ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 6, pp. 696–702. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © A.V. Shelud'ko, E.G. Ponomareva, O.E. Varshalomidze, E.P. Vetchinkina, E.I. Katsy, V.E. Nikitina, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 6, pp. 749–756.

EXPERIMENTAL ARTICLES

Hemagglutinating Activity and Motility of the Bacterium *Azospirillum brasilense* in the Presence of Various Nitrogen Sources

A. V. Shelud'ko[1], E. G. Ponomareva, O. E. Varshalomidze, E. P. Vetchinkina, E. I. Katsy, and V. E. Nikitina

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410049 Russia

Received November 24, 2008

Abstract—Hemagglutinating activity of the *Azospirillum brasilense* strain Sp245 grown in liquid media and the swarming motility of those bacteria grown in semisolid media vary significantly depending on the nitrogen source. In media with nitrate or nitrite, an increase in the hemagglutinating activity and a decrease in the swarming circles' diameter of Sp245 were observed, compared to bacteria grown in the presence of ammonium or N_2. A ~67-kDa hemagglutinin exhibiting affinity to the O-specific polysaccharide, an acidic D-rhamnan (OPS-I), was isolated from the surface of Sp245 cells. Introduction of the hemagglutinin into the media resulted in a decrease in the Sp245 cell motility while not affecting its mutants lacking the acidic D-rhamnan or the Sp245.5 mutant with a different OPS structure. Cells of strain Sp245.5 demonstrated hemagglutinating activity two times higher than that of the parent Sp245 strain and formed "diffuse" colonies, rather than distinct swarming circles Sp245 formed when grown in a semisolid medium. The data obtained demonstrate that intercellular contacts mediated by the interaction between the surface hemagglutinin and OPS-I, which is sensitive to environmental factors, affect the collective motility of cells.

Key words: Azospirillum brasilense, motility, polysaccharides, bacterial hemagglutinins, intercellular contacts.

DOI: 10.1134/S0026261709060058

Associative bacteria *Azospirillum brasilense* attract the attention of investigators due to their ability to stimulate growth of a wide variety of plants [1]. Apparently, active motility of azospirilla in liquid and viscous media accelerates their penetration into the favorable ecological niches including the rhizosphere [1]. In liquids, *A. brasilense* motility is provided by a single polar flagellum, while in viscous and semisolid media azospirilla form concentric colonies called swarming circles; both the polar and the numerous lateral flagella are used for movement [1, 2]. Capacity for flagellar-independent movement in semisolid media with formation of disks comprising granular clusters (microcolonies) of bacteria was revealed in a number of strains of *A. brasilense* [3, 4]. Patterns of azospirilla spreading and the morphology of their colonies formed in semisolid media are defined by intercellular communication [4]. Intercellular communication is mediated by specific compounds which are not thoroughly studied in the case of azospirilla; yet, it has been shown that they may be surface polysaccharides [4, 5]. On the other hand, interactions between polysaccharides and hemagglutinins of azospirilla modulate the aggregation of azospirilla cells [6, 7]. The level of polysaccharide synthesis and hemagglutinin activity in azospirilla vary depending on the bound nitrogen present in the media and its chemical nature [6–8], which in turn may influence the formation of intercellular contacts.

The aim of the present work was to study the motility and hemagglutinating activity of *A. brasilense* in the presence of various nitrogen sources and to characterize hemagglutinin of Sp245 and its effect upon bacterial motility.

MATERIALS AND METHODS

Strains and cultivation conditions. In the work, *A. brasilense* strains Sp245 [9] and Sp7 [2] were used along with the mutants of Sp245 with modifications in the lipopolysaccharide (Lps) structure and in production of calcofluor(Cal)-binding polysaccharides [10, 11] namely an $LpsI^-$ Cal^- KM252, $LpsI^-$ KM348, $LpsII^-$ KM139, and $LpsI^-$ $LpsII^-$ Cal^- Sp245.5.

A. brasilense strains were grown on a malate salt medium (MSM) at 28°C [12]. Bacto Agar was added to the medium when necessary. Time of cultivation in liquid media was 18 h, and in semisolid and solid media, 36 and 72 h.

[1] Corresponding author; e-mail: shel71@yandex.ru

Hemagglutinating activity of bacteria cultivated in liquid media. Aliquots of 50 μl of either (a) bacterial cultures (A_{590} = 0.5); (b) suspensions of bacteria rinsed with 0.05 M phosphate buffered saline (PBS, pH 7.0; A_{590} = 0.5; A_{590} = 1.0); (c) bacterial suspensions (A_{590} = 0.5) in PBS after 8-h incubation at 37°C in the presence of 2 mg/ml trypsin; or (d) bacteria-free culture liquid (cultures of A_{590} = 0.5 were used) were introduced into the wells of a 96-well immunoassay plate. Series of twofold dilutions in PBS were prepared for each sample. To the dilutions, 50-μl aliquots of 2% suspension of trypsinized or native rabbit erythrocytes in PBS were added; the hemagglutination titer was assessed after 18-h incubation at 4°C.

Hemagglutinin isolation, purification, and determination of specificity. Hemagglutinin (HA) isolation from the bacterial cell surface was performed according to the procedure described previously [7]. Hemagglutinin was purified by gel-filtration on a Sephadex G-75 column. Acetic acid (0.1 M; pH 4.8) or 0.05 M phosphate buffer (PB; pH 7.0) containing 0.15 M or 0.85 M NaCl were used as eluents [7]. Electrophoresis in 10% polyacrylamide gel under native conditions and in the presence of SDS was used to assess the homogeneity of the purified HA [13]. A silver nitrate staining technique and a technique with an additional step of oxidation of monosaccharide moieties were used to visualise proteins [13, 14]. To oxidize the monosaccharide moieties in the samples, the fixed gels were incubated for 5 min in the solution containing the following: 150 ml H_2O; 1.05 g periodic acid; and 4 ml of 25% isopropanol solution in 7% acetic acid. To stain the protein, 10 ml of 40% ethanol solution in 5% acetic acid was added to the periodate solution at this stage [14]. Protein concentration in the HA preparation was determined according to Bradford [13].

To determine the HA specificity, 0.3 M solutions (pH 7.0) of the following compounds were used: D-glucose, D-(+) lactose, D-(+) maltose, D-(–) mannitol, dulcite, L-(+) arabinose, inositol, L-(+) rhamnose, D-(–) mannose, D-(+) sorbitol, fructose, fucose, D-(+) galactose, D-(+) ribose, D-(+) cellobiose, D-(+) xylose, raffinose, L-lyxose, adonitol, *N*-acetyl-D-glucosamine, D-(+) glucosamine, *N*-acetyl-D-galactosamine, 2-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-α-D-mannopyranoside, 2-nitrophenyl-α-D-galactopyranoside, D-(+) glucose-6-monophosphoric acid barium salt, fructose-1,6-diphosphate monocalcium salt, D-galactosamine hydrochloride, neuraminic acid, glucuronic acid, and D-galacturonic acid.

Besides the listed compounds, solutions of the polymers isolated from the surface of Sp245 bacterial cells, including the LPS, O-specific polysaccharides (OPS) OPS-I and OPS-II, a capsule lipopolysaccharide–protein complex (LPPC), and polysaccharide–lipid complex (PSLC) [15–17] were also used to study HA specificity. The final polymer concentrations are given in the Results and Discussion section.

Aliquots (50 μl) of these solutions were introduced into the plate wells, twofold dilution series were obtained, and 50 μl of HA solution was added to each dilution. After 30-min incubation at 28°C, 50 μl of 2% trypsinized erythrocyte suspension in PBS was added into each well and the hemagglutination titer was evaluated.

Study of bacterial motility in liquid media. Bacterial cell suspensions in PBS (A_{590} = 0.5) were mixed with HA, LPS, OPS-I, OPS-II, LPPC, or PSLC solution in PBS on a coverslip (final concentrations of the polymers are presented in the Results and Discussion section) at 1 : 1 ratio and incubated for 1 or 30 min. The percentage of motile cells and their swimming speed were determined with computer-based video image analysis software as described elsewhere [4, 5]. To elucidate the possible inhibition of HA binding to bacterial cells, up to 0.1 mg/ml LPS, OPS-I, OPS-II, LPPC, or PSLC were added to the 10 μg/ml HA solution. After 1-h incubation at 28°C, solutions were mixed with the cell suspension at a 1 : 1 ratio, and bacterial motility was determined. PBS without polymer additon was used in the control experiments.

Study of bacterial motility on agar media. Bacterial motility was studied on semisolid media containing 0.2–0.6% agar [3, 4]. Cells from solid MSM were stab-inoculated into the soft media with a microbiological loop. The morphology of the bacteria spreading zones was evaluated with a naked eye.

All quantitative data were treated statistically with the Microsoft Office Excel 2003 software package (11.6355.6360) SP1. Confidence intervals were determined at a 95% significance level.

RESULTS AND DISCUSSION

Study of *A. brasilence* Sp245 cell behavior in semisolid media with varying nitrogen content. Under aerobic conditions in nitrogen-free semisolid media with all agar concentrations studied (0.2–0.6%), cells of the Sp245 strain formed colonies of greater diameter than those formed in the presence of ammonia, nitrate, or nitrite (Fig. 1a). The edge of the colony is formed by bacterial cells migrating within the medium (2–3 mm from the surface). Disks of smaller diameter within the colony area are formed on the agar surface. After 36 h of incubation in a nitrogen-free medium, bacterial cells migrate 3.7 times faster in the depth of the medium than on the surface, and while in the presence of ammonia, nitrate, or nitrite, this difference is only a factor of 1.4–1.6 (Fig. 1b). After 72 h of incubation in a medium lacking bound nitrogen, motility in the depth differs from that on the surface by a factor of 2.3, while in the presence of nitrogen sources the difference remains at the same level of 1.4–1.6. Apparently, since azospirilla are microaerophilic [2], in the

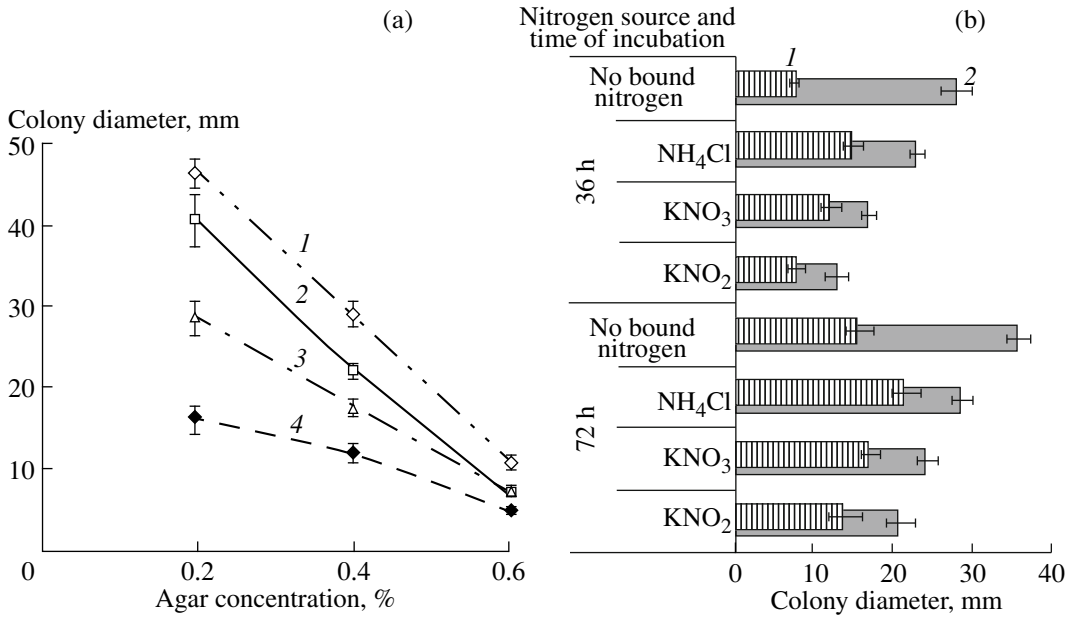

Fig. 1. Characteristics of the colonies formed by *A. brasilense* Sp245 after stab inoculation of cell suspensions in semisolid media containing various nitrogen sources (1 g/l): (a) 0.2, 0.4, and 0.6% agar media with no nitrogen sources (*1*), or in the presence of NH_4Cl (*2*), KNO_3 (*3*), and KNO_2 (*4*); incubation time was 36 h; (b) 0.4%-agar media: colony diameter on the agar surface (*1*) and colony diameter in the agar interior (*2*); incubation time was 36 and 72 h.

absence of nitrogen they form colonies mainly in the microniche favorable for nitrogenase functioning, which is in the agar interior. The presence of bound nitrogen including its accumulation during dinitrogen fixation promotes the development of colonies on the surface.

In the media containing 0.2–0.4% agar, a decrease in colony size was observed in the series ammonium–nitrate–nitrite (Figs. 1a, 1b). Maximum colony diameter in semisolid MSM (agar concentration 0.4%) containing ammonium chloride or potassium nitrate is concentration-independent within the range of 1–3 g/l salt.

The change in ammonium or nitrate concentration in the medium doesn't influence the individual motility of Sp245 cells. At 1 or 3 g/l salt, the rate of individual cell motility was 28.6 ± 1.5 and 28.6 ± 1.4 μm/s, respectively in the presence of ammonium chloride and 29.9 ± 1.5 and 29.8 ± 1.4 μm/s in the presence of potassium nitrate. Motility of the cells grown on a medium containing potassium nitrite decreased slightly with increasing the nitrite concentration. Cells grown in the presence of 1 and 3 g/l KNO_2 moved with an average individual speed of 24.4 ± 1.2 and 22.4 ± 1.4 μm/s, respectively. However, the colony diameter wasn't influenced by the salt concentration.

Therefore, in an MSM containing 0.2–0.4% agar and ammonium, nitrate, or nitrite, individual cell motility isn't the only factor determining the colony size. Probably, the nitrogen source present in the medium affects the interactions responsible for the contacts between migrating bacteria, and therefore, the colony diameter. In the medium with 0.6% agar, the differences in colony size were leveled (Fig. 1a), which is probably due to a tighter cell contact in dense media compared to the 0.2–0.4% agar media, and a decreased role of the interactions modulated by the nitrogen source.

Behavior of the $LpsI^-$ $LpsII^-$ Cal^- Sp245.5 [11] mutant in a semisolid medium is of particular interest. Novel OPS of the mutant consists of disaccharide units lacking rhamnose (according to a personal communication by Fedonenko et al.). Mutant cells form "diffuse" colonies while wide type bacteria form distinct swarming circles (Fig. 2). Most probably, the colony phenotype of strain Sp245.5 is caused by the principal change in the structure of surface polysaccharides which unbalances important carbohydrate–carbohydrate and protein–carbohydrate interactions.

Intercellular contacts are mediated by the components of the bacterial surface, in particular, protein and polysaccharide structures. The level of production and properties of the surface polymers in azospirilla may vary depending on the nitrogen source in the medium; this, in turn, affects the ability of cells to induce hemagglutination [7, 8].

Hemagglutinating activity of *A. brasilense* Sp245 and its mutants with various alterations in polysaccharide synthesis. Agglutination of trypsinized erythrocytes was induced by liquid bacterial cultures of Sp245 ($A_{590} = 0.5$) grown under stationary conditions in the medium with 1 g/l ammonium chloride, by cell suspensions in PBS ($A_{590} = 0.5$), and by a bacteria-free liq-

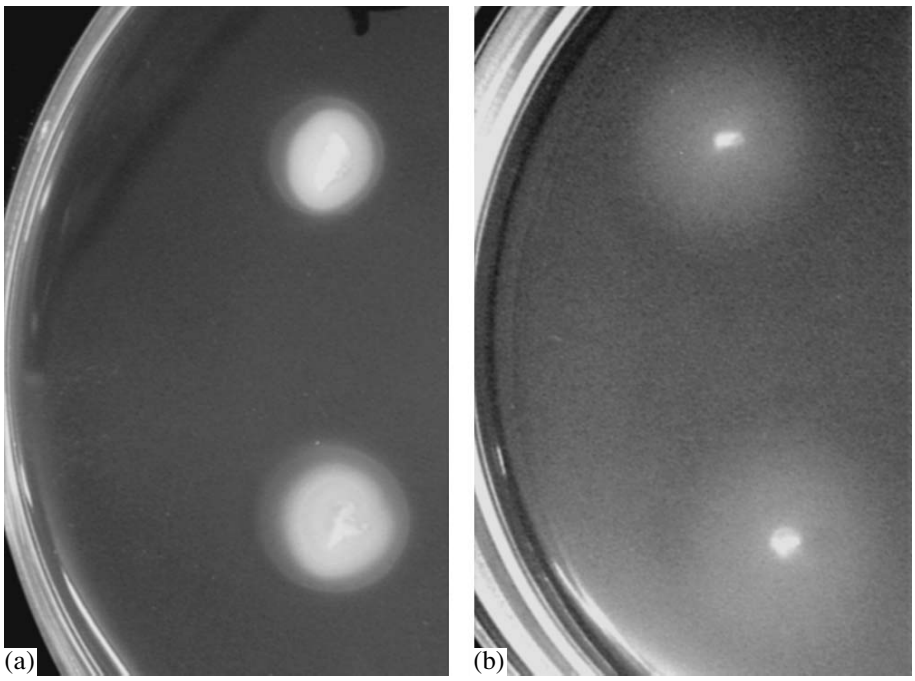

Fig. 2. Colonies formed by strain *A. brasilense* Sp245 and its LpsI$^-$ LpsII$^-$ Cal$^-$ mutant Sp245.5 after stab inoculation of cell suspensions in semisolid media containing ammonium chloride (1 g/l): Sp245 (a) and Sp245.5 (b). Agar concentration was 0.4%; incubation time was 36 h.

uid culture. The agglutination titer was 1 : 4 in each case.

The same value of hemagglutination titer may be due to the differences in the binding degree of hemagglutinins to the bacterial cell surface and to the characteristics of the complexes formed by hemagglutinins with other molecules, which do not participate in the reaction directly, but affect the activity of agglutinins.

Cells of strain Sp245 (A_{590} = 0.5) treated with trypsin for 8 h lost their ability to agglutinate trypsinized erythrocytes. Bacterial agglutination activity towards native erythrocytes carrying protein receptors sensitive to trypsin is affected by mutations in polysaccharide synthesis of strain Sp245. In wild type strain Sp245, LpsI contains acidic OPS-I and LpsII contains neutral OPS-II, while both OPS are identical linear polymers of D-rhamnose [15]. Supposedly, the negative charge of OPSI is due to labile components which may be destroyed in the process of the OPS isolation and purification during its preparation for chemical analysis or to admixtures of the core oligosaccharide which are difficult to remove (according to personal communication by Fedonenko et al.). In cell suspensions (A_{590} = 1.0 in PBS) of LpsI$^-$ Cal$^-$ mutant KM252 and LpsI$^-$ mutant KM348, as well as in the parent strain, a titer of the agglutination reaction was 1 : 16. Agglutination titers of the mutants LpsII$^-$ KM139 and LpsI$^-$ LpsII$^-$ Cal$^-$ Sp245.5 were 1 : 4 and 1 : 32, respectively. Therefore, loss of merely LpsI$^-$ did not affect the bacterial hemagglutination activity, while bacteria lacking LpsII$^-$ exhibited lower hemagglutinating activity than Sp245; principal changes in the LPS structure led to an increase in hemagglutinating activity. Defects in the synthesis of polysaccharides responsible for calcofluor binding do not affect agglutinating activity if LpsII synthesis is preserved. The difference in hemagglutinating activity of the mutants is probably determined not only by the differences in their polysaccharide structure, but also by the different effect of these polysaccharides on the activity of hemagglutinins. The data obtained is evidence that hemagglutinins of Sp245 are both localized on the surface of cells and secreted into the environment, and cells' hemagglutinating activity is affected by the structures of a protein and polysaccharide nature.

The ability of Sp245 cultures to cause erythrocyte agglutination depends upon the source of bound nitrogen present in the growth medium. A titer of agglutination of trypsinized erythrocytes by liquid cultures of Sp245 (A_{590} = 0.5) grown under stationary conditions in the presence of 1 g/l potassium nitrate or nitrite was 1 : 16, and in the presence of 1 g/l ammonium chloride, it was 1 : 4. Change in the content of ammonium chloride in the medium (1, 3, or 0.05 g/l) did not affect the agglutinating activity of strain Sp245. Earlier, it was reported [18] that in case of strain Sp245, exclusion of ammonium from the growth medium led to a decrease in bacterial hemagglutinating activity.

On the contrary, among *A. brasilense* Sp7 cultures only the cells grown in media with a low nitrogen con-

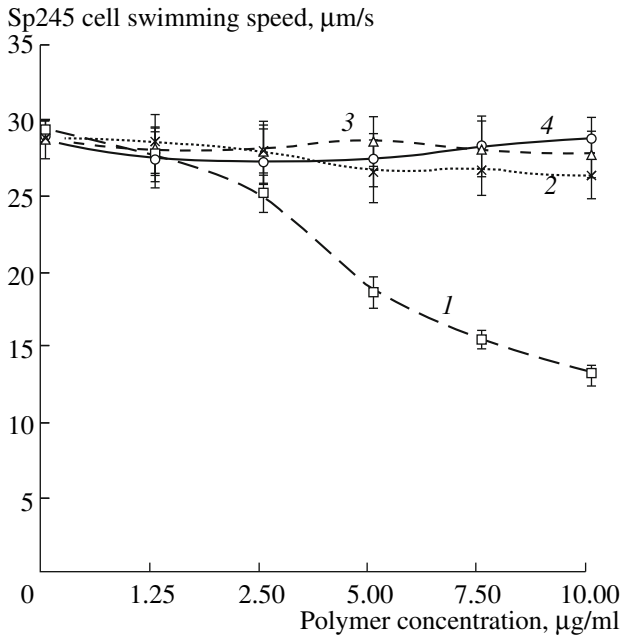

Fig. 3. Changes in cell motility of *A. brasilense* Sp245 after the addition of polymers isolated from the surface of Sp245 cells into the PBS: HA (*1*), LPS (*2*), PSLC (*3*), and LPPC (*4*). Time of incubation was 1 min.

tent (for example, 0.05 g/l KNO_3) were capable of hemagglutination [7]. Bacteria of the strain grown in nitrogen-rich media also synthesized the L-fucose and D-galactose-specific agglutinin with a blocked hemagglutinating activity [7]. One may assume that strains Sp245 and Sp7 differ in the mechanisms of agglutinin synthesis and activity regulation.

Isolation of *A. brasilense* Sp245 hemagglutinin and evaluation of its specificity. Hemagglutinin isolated from the surface of *A. brasilense* Sp245 cells and purified with gel-filtration was a homogeneous polymer of 67 kDa. When a technique used to stain the gel with silver nitrate included the step of monosaccharide oxidation [13], staining of the HA band was observed, indicating that HA is probably a glycoprotein which is typical of azospirilla lectins [7].

Specificity of Sp245 agglutinin was determined by carbohydrate inhibition of hemagglutination reaction. None of the 35 used mono- and disaccharides and amino sugars inhibited the hemagglutinating activity of the protein. However, LPPC and LPS isolated from the surface of Sp245 cells at a concentration of 50.5 μg/ml suppressed the activity of 1.0 μg/ml HA towards trypsinized erythrocytes. Among two OPS discovered in Sp245 [10, 15], only the acidic OPS-I at 25 μg/ml inhibited the HA activity (1.0 μg/ml). It should be noted here that LPPC, LPS, OPS-I, and OPS-II in the concentration range of 500–25 μg/ml did not induce agglutination of trypsinized erythrocytes. Only 500 μg/ml PSLC induced agglutination of trypsinized erythrocytes. When its concentration was decreased to 250–25 μg/ml, no erythrocyte agglutination occurred. However, at concentrations of 250–25 μg/ml PSLC did not block the activity of 1.0 μg/ml HA.

Therefore, HA isolated from the surface of Sp245 cells is a lectin exhibiting affinity towards OPS-I (an acidic D-rhamnan) and towards OPS-I-containing LPPC and LPS.

Increased hemagglutinating activity and decreased diameter of swarming circles in media containing nitrate or nitrite are in accordance with the supposition that colony size is influenced by intercellular contacts mediated by interactions between the HA and polysaccharides.

Determination of *A. brasilense* motility in the presence of surface polymers of strain Sp245. The addition of HA to a suspension of Sp245 cells led to a significant decrease in bacterial motility in liquid media (Fig. 3). The effect was retained during incubation for over 30 min. HA decreased the bacterial migration rate and affected the quantity of motile cells. At the HA concentration of 0, 5, 7.5, and 10 μg/ml, the number of motile cells was 83.3 ± 1.7, 60.2 ± 3.3, 29.1 ± 3.1, and 25.5 ± 3.1 percent, respectively. The presence of LPS, LPPC, or PSLC did not affect bacterial motility (Fig. 3).

Mobility investigation in Sp245 mutants with impaired polysaccharide production in the presence of 5 μg/ml HA demonstrated that it did not affect the mobility of the Sp245.5 mutant characterized by total rearrangement of the polysaccharide structure. HA induced an insignificant decrease in the motility rates of KM252 and KM348 mutants lacking OPS-I and decreased the motility of KM139 mutant cells which had lost OPS-II (Fig. 4a).

HA preincubation with LPS, LPPC, and OPS-I inhibited its influence on cell motility. In the presence of PSLC or OPS-II the effect of HA on the cell's motility rate was by 9.3 and 12.6% less pronounced in comparison with the unblocked protein (Fig. 4b). In strain Sp245, similar monosaccharides and antigens identical to those of OPS-I comprise LPS, LPPC, and PSLC [10, 11, 15, 16]. Antigen determinants of the polar flagellum sheath of the Sp245 strain are identical to those of the LPS [5]. Apparently, the decrease in cellular motility in the presence of HA is stipulated not only by its interaction with the cell, but also, with the polar flagellum responsible for bacterial movement. An additional proof of the specificity of HA interaction with the Sp245 cell polysaccharides and flagellum sheath is the absence of motility changes upon HA addition to the cells of *A. brasilense* Sp7 (Fig. 4a). The antigen structure of the LPS and flagellum sheath of strains Sp7 and Sp245 differ [5].

The results of the mutants' motility measurements and data of the inhibitory analysis strongly suggest considerable affinity between the HA and LPS of Sp245. Monosaccharides rhamnose, glucose, galactose, and glucosamine comprising LPS, LPPC, and PSLC [15–

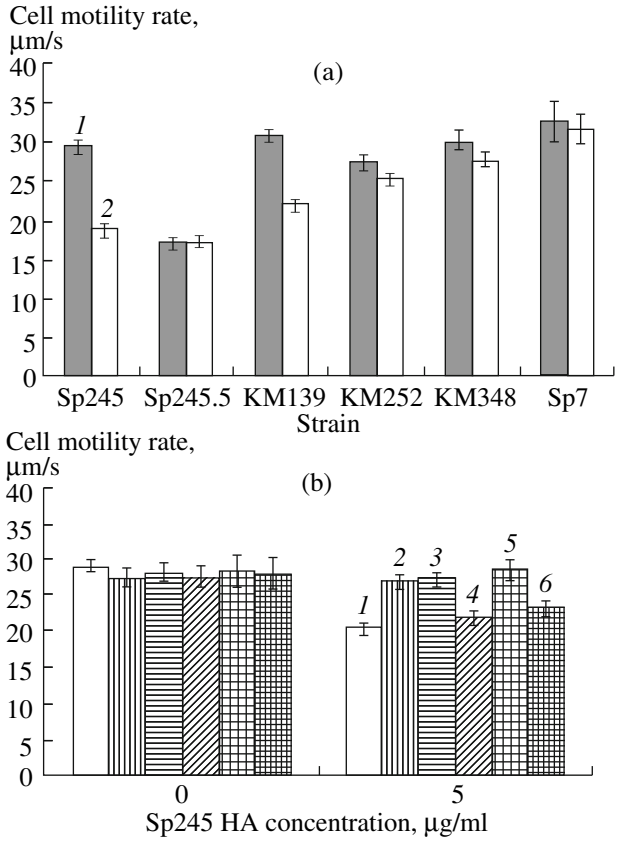

Fig. 4. Effect of the polymers isolated from the surface of Sp245 cells on the motility of *A. brasilense* in liquid media: (a) cellular motility of various azospirilla strains in PBS (*1*), PBS + HA (*2*), HA concentration was 5 μg/l, time of incubation, 1 min, (b) imotility of Sp245 cells in PBS (*1*), PBS + LS (*2*), PBS + LPPC (*3*), PBS + PSLC (*4*), PBS + OPSI (*5*), and PBS + OPSII (*6*).

17, 19] did not inhibit the influence of HA on motility. It is noteworthy that rhamnose was in its L-form. Meanwhile, OPS-I and OPS-II being linear D-rhamnans [15], suppressed (to a varying extent) the influence of HA on the motility of strain Sp245. The degree of HA affinity to OPS is probably defined by the conformation and charge of the latter. Acidic OPS-I completely neutralized the effect of HA on Sp245 motility, while the neutral OPS-II inhibited the HA activity only by 12.6%. The absence of OPSII in the KM139 mutant did not allow HA to decrease the mutant's cell motility to the same extent as in the wild type strain. The effect of agglutinin on the mutant's motility was by 12% less pronounced compared to the wild type strain (Fig. 4a). Only in the case of the Sp245.5 mutant lacking the OPS composed of D-rhamnan, cell motility did not change in the presence of HA (Fig. 4). In the semisolid media Sp245.5 formed "diffuse" colonies while the wild type strain formed distinct swarming circles (Fig. 2). "Diffuse" colonies were formed in spite of the fact that the hemagglutinating activity of the Sp245.5 cell suspension in PBS exceeded the value for Sp245 by a factor of 2 (1 : 32 in the mutant and 1 : 16 in the parent strain).

Therefore, intensity of the cellular motility of azospirilla in semisolid media is controlled by both the cell migration rate and the intercellular contacts mediated by interactions between HA and OPS.

Literature data are evidence that contacts between bacteria cells spreading on agar media are mediated by protein structures and LPS. For example, in *Myxococcus xanthus* carbohydrate–protein interactions are necessary for a collective, coordinated gliding of the cells and formation of supracellular structures (during aggregation and sporulation) [20, 21].

The results of the present work for the first time describe the role of intercellular contacts mediated by the interactions between HA and OPS in azospirilla spreading which employs the movement of flagella. Such interactions between bacteria are modulated in particular by the nature of the nitrogen source in the medium and availability of oxygen, which in turn may be of importance in the process of bacterial colonization of the host-plant's root system.

ACKNOWLEDGMENTS

The work was supported by a grant from the Russian Foundation for Basic Research (project no. 06-04-48204-a) and a grant from the President of the Russian Federation (project no. NSh-3171.2008.4).

REFERENCES

1. Katsy, E.I., *Molekulyarnaya genetika assotsiativnogo vzaimodeistviya bakterii i rastenii: sostoyanie i perspektivy issledovanii* (Molecular Genetics of Associative Interactions between Bacteria and Plants: Current Status and Prospects of the Studies), Ignatov, V.V,, Ed., Moscow: Nauka, 2007.

2. Tarrand, J.J., Krieg, N.R., and Dobereiner, J., A Taxonomic Study of the *Spirillum lipoferum* Group with Description of a New Genus, *Azospirillum* gen. nov., and Two Species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum braslense* sp. nov., *Can. J. Microbiol.*, 1978, vol. 24, no. 8, pp. 967–980.

3. Shelud'ko, A.V. and Katsy, E.I., Formation of Polar Bundles of Pili and the Behavior of *Azospirillum brasilense* Cells in a Semiliquid Agar, *Mikrobiologiya*, 2001, vol. 70, no. 5, pp. 662–667 [*Microbiology* (Engl. Transl.), vol. 70, no. 5, pp. 570–575].

4. Shelud'ko, A.V., Borisov, I.V., Krestinenko, V.A., Panasenko, V.I., and Katsy, E.I., Effect of Congo Red on the Motility of the Bacterium *Azospirillum brasilense, Mikrobiologiya*, 2006, vol. 75, no. 1, pp. 62–69 [*Microbiology* (Engl. Transl.), vol 70, no. 1, pp. 48–55].

5. Burygin, G.L., Shirokov, A.A., Shelud'ko, A.V., Katsy, E.I., Shchegolev, S.Yu., and Matora, L.Yu., Detection of a Sheath on *Azospirillum brasilense* Polar Flagellum, *Mikrobiologiya*, 2007, vol. 76, no. 6, pp. 822–829 [*Microbiology* (Engl. Transl.), vol 76, no. 6, pp. 728–734].

6. Nikitina, V.E., Ponomareva, E.G., Alen'kina, S.A., and Konnova, S.A., The Role of Cell-Surface Lectins in the Aggregation of Azospirilla, *Mikrobiologiya*, 2001, vol. 70, no. 4, pp. 471–476 [*Microbiology* (Engl. Transl.), vol 70, no. 4, pp. 408–412].

7. Nikitina, V.E., Alen'kina, S.A., Ital'yanskaya, Yu.V., and Ponomareva, E.G., Purification and Comparison of Lectins from the Cell Surface of Azospirilla Cells Active and Inactive in Hemagglutination, *Biokhimiya*, 1994, vol. 59, no. 5, pp. 656–662.

8. Yagoda-Shagam, J., Barton, L.L., Reed, W.P., and Chiovetti, R., Fluorescein Isothiocyanate-Labeled Lectin Analysis of the Surface of the Nitrogen-Fixing Bacterium *Azospirillum brasilense* by Flow Cytometry, *Appl. Environ. Microbiol.*, 1988, vol. 54, no. 7, pp. 1831–1837.

9. Baldani, V.L.D., Baldani, J.I., and Döbereiner, J., Effects of *Azospirillum* Inoculation on Root Infection and Nitrogen Incorporation in Wheat, *Can. J. Microbiol.*, 1983, vol. 29, no. 8, pp. 924–929.

10. Katzy, E.I., Matora, L.Yu., Serebrennikova, O.B., and Scheludko, A.V., Involvement of a 120-MDa Plasmid of *Azospirillum brasilense* Sp245 in the Production of Lipopolysaccharides, *Plasmid*, 1998, vol. 40, no. 1, pp. 73–83.

11. Katsy, E.I., Borisov, I.V., Petrova, L.P., and Matora, L.Yu., The Use of Fragments of the 85- and 120-MDa Plasmids of *Azospirillum brasilense* Sp245 to Study the Plasmid Rearrangement in This Bacterium and to Search for Homologous Sequences in Plasmids of *Azospirillum brasilense* Sp7, *Genetika*, 2002, vol. 38, no. 2, pp. 182–189 [*Russ. J. Genetics* (Engl. Transl), vol. 38, no. 2, pp. 124–131].

12. Döbereiner, J. and Day, J.M., Associative Symbiosis in Tropical Grasses: Characterization of Microorganisms and Dinitrogen Fixing Sites, in *Symposium on Nitrogen Fixation*, Newton, W.E. and Nijmans, C.J., Eds., Pullman: Washington State Univ. Press, 1976, pp. 518–538.

13. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual. Second ed.*, New York: Cold Spring Harbor Lab. Press, 1989, vol. 1–3.

14. Tsai, C.M. and Frasch, C.E., A Sensitive Silver Stain for Detecting Lipopolysaccharides in Polyacrylamide Gels, *Anal. Biochem.*, 1982, vol. 119, no. 1, pp. 115–119.

15. Fedonenko, Yu.P., Zdorovenko, E.L., Konnova, S.A., Ignatov, V.V., and Shlyakhtin, G.V., A Comparison of the Lipopolysaccharides and O-Specific Polysaccharides of *Azospirillum brasilense* Sp245 and Its Omegon-Km Mutants KM018 and KM252, *Mikrobiologiya*, 2004, vol. 73, no. 2, pp. 180–187 [*Microbiology* (Engl. Transl.), vol 73, no. 2, pp. 143–149].

16. Konnova, S.A., Makarov, O.E., Skvortsov, I.M., and Ignatov, V.V., Exopolysaccharides of the Bacterium *Azospirillum brasilense* Sp245 and Sp107, *Mikrobiol. Zh.*, 1992, vol. 54, no. 1, pp. 31–42.

17. Fedonenko, Yu.P., Egorenkova, I.V., Konnova, S.A., and Ignatov, V.V., Involvement of the Lipopolysaccharides of Azospirilla in the Interaction with Wheat Seedling Roots, *Mikrobiologiya*, 2001, vol. 70, no. 3, pp. 384–390 [*Microbiology* (Engl. Transl.), vol 70, no. 3, pp. 329–334].

18. Antonyuk, L.P., Plant Lectins as Communication Factors in Symbioses, in *Molekulyarnye osnovy vzaimootnoshenii assotsiativnykh mikroorganizmov s rasteniyami* (Molecular Basis of the Relationships between Associative Microorganisms and Plants), Ignatov, V.V., Ed., Moscow: Nauka, 2005, pp. 118–159.

19. Skvorsov, I.M. and Ignatov, V.V., Extracellular Polysaccharides and Polysaccharide-Containing Biopolymers from *Azospirillum* Species: Properties and the Possible Interaction with Plant Roots, *FEMS Microbiol. Letts.*, 1998, vol. 165, no. 2, pp. 223–229.

20. Bowden, M.G. and Kaplan, H.B., The *Myxococcus xanthus* Lipopolysaccharide O-Antigen Is Required for Social Motility and Multicellular Development, *Mol. Microbiol.*, 1998, vol. 30, no. 2, pp. 275–284.

21. Will, D., Wu, S.S., and Kaiser, D., Contact Stimulation of Tgl1 and Type IV Pili in *Myxococcus xanthus, J. Bacteriol.,* 1998, vol. 180, no. 3, pp. 759–761.