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EXPERIMENTAL ARTICLES

Detection of a Sheath on *Azospirillum brasilense* Polar Flagellum

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Abstract—The presence of a polysaccharide sheath on the surface of the polar flagellum of *Azospirillum brasilense* was revealed by immunoelectron microscopy and immunodiffusion analysis with strain-specific antibodies to lipopolysaccharides (LPS). The antigenic identity of *A. brasilense* Sp245 sheath material and one of the two O-specific polysaccharides of its somatic LPS was demonstrated. The screening effect of the sheath in respect to flagellin was determined by agglutination tests and by the inhibition of azospirilla motility in liquid and semisolid agarized media caused by strain-specific antibodies to LPS; no pronounced effect of genus-specific antibodies to flagellin was observed.

Key words: Azospirillum brasilense, polar flagellum, sheath, motility, lipopolysaccharides.

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The existence of a protein or polysaccharide sheath on the surface of a polar bacterial flagellum in *Burkholderia, Caulobacter, Helicobacter, Vibrio*, etc. has been confirmed in a number of publications [1–4]. The role of lipopolysaccharides (LPS) in formation of the sheath surrounding a polar flagellum was confirmed in some cases [1, 5]. Experiments on *Helicobacter pylori* revealed the structural differences between the flagellar sheath and the outer membrane; this structure was therefore not a simple extension of the outer bacterial membrane. The flagellar sheath of the bacteria investigated interacted with only two types of LPS-specific monoclonal antibodies (Ab), and not with the antibodies to other surface macromolecules [1].

The sheath of the polar flagellum is very important for the interaction with bacterial partners and with the environment. For example, some authors suggest an understanding of the *Vibrio cholerae* polar flagellum not as an adhesin proper (participating in colonization processes), but rather as a carrier for the LPS acting as adhesin [5]. Experiments with *V. anguillarum* revealed that in these bacteria the lipopolysaccharide sheath acts as a virulence factor [6]. All the bacteria mentioned above with sheathed polar flagella are pathogens or opportunistic pathogens of humans, animals [1, 3, 6], or plants [2].

Azospirillum brasilense, bacteria stimulating plant growth [7], were the object of the present work. When grown in liquid media, azospirilla have one polar flagellum; it is responsible for motility and chemotaxis required for efficient plant colonization [8]. It has been stated that the polar flagellum of azospirilla directly participates in bacterial adsorption on plant roots [9, 10]. However, the degree of azospirilla adsorption did not decrease after treatment of the roots with the preparations of isolated bacterial polar flagella or of bacteria with the antiserum to flagellin [10].

This paper presents the indication that the polar flagellum of dinitrogen-fixing soil bacteria *Azospirillum brasilense* is covered with a (lipo)polysaccharide sheath, which isolates flagellin from the environment.

MATERIALS AND METHODS

The following strains were used in this work: *Azospirillum brasilense* Sp245, Sp107 [11], and Sp7 [12] (wild type); *A. brasilense* Sp245.5 (a spontaneous mutant of strain Sp245 with a cardinally changed LPS [13]); *A. brasilense* KM018 and KM252 (Omegon-Km mutants of strain Sp245 deficient in the synthesis of O-specific polysaccharides, O-PS [14]), and *Escherichia coli* K-12 [15]. *A. brasilense* strains were grown at 30°C in a liquid synthetic malate medium [16] supplemented with 1 g/l of NH_4Cl (MSM) until the late exponential phase. *E. coli* K-12 was grown on LB medium at 37°C [17].

LPS were extracted with EDTA [18] by a modified method. Bacterial cells were washed with phosphate buffer saline (PBS) and centrifuged. LPS were extracted for 30 min at room temperature; the extraction buffer (pH 8.5) contained Tris–HCl, 0.1 M; EDTA, 10 mM; PMSF (phenylmethylsulfonyl fluoride),

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0.1 mM; and Triton X-100, 1%. The EDTA concentration was 0.05 mM per 1 g of wet biomass. The cells were removed from the extract by centrifugation.

To obtain the preparations of the polar flagellum, the washed cell precipitate from 1000 ml of an 18-h liquid culture was resuspended in 40 ml of PBS. The suspension was homogenized for 90 s. To precipitate the cells, the suspension was centrifuged twice for 15 min at 3000 *g*. The supernatant was treated by ultracentrifugation for 1.0 h at 100000 *g*; the pellet was dissolved in 1.0 ml of distilled water. A sucrose density gradient was used in some instances of ultracentrifugation of the flagellum preparation.

To obtain Ab to flagellin, the subunits of the polar flagellum were separated by electrophoresis in PAG and isolated from the gel by special diffusion [19]. After electrophoresis, the flagellin subunits were visualized by staining with Coomassie R-250 water suspension (without fixation); the corresponding bands were then excised from the gel. The gel was homogenized in a mortar and the protein was eluted by diffusion into a solution containing 50 mM Tris–HCl (pH 8.0), 5% 2-mercaptoethanol, and 2% sodium dodecyl sulfate (SDS). The suspension was dialyzed against distilled water and used for rabbit immunization. The antigen was mixed with Freund's adjuvant (1 : 1) and injected into the popliteal lymph nodes. The procedure was carried out four times with two-week intervals. The complete Freund's adjuvant was used for the first immunization, the incomplete adjuvant for the subsequent ones.

Ab to LPS were obtained as described previously [20].

SDS electrophoresis was carried out in 12.5% PAG. For protein visualization, the gels were stained with Coomassie R-250.

Electrical transfer of the isolated components to nitrocellulose filters was carried out in the course of immunoblotting. For antigen immunodetection, rabbit antibodies to flagellin (75 μg/ml) were used as primary antibodies. Antirabbit goat antibodies (Sigma) labeled with horseradish peroxidase were used as secondary antibodies [21].

Double immunodiffusion was carried out in 1% agarose gels according to the standard procedure. The gels were stained with Coomassie R-250.

For the agglutination reaction, the cells of an 18-h culture were washed by centrifugation and resuspended in PBS to the final A_{660} = 1–1.2 (l = 1 cm). Cell samples (50 μl) were introduced in 96-well plates for immunological reactions on top of equal volumes of antiflagellin and anti-LPS antibodies titrated in PBS (initial concentrations, 2000 and 100 μg/ml, respectively). Precipitation of an irregular shape at the bottom of a well was an indication of the positive reaction.

In order to study the cell motility in liquid cultures, the cells were prepared as described above, resuspended to A_{660} = 0.5, mixed with Ab (1 : 1; the final Ab concentrations are given in the Results and Discussion section), and examined as hanging drops or wet mounts. The specimens were investigated under a Jenaval microscope (Carl Zeiss, Jena, GDR) under phase contrast; a wide-range objective lens was used. A DCR-TRV900E video camera (Sony, Japan) was used for video recording. Motility of all the cells within a microscope field was assayed; average swimming rates were determined for 50–150 randomly chosen single motile cells. The video images were analyzed with a software package by V.A. Krestinenko (Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences); it enables monitoring Cartesian coordinates of a cell in the frame-by-frame mode. Confidence intervals were determined for the 95% level.

Collective spreading of bacterial cells was studied in semisolid MSM with 0.35% agar supplemented with antiflagellin and anti-LPS antibodies (final concentrations, 10 μg/ml).

Electron microscopic analysis was carried out on a BS-500 transmission electron microscope (Tesla, Czechoslovakia) at 80 kV accelerating voltage. The cells of 18-h cultures of *A. brasilense* Sp245 and Sp7 were resuspended in PBS to A_{660} = 0.5 and used to investigate the interaction with anti-LPS Ab. A conjugate of colloidal gold with protein A was used for antibody labeling.

Absorption spectra of the products of the phenol–sulfuric acid reaction of carbohydrate-containing preparations were determined according to the standard procedure [22].

RESULTS AND DISCUSSION

Interaction of anti-LPS antibodies with the surface of *A. brasilense* polar flagella. Electron microscopic analysis of the interaction between anti-LPS antibodies and the cells of *A. brasilense* Sp7 and Sp245 was performed. Conjugates of colloidal gold with protein A with an average particle diameter of 20 nm were used for antibody labeling. Electron microscopy revealed the presence of lipopolysaccharide determinants on the polar flagella of bacteria under investigation. The distribution of these determinants suggested the existence of a sheath covering the polar flagellum of azospirilla strains. The interaction of strain-specific Ab to LPS with the *A. brasilense* Sp245 cell surface is presented in Fig. 1. The particles of colloidal gold revealed the presence of Ab both on the cell proper and on the polar flagellum; the anti-LPS Ab interacted with the sheath substance rather than with the surface of the flagellum. The microphotograph illustrates the correlation between the number of attached colloidal gold particles and the volume of the flagellar sheath material.

Reaction of comparative immunodiffusion with the antibodies to the lipopolysaccharides of strain Sp245 revealed the formation of a common precipitation band

Fig. 1. Interaction of *A. brasilense* Sp245 cells and Ab with the LPS of this strain labeled with conjugates of protein A with colloidal gold. Scale bar, 200 nm.

by the native flagellum preparation (not separated from the sheath) and O-PS1, one of the two O-specific polysaccharides of the strain's LPS (Fig. 2a) [14]. The second characteristic O-PS of this strain was also present in the flagellum preparation, although the corresponding precipitation line was considerably weaker.

Comparison of light absorption by the products of a phenol–sulfuric acid reaction of the LPS preparations and of *A. brasilense* Sp245 sheathed polar flagellum. The colorimetric method based on the yellow to orange staining of mono-, oligo-, and polysaccharides and their derivatives in the presence of phenol and concentrated sulfuric acid was used to compare the overall monosaccharide composition of these carbohydrate-containing polymers [22]. For the LPS preparation of strain Sp245 and the preparation of the native flagella of this strain including polysaccharide sheaths, absorption spectra (wavelength range from 400 to 520 nm) of phenol–sulfuric acid reaction products were compared. The similarity between the spectra of the LPS preparations and of the native polar flagellum from strain Sp245 compared to the spectra of the LPS preparations from *A. brasilense* Sp7 and *E. coli* K-12 (Fig. 3) suggests the similarity of the monosaccharide composition of the first two structures.

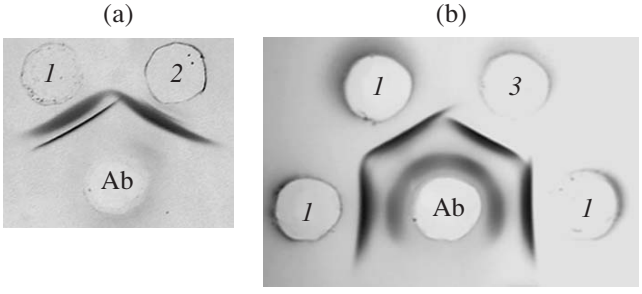

Fig. 2. Results of immunodiffusion analysis with Ab to the LPS of strain Sp245 with Sp245 LPS preparations (*1*), a preparation of Sp245 native flagellum (*2*), and a preparation of Sp107 LPS (3).

Since we have previously confirmed the identity of the antigenic determinants of the capsular polysaccharides, exopolysaccharides, and lipopolysaccharides of azospirilla [23], detailed structural analysis of the sheath material of the azospirilla polar flagellum is required for unequivocal statements concerning its chemical nature.

Production of antibodies to the sheath-free flagellin of the polar flagellum of *A. brasilense* Sp7 type strain. The flagellar sheath is strongly bound to the flagellum; even ultracentrifugation of the polar flagellum preparation in sucrose density gradient did not separate the sheath. An electrophoretically purified flagellin preparation was therefore eluted from the gel and used to obtain the antibodies to the sheath-free flagellin of *A. brasilense* Sp7 type strain. The specificity of the antibodies thus produced was assayed with immunoblotting. Ab to the flagellin of the type strain exhibited cross-reaction with the flagellin of strain Sp245 (Fig. 4); this finding indicates that the *A. brasilense* H antigen has no pronounced strain specificity. Thus, the Ab thus obtained could be used to reveal flagellin in the azospirilla strains under investigation.

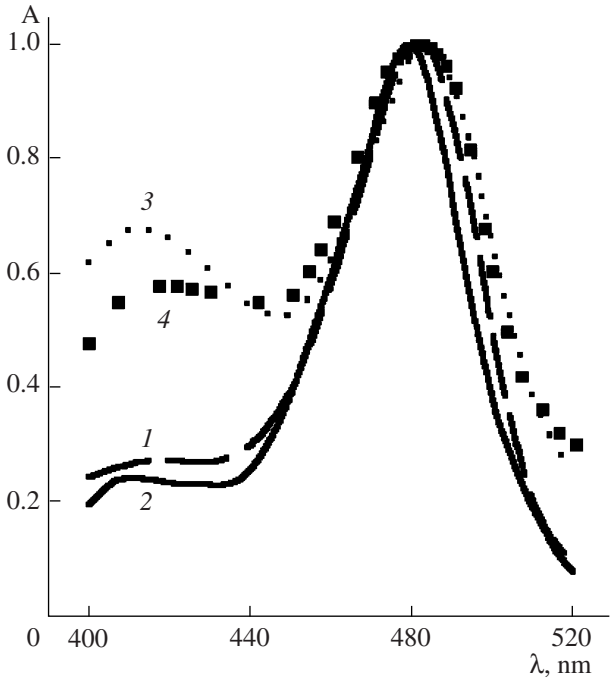

Fig. 3. Spectra of light absorption by the products of the phenol–sulfuric acid reaction of *A. brasilense* Sp245 LPS preparation (*1*) and of the preparation of the native polar flagellum of this strain (*2*); the spectra of LPS from *A. brasilense* Sp7 LPS (*3*) and *E. coli* K-12 (*4*) are shown for comparison.

Interaction between the antibodies to flagellin and the determinants of native flagella. In order to confirm the presence of sheaths around the flagellin of the azospirilla polar flagella, an agglutination reaction of Ab was carried out with both *A. brasilense* Sp245 cells and the mutant cells of this strain with an altered LPS structure. The results presented in Table 1 indicate that the flagellin determinants of the native flagellum are not easily available for antiflagellin antibodies. It should be mentioned that Ab to flagellin were used in significantly higher concentrations (their concentrations in the first wells of the plate were 2000 and 100 μg/ml, respectively). A weakly positive agglutination reaction with antiflagellin Ab was detected with Sp245 (wild type LPS, well 1), Sp245.5 (radically changed LPS, well 2), and KM252 (O-PS1$^-$, well 1). The cells of the mutant strain KM018, with only O-PS1 among their LPS, exhibited no agglutination reaction in the presence of antiflagellin Ab. It shoud be noticed that a sheath covering the flagellin was found in the mutant strain KM252 deficient in O-PS1 synthesis. According to our preliminary data [14], O-PS1 is an intermediate product in the synthesis of the second polysaccharide; in O-PS1$^-$ mutants of strain Sp245 the flagellar sheath probably consists of the modified O-PS2.

Motility of *A. brasilense* in the presence of anti-LPS and antiflagellin antibodies. In order to investigate motility of different strains of azospirilla in the presence of strain-specific antibodies to LPS and the antibodies to the flagellin of the type strain Sp7, two series of experiments were performed. In the first series, the interaction of these antibodies with the polar flagellum of azospirilla was assessed by the motility of all the cells in the microscope field and by the average swimming speed of randomly chosen motile single cells; cell aggregates resulting from the interaction between the antibodies and corpuscular antigens were not considered. In the second series, the character of bacterial spreading was assessed on semisolid media (0.35% agar) supplemented with the antibodies to flagellin and LPS.

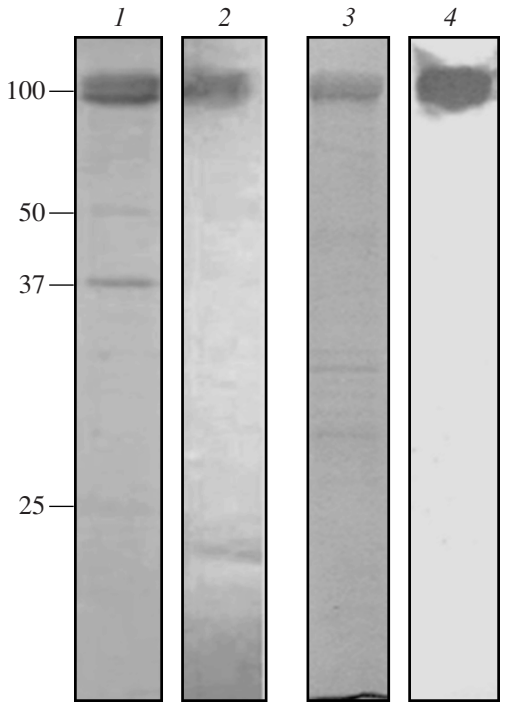

Fig. 4. PAG electrophoresis of the preparations of polar flagella from *A. brasilense* Sp7 (*1*) and SP245 (*3*) and of immunoblotting of these preparations with polyclonal antibodies to *A. brasilense* Sp7 flagellin subunits isolated from the gel (*2* and *4*, respectively).

It can be seen from the diagram of dependence of the ratio of *A. brasilense* Sp7 motile cells and their average swimming speed on the concentration of strain-specific Ab to LPS (Fig. 5) that elevated concen-

Table 1. Agglutination reaction of *A. brasilense* Sp245 (wild type cells and the mutants with an altered LPS structure) with anti-LPS and antiflagellin antibodies

Strain	Characterization	Ab/LPS$_{Sp245}$ (Ab concentration in the first well is 100 μg/ml)	Ab/flagellin$_{Sp7}$ (Ab concentration in the first well is 2000 μg/ml)
Sp245	Wild type	+ + + + + + + – – –	+ – – – – – – – – –
KM018	Sp245 Omegon mutant (only O-PS1 is present in the LPS) [14]	+ + + – – – – – – –	– – – – – – – – – –
KM252	Sp245 Omegon mutant (only O-PS2 is present in the LPS) [14]	+ + + + + + – – – –	+ – – – – – – – – –
Sp245.5	Sp245 Spontaneous mutant (LPS is radically changed) [13]	– – – – – – – – – –	+ + – – – – – – – –

"+" Stands for precipitation in the corresponding well; "–", no precipitation.

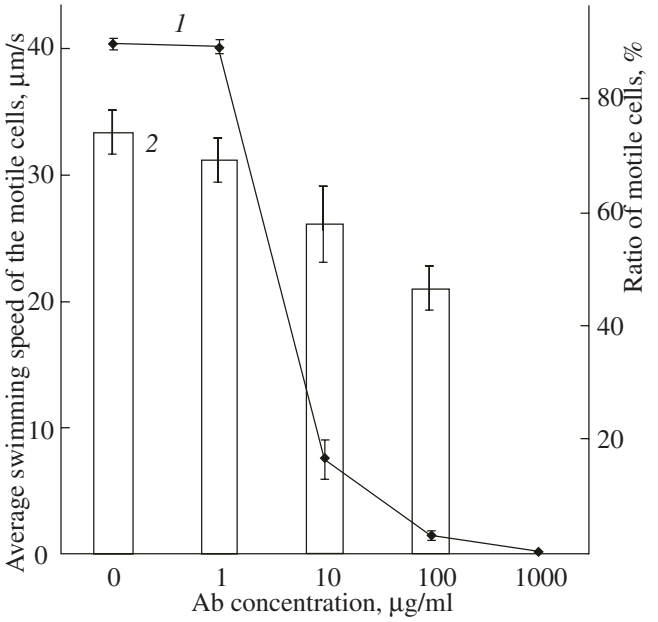

Fig. 5. The ratio of *A. brasilense* Sp7 motile cells and their average swimming speed depending on the concentration of the Ab to LPS of this strain.

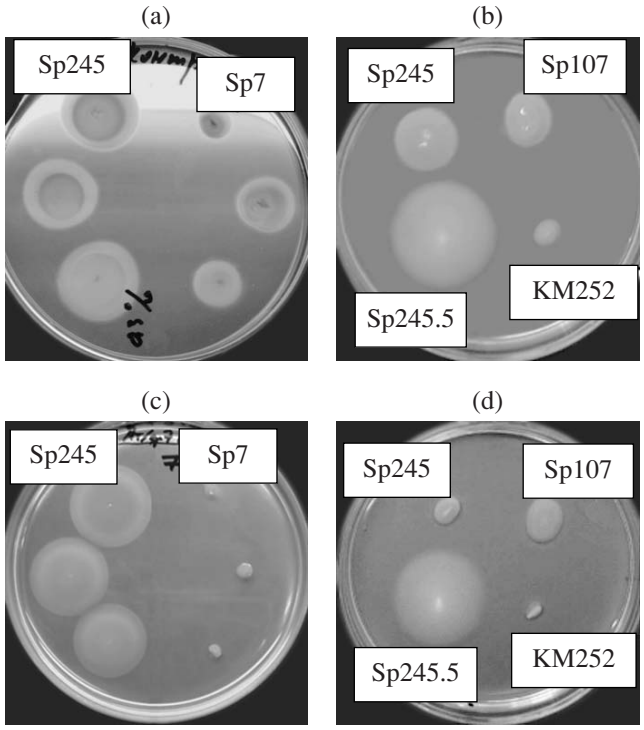

Fig. 6. Specific inhibition of *A. brasilense* motility in semisolid media in the presence of Ab to LPS (10 μg/ml): MSM without Ab (a, b); MSM with the Ab to the LPS of strain Sp7 (c); and MSM the Ab to the LPS of strain Sp245 (d). Bacteria were point-inoculated and incubated at 28°C for 72 h.

trations of these antibodies resulted in complete arrest of bacterial movement. The decreased swimming speed in the presence of Ab, as well as visually observed abrupt stopping of *A. brasilense* free cells or changes from forward motion to tumbling suggest that bacterial motility was suppressed by the interaction of antibodies with bacterial polar flagella. Neither the Ab to the flagellin of this strain, nor the Ab to the LPS of other strains had this effect (Table 1).

Unlike the antibodies to flagellin, the antibodies to LPS significantly decreased the rate of bacterial spreading in semisolid media. When strain-specific anti-LPS Ab were added into the media, bacterial colonies formed only on the agar surface; the spreading within semisolid media was completely blocked (Fig. 6c). The antibodies to LPS added to the medium probably interacted with the sheath material and created steric obstacles to the rotation of the polar flagellum. Since bacterial spreading in semisolid media was additionally retarded by the immune aggregates formed along the front line, the inhibitory effect of anti-LPS Ab was more pronounced in semisolid media.

The test based on motility inhibition in the presence of strain-specific antibodies to LPS may be useful for selection of motile mutants with completely altered LPS or as a rapid serological test (Fig. 6). Fig. 6d illustrates the character of distribution of strain Sp245 mutants with an altered LPS structure in the presence of Ab to the wild type LPS. The mutant Sp245.5 with completely altered LPS retained the dimensions of its swarming in the presence of these Ab. These results correlated with the results of agglutination reaction (Table 1). Addition of antiflagellin Ab did not change the spreading pattern of the strains tested (data not shown). The comparative results on motility of two closely related *A. brasilense* strains, Sp245 and Sp107, are also presented in Fig. 6d. Immunodiffusion tests revealed only minor antigenic differences, i.e., a small spur at the confluence of precipitation bands (Fig. 2b). The presence of this spur indicated the absence in the O-PS1 of strain Sp107 of certain antigenic determinants present in the O-PS1 of strain Sp245. Ab to the LPS of strain Sp245 in the concentrations inhibiting the motility of this strain therefore had only an insignificant effect on Sp107 motility in liquid and semisolid media (Table 2, Fig. 6). The inhibitory effect resulted from an increase in the concentration of Ab to strain Sp245 LPS; the amount of the antibodies to the determinants common to strains Sp245 and Sp107 increased in this case. The comparative results on strain motility unequivocally demonstrated the major role of O-PS1 in the formation of flagellar sheaths in strain Sp245.

The results obtained in the present work suggest the conclusion that in *A. brasilense* flagellin monomers are covered with a (lipo)polysaccharide sheath with the antigenic determinants identical to those of somatic LPS. Together with the known description of the role of the polar flagellum for the initial stages of plant–microbial interactions [9, 10], detection of the flagellin

Table 2. Effect of the antibodies of different specificity* on the ratio of *A. brasilense* motile cells and on their average swimming speed

Strain	Ratio of motile cells, %				Average swimming speed, μm/s			
	Control**	Ab/LPS_{Sp7}	Ab/LPS_{Sp245}	Ab/$flagellin_{Sp7}$	Control**	Ab/LPS_{Sp7}	Ab/LPS_{Sp245}	Ab/$flagellin_{Sp7}$
Sp7	85.5 ± 2.2	0	84.0 ± 3.3	62.7 ± 2.7	36.7 ± 2.8	0	36.0 ± 3.7	28.5 ± 1.0
Sp245	85.8 ± 3.8	84.0 ± 3.3	0	82.8 ± 2.7	29.9 ± 3.5	32.7 ± 1.8	0	32.3 ± 2.9
Sp107	84.0 ± 2.8	81.2 ± 2.9	63.8 ± 4.0	77.8 ± 2.6	31.3 ± 5.2	31.9 ± 3.2	26.3 ± 1.5	31.6 ± 2.3

Notes: * Concentration of all the antibodies was 1000 μg/ml.
** Bacterial suspensions without antibodies were used as controls.

sheaths suggests that the flagellar surface polysaccharides of the azospirilla flagellum may be considered among the dominant substances determining absorption of these bacteria on plant roots.

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