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EXPERIMENTAL ARTICLES

Tyrosinases of Motile *Azospirillum* Strains

E. P. Vetchinkina[1], E. G. Ponomareva, Yu. V. Gogoleva, and V. E. Nikitina

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia

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Abstract—A number of motile strains of *Azospirillum brasilense, A. lipoferum*, and *A. irakense*, were found to possess tyrosinase activity both on the surface of and inside the cells. *A. brasilense* Sp245, Sp7, and *A. irakense* KBC-1 each possessed two forms of tyrosinase of different molecular masses; *A. lipoferum* 43, *A. lipoferum* 59b, and *A. irakense* KA-3 each had a single tyrosinase form of approximately the same molecular mass; and *A. brasilense* Sp107 possessed a single form of tyrosinase different from all the other forms.

Keywords: soil bacteria, motile *Azospirillum* strains, tyrosinase

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Bacteria of the genus *Azospirillum* are free-living nitrogen-fixing microorganisms capable of association with plants and stimulation of plant growth and development. They mainly colonize the root surface, although cases of invasion inside the roots were also reported [1]. The mechanism of invasion is not clear; invasiveness of azospirilla and their adaptation to survival and proliferation inside the roots is probably associated with active enzyme systems. Some researchers report the presence of phenol oxidase activity in bacteria, including the nitrogen-fixing ones [2–5].

Tyrosinase, one of the enzymes of the polyphenol oxidase complex, is capable of oxidative polymerization of the phenolic compounds of the plant cell wall. In the literature, there are very few data on microbial tyrosinases [6]. Tyrosinases of *Streptomyces glaucescens* [7] and *Pseudomonas putida* [8] were isolated and characterized. Solano et al. demonstrated the presence of tyrosinase activity in a marine bacterium, MMB-1 [9]. Tyrosinase activity was detected in some nitrogen-fixing rhizobacteria of the genera *Azospirillum, Rhizobium,* and *Azotobacter*. The work of Nikitina et al. was devoted to detection of phenol oxidase activity—including tyrosinase activity—in a number of strains of several species of the genus *Azospirillum* [10]. Tyrosinase-like activity was determined in a number of *Rhizobium leguminosarum* strains and in *R. meliloti* GR4 [11, 12]. Shivprasad and Page detected tyrosinase in *Azotobacter chroococcum* [13].

In practically all cases, tyrosinase activity is associated with the formation of pigments. Synthesis of brown pigments, melanins, occurs in parallel with oxidative polymerization of phenolic compounds. Under unfavorable environmental conditions, the pigments may perform a protective function by providing a screen from excessive light and promoting a decrease in cell wall permeability for toxic compounds and pathogens.

Some researchers indicated the ability of mutant strains of *Azospirillum lipoferum*, which are characterized by nonmotility and production of brown pigments, to exhibit polyphenol oxidase activity [14–16]. In the nitrogen-fixing azospirilla, melanization is not a prerequisite for growth but may be an alternative protection mechanism of aeroadaptation increasing the survival and competitiveness of a species.

There is no data on the ability of motile forms of azospirilla to produce tyrosinases. In this connection, the question arises whether only pigmented and nonmotile bacteria of the genus *Azospirillum* may produce tyrosinases or the motile and nonpigmented strains belonging to various species possess active tyrosinases as well.

The goal of the present work was to detect and investigate tyrosinase activity on the surface of and inside the cells of motile *Azospirillum* strains of three species, as well as to visualize the protein forms of the enzyme under study by electrophoresis and specific staining.

MATERIALS AND METHODS

Organisms and cultivation conditions. Seven strains of azospirilla were the subjects of the study: *A. brasilense* Sp245 [17], Sp7 [18], and Sp107 [19]; *A. lipoferum* 43 [20] and 59b [18]; and *A. irakense* KA-3 and Kbc-1 [21].

Bacteria were grown on an agarized 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; glucose, 1,44; agar-agar, 20; pH 6.8 [22], or the same medium supplemented with a 10% aqueous

[1] Corresponding author; e-mail: elenavetrus@yandex.ru

extract of germinated wheat sprouts. Apart from these, the following agarized media were used: water–potato medium at 10 : 1 concentration and beer wort (4° B). Azospirilla were cultured at 27°C.

Qualitative detection of tyrosinase activity in the process of bacterial growth. The presence of tyrosinase activity in growing *Azospirillum* cultures was revealed at room temperature (18°C) in 10-cm petri dishes after 12, 24, and 36 h of cultivation by their ability to degrade L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA; Acros, Germany) with the formation of dark-brown oxidation products. For this purpose, 0.5 mL of 2 mM L-DOPA solution in 50 mM Tris-HCl buffer (pH 7.5) was applied all over the surface of 12-, 24-, and 36-h growing bacterial colonies. The presence of tyrosinase activity was assessed after 3 h of incubation by the appearance of dark-brown coloration of bacteria and culturing medium [23].

Conditions of enzyme isolation. Bacterial biomass was washed off the agar surface with 20 mM Na–K phosphate buffer (pH 6.0), the cells were washed several times and resuspended in a minimum volume (2 mL) of the same buffer. In order to obtain the fraction of enzymes residing on the surface of bacterial cells, the method of cell shearing was used: the cell suspension was passed several times through a syringe with a 0.8 × 38 needle, the suspension was centrifuged at 12000 *g* for 15 min, and the supernatant was separated from the pellet and filtered [24].

To obtain intracellular enzymes remaining after the isolation of surface proteins, the cell suspension in the same buffer was sonicated on a UD 20 "Techpan" (Poland) disintegrator under maximum power in three runs of 3 min each and centrifuged at 12000 *g* for 15 min; the supernatant was then separated from the pellet and filtered.

Quantitative assessment of enzymatic activity. Tyrosinase activity was determined by the rate of oxidation of 2 mM L-DOPA in 50 mM Tris-HCl buffer (pH 7.5) at room temperature (18°C). The solution of a phenolic substrate was added to the intracellular and extracellular bacterial extracts and left at 4°C for 3, 24, and 48 h. After 3 h, absorption spectra were recorded for each sample and they were left to incubate for another 21 h, then the procedure was repeated and the mixtures were left for another day in order for a spectrum to be measured after a total of 48 h of incubation. Oxidation of L-DOPA to "DOPA quinone" was measured by an increase in absorbance at 475 nm (ε_{475} 3700 M^{-1} cm^{-1}) using a Specord M 40 (Carl Zeiss, Germany) spectrophotometer. The amount of enzyme catalyzing the formation of 1 μmol product per 1 h was accepted as the unit of enzymatic activity and expressed as μmol h^{-1} mg^{-1} protein [23].

Protein determination. Protein concentration was determined according to Bradford [25].

PAGE. Composition of the enzymes was investigated by the method of non-denaturing electrophoresis according to Laemmli in 7% polyacrylamide gel [26] on a VE-4M (Helicon, Russia) vertical gel electrophoresis system with 150 × 150 mm plates and 1 mm thick gel. The sample (50 μL) containing 250 μg/mL protein in 50 mM Tris-HCl buffer (pH 6.8) supplemented with 10% glycerol, without SDS or β-mercaptoethanol, were introduced into each lane.

Specific staining of the gel for tyrosinase activity was performed with a reaction mixture of the following composition: 2 mM L-DOPA (Serva, Germany) in 50 mM Tris-HCl buffer (pH 7.5) [23]. Dark-brown bands corresponding to tyrosinase developed within 10 min.

RESULTS AND DISCUSSION

Tyrosinase activity in the course of cultivation of *Azospirillum*. We established experimentally that the optimal medium for growth of all azospirilla strains and production of intense qualitative tyrosinase reactions was agarized potato medium. For this purpose, different media were inoculated with equal concentrations of bacterial material and biomass increase was evaluated visually, while tyrosinase activity was determined qualitatively several hours after reagent application, as colored products of phenol substrate oxidation accumulated. Coloration intensity of the colonies and cultivation medium, differences in the area of colored surface, and different rates of coloration development were distinct. Activity increased with culture age and accumulation of the biomass. Maximum growth was observed after 36 h, which coincided with the maximum tyrosinase activity.

As a result, it was found that after 24 h of cultivation all strains under study exhibited tyrosinase activity at a varying degree; the maximum activity was observed after 36 h. The most active L-DOPA degraders were the strains *A. brasilense* Sp107 and *A. lipoferum* 59b. A less intense reaction was observed in the case of *A. irakense* KA-3 and KBC-1. The remaining strains possessed insignificant tyrosinase activity.

Isolation and dynamics of tyrosinase activity in material washed off the bacterial surface and in the intracellular extracts. At the next stage of the work, we used spectrophotometric methods to explore the dynamics of the activity of surface and intracellular tyrosinases in the *Azospirillum* strains under study.

We observed that bacterial oxidases worked rather "slowly" if compared, for example, to fungal enzymes of this class. Bacterial cells decomposing a phenolic substrate take several hours—sometimes days—to accumulate the amount of colored oxidation products sufficient for spectrophotometric detection. Therefore, we recorded and analyzed absorption spectra after 3 h, 1 day, and 2 days of enzyme incubation with L-DOPA. Quantitative characteristics and dynamics of activity of the extracellular and intracellular tyrosinases produced by azospirilla are presented in Fig. 1.

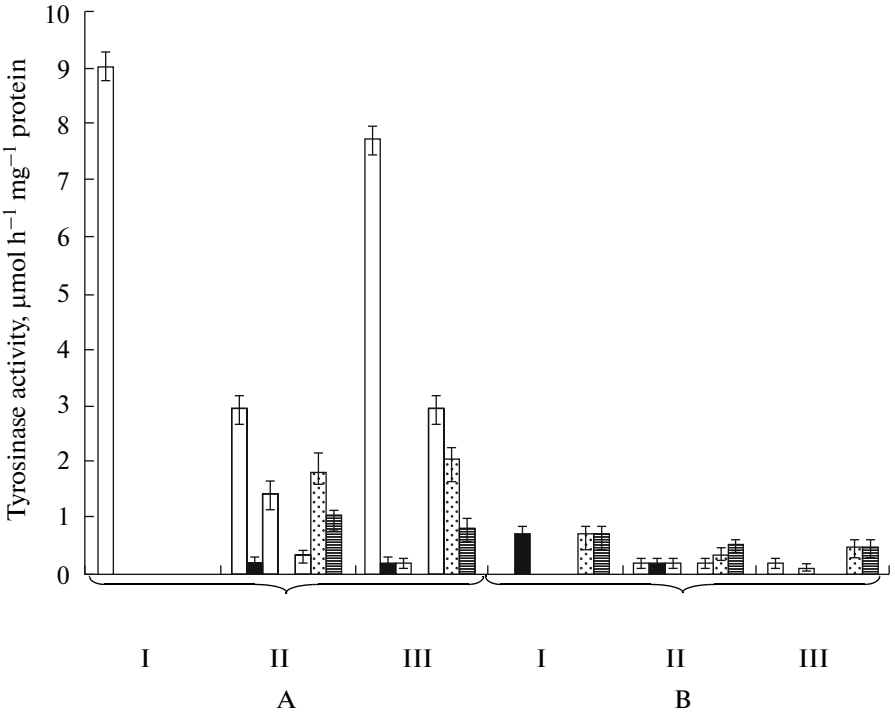

Fig. 1. Dynamics of tyrosinase activity in bacteria of the genus *Azospirillum*; A. *brasilense* Sp107 (*1*), *A. brasilense* Sp245 (*2*), *A. brasilense* Sp7 (*3*), *A. lipoferum* 43 (*4*), *A. lipoferum* 59b (*5*), *A. irakense* KA-3 (*6*), and *A. irakense* KBC-1 (*7*). I, II, and III indicate 3, 24, and 48 h, respectively, of the enzyme incubation with the substrate. A and B stand for extracellular and intracellular enzymes, respectively.

Three-hour incubation of cell-surface enzymes with the specific substrate did not reveal tyrosinase activity in any of the strains under study except for *A. brasilense* Sp107. Tyrosinase activity could be determined after incubation for 24 h; the highest rates of substrate oxidation were noted in the case of *A. brasilense* Sp107 and *A. irakense* KA-3, with activities of 2.9 and 1.85 μmol h^{-1} mg^{-1}, respectively. The lowest rate was found for *A. brasilense* Sp245 (0.2 μmol h^{-1} mg^{-1}). After 48 h, the activity decreased only in *A. brasilense* Sp7 (from 1.45 to 0.2 units), and in *A. irakense* KBC-1 (insignificantly). In the rest of the strains, the surface tyrosinase retained its activity or even increased it: the enzyme continued to oxidize L-DOPA to "DOPA quinone", which was especially pronounced in *A. brasilense* Sp107, where tyrosinase activity increased to 7.8 units. Unlike other strains, tyrosinase activity of *A. lipoferum* 43 could not be detected spectrophotometrically throughout the experiment. It should be noted that a similar picture was observed with respect to other enzymes of the phenol oxidation complex (laccases and Mn peroxidases) of *A. lipoferum* 43 culture [10]. The oxidases of this strain were probably unstable and did not exhibit activity under the experimental conditions.

Studies of the intracellular enzymes demonstrated that although all strains exhibited tyrosinase-specific activity, it was much lower than that of the enzymes isolated from cell surfaces. This resulted from higher protein content in the intracellular extracts, which was 10–50 times higher than the extracellular concentration. Increase in the time of incubation led to an increase in the yield of the substrate oxidation products in practically all strains. Specific activity of intracellular tyrosinase was the highest (0.8 μmol h^{-1} mg^{-1}) in *A. brasilense* Sp245 and *A. irakense* KA-3 and KBC-1.

Study of tyrosinases by PAGE. Investigation of the enzyme composition was performed by non-denaturing electrophoresis in polyacrylamide gel. Specific staining (L-DOPA) of tyrosinase made it possible to identify the protein bands corresponding to the enzymes of the intracellular extracts of all *Azospirillum* strains under study, but not in the material washed off the cell surfaces (Fig. 2). In *A. brasilense* Sp245, Sp7, and *A. irakense* KBC-1, two forms of tyrosinase of different molecular masses were revealed. *A. lipoferum* strains 43 and 59b and *A. irakense* KA-3 each possessed a single form of approximately the same molecular mass, while *A. brasilense* Sp107 possessed a single tyrosinase form of a different molecular mass. Unfortunately, electrophoresis failed to visualize the surface tyrosinases in any of the strains under study. The surface enzymes are probably unstable and lose their activity in the process of separation, which requires

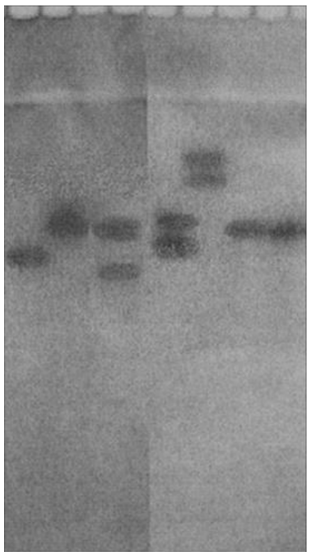

Fig. 2. Native electrophoresis and specific staining (L-DOPA) of tyrosinase of the intracellular extracts of various bacterial strains of the genus *Azospirillum*: *A. brasilense* Sp107 (*1*), *A. irakense* KA-3 (*2*), *A. irakense* KBC-1 (*3*), *A. brasilense* Sp245 (*4*), *A. brasilense* Sp7 (*5*), *A. lipoferum* 59b (*6*), and *A. lipoferum* 43 (*7*).

further investigation and optimization of electrophoresis conditions for these proteins.

We found that all motile *Azospirillum* strains under study possessed tyrosinase activity at a varying degree. For the first time, molecular forms of the enzymes were visualized in zymograms.

In their studies, Givaudan et al. [14] and Faure et al. [15] demonstrated the presence of polyphenol oxidase activity in the nonmotile forms of *A. lipoferum* isolated from the rice rhizosphere, which also correlated with the production of a dark-brown pigment, supposedly melanin. In contrast to that, no phenol oxidase activity was detected in any of the motile wild strains of *Azospirilla* studied in the present work. In our study we managed to detect tyrosinase both on the surface of and inside bacterial cells and to determine its activity in different species and strains of azospirilla. All strains used in the work were motile, which was verified by microscopy immediately before the experiments. Visual observation confirmed that bacterial cells were not pigmented.

The question of tyrosinase's functional role in azospirilla remains open and requires further investigation, although several hypotheses may be proposed. Since azospirilla are nitrogen-fixing bacteria and are in close association with plant roots, they use these enzymes to oxidize and polymerize the toxic phenolic compounds present in the rhizosphere, which increases their survival and competitiveness. Moreover, for some *Azospirillum* strains, for example, for *A. brasilense* Sp245, it was found that they colonize not only the surface of the plant root system, but are also capable of penetration inside the root. One cannot exclude that tyrosinases, together with other enzyme systems, take a part in the mechanisms of invasiveness of azospirilla by degrading of the root cell envelope and thus promoting survival and proliferation of the bacteria inside the root.

This is the first report on tyrosinase activity in motile forms of *Azospirillum brasilense, A. lipoferum,* and *A. irakense* and on determination of molecular forms corresponding to the intracellular tyrosinase in the strains under study. The ability of azospirilla to synthesize the enzymes described herein may evidence important adaptational abilities and activity of the microorganisms in the rhizosphere.

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