and *A. amazonense* 14 were much less efficient in phenol red degradation. The rest of the strains were not able to decompose the substrate under the given conditions, so the medium had the same color as the control.

Phenol oxidase activity of bacterial surface washes. Thus, we established that, upon surface cultivation on agarized potato medium, the following strains were able to produce the phenol oxidase complex: *A. brasilense* Sp245, Sp7, Sp107, and Cd; *A. lipoferum* 43 and 59b; *A. irakense* KA-3 and KBC-1; and *A. amazonense* 14. At the next stage of our study, we determined the dynamics of laccase, Mn-peroxidase, lignin peroxidase, and tyrosinase activity of the strains in a spectrophotometric assay. Studying phenol oxidase activity of surface washes off *Azospirillum* cultures, we found that the oxidases worked fairly slowly in comparison to fungal enzymes of the same class. The bacterial cell required from several hours to one day to decompose a phenolic substrate and accumulate the amounts of colored oxidation products sufficient for spectrophotometric measurements. Therefore, we recorded absorbance spectra after 3 h, 1 day, and 2 days of enzyme incubation with the relevant substrate. The quantitative characteristics and dynamics of activity of extracellular and intracellular phenol oxidases produced by azospirilla are presented in Figures 2–5.

High laccase activity was demonstrated already after 3 h of incubation by *A. brasilense* Sp107 and Cd and *A. irakense* KA-3 (Fig. 2). *A. brasilense* Sp245 and Sp7 and *A. lipoferum* 43 and 59b exhibited laccase activity two to three times lower than that of the above-mentioned bacteria. The enzyme activity in *A. irakense* KBC-1 was insignificant—more than 30 times lower than that of *A. brasilense* Sp107—and in *A. amazonense* 14 no laccase activity was detected after 3 h incubation. However, after 24 h of incubation, laccase activity in this strain was detected at the level of 0.5 U/mg. In other strains, laccase activity decreased, although it did not disappear completely (the enzyme continued substrate decomposition) after 1 day of incubation. After the second day of reaction, the dynamics was similar, laccase activity decreasing down to zero only in strains *A. lipoferum* 43 and *A. irakense* KBC-1.

After 3-h incubation, the level of Mn-peroxidase activity in washes off the bacterial surface was comparable to that of laccase activity in *A. brasilense* Sp107, but was 1.5–2.5 times higher in *A. brasilense* Cd and *A. irakense* KA-3 and 67 times higher in *A. amazonense* 14, taking into account that the Amazon strain exhibited no laccase activity after 3 h incubation

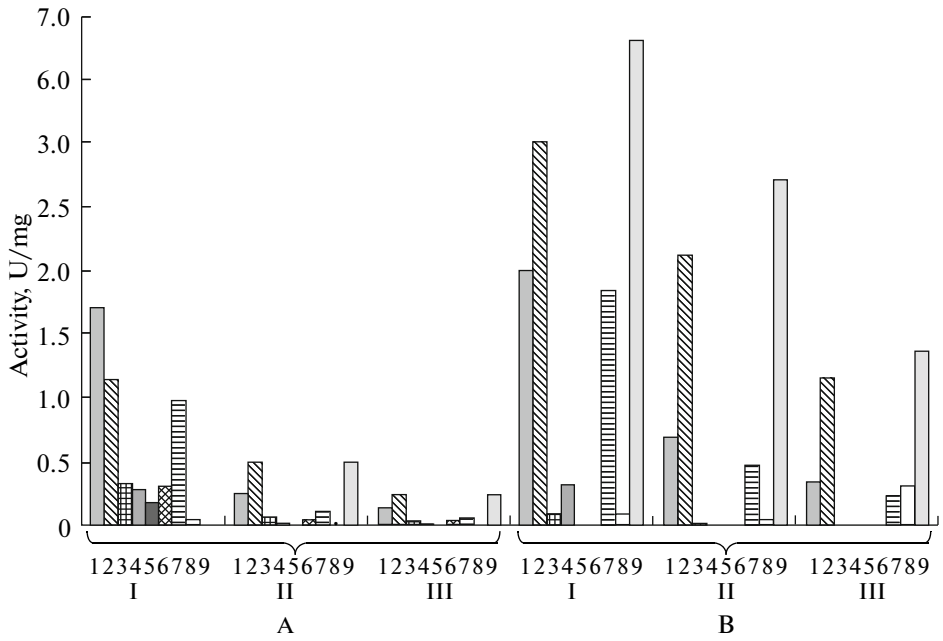

Fig. 2. Dynamics of laccase and Mn-peroxidase activity in washes of *Azospirillum* cell surface. *A. brasilense* Sp107 (*1*), *A. brasilense* Cd (*2*), *A. brasilense* Sp245 (*3*), *A. brasilense* Sp7 (*4*), *A. lipoferum* 43 (*5*), *A. lipoferum* 59b (*6*), *A. irakense* KA-3 (*7*), *A. irakense* KBC-1 (*8*), and *A. amazonense* 14 (*9*). I, II, and III indicate 3, 24, and 48 h of enzyme incubation with substrate. A, laccase and B, Mn-peroxidase.

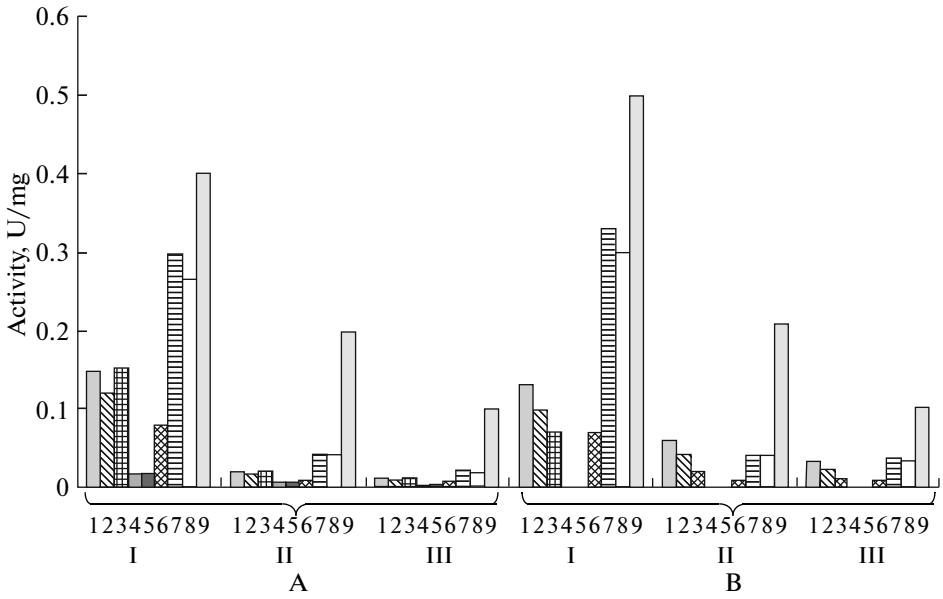

Fig. 3. Dynamics of laccase and Mn-peroxidase activity in intracellular extracts of *Azospirillum* strains. See the caption to Fig. 2 for the legend.

(Fig. 2). No Mn-peroxidase activity was revealed in either *A. lipoferum* strain (43 and 59b) during the whole experiment. In two *A. brasilense* strains (Sp245 and Sp7), Mn-peroxidase activity disappeared after 24-h incubation. In the rest of the strains, Mn-peroxidase activity after 24 and 48 h of incubation was at a significantly high level, which is considerably different from laccase activity dynamics for the same strains.

Among the strains under study, only *A. brasilense* Sp107 exhibited extracellular tyrosinase activity against the specific substrate after 3-h incubation (Fig. 4). The activity was detected after 24 h incuba-

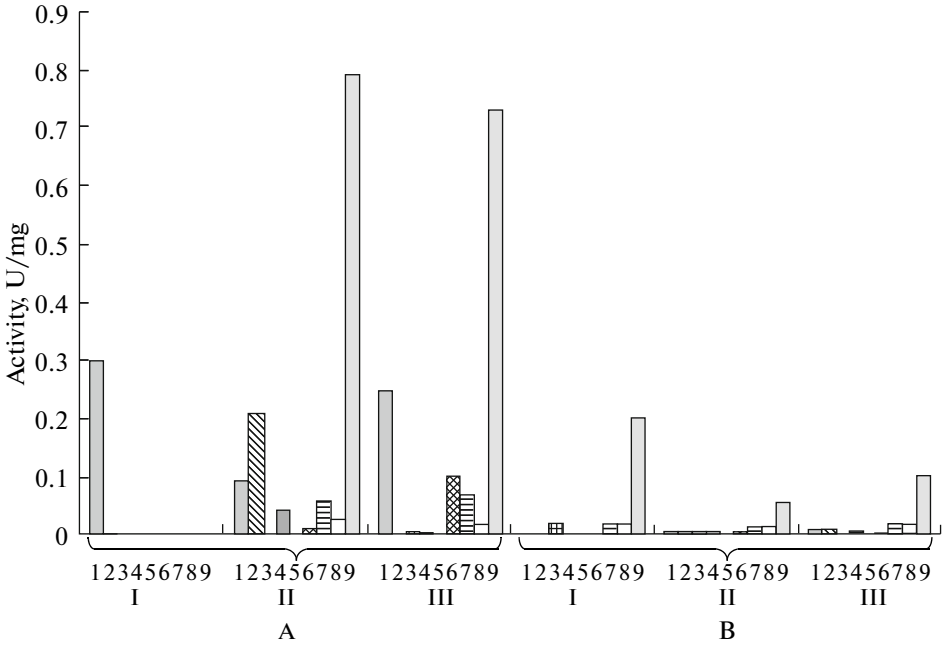

Fig. 4. Dynamics of tyrosinase activity of *Azospirillum* strains. See the caption to Fig. 2 for the legend. Extracellular (A) and intracellular (B) enzymes.

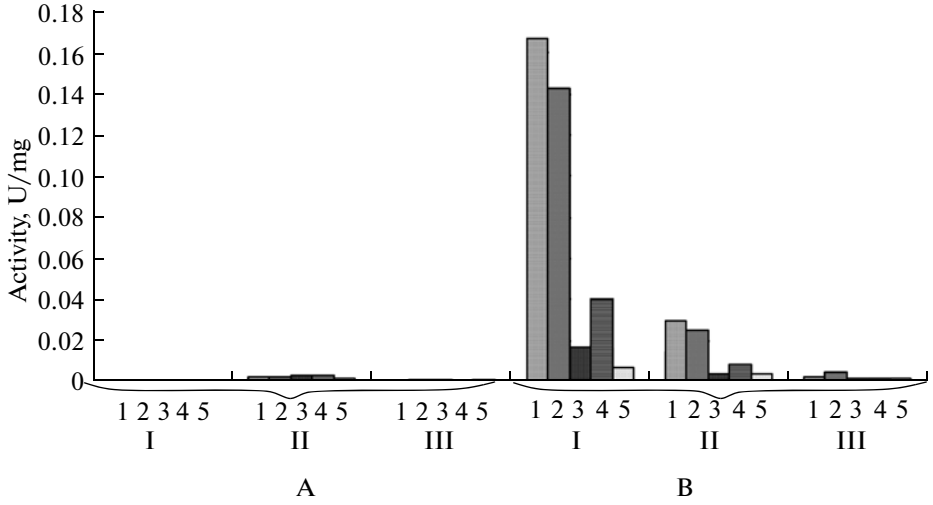

Fig. 5. Dynamics of lignin peroxidase activity of *Azospirillum* strains. *A. irakense* KBC-1 (1), *A. irakense* KA-3 (2), *A. lipoferum* 43 (3), *A. brasilense* Sp7 (4), and *A. amazonense* 14 (5). I, II, and III indicate 3, 24, and 48 h of enzyme incubation with the substrate. Extracellular (A) and intracellular (B) enzymes.

tion with the maximum value for *A. amazonense* 14 and values four to eight times lower for *A. brasilense* Cd and Sp107, respectively. On the second day of incubation, tyrosinase activity increased in the cultures of *A. brasilense* Sp107, *A. irakense* KA-3, and *A. lipoferum* 59b, while *A. amozonense* 14 retained its activity. Thus, tyrosinase activity in some strains did not decrease and even increased with time, and the enzyme continued to oxidize L-DOPA to DOPA-quinone. This effect was not observed in the cases of laccase and Mn-peroxidase. However, despite its high stability, tyrosinase activity was by an order of magnitude lower than that of laccase and Mn-peroxidase. Tyrosinase activity in *A. lipoferum* 43 was not determined. It is important that the extracellular phenol oxidases under study (laccases, Mn-peroxidases, and tyrosinases) in *A. lipoferum* 43 culture lost their activity after 24 h of incubation (Figs. 2 and 4). This distinguished the strain from the others.

We found that lignin peroxidase activity in some *Azospirillum* strains (*A. irakense* KA-3 and KBC-1, *A. lipoferum* 43, *A. brasilense* Sp7, and *A. amazonense* 14) was significantly (up to 670 times in some cases) lower than Mn-peroxidase activity, while in other strains it was not detected at all (Fig. 5). Lignin peroxidase activity in washes off bacterial cell surface was very low and could be revealed only after 24 h of incubation. The highest lignin peroxidase activity was observed for *A. amazonense* 14.

Phenol oxidase activity of bacterial intracellular enzymes. Study of the enzymes demonstrated that, except for *A. lipoferum* 43 and *A. brasilense* Sp7, all strains exhibited laccase and Mn-peroxidase activity of practically the same level, which was significantly lower than that of the extracellular enzymes (Fig. 3). The enzymes were most active in strains *A. irakense* KA-3 and KBC-1 and *A. amazonense* 14. Intracellular phenol oxidases in *A. brasilense* Sp107, Cd, and Sp245 and *A. lipoferum* 59b were two to six times less active, although their activity was still sufficiently high. Intracellular laccase activity in *A. lipoferum* 43 and *A. brasilense* Sp7 was at an extremely low level, and Mn-peroxidase activity was completely absent. A longer incubation time decreased the yield of the oxidation products in all *Azospirillum* strains, although intercellular laccases and Mn-peroxidases remained active even after 48-h incubation and continued decomposition of phenolic substrates, although less intensely.

Very low intracellular tyrosinase activity after 3-h incubation was observed only in the strains *A. brasilense* Sp245, *A. irakense* KA-3 and KBC-1, and *A. amazonense* 14 (Fig. 4). After 24 and 48 h of incubation, the enzyme activity dynamics did not change significantly. All *Azospirillum* strains, except for the Amazonian one, either did not exhibit any intracellular tyrosinase activity or the activity was quite low in comparison to the enzyme activity in the washes off bacterial cell surfaces.

In the case of intracellular lignin peroxidase activity dynamics, the pattern was reversed in comparison to other phenol oxidases under study. Intracellular enzyme activity was detected at the maximum level already after 3-h incubation with the substrate, while lignin peroxidase activity in bacterial surface washes was revealed only after 1 day of incubation (see above). With time, the activity decreased but did not disappear completely and the enzyme continued to oxidize phenol red. The coloration became more intense with time, which was confirmed with absorbance spectra analysis evidencing accumulation of phenol red oxidized products, that is, continuous enzyme functioning. The highest activity of lignin peroxidase was observed in strains *A. irakense* KBC-1 and KA-3.

The results of the experiments demonstrated that the azospirilla strains under study possessed various degrees of phenol oxidase activity carried out by an enzyme complex. Combination of motility and phenol oxidase activity in bacterial strains under study does not agree with the results obtained in [16], which evidenced that polyphenol oxidase activity was only present in nonmotile *A. lipoferum* forms isolated from the rice rhizosphere while wild strains did not possess such activity.

The functional importance of phenol oxidases in azospirilla remains an open question. However, some assumptions can be made. *Azospirillum* are nitrogen-fixing bacteria and thus are in close association with the roots of some groups of plants. The established phenol oxidase activity of azospirilla may be related to the adaptation mechanisms that increase survivability and competitiveness of bacteria in the rhizosphere due to their ability to oxidize and polymerize toxic phenolic compounds. Moreover, the ability of some strains, for example, *A. brasilense* Sp245, not only to colonize root surfaces, but also to invade the root, has been established [1]. It is not impossible that the mechanism of azospirilla invasion includes, along with other enzyme complexes, bacterial phenol oxidases that degrade the root cell envelope and thus promote survival and proliferation of azospirilla inside the root. Participation of phenol oxidases—specifically, laccases and tyrosinases—in melanin synthesis and formation of bacterial cell pigments is also quite possible; this increases the chances of the culture's survival under unfavorable conditions, as was demonstrated for fungi [29, 30]. Therefore, ability of azospirilla to synthesize various phenol oxidases revealed in the study may evidence significant adaptative capabilities and activity of microorganisms in the rhizosphere.

ACKNOWLEDGMENTS

This work was supported by a grant of the President of the Russian Federation, project no. Nsh-3171.2008.4.

REFERENCES

1. Dobereiner, J., Ten years *Azospirillum*, in *Azospirillum II: Genetics, Physiology, Ecology*, Klingmuller, W., Ed., Berlin: EXS48, 1983, pp. 9–23.
2. Castro-Sowinski, S., Martinez-Drets G, and Okon, Y., Laccase Activity in Melanin-Producing Strains of *Sinorhizobium meliloti, FEMS Microbiol. Letts.*, 2002, vol. 209, pp. 119–125.
3. Hullo, M.F., Moszer, I., Danchin, A., and Martin-Verstraete, I., CotA of *Bacillus subtilis* Is a Copper-Dependent Laccase, *J. Bacteriol.*, 2001, vol. 183, no. 18, pp. 5426–5430.
4. Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H., and Henriques, A.O., Molecular and Biochemical Characterization of a Highly Stable Bacterial Laccase That Occurs as a Structural Component of the *Bacillus subtilis* Endospore CotA, *J. Biol. Chem.*, 2002, vol. 277, no. 21, pp. 18849–18859.
5. Enguita, F.J., Marc, D., Martins, L.O., Grenha, R., Henriques, A.O., Lindley, P.F., and Carrondo, M.A.,

Substrate and Dioxygen Binding to the Endospore CotA Laccase from *Bacillus subtilis, J. Biol. Chem.*, 2004, vol. 279, no. 22, pp. 23472–23476.

6. Ruijssenaars, H.J. and Hartmans, S., A Cloned *Bacillus halodurans* Multicopper Oxidase Exhibiting Alkaline Laccase Activity, *Appl. Microbiol. Biotechnol.*, 2004, vol. 65, pp. 177–182.

7. Rosconi, F., Fraguas, L.F., Martínez-Drets, G., and Castro-Sowinski, S., Purification and Characterization of a Periplasmic Laccase Produced by *Sinorhizobium meliloti, Enz. Microbial. Technol.*, 2005, vol. 36, pp. 800–807.

8. McMahon, A.M., Doyle, E.M., Brooks, S., and O'Connor, K.E., Purification and Characterisation of Tyrosinase and Laccase from *Pseudomonas putida, Enz. Microbial. Technol.*, 2007, vol. 40, pp. 1435–1441.

9. Solano, F., Garcia, E., Perez-De-Egea, E., and Sanchez-Amat, A. Isolation and Characterization of Strain MMB-1 (CECT 4803), a Novel Melanogenic Marine Bacterium, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 3506–4399.

10. Shivprasad, S. and Page, W.J., Catechol Formation and Melanization by Na-Dependent *Azotobacter chroococcum*: A Protective Mechanism for Aeroadaptation?, *Appl. Environ. Microbiol.*, 1989, vol. 55, pp. 1811–1817.

11. Hawkins, F., Kennedy N., Johnston A.W.B. A *Rhizobium leguminosarum* Gene Required of Symbiotic Nitrogen Fixation, Melanin Synthesis and Normal Growth on Certain Growth Media, *J. Gen. Microbiol.*, 1991, vol. 137, pp. 1721–1728.

12. Lerch, K. and Ettinger, I., Purification and Characterization of a Tyrosinase from *Streptomyces glaucescens, Eur. J. Biochem.*, 1972, vol. 31, pp. 427–437.

13. Freeman, J.C., Nayar, P.G., and Begley, T.P., Stoichiometry and Spectroscopic Identity of Copper Centers in Phenoxazonine Synthase: A New Addition for the Blue Cooper Oxidase Family, *Biochemistry*, 1993, vol. 32, pp. 4826–4830.

14. Endo, K., Hosono, K., Verru, T., and Ueda, K., A Novel Extracytoplasmatic Phenol Oxidase of *Streptomyces*: Its Possible Involvement in the Onset of Morphogenesis, *Microbiology* (UK), 2002, vol. 148.

15. Mercado-Blanco, J., Garcia, F., Fernandez-Lopez, M., and Olivares, J., Melanin Production by *Rhizobium meliloti* GR4 Is Linked to Nonsymbiotic Plasmid pRme GR4b, *J. Bacteriol.*, 1993, vol. 175, pp. 5403–5410.

16. Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M.L., and Bally, R., Polyphenol Oxidase in *Azospirillum lipoferum* Isolated from Rice Rhizosphere: Evidence for Laccase Activity in Non-Motile Strains of *Azospirillum lipoferum, FEMS Microbiol. Lett.*, 1993, vol. 108, pp. 205–210.

17. Faure, D., Bouillant, M.L., and Bally, R., Isolation of *Azospirillum lipoferum* 4 T Tn5 Mutants Affected in Melanization and Laccase Activity, *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 9, pp. 3413–3415.

18. Diamantidis, G., Effosse, A., Potier, P., and Bally, R., Purification and Characterization of the First Bacterial Laccase in the Rhizospheric Bacterium *Azospirillum lipoferum, Soil Biol. Biochem.*, 2000, vol. 32, pp. 919–927.

19. Sadasivan, L. and Neyra, C.A., Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: Exopolysaccharides and Cyst Formation, *J. Bacteriol.*, 1985, vol. 163, no. 2, pp. 716–723.

20. Niku-Paavola, M., Karhunen, E., Salola, P., and Paunio, V., Ligninolytic Enzymes of the White Rot Fungus *Phlebia radiata, Biochem. J.*, 1988, vol. 254, pp. 877–302.

21. Slomczynski, D., Nakas, J., and Tanenbaum, S., Production and Characterization of Laccase from *Botrytis cinerea, Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 907–912.

22. Leonowicz, A. and Crzywnowicz, K., Quantitative Estimation of Laccase Forms in Some White-Rot Fungi Using Syringaldazine as a Substrate, *Enzyme Microbiol. Technol.*, 1981, vol. 3, pp. 55–58.

23. Glenn, J. and Gold, M., Purification and Characterization of an Extracellular Mn(II)-Dependent Peroxidase from the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium, Arch. Biochem. Biophys.*, 1985, vol. 242, pp. 329–341.

24. Pomerantz, S.M. and Murthy, V.V., Purification and Properties of Tyrosinases from *Vibrio tyrosinaticus, Arch. Biochem. Biophys.*, 1974, vol. 160, no. 1, pp. 73–82.

25. Kuwahara, M., Glenn, J., Morgan, M., and Gold, M., Separation and Characterization of Two Extracellular H_2O_2-Dependent Oxidases from Ligninolytic Cultures of *Phanerochaete chrysosporium, FEBS Lett.*, 1984, vol. 169, pp. 247–250.

26. Nikitina, V.E. and Ital'yanskaya, Yu.V., USSR Inventor's Certificate no. 1312773, 1987.

27. Eshdat, Y., Ofek, I., Yachow-Yan, Y., and Sharon, N., Isolation of a Mannose-Specific Lectin from *Escherichia coli* and Its Role in the Adherence of the Bacteria to Epithelial Cells, *Biochem. Biophys. Res. Commun.*, 1978, vol. 85, pp. 1551–1559.

28. Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem.,* 1976, vol. 72, pp. 248–254.

29. Thurston, C.F., The Structure and Function Fungal Laccase, *J. Microbiol.*, 1994, vol. 140, pp. 19–26.

30. Galhaup, C. and Haltrich, D., Enhanced Formation of Laccase Activity by the White-Rot Fungus *Trametes pubescens* in the Presence of Copper, *Appl. Microbiol. Biotechnol.*, 2001, vol. 56, pp. 225–232.