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EXPERIMENTAL ARTICLES

Immunoelectron Microscopy Investigation of the Cell Surface of *Azospirillum brasilense* Strains

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Abstract—The genus-specific surface protein antigens of *Azospirillum brasilense* strains were visualized immunochemically. The procedure used for cell sample preparation was optimized to ensure that the surface protein structures were detected on cells in situ. Gold and gold-silver nanoparticles were conjugated to antibodies raised against the flagellin of *A. brasilense* type strain Sp7, against the lipopolysaccharide of *A. brasilense* Sp245, and against the genus-specific protein determinants of *A. brasilense* Sp7. Electron microscopic analysis using nanoparticle-labeled antibodies revealed antigenic determinants of the polar flagellum on the *A. brasilense* Sp245 cell surface, which in these bacteria are normally screened from the surroundings by a lipopolysaccharide sheath. Pili-like structures were detected on the Sp245 wild-type strain and on its Fla$^-$ Swa$^-$ Omegon-Km mutant SK048, which are presumably involved in microcolonial spreading in these bacteria.

Keywords: Azospirillum brasilense, flagellin, polar flagellum, lipopolysaccharide, pili-like structures, antibodies
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Components of the bacterial cell surface are usually studied after their separation from the cell mass. Few works deal with the cell surface architecture and exposure or isolation of specific surface structures of bacterial cells. Understanding the properties of cell surfaces is, however, required for adequate control of such processes as bacterial adhesion and adsorption. In the present work, such an approach was applied to *Azospirillum* strains, which for 40 years have been a model object for research in plant-microbe associations due to their ability to increase production of cultivated plants (Bashan and de-Bashan, 2010). Investigation of the structures of azospirilla acting as the major surface antigens makes it possible to determine the overall organization of the surface of bacterial cells functioning in the plant rhizosphere. This knowledge is required for development of specific probes for ecological and taxonomic investigation of azospirilla.

The bacterial surface plays the key role in establishment of plant-microbe associations. In many gram-negative bacteria attachment to plant roots is mediated by flagella, fimbriae, or pili (De Weger et al., 1987; Croes et al., 1993; De Troch and Vanderleyden, 1996; Dörr et al., 1998).

When maintained in liquid media, azospirilla have one polar flagellum, which is responsible for motility and chemotaxis (Zhulin and Armitage, 1992). The polar flagellum was reported to participate in the phase of azospirilla adsorption on plant roots (Michiels et al., 1991; Croes et al., 1993). However, no decrease in the level of azospirilla adsorption was shown when the roots were pretreated with preparations of an isolated polar flagellum, or when bacteria were treated with an antiserum against flagellin (Croes et al., 1993). This fact was explained by detection of a (lipo)polysaccharide sheath on the surface of *Azospirillum brasilense* flagellin which carries antigenic determinants identical to those of somatic lipopolysaccharide (LPS) (Burygin et al., 2007). The latter work showed the polysaccharide sheath to isolate the filament of the polar flagellum from the environment, while the antigenic determinants of *Azospirillum* flagellin were genus-specific.

While some works reported formation of fibrillar material in the course of azospirilla attachment to plant roots, the nature of this material remains unknown (De Troch and Vanderleyden, 1996). Bashan and Levanony (1988) investigated attachment of *A. brasilense* strain Cd to sand grains and hypothesized that fibrillar material of a proteinaceous nature was responsible for this process. This conclusion was supported by the fact that treatment with proteinase or ethylenediaminetetraacetic acid resulted in a significant decrease in the number of attached bacteria.

Madi et al., (1988) revealed formation of fibril-like structures by *A. brasilense* under stationary conditions (on the surface of *A. brasilense* Cd culture grown in semiliquid medium with 0.05% agar for 48 h). The authors observed no such structures in bacteria grown in liquid medium under stirring. The nature of the fimbriae was not discussed in that work. Del Gallo et al., (1989) showed the presence of cellulose in aggregates of flocculating *Azospirillum* cells and suggested the role of cellulose fibrils in the preservation of floc stability. However, Madi and Henis (1989) did not detect cellulose in *Azospirillum* aggregates.

Shelud'ko et al., (2010) found for *A. brasilense* Sp245 that the content of genus-specific surface antigens involved in microcolonial spreading of these bacteria increased in the course of their adaptation to existence on wheat roots. We expect that proteinaceous pili or pili-like structures are involved in this mode of collective motility of azospirilla and in their colonization of wheat roots.

The goal of the present work was to perform immunomicroscopic visualization of flagellin determinants and surface proteins involved in establishment of plant-microbial interaction on the surface of *Azospirillum brasilense* cells.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The following *A. brasilense* strains from the Collection of Rhizosphere Microorganisms, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (IBPPM), were used: strains Sp7 (IBPPM 150), Sp245 (IBPPM 219), and SK048, a Fla$^-$ Swa$^-$ Omegon-Km mutant of strain Sp245 (Scheludko et al., 1998). The cells were grown at 30°C to the late exponential growth phase in a synthetic malate medium (Döbereiner and Day, 1976) supplemented with NH_4Cl (1 g/L).

Antibodies. Genus-specific rabbit antibodies to a flagellin preparation from the type strain *A. brasilense* Sp7 (electrophoretically purified and eluted from the gel) (Burygin et al., 2007), strain-specific antibodies to *A. brasilense* Sp245 LPS (Matora et al., 1998), and antibodies to whole cells of the type strain *A. brasilense* Sp7 (Shirokov et al., 2015) were used.

Preparation of nanoparticles and their conjugates with antibodies. Spherical gold nanoparticles (15 nm) were obtained by citrate reduction of $HAuCl_4$ (Frens, 1973). Gold-silver nanocages were produced from silver nanocubes by galvanic replacement (Skrabalak et al., 2007) with minor modifications (Khlebtsov et al., 2011). Conjugation of nanoparticles with immunoglobulin molecules was carried out as described by Khanadeev et al., (2011).

Immunodot analysis of bacterial suspensions. Bacterial suspension (1 μL, A_{660} = 0.5) treated as described in the Results and Discussion section was applied to the center of a 5-mm square on the surface of a nitrocellulose membrane and incubated in a dry-air thermostat at 50°C. After blocking of the site of nonspecific sorption for 1 h at room temperature in a 1% solution of bovine serum albumin (BSA), the membrane was immersed into a solution of rabbit antiflagellin antibodies conjugated with 15-nm spherical gold nanoparticles supplemented with 0.01% BSA and 0.02% Tween-20 (A_{520} = 0.5). The incubation was carried out overnight at 4°C.

Immunoelectron microscopy. For electron microscopy, the cells were applied to formvar-coated nickel grids and incubated in a dry-air thermostat for 1 min at 50°C. After application of the preparation, it was blocked with 1% BSA solution, dried, immersed into a solution of rabbit antibodies conjugated with metal nanoparticles, and incubated for 60 min at 25°C. The preparation was then washed with phosphate-buffered saline (PBS) supplemented with 0.01% BSA and 0.02% Tween-20, the grids were dried and examined under a LIBRA 120 electron microscope (Carl Zeiss, Germany) at the Simbioz Center for the Collective Use of Research Equipment, IBPPM.

RESULTS AND DISCUSSION

Detection of flagellin determinants of *A. brasilense* Sp245 sheathed flagella. High sensitivity of *Azospirillum* polar flagella to mechanical disturbance, which is inevitable during preparation of bacterial samples, limits applicability of the microscopic approach to the detection of determinants of these structures.

Optimal conditions of sample preparation were determined for electron microscopic detection of sheathed flagellin among the surface antigens of the model strain *A. brasilense* Sp245. In particular, the procedure was optimized for release of the capsular and sheath material from bacterial cells, which resulted in minimal loss (detachment) of flagella. Dot analysis was used as a test indicating the optimal conditions of cell preparation. Three-time treatment of bacterial suspensions with PBS, in combination with gentle resuspension and centrifugation at 3000 rpm was shown to result in the best interaction between cells and colloidal gold-labeled genus-specific antibodies to flagellin (data not shown).

Microphotographs (Fig. 1) and schematic representation of bacterial cells (Fig. 2) may be used for comparison of the results of immunolabeling of the cells of strain Sp245 with and without the polysaccharide sheaths with genus-specific antibodies to flagellin and strain-specific antibodies to LPS labeled with 15-nm spherical gold nanoparticles. It can be seen that antibodies to flagellin (Figs. 1a and 2a) interacted directly with the flagella of sheath-free cells, while the anti-LPS antibodies recognized material antigenically related to the lipopolysaccharide on bacteria with sheathed flagella (Figs. 1b and 2b). Thus, conditions

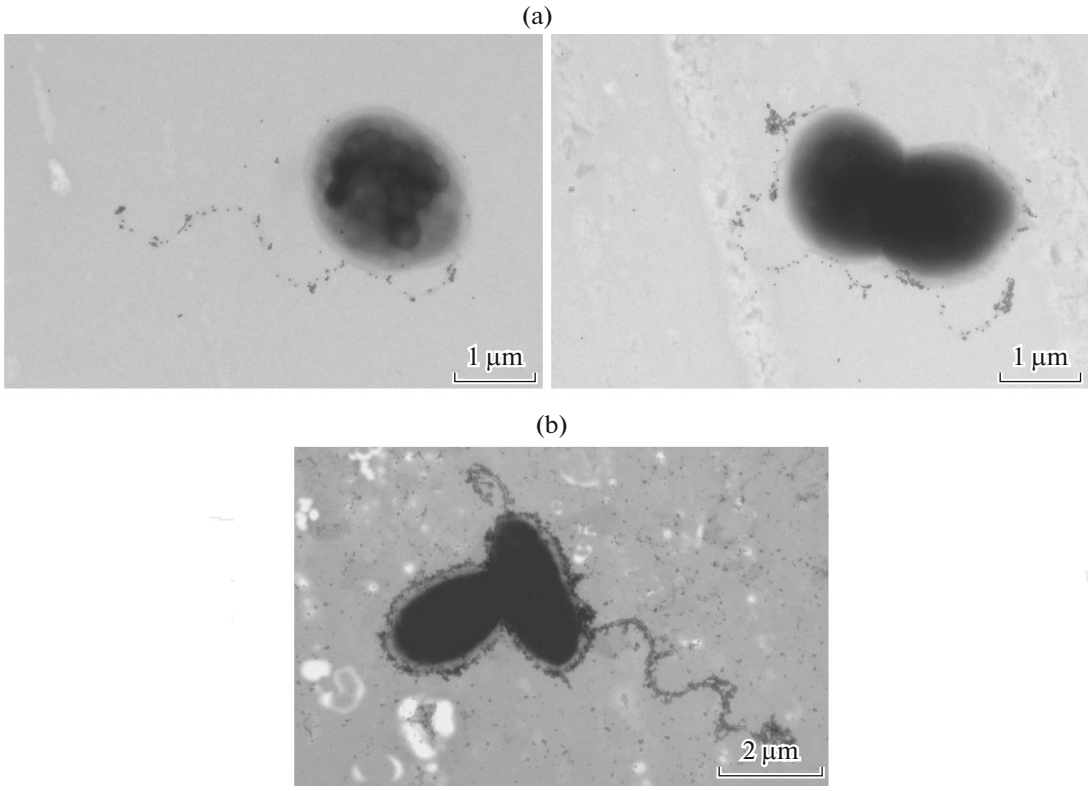

Fig. 1. Immunoelectron microscopy of the cells of *A. brasilense* Sp245 by using antibodies to flagellin (a) and to LPS (b) labeled with spherical gold nanoparticles.

were determined for visualization of the flagellin determinants of the studied bacteria, in which the polar flagellum is normally covered with a (lipo) polysaccharide sheath shielding flagellin from the environment. Importantly, visualization of these determinants was carried out for the whole cell surface with nondetached flagella.

This observation clearly indicates that the flagellin of azospirilla, which is shielded from the environment by a polysaccharide sheath, may act in the processes of attachment only as a carrier of a substance responsible for adsorption of these bacteria on plant roots. This suggestion is in good agreement with the literature data showing no decrease in *Azospirillum* adsorption to plant roots after root treatment with preparations of an isolated polar flagellum or after treatment of bacteria with an antiserum to flagella (Croes et al., 1993).

Simultaneous detection of different antigens on the surface of *A. brasilense* Sp245. For simultaneous electron microscopic detection of the somatic and flagellar antigens on the bacterial surface, two types of nanoparticles were used: 15-nm spherical gold nanoparticles conjugated with antibodies to flagellin and 50-nm gold-silver nanocages conjugated with antibodies to LPS (somatic antigen). For immunolabeling, the cells were released from their capsules and polysaccharide sheaths. Detection of flagellar deter-

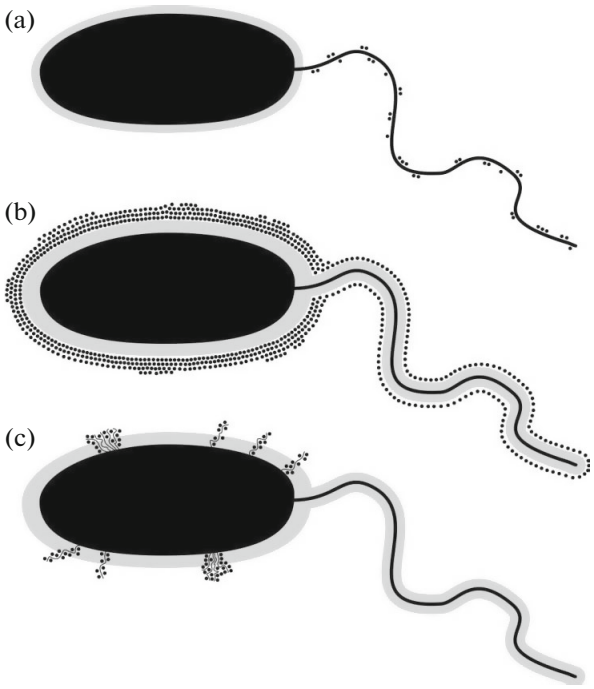

Fig. 2. Schematic representation of the results of immunoelectron microscopy of the cells of *A. brasilense* Sp245 with antibodies to flagellin (a), LPS (b), and genus-specific antibodies to proteinaceous surface determinants (c).

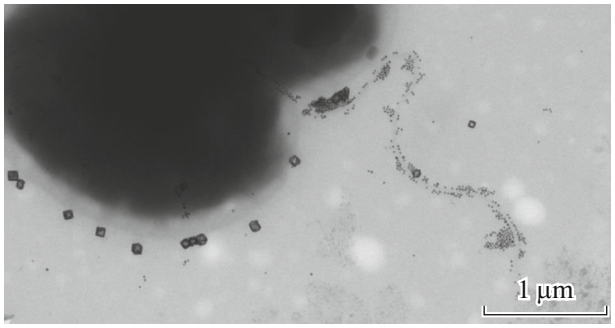

Fig. 3. Simultaneous visualization on the surface of *A. brasilense* Sp245 of determinants of the polar flagellum (with flagellin antibodies labeled with gold nanospheres) and of the somatic antigen (with LPS antibodies labeled with gold-silver nanocages).

minants with spherical nanoparticles and of the LPS determinants with nanocages on the cell surface of *A. brasilense* Sp245 is shown in Fig. 3. It can be seen that LPS nanocages was detected only on the outer membrane of *washed* cells and barely shielded the determinants of sheath-free flagellin (nanospheres).

Detection of proteinaceous pili on *A. brasilense* Sp245 and on the *A. brasilense* mutant strain SK048. We have previously shown an intensified synthesis of proteinaceous surface structures involved in microcolonial spreading of azospirilla in the course of their interaction with wheat roots (Shelud'ko et al., 2010). Antibodies to the pool of outer membrane protein antigens (Shirokov et al., 2015) were used to determine whether proteinaceous pili or pili-like structures were involved in this mechanism. The antibodies were obtained by using intact cells of *A. brasilense* type strain Sp7, which is in a different serological group than *A. brasilense* Sp245 according to characteristics of its LPS or the O antigen (Matora et al., 2008). Shirokov et al., (2015) used linear immunoelectrophoresis and immunodiffusion analysis with these antibodies to detect at least three common proteinaceous antigens as part of the outer membranes of the wild-type strains Sp7 and Sp245. The data on the absence of strain specificity of the proteinaceous determinants of azospirilla made it possible to use antibodies to whole cells of the serologically different strain for cross-detection of proteinaceous antigens in *A. brasilense* Sp245.

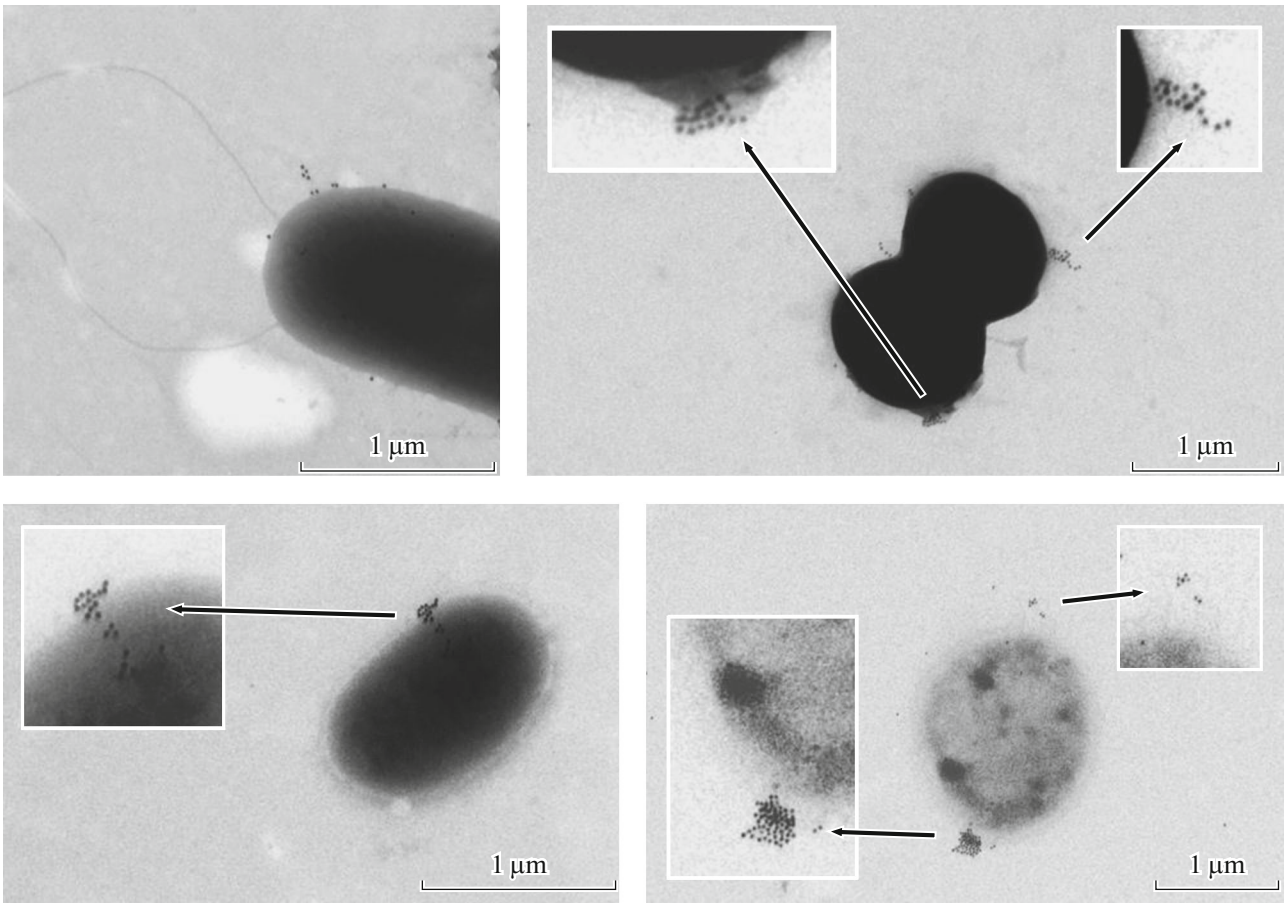

Fig. 4. Immunoelectron microscopy of the cells of *A. brasilense* Sp245 by using genus-specific antibodies to the surface proteinaceous determinants labeled with spherical gold nanoparticles.

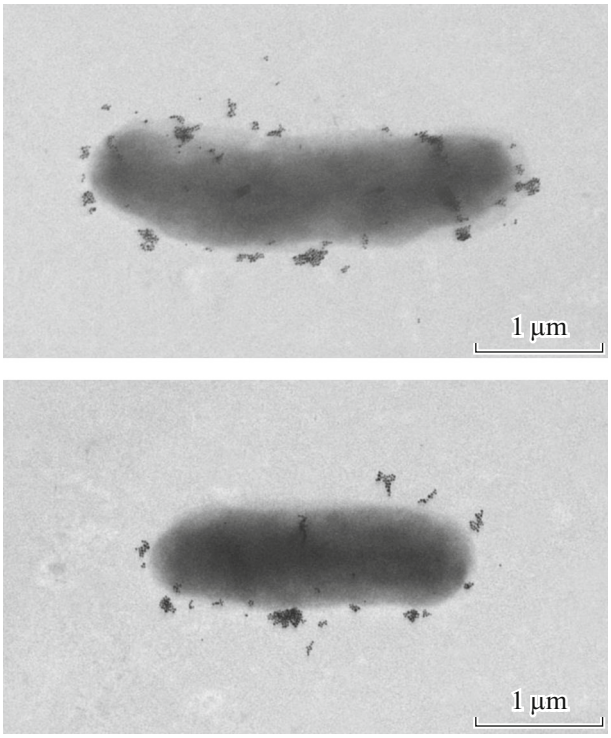

Fig. 5. Immunoelectron microscopy of the cells of the Gri^+ mutant of *A. brasilense* SK048 by using genus-specific antibodies to the surface proteinaceous determinants labeled with spherical gold nanoparticles.

Results of immunoelectron microscopy of strain Sp245 with antibodies to proteinaceous determinants are shown in Fig. 4 and Fig. 2c (schematic representation of the bacterium). Gold nanoparticles detected an interaction between the antibodies and the pili-like surface structures, which differed in morphology.

To confirm the involvement of these structures in the process of microcolonial spreading of azospirilla, immunolabeling of the cells of strain SK048 was carried out. The latter, a mutant of *A. brasilense* Sp245, is a convenient object for investigation of collective migration with the formation of microcolonies. The cells of strain SK048 have no polar flagellum and spread in semiliquid media not by swarming but by forming granule-shaped microcolonies (this phenotype was termed Gri^+, from granular inclusions) (Shelud'ko and Katsy, 2001). Fig. 5 shows numerous pili on the surface of the mutant strain *A. brasilense* SK048, which look more massive than those of strain Sp245. This is in agreement with the results presented by Shelud'ko et al., (2010). The dynamics of wheat root colonization by the Gri^+ mutants and the parent strain studied in that article showed that the spreading mechanism with microcolony formation (Gri^+ phenotype) provided for relatively efficient colonization of growing roots by bacteria, while synthesis of preexisting proteinaceous surface structures (probably pili, responsible for Gri-type motility) was intensified during the interaction of azospirilla with wheat roots.

Molecular genetic investigation is required for accurate classification of proteinaceous structures revealed in the present work. Summarizing our results, it should be noted that no mention was found in the available literature of detection of pili or pili-like structures on the surface of *Azospirillum* cells by immunoelectron microscopy.

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