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EXPERIMENTAL ARTICLES

The Role of Cell-Surface Lectins in the Aggregation of Azospirilla

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Abstract—The mutant strain *Azospirillum brasilense* Sp7.2.3 with impaired lectin activity exhibited poorer cell aggregation than its parent strain *A. brasilense* Sp7(S) both in the exponential and stationary growth phases. The pretreatment of bacterial cells with the specific haptens (L-fucose and D-galactose) of a lectin located at the cell surface of the mutant strain was found to inhibit the aggregation of azospirilla. The specific binding of the *A. brasilense* Sp7(S) lectin to the extracellular polysaccharide-containing complexes of this strain was revealed by dot immunoblotting on nitrocellulose membrane filters. The interaction of the lectins of *A. brasilense* 75, *A. brasilense* Sp7, and *A. lipoferum* 59b with the polysaccharide-containing complexes that were isolated from these strains was not specific. No interstrain cross-interaction between the exopolysaccharides and lectins of azospirilla was found. A coflocculation of *A. brasilense* Sp7 cells with *Bacillus polymyxa* 1460 cells was shown. The involvement of autogenous lectins in the aggregation of bacterial cells is discussed.

Key words: lectin, azospirilla, polysaccharides, aggregation.

The capability for aggregation is widespread among microorganisms including free-living nitrogen-fixing bacteria of the genus *Azospirillum* which aggregate and flocculate under certain conditions [1, 2]. Azospirilla can form cysts and cell aggregates, which provide for their viability in soil under unfavorable conditions. The formation of bacterial aggregates (the so-called flocculation) also promotes the inoculation of plants and is useful in biotechnological practice, facilitating the separation of cells from the cultivation medium. The mechanism of intercellular adhesion and the molecules involved in this process have been extensively investigated in recent years. The important role of capsular polysaccharides, exopolysaccharides, and proteins in the aggregation of azospirilla have been reported [3, 4]. The importance of extracellular proteins was also shown in the adhesion of azospirilla to abiotic surfaces and in the aggregation of various bacterial species isolated from different sources. Some proteins (presumably lectins) of the outer membrane of azospirilla may be involved in the aggregation of *A. brasilense* cells [5, 6]. Earlier, we isolated lectins from the outer membranes of *A. brasilense* Sp7, *A. brasilense* 75, *A. lipoferum* 43, and *A. lipoferum* 59b, which were not associated with the pili [7]. These lectins differed in their specificity towards carbohydrates and represented glycoproteins with subunit molecular masses ranging from 36 to 43 kDa.

The aim of the present work was to study the role of these lectins in the aggregation of bacteria.

MATERIALS AND METHODS

Azospirillum brasilense Sp7 was obtained from the Institute of Microbiology of the Russian Academy of Sciences in Moscow. This strain underwent a spontaneous mutation as a result of its long-term maintenance on rich media [8] and is designated in this work as *A. brasilense* Sp7(S). The strain *A. brasilense* Sp7 (ATCC 29145) was kindly provided by D. Janssens (Rijksuniversiteit, Gent. Lab. Voor. Microbiol. in Gent, Belgium); the strain *A. lipoferum* 59b (B-1519) was obtained from the VKM (All-Russia Collection of Microorganisms); the strains *A. brasilense* 75 and *A. lipoferum* 43 were isolated from the wheat cultivar Saratovskaya 29 roots at the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences; the strain *A. brasilense* Sp7.2.3 was derived by transposon mutagenesis from the type strain *A. brasilense* Sp7.

To obtain lectins, the strain *A. brasilense* Sp7 and its mutant *A. brasilense* Sp7.2.3 were cultivated in synthetic medium [9] at 37°C. The cell-surface lectins were isolated and purified as described earlier [10].

To obtain polysaccharide-containing biopolymers, namely, lipopolysaccharide–protein complexes (LPPCs), polysaccharide–lipid complexes (PLCs), and lipopolysaccharides (LPSs), azospirilla were grown in a malate-

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containing synthetic medium in an ANKUM-2M fermentor (Soviet Union) to the end of the exponential growth phase. The medium composition and the cultivation conditions were described earlier [11]. The polysaccharide-containing material was stripped from the surface of cells by incubating them in 0.15 M NaCl under shaking for 72 h. To remove the low-molecular-weight impurities, the extract was dialyzed through a membrane with a molecular weight cutoff of 12–14 kDa. The nondiffusible material was subjected to gel filtration on a column with Sepharose C1-4B as described earlier [11]. The PLC fraction was additionally purified on a Q-2 cation exchanger in H^+ form and then by ion-exchange chromatography on a column with DEAE-Toyopearl 650M (Toyo Soda, Japan). LPSs were extracted from bacterial cells with a phenol solution and purified by chromatographic methods to an apparent homogeneity. The resultant preparations of polysaccharide-containing complexes were lyophilized.

To determine the monosaccharide composition of the complexes, they were hydrolyzed in 4 N CF_3COOH in sealed ampoules at 105°C for 4 h and the hydrolysates were evaporated to remove the acid. The residues were subjected to ascending thin-layer chromatography on cellulose plates in a pyridine–ethyl acetate–acetic acid–water (5 : 5 : 1 : 3) mixture. Sugars were visualized by spraying the developed plates with a solution of anisidine phthalate in butanol followed by their heating at 115°C.

The interaction of lectins with the polysaccharide-containing complexes was studied by dot immunoblotting on Synpor (Czech Republic) nitrocellulose membrane filters with a pore diameter of 1.5 μm. The immune complexes formed were detected using the protein A conjugate with colloidal gold [12].

Antibodies against azospirilla lectins were obtained by the hypodermic injection of rabbits with a mixture of purified lectin (80 μg/ml) with Freund's adjuvant. The injection was repeated three times at two-week intervals. The antiserum was purified by the precipitation of lipoproteins with ammonium sulfate [13].

Cell aggregation was evaluated according to Madi and Henis [14].

The coflocculation of azospirilla with other bacteria was studied using the method described by El-Behhari *et al.* [15].

The results were statistically processed by the Student's *t*-test.

RESULTS AND DISCUSSION

The comparative study of the strain *A. brasilense* Sp7(S) and its mutant *A. brasilense* Sp7.2.3 with impaired lectin activity showed that their levels of cell aggregation are 38 and 23%, respectively; i.e., the aggregation of the mutant cells is about 40% weaker than that of the parent cells.

Table 1. Percentage of the cell aggregation of *A. brasilense* Sp7 and its lectin-deficient mutant *A. brasilense* Sp7.2.3 as a function of the culture age

Strain	Culture age, h			Significance level, *P*
	12	24	48	
A. brasilense Sp7	53 ± 1	38 ± 2	24 ± 2	<0.05
A. brasilense Sp7.2.3	19 ± 1	23 ± 2	18 ± 1	>0.05

The effect of the culture age on the composition of the cell surface of bacteria has been reported by many researchers. In view of this, we studied cell aggregation in the mid-exponential (12 h of cultivation), the late-exponential (24 h), and the stationary (48 h) growth phases. As can be seen from Table 1, the aggregation of *A. brasilense* Sp7 cells was as high as 53% after 12 h of cultivation and then gradually decreased to 24% in the 48th h. The aggregation of *A. brasilense* Sp7.2.3 cells was poor within 12 h of growth, reached a maximum (24%) after 24 h of cultivation, and decreased to 18% in the 48th h. Therefore, the aggregation of *A. brasilense* Sp7(S) is more intense than that of *A. brasilense* Sp7.2.3 and changes with the culture age. This finding indicates that the surface lectin of *A. brasilense* Sp7(S) cells promotes their aggregation. The pretreatment of *A. brasilense* Sp7(S) cells with L-fucose and D-galactose, the specific lectin haptens of this strain, resulted in a considerable inhibition of cell aggregation. However, after cell aggregates have been formed, the addition of the haptens did not lead to the breakup of the aggregates.

These results indicate the involvement of the *A. brasilense* Sp7(S) lectins in the aggregation of bacterial cells. Taking into account the ability of all lectins to bind to carbohydrates, it can be assumed that the mechanism of the aggregation of azospirilla may involve the interaction between the cross-reactive antigens (lectins and polysaccharides) occurring on the bacterial surface and in the capsular material. According to our earlier observations, the polysaccharide-containing complexes LPPC and PLC are components of the capsular material of azospirilla and are released into the environment [11]. The carbohydrate moiety of the LPS of gram- negative bacteria is also exposed to the environment. The complexes contain various heteropolysaccharides; for instance, the polysaccharide PS-1 (Table 2) with a molecular mass of about 20 kDa was derived from the PLC by means of its hydrolysis. At the same time, the polysaccharide PS-2 with a molecular mass of about 10 kDa was produced by azospirilla in free form. The monosaccharide composition of all the glycans studied is given in Table 2.

The interaction of the lectins of *A. brasilense* Sp7(S), *A. brasilense* 75, and *A. lipoferum* 59b with different polysaccharide-containing complexes was studied by using the dot immunoblotting technique. The

Table 2. Characteristics of the lectins and polysaccharide-containing complexes of azospirilla

Strains	Specific haptens of lectins	Complexes	Monosaccharide composition of polysaccharides	Minimal concentration of polysaccharides, μg/ml	Specific inhibitor
A. brasilense Sp7(S)	*L*-Fucose,	LPPC		50	Gal
	D-Galactose	PLC		12.5	Gal
		PS-1	Rhamnose, galactose, galacturonic acid	6.25	Gal
		LPS		No binding	–
		PS-2	Galactose, glucose	1.56	Gal
A. brasilense 75	*L*-Arabinose	LPPC	Rhamnose, galactose, glucosamine, galactosamine	0.06	–
	L-Rhamnose, *D*-Mannose	PLC	Rhamnose, galactose, galacturonic acid, glucosamine, galactosamine	0.012	–
A. lipoferum 59b	*L*-Arabinose	LPPC	Rhamnose, glucose, glucosamine, galactosamine	0.20	–
	D-Mannose Glucuronic acid	PLC	Rhamnose, glucose, glucosamine	0.78	–

cells of all strains studied were found to be able to bind to their own polysaccharide complexes (except for LPS), which is indicative of the occurrence of at least one common antigen determinant. However, the interactions of different strains had some specific features. For instance, the interaction of the *A. brasilense* Sp7(S) lectin with the LPPC, PLC, PS-1, and PS-2 was completely inhibited by D-galactose, one of the two carbohydrate haptens of the lectin, indicating the specific character of binding. On the other hand, the interactions of the *A. brasilense* 75 and *A. lipoferum* 59b lectins with the LPPC and PLC were not inhibited by any of the specific haptens and, therefore, were not specific. The LPSs that were isolated from the bacterial membranes did not interact with the lectins of the azospirilla, which might be due to a large lipid component of the LPS molecule, hindering the LPS–lectin interactions. The fact that the lectins interact with the native LPS derived from the extracellular LPPC but do not interact with the LPS extracted from the outer bacterial membranes with a phenol solution indicates that these two types of LPSs are structurally different. The cross-binding between the lectins and the polysaccharide complexes of different *Azospirillum* strains was not observed, although some polysaccharides contained the lectin-specific carbohydrates. In particular, the polysaccharide complexes of *A. brasilense* Sp7, which contain considerable amounts of fucose [11], did not interact with the fucose-specific lectin of *A. brasilense* Sp7(S). This was obviously because fucose in the heteropolysaccharide molecule is not a terminal monosaccharide.

Thus, the lectins of azospirilla promote cell aggregation by binding, either specific or nonspecific, to only their own exopolysaccharides. The lower (in comparison with the PLC) necessary concentration of the PS-1 of *A. brasilense* Sp7(S) in the dot immunoblotting test suggests that the interaction between the PLC and bacterial lectins involves their carbohydrate components. According to earlier observations, the lectin of *A. brasilense* Sp7 taken at high concentrations is able to form high-molecular-weight aggregates. In this case, the interaction of the lectin with L-fucose and D-galactose is inhibited. The binding of the lectin molecules seems to occur at the sites of the D-galactose residues.

The aggregation of *A. brasilense* Sp7 cells was minimal at neutral pH values and became more intense when the pH was decreased to 5.0 or increased to 9.0 (Fig. 1). These results are in agreement with the observations made with *A. brasilense* Cd cells [13]. To elucidate whether or not the lectins of azospirilla can promote the aggregation and flocculation of other bacteria, we studied the coflocculation of *A. brasilense* Sp7 and *A. brasilense* Sp7.2.3 cells with *Micrococcus citreus* 27/1M, *B. polymyxa* 1460, and *Rhizobium leguminozarum* 1003 cells. The suspensions of bacterial cells in a phosphate-buffered saline containing 30 mg of wet biomass per 1 ml were mixed, and the degree of cell flocculation was measured after 24 h of incubation. No cell flocculation was observed when we mixed *A. brasilense* Sp7 and *M. citreus* 27/1M cells or *A. brasilense* Sp7.2.3 and *B. polymyxa* 1460 cells. The mixture of *A. brasilense* Sp7 and *R. leguminosarum* 1003 cells exhibited slight coflocculation. The most intense coflocculation was observed in a mixture of *A. brasilense* Sp7 and *B. polymyxa* 1460 cells (Fig. 2). These data show a high ability of *A. brasilense* Sp7 cells to coflocculate with *B. polymyxa* 1460 cells and the involvement of lectins in the coflocculation.

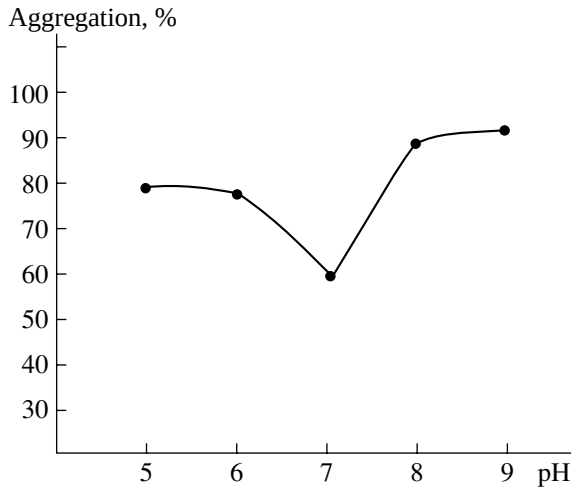

Fig. 1. Effect of pH on the aggregation of *A. brasilense* Sp7 cells.

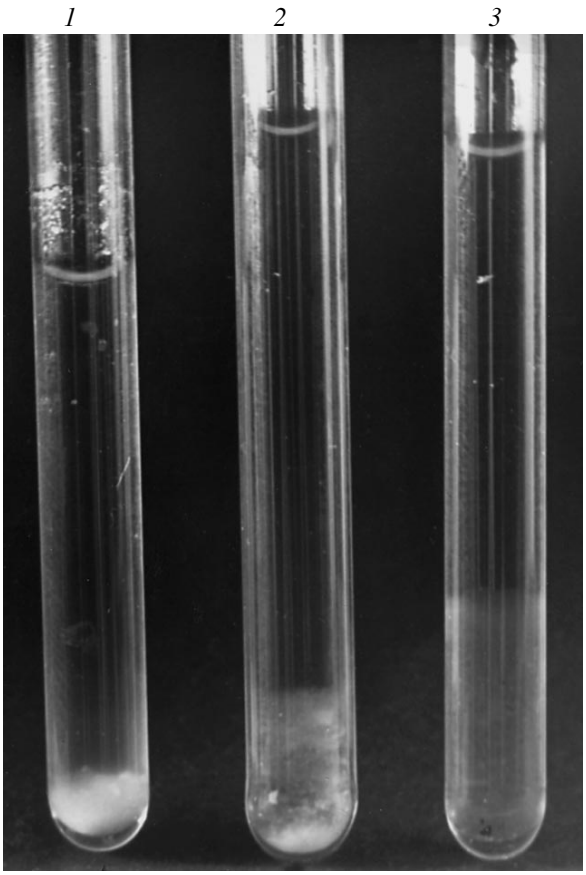

Fig. 2. Coflocculation of *A. brasilense* Sp7 cells with *B. polymyxa* 1460 cells: (1) a suspension of *A. brasilense* Sp7 cells; (2) a mixed suspension of *A. brasilense* Sp7 and *B. polymyxa* 1460 cells; and (3) a suspension of *B. polymyxa* 1460 cells.

Thus, this study showed that (1) the aggregation capacities of *A. brasilense* Sp7(S) and its mutant *A. brasilense* Sp7.2.3 considerably differ, (2) the treatment of bacterial cells with carbohydrate haptens inhibits their aggregation, (3) the lectin of *A. brasilense* Sp7(S) specifically binds to the extracellular polysaccharide-containing complexes of this strain but not of other *Azospirillum* strains, and (4) *A. brasilense* Sp7 cells readily coflocculate with *B. polymyxa* 1460 cells. These results suggest that the lectins of azospirilla may be involved in the cell aggregation. This suggestion is in agreement with the following data (which was reported earlier).

The lectins of azospirilla were found to stimulate the specific adhesion of cells to plant roots [16], suggesting that they may also be involved in the agglutination of bacterial cells. The lectin activity of azospirilla was found to depend on the nitrogen content of the cultivation medium, as high nitrogen concentrations inhibited the lectin activity [15]. Burdman and his coworkers demonstrated that the increased amounts of nitrogen-containing compounds in the medium (i.e., low C/N ratios) inhibit the aggregation of *A. brasilense* Cd and *A. brasilense* Sp7 cells [4].

In our earlier studies, we also found that the lectin activities of *A. brasilense* Sp7 and *A. lipoferum* 59b are stimulated under unfavorable growth conditions and, conversely, are inhibited under beneficial growth conditions [17, 18]. Based on these results, an important role of lectins in the adaptation of bacterial cells to unfavorable conditions was assumed. It should also be noted that *Azospirillum* cells tend to flocculate under stressful conditions [19].

To conclude, the involvement of the lectins of azospirilla in the cell aggregation seems to be justified, however, the involvement of other mechanisms cannot be excluded.

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