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EXPERIMENTAL ARTICLES

Involvement of the Lipopolysaccharides of Azospirilla in the Interaction with Wheat Seedling Roots

Yu. P. Fedonenko[1], I. V. Egorenkova, S. A. Konnova, and V. V. Ignatov

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410015 Russia

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Abstract—The present study was undertaken to comparatively investigate the attachment capacities of *Azospirillum brasilense* Sp245 and its lipopolysaccharide-defective Omegon-Km mutants KM018 and KM252, as well as their activities with respect to the alteration of the morphology of wheat seedling root hairs. The adsorption dynamics of the parent Sp245 and mutant KM252 strains of azospirilla on the seedling roots of the soft spring wheat cv. Saratovskaya 29 were similar; however, the attachment capacity of the mutant KM252 was lower than that of the parent strain throughout the incubation period (15 min to 48 h). The mutation led to a considerable decrease in the hydrophobicity of the *Azospirillum* cell surface. The lipopolysaccharides extracted from the outer membrane of *A. brasilense* Sp245 and mutant cells with hot phenol and purified by chromatographic methods were found to induce the deformation of the wheat seedling root hairs, the lipopolysaccharide of the parent strain being the most active in this respect. The role of the carbohydrate moiety of lipopolysaccharides in the interaction of *Azospirillum* cells with plants is discussed.

Key words: *Azospirillum brasilense*, adsorption, lipopolysaccharides, root hair deformation.

Investigations of the molecular mechanisms of contact interactions between *Azospirillum* cells and cereal plants are mainly concerned with the extracellular polysaccharide-containing biopolymers involved in these interactions. At the same time, little is known about the role of surface bacterial lipopolysaccharides (LPSs) in the formation of plant–microbial associations [1, 2]. The function of surface cell structures can be efficiently studied using mutants defective in the synthesis of a particular surface constituent.

Katzy *et al.* [3] derived two kanamycin-resistant mutants, KM252 and KM018, from *A. brasilense* Sp245. The double-diffusion immunoelectrophoresis of the crude LPS extract of the parent strain gave two precipitin lines, outer and inner, whereas the immunoelectrophoresis of the LPS preparations of the mutants KM252 and KM018 gave only one precipitin line, inner and outer, respectively.

Age-related structural changes in the surface of *Azospirillum* cells, as well as the removal of capsular material, alter the cell's ability to attach to wheat seedling roots [4, 5].

The aim of the present work was to study the involvement of the LPSs of azospirilla in the initial stages of their interaction with wheat roots. In particular, we compared the abilities of the parent and mutant strains of azospirilla to attach to wheat seedling roots and evaluated the ability of isolated azospirillary LPSs to alter the morphology of the wheat seedling root hairs.

MATERIALS AND METHODS

Cultivation conditions. The strain *Azospirillum brasilense* Sp245, which was isolated from the surface-sterilized wheat roots [6], was a gift from J. Döbereiner, Empresa Brasileira de Pesquisa Agropecuaria in Rio de Janeiro, Brazil. The kanamycin-resistant mutants KM252 and KM018 of this strain with the Omegon-Km insertion in their 120-MDa plasmids were derived in the Laboratory of Microbial Genetics at the Institute of Biochemistry and Physiology of Plants and Microorganisms [3].

The parent strain was cultivated in liquid synthetic medium with malate as the source of carbon and energy [7]. The mutant strains were grown in the same medium supplemented with 100 μg/ml kanamycin sulfate as the selective factor. The strains were cultivated at 33°C in Erlenmeyer flasks on a shaker to the late exponential growth phase. Cells were harvested by centrifugation at 3000 *g* for 40 min. To remove capsular material, cells were incubated in 0.15 M NaCl with 0.2% NaN_3 for 6 days. At 24-h intervals, cells were separated from the medium by centrifugation and placed in a fresh incubation medium. The removal of the capsular material was controlled by analyzing the saline solution used by double-diffusion immunoelectrophoresis with homolo-

[1]Corresponding author; e-mail: root@ibppm.saratov.su

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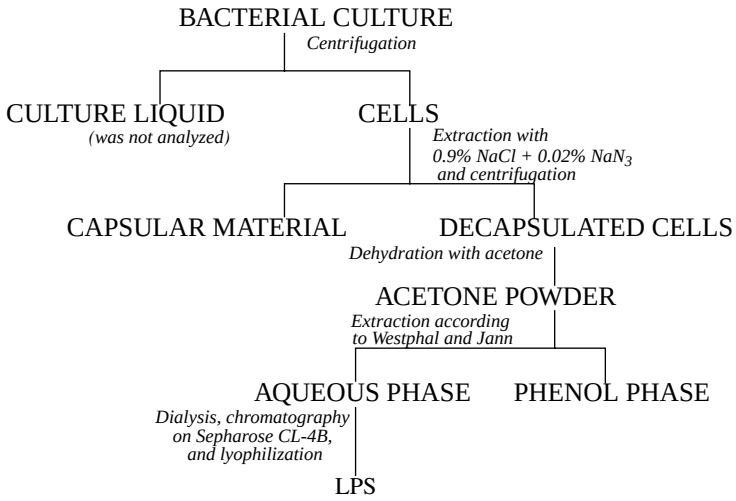

Fig. 1. Isolation and purification of lipopolysaccharide (LPS) from the outer membrane of *A. brasilense* Sp245 cells.

gous antibodies [8]. Decapsulated cells were dehydrated with acetone.

Germination of wheat seeds. Seeds of the soft spring wheat *Triticum aestivum* cv. Saratovskaya 29 (1995 crop) were obtained from the Research Agricultural Institute of Southeastern Russia in Saratov. The seeds were thoroughly washed with water, sterilized in ethanol for 30 s and in diacide for 5 min, washed repeatedly with sterile distilled water, and placed on the surface of nutrient agar in petri dishes. The dishes were incubated at 25°C in the dark for 48 h. Axenic wheat seedlings were transferred onto wet paper filters and incubated for 12 h.

Bacterial adsorption on wheat seedling roots was studied as described earlier [4].

The surface hydrophobicity of *Azospirillum* cells was evaluated by the salting-out method (the results were expressed as the minimum sulfate ammonium concentration causing cell aggregation) and by the method based on the cell partition in a two-phase system [9].

Preparation of LPSs. LPSs were extracted from the ground dry biomass with a hot phenol–water mixture [10] (the experimental protocol is shown in Fig. 1). The aqueous fraction of the extract was dialyzed through a membrane with a molecular weight cut off of 12–14 kDa first against tap water for 5 days and then against distilled water for 2 days. The dialyzed extract was concentrated with a rotary vacuum pump and fractionated by gel filtration on a (75×3.7 cm) column and then on a (45×1.8 cm) column both packed with Sepharose CL-4B (Pharmacia, Sweden). The elution buffer was 25 mM ammonium bicarbonate (pH 8.25) at a flow rate of 0.5 ml/min. The process of elution was monitored using an LKB 2142 differential refractometer (LKB, Sweden). Alternatively, elution profiles were constructed by measuring the concentration of carbohydrates in the eluate with phenol and sulfuric acid (the reaction products were measured spectrophotometrically at 490 nm [11]) or the concentration of proteins through their absorbance at 280 nm. Spectral measurements were performed using an SF-26 spectrophotometer (LOMO, Russia).

The chromatographically purified LPSs were concentrated and lyophilized. The monosugar composition of the LPSs was determined by the thin-layer chromatography of their hydrolysates on cellulose plates [7].

Effect of LPSs on the morphology of wheat seedling root hairs was studied by a modification of Fahraeus' method [12]. LPS preparations were added to liquid or semiliquid incubation medium at a concentration of 0.125 mg/ml [12]. Each experiment was performed in quadruplicate using 18–20 two-day-old wheat seedlings. The seedlings were incubated with LPS for 24 h, after which the number of deformed root hairs within the first centimeter of the zone of root differentiation was determined using a light microscope.

Light microscopy. The adsorption of *A. brasilense* cells on wheat roots and the deformation of their hairs under the action of LPSs were studied using a Biolar PI polarization–interference microscope (Poland) with a 12× eyepiece and a 10×, 20×, or 40× objective.

Experimental results were statistically processed using formulas presented in Lakin's manual [13]. Confidence intervals are given for a 95% confidence level.

RESULTS AND DISCUSSION

Adsorption experiments were carried out with the parent *A. brasilense* Sp245 strain and its mutant KM252. Cells of the mutant strain KM018 were not used in these experiments for reasons of their nonmotility, since this could considerably complicate the interpretation of experimental results. Adsorption was measured beginning with a 15-min incubation of azospirilla with roots and ending with 48-h incubation

(Fig. 2). As is evident from this figure, both parent and mutant cells exhibited similar dynamics of their adsorption on the roots: the number of attached cells increased within the first 3 h of incubation and then changed very little. However, mutant cells were characterized by a weaker attachment ability throughout the incubation period: on the average, the number of attached KM252 cells was 4–8 times lower than that of attached *A. brasilense* Sp245 cells (cf. curves *1* and *2* in Fig. 2). The statistical processing of the results of these experiments (as a whole, we conducted seven quintuplicate experiments) showed that the observed differences in the adsorption of parent and mutant cells were statistically significant.

It is known that the attachment of azospirilla to wheat roots is driven both by nonspecific interactions due to the charge and hydrophobicity of the bacterial cell surface and by specific interactions involving the surface proteins and polysaccharides of bacterial cells [14]. The evaluation of the surface hydrophobicity of parent and mutant *Azospirillum* cells by two methods showed that the hydrophobicity of the parent cells was higher than that of the mutant cells. The difference was especially pronounced by the data of the method of salt-induced cell aggregation: the parent cells aggregated at an ammonium sulfate concentration of 39%, whereas the KM252 and KM018 cells aggregated at salt concentrations of 46 and 60%, respectively. Taking into account that the hydrophobicity of azospirilla correlates with the amount of their surface proteins and the adhesiveness of cells [15], the observed decrease in the adhesiveness of mutant cells could not be attributed to only changes in the LPS composition. For this reason, we directly evaluated the activity of azospirillary LPSs with respect to the alteration of the morphology of wheat seedling root hairs.

The fact that soil bacteria can alter the morphology of root hairs has long been known, and this phenomenon has been sufficiently studied as concerned with the legume–rhyzobial symbiosis [16]. Furthermore, Okon and Kapulnik showed that the inoculation of several wheat, corn, and sorghum cultivars with certain *Azospirillum* strains led to changes in the morphology of their roots, particularly to an increase in the numbers of root hairs, branches, and lateral roots [17]. Experiments with wheat seedlings showed that the number of the tuning folk-type root deformations induced by azospirilla decreases in the order: *A. brasilense* Sp245, Sp107, Sp7, and Sp242 [18]. In the experiments of such type, it is difficult to ascertain what particular bacterial substance(s) is/are responsible for morphological changes in the plant root system otherwise than to obtain and investigate these substances in individual form. It is presently known that root deformation is caused by indolylacetic acid, a growth-promoting hormone produced by azospirilla [19], and by polysaccharide-containing complexes, which are localized in the capsular material of these bacteria and liberated into the environment in the course of their growth [20]. The

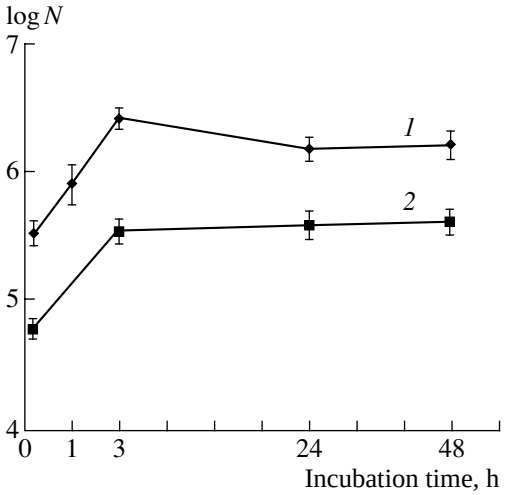

Fig. 2. Adsorption of (*1*) *A. brasilense* Sp245 and (*2*) *A. brasilense* KM252 cells on the wheat cv. Saratovskaya 29 seedling roots. *N* is the number of bacterial cells adsorbed on 1 cm of the root length; the initial concentration of bacterial cells in the suspension was 6.5×10^8 cells/ml.

effect of azospirillary LPSs on the cereal root system is studied poorly.

To fill this gap and investigate the root-deforming activity of the LPSs, the parent *A. brasilense* Sp245 strain and its mutants KM252 and KM018 were grown to the late exponential phase (the growth rates of these strains were nearly the same). During growth, cells were regularly tested by immunodiffusion with antibodies against the *Azospirillum* cells treated with glutaraldehyde. After decapsulation, the cells were dehydrated with acetone and extracted by the method of Westphal and Jann [10] (the preparation of LPSs is schematically shown in Fig. 1). LPSs were localized in the aqueous fraction, indicating that they are fairly hydrophilic. After the removal of phenol by dialysis, the aqueous fraction was subjected to gel filtration on Sepharose CL-4B. This procedure yielded homogeneous LPS preparations eluted in the void volumes of the columns and allowed partially degraded LPS molecules and low-molecular-weight impurities to be removed.

Chemical composition of the lipopolysaccharides of *A. brasilense* Sp245 (LPS1) and its mutants KM252 (LPS2) and KM018 (LPS3)

Lipopolysaccharide	Constituents, wt %			
	carbohydrates	proteins	nucleic acids	KDO
LPS1	65.8 ± 2.1	0.7 ± 0.06	0.004	0.6 ± 0.02
LPS2	82.8 ± 2.5	1.3 ± 0.10	0.002	0.4 ± 0.02
LPS3	67.9 ± 1.8	0.9 ± 0.05	0.003	0.8 ± 0.05

Note: KDO, 2-keto-3-deoxyoctonate.

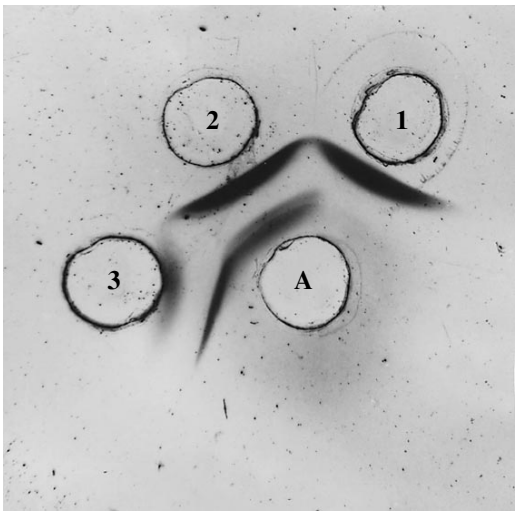

Fig. 3. Double-diffusion immunoelectrophoresis of the LPSs of (1) *A. brasilense* KM018, (2) Sp245, and (3) KM252 with (A) antibodies against the glutaraldehyde-treated *A. brasilense* Sp245 cells.

The yield of freeze-dried preparations of LPS from the outer membrane of *A. brasilense* Sp245, KM252, and KM018 cells (they were designated LPS1, LPS2, and LPS3, respectively) was from 1.8 to 2.5% of the dry cell biomass. Low contents of proteins and nucleic acids in the LPS preparations were indicative of a high purity of these preparations (see table). Thin-layer chromatography showed that the carbohydrate moieties of LPS molecules contained rhamnose, glucose, and glucosamine.

Analysis of LPS1 by double-diffusion immunoelectrophoresis with antibodies isolated from the blood serum of a rabbit immunized with the glutaraldehyde-treated cells of strain Sp245 showed the presence of two precipitin lines, outer and inner (Fig. 3). Similar analysis of LPS2 and LPS3 showed that they form only one precipitin line, either inner (LPS2) or outer (LPS3). Therefore, the chromatographically purified LPS preparations behaved, in the immunodiffusion test, in a way similar to crude LPS preparations [3].

Microscopic observations of the effect of LPSs on the morphology of the root hairs of 2-day-old wheat seedlings were carried out by adding LPS preparations to the mineral medium in which the seedlings were grown. The concentration of added LPSs (0.125 μg/ml) was chosen to be equal to the working concentration of the capsular polysaccharide-containing complexes of azospirilla [12]. Observations showed that LPSs caused the deformation of wheat root hairs in the form of symmetric and asymmetric branching folks, twistings, swellings, etc. Figure 4 presents the micrographs of the most typical LPS-induced morphological alterations: branching (panel a) and asymmetric folk (panel b).

Quantitative analysis showed that all three LPS preparations caused the deformation of the wheat seedling root hairs (Fig. 5); however, the effects of LPS2 and LPS3 were weaker than that of LPS1: the latter increased the number of deformed hairs by 133% as compared with the control (the number of root deformations observed in the absence of LPS was taken to be 100%), whereas LPS2 and LPS3 increased the number of deformed root hairs only by 44 and 26%, respec-

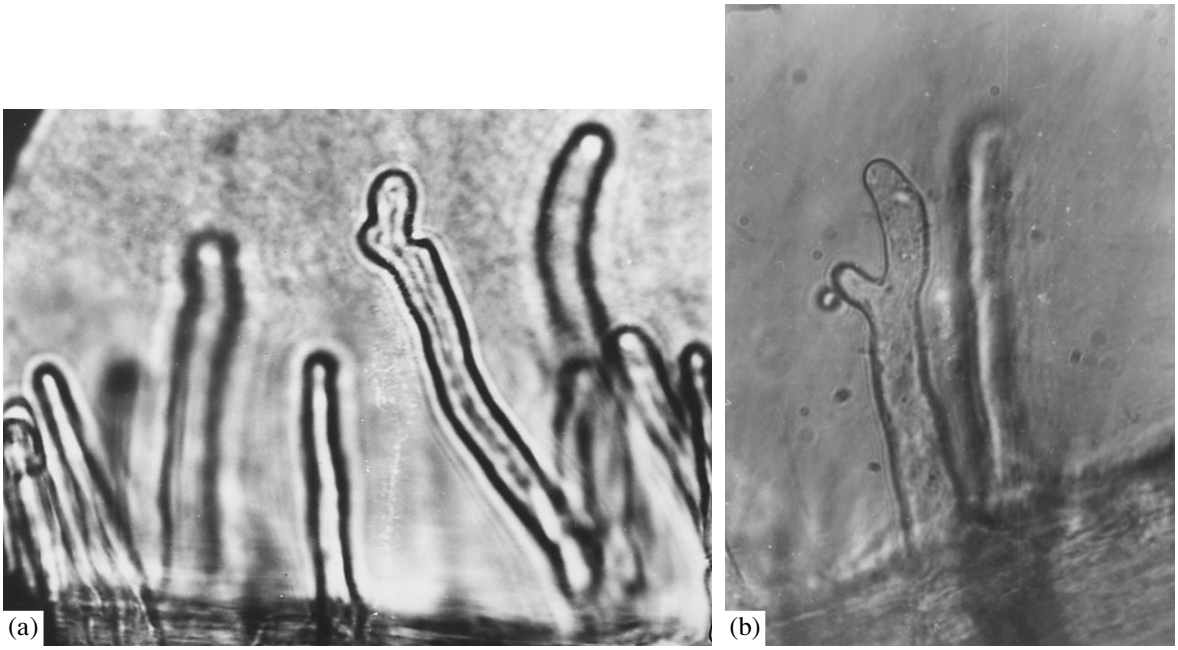

Fig. 4. Two types of the deformed root hairs of 3-day-old wheat cv. Saratovskaya 29 seedlings induced by the surface LPS of *A. brasilense* Sp245: (a) hair branching and (b) asymmetric folk. Magnification, 670×.

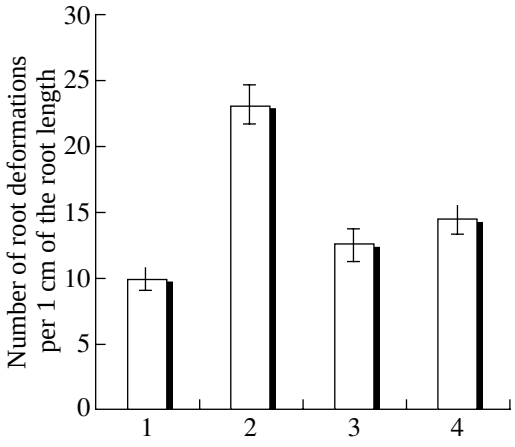

Fig. 5. Effect of the LPS of (2) *A. brasilense* Sp245, (3) KM252, and (4) KM018 on the deformation of the root hairs of 2-day-old wheat cv. Saratovskaya 29 seedlings. Bar 1 represents the number of root deformations in the absence of added LPS.

tively. The decrease in the root-deforming activity of LPS2 and LPS3 can be related to the absence of one of the two carbohydrate subunits in these lipopolysaccharides, as indicated by the double-diffusion immunoelectrophoresis test (see above). The mechanism of the root deformation is as yet poorly understood, although it has been found that (1) there is a correlation between the degree of the root deformation and the intensity of the rhizosphere colonization by soil bacteria and (2) lectin–carbohydrate interactions are involved in the root deformation induced by extracellular polysaccharide-containing complexes [4]. Of interest is the fact that the LPS built in the bacterial membrane does not interact with the wheat germ agglutinin [5, 21], but it acquires such ability after having been isolated from the membrane (unpublished data). The affinity of LPS for wheat lectin is weaker than that of extracellular polysaccharide-containing complexes [12] and is nonspecific, as judged from the absence of the inhibition of the lectin–LPS interactions by monosugars present in the LPS.

The results of experiments with the parent *Azospirillum* cells and their mutants defective in the synthesis of LPS and with the LPS preparations derived from such cells allow the suggestion to be made that the Omegon insertion in the 120-MDa plasmid alters the LPS of the outer membrane of *A. brasilense* Sp245. As a result, the mutant LPS2 and LPS3 have lowered activity with respect to wheat seedling roots. The data presented indicate that the membrane and capsular LPSs of soil bacteria are involved in the bacterial colonization of plant roots.

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