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EXPERIMENTAL ARTICLES

Wheat Root Colonization by *Azospirillum brasilense* Strains with Different Motility

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Abstract—Migration of associative bacteria *Azospirillum brasilense* in semisolid media is performed mainly by swarming (Swa^+ phenotype), which depends on the flagellar functioning and intercellular contacts. Non-swarming mutants of *A. brasilense* Sp245 lacking a polar flagellum migrate in semisolid media with microcolony formation using a unrevealed mechanism (Gri^+ phenotype). The study of wheat root colonization dynamics demonstrated that *A. brasilense* Sp245 Gri^+ mutants exhibited lower capacity for wheat root adsorption. However, after "anchoring" has occurred, both *A. brasilense* Sp245 and its Swa^-Gri^+ mutants colonized the growing roots with virtually the same efficiency. All strains under study formed microcolonies on the surface of roots, stimulated root branching, and exhibited changes in the composition of protein antigens exposed on the bacterial cell surface. Indirect evidence was obtained for enhanced production of genus-specific protein antigens in the process of *A. brasilense* Sp245 adaptation to growth on plant roots.

Key words: Azospirillum brasilense, motility, plant colonization, antigens, immunofluorescence microscopy, enzyme-linked immunoassay

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Bacteria *Azospirillum brasilense* exhibit a number of social motility mechanisms potentially important for the formation of associations with plants. In liquid media, azospirilla swim by means of a single polar flagellum (Fla). Through colonization of semisolid media, the bacteria migrate in fluxes of swarming bacteria (Swa^+ phenotype) using Fla together with numerous lateral flagella (Laf) [1, 2]. *A. brasilense* cells may migrate in semisolid media with formation of granular inclusions (microcolonies) (Gri^+ phenotype) [3–5]. Probably, Gri^+ migration depends upon polar bundle-forming pili found sometimes on *A. brasilense* cells instead of the polar flagellum [3]. In mutants with impaired production or functioning of the polar flagellum, the Gri^+ phenotype is stable [2, 3, 5] and their derivatives with restored production of the polar flagellum are capable of swarming [5]. Earlier, we demonstrated that wheat root exudates stimulate swarming in *A. brasilense* Sp245 [5], while the presence of wheat germ agglutinin in the cultivation medium facilitates the transition of part of the Sp245 population to microcolonial spreading [4].

The ability of azospirilla to migrate toward plant roots in soil and their sensitive chemotaxis system "tuned" to recognize certain components of the exudates of their preferential partners facilitate plant colonization by the bacteria [6–8]. Specific features of the process of *A. brasilense* biofilm formation on the surface of plant roots are poorly studied. Some data were obtained on the initial stages of root system colonization by azospirilla, that is, cell adsorption and anchoring. Polysaccharides, proteins with lectin activity, and the polar flagellum were found to be responsible for the first stages of bacterial interaction with root surface [9–14]. However, the mechanisms of migration allowing *A. brasilense* cells to form biofilms upon anchoring on the root surface and relative contributions of various cell surface polymers of *A. brasilense* into the process are not known.

The aim of the present work was comparative analysis of root colonization dynamics and changes in cell-surface antigen determinants *in planta* in *A. brasilense* Sp245 and its original social motility mutants.

MATERIALS AND METHODS

Strains and cultivation conditions. In this work, *A. brasilense* strain Sp245 isolated form wheat roots [15], as well as its Swa^- Gri^+ mutants (Km_R) Fla^- SK048, Fla^- Laf^- SK051, and Fla^- leaky Laf^- SK454 [2, 3], were used. The mutants contain the insertion of an artificial transposon Omegon-Km (SK048, SK454) or mutagenesis vector pJFF350 (SK051) in the chromosome (SK454) or in the plasmids of 85 MDa (SK051) and 120 MDa (SK048) [2, 16].

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The bacteria were grown in malate–salt medium (MSM) [17] and Luria–Bertani (LB) medium [18] at 30°C. Kanamycin (Km) was added to the medium when necessary at a final concentration of 50 μg/ml.

Plant inoculation with the bacteria. Soft spring wheat seeds of Saratov 29 cultivar (Agricultural Research Institute of the Southeast Region, Russian Academy of Agricultural Sciences, Saratov) were sterilized and sprouted for 3 days as described in [9]. Plant inoculation was performed by incubating the seedlings at constant shaking (25 rpm) in a bacterial suspension (A_{590} = 0.5) in 50 mM phosphate-buffered saline (PBS, pH 7.2). To prepare the suspensions, 18-h bacterial cultures grown on MSM and washed with PBS were used. After 3 h, wheat seedlings were washed once with sterile PBS and placed on top of the liquid layer in tubes with 10 ml of the medium containing the following (g/l): KH_2PO_4, 4; $CaCl_2$, 1.25; H_3BO_3, 0.0016; $CuSO_4 \cdot 5H_2O$, 0.028; $MgSO_4$, 0.09; $Na_2MoO_4 \cdot 2H_2O$, 0.0025; KI, 0.008; $ZnSO_4$, 0.015; $FeSO_4 \cdot 7H_2O$, 0.028; and ethylenediaminetetraacetate, disodium salt, 0.037 (pH 6.0). The plants were grown for 7 days at 22°C under natural light conditions. Bacterial cell distribution over the roots was determined with a Jenaval phase contrast microscope and a DMLB fluorescence microscope (Leica, Germany).

Bacterial counts on wheat seedling roots. Weighed amounts of 3- and 10-day seedling roots washed under sterile conditions were homogenized. In homogenates, the number of colony-forming units (CFUs) was determined by plating of serial dilutions on solid MSM. The number of bacteria collected from roots was calculated per plant. In parallel, root homogenates were used in an enzyme-linked immunoassay (ELISA) (see below). To control the presence of foreign microflora, dilutions of root homogenates were also plated on LB without antibiotics or with kanamycin. The latter control was meant to detect microflora resistant to kanamycin similar to the mutants. Root samples from which colonies of atypical morphology were retrieved upon plating on rich medium were rejected.

Antibodies used in the work. Strain-specific antibodies (Ab) against polysaccharide antigens of *A. brasilense* strains Sp245 (Ab1) and Sp7 (Ab3) were obtained as previously described [19]. To reveal genus- and species-specific surface antigen proteins, polyclonal antibodies against the intact *A. brasilense* Sp7 cells (Ab2) were used. These antibodies recognize the pool of Sp7 antigens and interact with Sp245 protein antigens, but not with the polysaccharide determinants [19]. The concentrations of Ab1–Ab3 used at all cases were 50 μg/ml. The secondary antibodies were goat antirabbit antibodies conjugated with horse radish peroxidase (Sigma) and donkey antirabbit antibodies conjugated with fluorescein isothiocyanate (DARAb-FITC) (Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medicinal Sciences, Moscow).

Indirect solid-phase enzyme-linked immunoassay. Sterile (control) or bacteria-colonized root homogenates were diluted 80 times in PBS (optimal dilutions of the samples were found experimentally), introduced by 50 μl into the wells of 96-well polystyrene plates and incubated for 30 min on a vibroshaker at room temperature and then for 18 h at 4°C without agitation. Further analysis was performed according to the recommendations proposed earlier [20]. Bacterial polysaccharide and protein antigens were revealed using Ab1 and Ab2, respectively. Goat antirabbit Ab conjugated with horse radish peroxidase were used at 1 μg/ml. Peroxidase activity was detected in 0.1 M sodium citrate buffer (pH 4.5) containing 0.03% *o*-phenylenediamine and 0.02% hydrogen peroxide. The optical density (A_{490}) of the samples was measured on an AIF-Ts-01S enzyme-linked immunoassay analyzer (ILIP, St. Petersburg). ELISA of bacterial cultures was performed in a similar manner. In the latter case, bacterial suspensions washed twice with PBS (A_{590} = 0.4) were diluted 100-fold and introduced into plate wells (50 μl per well).

Fluorescence microscopy of wheat seedling roots. The seedlings were washed once with PBS (5 min) and roots were cut into 10–15-mm long pieces. After 10-min blocking of nonspecific binding sites with 0.05% polyethylene glycol 2000, Ab1 and Ab2 solutions (50 μg/ml) were applied. After 20 min, the roots were washed three times with PBS (5 min) and treated with 100 μg/ml DARAb-FITC. After 15 min, the roots were washed twice with PBS (5 min). The pictures were registered using an MPS 60 microphotography fitting (Leica, Germany). Sterile roots, as well as roots of inoculated seedlings treated with either unlabeled Ab3 or DARAb-FITC, were used as controls.

Two-dimensional immunoelectrophoresis. To obtain the extracts of outer membranes, the bacteria were stab-inoculated into semisolid MSM (0.4% agar). After 36 h, cells from the colonies formed by motile bacteria were collected, washed with PBS, centrifuged, and treated with extraction buffer containing 0.1 M Tris–HCl, 10 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1% Triton X-100 (EDTA concentration was 0.05 mM per 1 g wet cells), pH 8.5, at room temperature for 30 min. The cells were removed from the extracts by centrifugation. Two-dimensional immunoelectrophoresis was performed in 1% agarose gels prepared on barbital–glycine–Tris buffer of ionic strength 0.02 and pH 8.8 according to the recommendations provided in [19] and using a Multiphor II (LKB, Sweden) equipment.

Relative hydrophobicity of cell surface of *A. brasilense* was determined by salting out taking into account that lower salt concentration causes aggregation of more hydrophobic cells [20, 21]. The minimal concentration of ammonium sulfate (%) at which bacterial aggregation was observed was determined. For

Table 1. Effect of inoculation with *A. brasilense* cultures on growth of seedling roots of Saratovskaya 29 soft spring wheat

A. brasilense strain	Seedling characteristics 7 days after inoculation		
	Roots		Above-ground part
	Length, mm	Branched roots, %	Length, mm
Sp245	49.8 ± 5.6	65.7 ± 7.1	122.1 ± 14.4
SK048	52.8 ± 7.2	63.0 ± 5.6	123.5 ± 15.2
SK051	57.4 ± 8.0	63.1 ± 10.0	122.4 ± 15.9
SK454	51.1 ± 7.2	62.3 ± 10.9	118.3 ± 20.6
Control without inoculation	55.1 ± 7.2	44.6 ± 5.4	115.2 ± 15.1

Table 2. Dynamics of *A. brasilense* cell anchoring on the roots of wheat seedlings

A. brasilense strain	Number of CFU × 10^6 in root homogenates of a single plant after incubation in bacterial suspension* during		
	3 h	4 h	6 h
Sp245	14.9 ± 1.7	15.2 ± 2.0	14.1 ± 1.6
SK048	2.2 ± 0.4	2.1 ± 0.3	2.3 ± 0.4
SK051	2.5 ± 0.5	ND**	2.4 ± 0.4
SK454	2.4 ± 0.4	ND	2.2 ± 0.4

Notes: * Azospirilla cultures containing 10^8 cells/ml were used for plant inoculation.
** ND, not determined.

all quantitative measurements, at least nine independent experiments were performed. The data were treated statistically using the Microsoft Office Excel 2003 software package (11.6355.6360) SP1. Confidence limits were determined at a 95% significance level.

RESULTS AND DISCUSSION

Dynamics of wheat root colonization by *A. brasilense* Sp245 and its Swa$^-$ Gri$^+$ mutants. Initial stages of colonization of the plant root system by azospirilla depend upon the ability of bacteria to migrate toward the roots [7, 8]. Therefore, we chose inoculation conditions (with tube rocking) providing both the wild-type strain and its mutants in flagellation and motility equal chances to contact the root surface. One day after inoculation, the number of cells anchored on the seedling roots was determined. On the seventh day after inoculation, wheat root system became more branched compared to control samples (Table 1), evidencing the positive effect of the bacteria [9].

Under the chosen conditions, after 3-h incubation of 3-day seedlings in bacterial suspensions, the number of cells attached to the roots stabilized (Table 2). Mutant strains SK048, SK051, and SK454 demonstrated lowered capacity for adsorption on root surface compared to the wild-type strain (Fig. 1a).

Earlier, we discovered no differences in production or antigenic structure of the surface carbohydrate polymers between *A. brasilense* Sp245 and its mutants used in the present work [3]. The only exception was the mutant strain SK051, which does not produce calcofluor-binding polysaccharides [2]. The hydrophobicity of the wild-type and mutant *A. brasilense* strains grown in liquid medium assessed by the salting-out technique did not vary much. Minimal ammonium sulfate concentration causing cell aggregation of Sp245, SK048, SK051, and SK454 was 16.4 ± 0.8, 15.9 ± 1.0, 16.3 ± 0.9, and 15.7 ± 0.8%, respectively. Therefore, in all *A. brasilense* strains under study, hydrophobic interactions and the polysaccharide components of the bacterial cell surface equally contribute to bacterial attachment to the root surface. Decreased ability of the mutants SK048, SK051, and SK454 to adsorb on root surfaces as compared to Sp245 may be due to their lack of the polar flagellar filament.

After 1-day incubation of inoculated seedlings, ~30% of Sp245 cells adsorbed on the roots were anchored. In the case of the mutants SK048, SK051, and SK454, ~59, 44, and 21% of adsorbed cells remained on the roots, respectively (Fig. 1a). Bacterial platings from 10-day seedlings with increased number and length of roots indicated that, after bacterial anchoring to the plant, the size of the populations of Sp245 and its mutants increased to about the same extent (Fig. 1a). The number of mutant bacteria on 10-day sprouts approached the level of initially adsorbed cells, while in the case of the wild-type strain the value was only about 40% (Fig. 1a). The observed decrease in azospirilla number on the growing seedling roots may be explained by migration of some of the adsorbed bacterial cells into the plant cultivation medium. The hypothesis was confirmed by the results of bacterial plating from the media after plant removal (data not shown).

Therefore, the dynamics of wheat seedling colonization by the mutant and parent strains evidences that, upon cell anchoring, the mechanism of migration with microcolony formation (Gri$^+$ phenotype) may provide efficient colonization of growing roots by the bacteria. It is important to note that an increase in branched roots number in 10-day seedlings inoculated with Sp245 and its mutants did not depend on the number of bacteria initially adsorbed/anchored on the plants (see Fig. 1a and Table 1).

Formation of bacterial aggregates in the process of wheat root colonization. The hypothesis on azospirilla migration with microcolony formation in the process of wheat seedling root colonization was confirmed by microscopic observations. As was mentioned above, in the process of the experiments on adsorption, bacteria

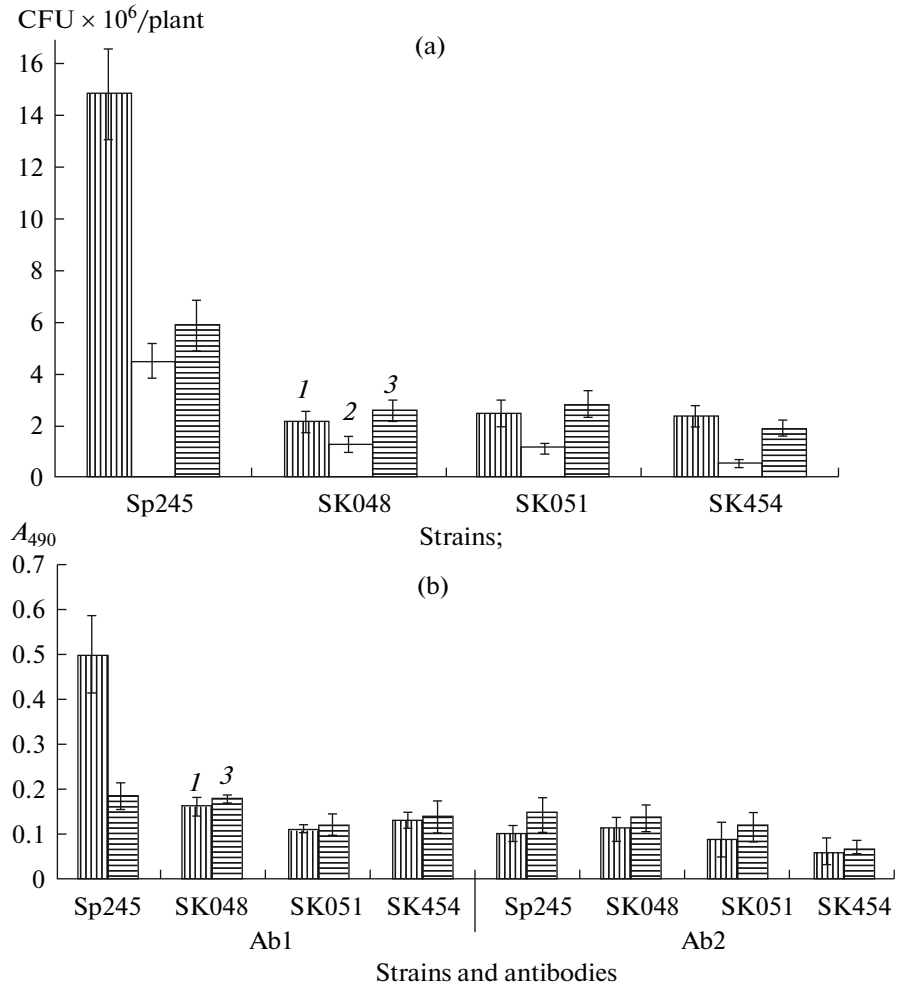

Fig. 1. Dynamics of wheat seedling roots colonization by *A. brasilense* strains: number of CFU per plant in root homogenates (a) and root homogenate binding efficiency with the antibodies against Sp245 polysaccharide antigens (Ab1) and antibodies against protein antigens (Ab2) revealed by ELISA (b). Three-day seedlings after 3-h incubation with bacterial suspension (*1*); the seedlings 24 h after inoculation (*2*); and the seedlings 7 days after inoculation (*3*).

and the seedlings were constantly shaken providing both motile and immotile bacteria equal chances to contact the root surface. As a result, attached cells of both the wild-type and the mutant strains were detected in the same root regions. At the inoculation stage, in the case of strain Sp245, the bacteria adsorbed on root hairs were spread evenly without preferential grouping in any zone (Fig. 2a, *1*). In the case of inoculation with strains SK048 (Fig. 2b *1*), SK051, and SK454, some root hairs were observed with bacterial cells gathered in aggregates along the hair.

As soon as 24–48 h after plant inoculation, together with evenly distributed Sp245 cells, cell aggregates were observed on the roots. Within the same time interval after the inoculation of the seedlings with the cultures of mutant strains, the number of bacterial aggregates of SK048, SK051, and SK454 increased and were residing on both the root hairs and the root surface. On the seventh day, cell aggregates remaining on the root surface formed clusters on the seedlings colonized with both strain Sp245 (Fig. 2a *2*) and its mutants SK048 (Fig. 2b *2*), SK051, and SK454 (Fig. 2c *2*).

Apparently, formation of aggregates by azospirilla in the process of their migration along the root surface of the plant is a result of the effect of certain external factors on the bacterial population. For example, in semisolid media containing wheat germ agglutinin, which slows down cell migration, part of the *A. brasilense* Sp245 population switches from swarming to migration with formation of microcolonies [4]. Oxygen limitation may be an additional stimulus for the change to microcolonial migration *in planta*, since in vitro preculturing under conditions of oxygen deficiency resulted in a somewhat increased share of Swa^- Gri^+ clones within *A. brasilense* Sp245 population [3].

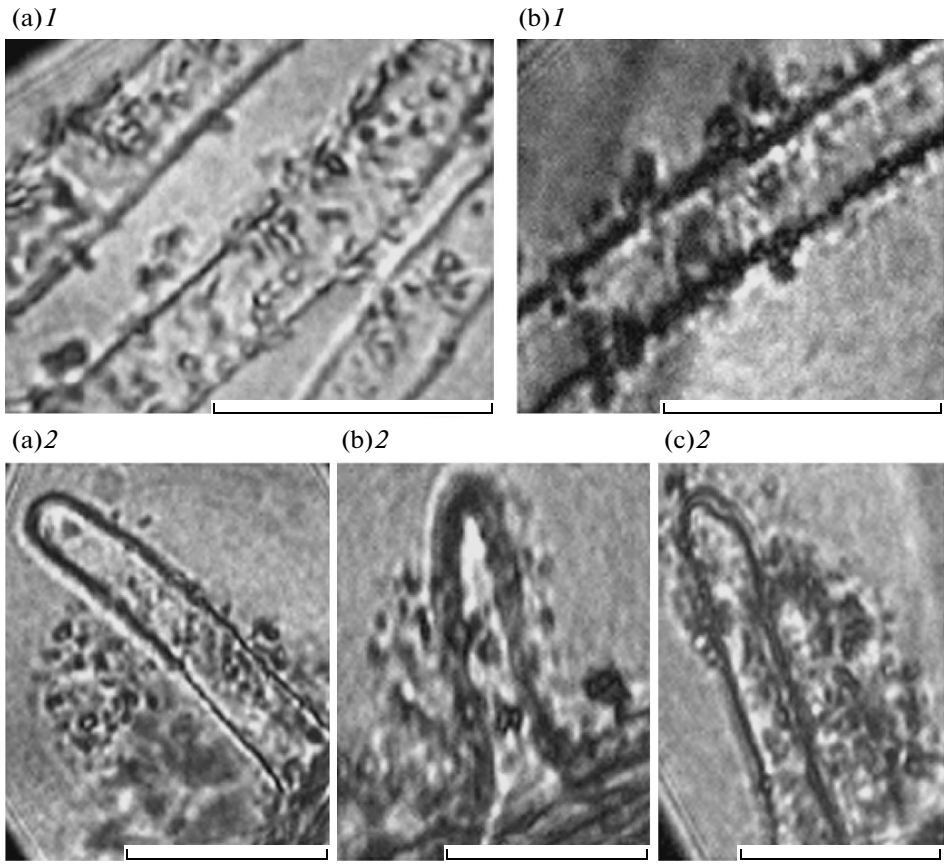

Fig. 2. Phase contrast microscopy of wheat seedling root hairs inoculated with *A. brasilense* cells of strain Sp245 (a), SK048 (b), and SK454 (c). After 3-h incubation in bacterial suspension, (*1*) and 7 days after inoculation (*2*).

One week after inoculation of wheat seedling roots, cell clusters of *A. brasilense* Sp245 or its mutants were also detected on the root surface and root hairs by immunofluorescence microscopy. Fluorescence of FITC-labeled antibodies in a complex with the antibodies against Sp245 polysaccharides (Ab1) or with the antibodies recognizing protein structures (Ab2) was viewed as glowing regions of the roots (Fig. 3). A complex of Ab1 or Ab2 with FITC-labeled antibodies allowed us to detect bacteria residing on root hairs of 10-day seedlings inoculated with the cells of both strain Sp245 (Fig. 3a *2*, b *1,2*, and 3d *2*) and SK048 (Fig. 3c *2* and 3e *2*), SK051, and SK454. In 3-day seedlings with freshly adsorbed cells, bacterial clusters could only be visualized with the antibodies against strain Sp245 polysaccharides (Ab1). When using the antibodies against bacterial protein antigens (Ab2), the fluorescence signal was only registered in rare bacterial groups among the large number of cells adsorbed on the root surface of 3-day seedlings. Therefore, in the case of anchored azospirilla, the protein structures exposed on the surface of cells are revealed by Ab2 to a lesser degree than in bacteria adapted to living on plants. It should be noted that the signal was never observed in the controls (Fig. 3f *2* and 3g *2*).

Therefore, immunofluorescence microscopy results demonstrate that polysaccharide and protein antigens may be involved in biofilm formation by azospirilla on both wheat roots and the boundary between water and hydrophilic or hydrophobic solid surface [20]. The root surface is more heterogeneous compared to the model glass or polystyrene surfaces used earlier to study in vitro biofilm formation by azospirilla [20]. In particular, the root surface contains plant cell walls and the mucigel formed by polysaccharides and the proteins secreted by the plant [22]. Apparently, bacterial glycopolymers and proteins not only promote adhesion of the bacterial biofilm to the root surface [9–11, 14], but also contribute to organizing its structure by involving factors of plant origin, including those facilitating the switch of azospirilla to microcolonial spreading [4]. One of the stages of biofilm formation on glass or polystyrene surface is the formation of cellular aggregates, which subsequently join to form an entire layer of cells [20]. On the contrary, after bacteria anchoring to the root surface, they preferentially reside in microcolonies (Fig. 2 and 3). Formation of cellular aggregates on plant roots is typical of azospirilla [23], yet the nature of cellular structures governing the process of aggregation is not clear.

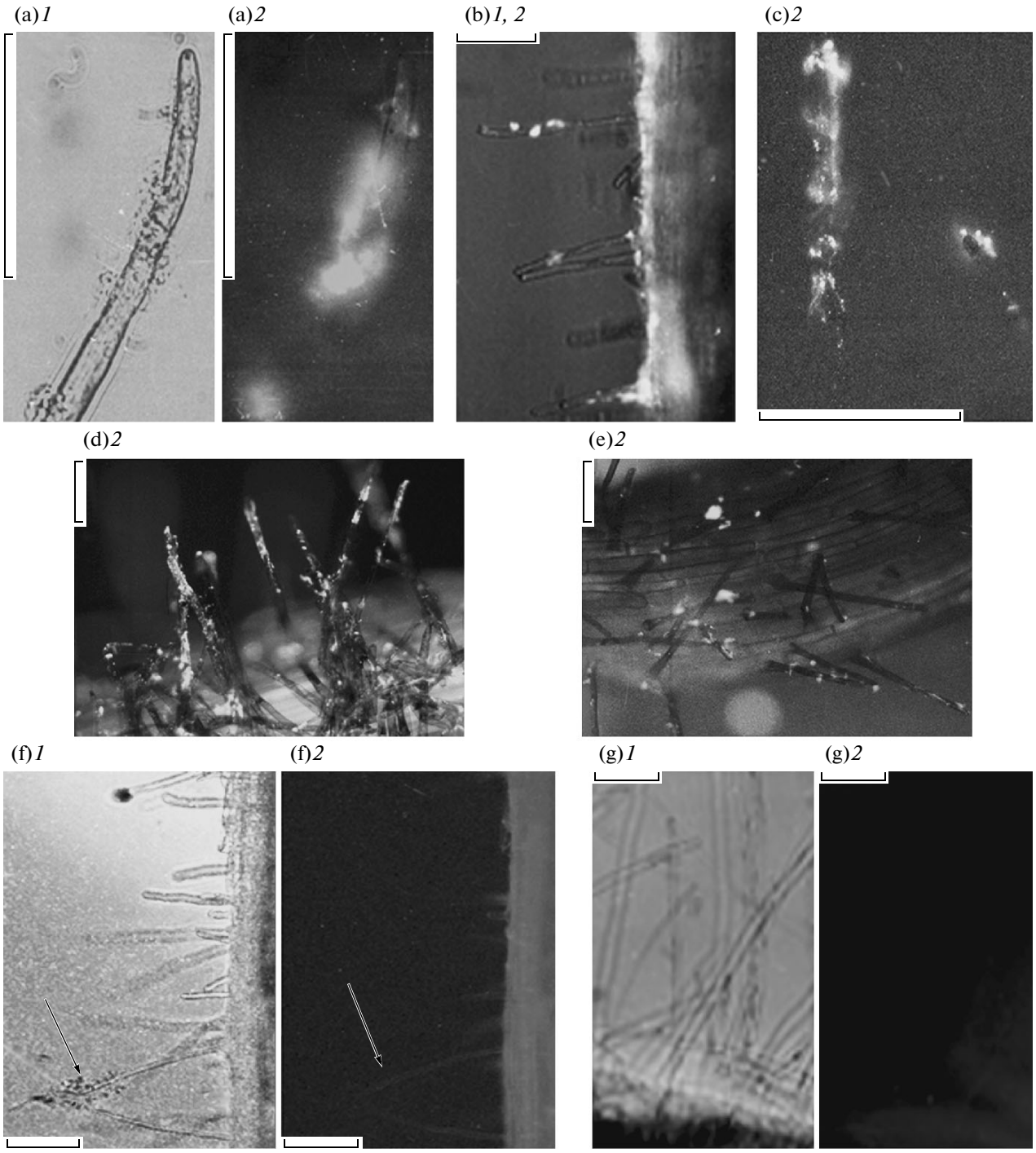

Fig. 3. Immunofluorescence microscopy of wheat seedling roots 7 days after inoculation with *A. brasilense* cultures Sp245 and SK048: Sp245 (a, b, d, f); SK048 (c, e); without inoculation (control) (g); with antibodies against Sp245 polysaccharides (Ab1) (a, b, c, g); with antibodies against protein antigens (Ab2) (d, e); with antibodies against *A. brasilense* Sp7 polysaccharides (Ab3) (control) (f). Transmitted light (*1*) and excitation mode (494 nm) (*2*). The arrow indicates a bacterial clustering on a root hair. Scale bars correspond to 10 μm.

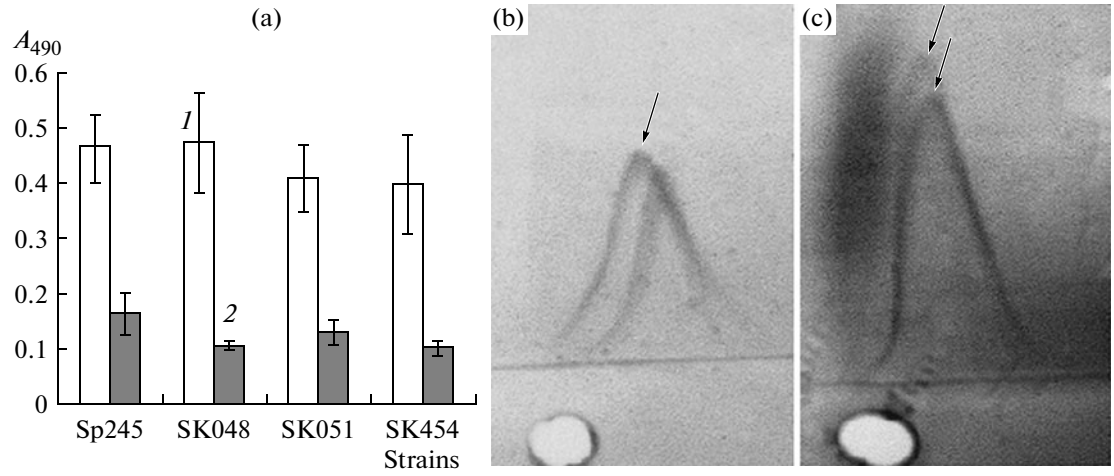

Fig. 4. Results of immunochemical analysis of *A. brasilense* culture antigens: (a) binding efficiency of the antibodies against Sp245 polysaccharide antigens (Ab1) (*1*) and antibodies against protein antigens (Ab2) (*2*) with the cells from 18-h cultures as revealed by ELISA; (b), (c), two-dimensional immunoelectrophoresis (with Ab2) of outer membrane extracts of *A. brasilense* strain Sp245 (b) and its Gri^+ mutant SK048 (c) migrating in semisolid medium. The arrows indicate precipitation bands evidencing the identity of the genus-specific antigen detected in the preparations and the presence of two isoforms of the antigen.

Capsule polysaccharides, exopolysaccharides, and proteins are proposed as possible structures of the kind [24].

Analysis of the polysaccharide and protein antigens exposed on the cell surface of *A. brasilense* Sp245 and its motility mutants. Immunofluorescence microscopy with antibodies against cell surface antigens made it possible to reveal the exposed polysaccharide and protein determinants in azospirilla cells residing directly on wheat seedling roots. Enzyme-linked immunoassay in parallel with counting of colony-forming units was used to detect possible changes in the content of polysaccharide and protein antigens during the process of azospirilla adaptation to living on plant roots.

In ELISA with Ab1, a similar amount of polysaccharide antigen determinants was detected on the cells of *A. brasilense* Sp245, SK048, SK051, and SK454 from the cultures used for plant inoculation (Fig. 4a). No difference in the immunochemical characteristics of lipopolysaccharides in the azospirilla strains under study was detected [3]. According to the results of immunofluorescence microscopy of the inoculated wheat seedling roots, quantification of CFU in homogenates of the inoculated roots (Fig. 1a), and ELISA of the homogenates (Fig. 1b), variations in the content of polysaccharides recognized by Ab1 correspond to the changes in the number of root-anchored *A. brasilense* cells of wild-type and mutant strains during a week of incubation.

Immunochemical analysis of the proteins isolated from the cell surface of *A. brasilense* Sp245 and its Fla^- Swa^- Gri^+ mutant SK048 migrating in semisolid agar (Fig. 4b and 4c) revealed the same major antigen component in both preparations. Moreover, in contrast to the Sp245 preparation, a double peak was observed on the immunoelectrophoresis picture (Fig. 4c) of the SK048 preparation, resulting from the precipitation bands, which may be due to the existence of two isoforms of the relevant antigen.

In ELISA with Ab1 of 18-h bacterial cultures used for plant inoculation, the amount of exposed protein determinants detected in *A. brasilense* strains Sp245 and SK051 was about the same and in SK048 and SK454 it was insignificantly lower. The increase in total amount of protein determinants exposed on the cells of *A. brasilense* strains Sp245, SK048, SK051, and SK454 7 days after wheat seedlings inoculation was also statistically insignificant (Fig. 1b). The number of *A. brasilense* Sp245 cells on the roots decreased by an order of magnitude within the same time interval, while in the case of SK048, SK051, and SK454 this value practically did not change (Fig. 1a). The data evidences indirectly that synthesis of the protein determinants recognized by Ab2 is significantly intensified in the process of *A. brasilense* Sp245 adaptation to plant roots.

Therefore, in the work we confirmed the data of other authors [13] on decreased capability of *A. brasilense* Fla^- mutants having lost the polar flagellum filament to adsorb on plant roots. It was demonstrated for the first time that, upon anchoring on root surface, both the wild-type *A. brasilense* cells and the Fla^- Swa^- Gri^+ mutants migrated over the growing root surface with approximately equal efficiency. Microcolony (cell aggregates) formation on root surface is typical of both the wild-type strain and its Fla^- Swa^- Gri^+ mutants. The content of genus-specific protein antigens on *A. brasilense* Sp245 cell surface was established to increase during the process of adaptation to living on plant roots.

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