CURRENT MICROBIOLOGY Vol. 36 (1998), pp. 241-244

Current Microbiology
An International Journal

Cell-Surface Lectins of *Azospirillum* spp.

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Received: 23 June 1997 /Accepted: 23 September 1997

Abstract. Cell-surface lectins were screened in seven strains of *Azospirillum brasilense* and *A. lipoferum*. The presence of lectins was determined by particle agglutination assays employing latex beads coated with neoglycoproteins and by Western blot with neoglycoproteins labeled with horseradish peroxidase as a probe. Seven strains were agglutinated with the assayed sugar residues. The highest agglutination was with fucose and glucose and to a lesser extent with mannose residues. Cell-wall proteins extracted from two *Azospirillum* spp. strains exhibit lectin-like activities. We believe that lectins are present in the cell-wall of *Azospirillum* spp.

Azospirillum spp. are widely distributed plant-growth-promoting bacteria (PGPB) that enhance the development of many important crop and noncrop plants [3]. Root colonization is a prerequisite for the PGPB interaction with plants [11]. To colonize roots, bacteria must first recognize them, possibly via lectins.

In PGPB, cell-surface lectins are far less studied. Some lectin studies focused on plant lectins interacting with polysaccharide residues on the bacterial surface [13]. In *Azospirillum*-plant association, wheat germ agglutinin (WGA) interacting with *A. lipoferum* revealed two putative receptors in the bacteria capsular fraction [9]. WGA bound to A. *brasilense* Sp-245 was proposed as a signal molecule in the association wheat-*Azospirillum* [2]. Rice embryo lectin bound specifically to the nitrogenfixing bacteria *Beijerinckia* spp. and *A. lipoferum* 4 B, but did not interact with other bacteria from the rhizosphere of maize [12]. However, to the best of our knowledge, cell-surface lectins of *Azospirillum* spp. have not been reported. The aim of this study was to reveal the presence of cell-surface lectins in seven strains of *Azospirillum* spp.

Bacterial strains, culture conditions, and preparation of cells for binding tests. The seven *Azospirillum* spp. strains used in this study were: *A. brasilense* Sp-245; *A. brasilense* Cd (DSM 7030); *A. brasilense* Sp-6 (our laboratory collection); *A. lipoferum* 1842 (DSM 1842); *A. lipoferum* 37 and 779; and *A. lipoferum* JA2. Bacteria were maintained and preserved by the standard techniques for this genus [5]. For experiments, *Azospirillum* spp. strains were cultured in 50 ml of nutrient broth (NB) medium (Difco Laboratories, Detroit, MI) in 250-ml Erlenmeyer flasks and incubated at 30 ± 1°C in a rotary shaker at 120 rpm for 24 h. Cells were harvested by centrifugation at 3500 g for 10 min and washed once in PBS buffer (0.1 M potassium phosphate buffer, pH 6.8, supplemented with 0.15 M NaCI). Washed bacterial cells were resuspended in PBS buffer at a concentration of approximately 10^{10} cfu/ml and were immediately used in binding assays.

Biochemical analysis. Preparation of neoglycoproteincoated latex beads was done according to Amini et al. [1]. The prepared latex bead suspension was divided into 200-µl aliquots, and each aliquot was mixed with 100 0 of the following neoglycoproteins (separately) (0.5 mg/ml in PBS): ESA-fucosylamide, BSA-glucosamide, BSA-*p*-aminophenyl-*N*-acetyl-β-D-glucosamine, BSA-p-aminophenyl-N-acetyl-β-D-galactosamine, and BSA-D-mannopyranoside. These mixtures were incubated at 30 ± 1°C for 12 h in a horizontal shaker at 50 rpm to ensure covering of the latex beads with the neoglycoprotein. The mixtures were then centrifuged at 9200 *g* for 5 min at 20 ± 2°C, and the supernatants were discarded. The pellets containing latex beads conjugated with neoglycoproteins were resuspended in 2 ml of PBS buffer and kept at 4 ± 1 °C until used.

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Table 1. Binding of neoglycoprotein-coated latex beads to *Azospirillum* spp. cells in particle agglutination assay

Strain	Control BSA[a] or latex beads	Neoglycoproteins				
		BSA-fucosylamide	BSA-glucosylamide	BSA-galactosamide	N-acetyl β-D-glucosamine	N-acetyl D-mannose
A. brasilense Sp 245	0	3	3	2	2	3
A. brasilense Cd	0	3	3	2	2	3
A. brasilense Sp 6	0	2	2	0	1	1
A. lipoferum 1842	0	3	3	1	2	2
A. lipoferum 779	0	3	3	1	1	3
A. lipoferum JA2	0	3	3	1	3	3
A. lipoferum 37	0	3	3	1	1	3

Reactions were scored with the following index: 0, no agglutination; 1, weak agglutination; 2, apparent agglutination; and 3, strong agglutination, where almost all cells were agglutinated [1].
[a]Bovine serum albumin.

Particle agglutination assay (PAA). PAA was done essentially as described by Arnim et al. [1]. The reactions were scored by use of an index: 0, no agglutination; 1, weak agglutination; 2, apparent agglutination; and 3, strong agglutination, where almost all cells were agglutinated [1]. As controls, (i) strains were tested for autoaggregation by mixing 20 µl of bacterial cell suspension with 20 p1 of PBS buffer, (ii) 20 µl of bacterial cell suspension was mixed with 20 µl of 1 mg/ml bovine serum albumin (BSA), (iii) 20 µl of bacterial cell suspension was mixed with 20 µl of latex bead suspension, and (iv) 20 µl of bacterial cell suspension was mixed with 20 µl of latex beads coated with BSA.

Particle-agglutination inhibition assay (PAIA). The assay was done as follows: 100 µl of bacterial cell suspension were preincubated (separately) with 100 µl of 0.1 M arabinose, fucose, galactose, glucose, mannose, N-acetyl glucosamine, and N-acetyl galactosamine (Sigma, St. Louis, MO, USA) for 30 min at 22 ± 1°C. These bacterial mixtures were employed in the PAA as described above.

Extraction of cell-surface proteins from *A. brasilense* Cd and *A. lipoferum* 1842. Cells from fresh cultures of *Azospirillum* spp. were harvested either from solid Nutrient Agar plate cultures (five plates) or from 250 ml Nutrient Broth medium. Cell-surface proteins were extracted by the urea extraction technique [7].

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell-wall proteins. Cell-wall proteins from *A. lipoferum* 1842 and *A. brasilense* Cd strains were separated by the discontinuous buffer system of Laemmli [10] in a Protein II apparatus (Bio Rad, Hercules, CA, USA) with low molecular weight standards (Bio-Rad) for SDS-PAGE of proteins.

Neoglycoprotein labeling with horseradish peroxidase (HRP). This was done according to Harlow and Lane [8].

Blotting. The separated proteins from SDS-PAGE and the previously isolated 43-kDa hydrophobic protein from *A. lipoferum* 1842 [6] were electrophoretically transferred to a immobilon-nitrocellulose membrane (0.45 µm) (Sigma) in a semidry Trans-blot cell (Bio-Rad, Richmond, CA) [8].

Azospirillum spp., in general, are not plant-specific bacteria. Currently, it is proposed that there is only an affinity between the bacteria and the plant genotype. The possible involvement of lectins in the attachment process was proposed but not confirmed [4].

The seven *Azospirillum* strains were agglutinated by the five neoglycoproteins tested (Table 1). However, the levels of agglutination varied. Neoglycoproteins with fucose and glucose had the highest agglutination level followed by neoglycoproteins with mannose. BSA and the latex beads themselves, serving as controls, did not agglutinate *Azospirillum* spp. cells, and the cells themselves did not autoaggregate in this assay.

Results of agglutination inhibition assays were variable among the three selected strains tested (*A. brasilense* Sp-245, *A. lipoferum* 1842, and *A. lipoferum* 779). Complete inhibition of the agglutination of *A. brasilense* Sp-245 with the five neoglycoproteins was achieved only by arabinose. Other monosaccharides inhibited the agglutination only by D-mannopyranoside, N-acetyl-β-D-galactosamine and N-acetyl-β-D-glucosamine (Fig la). In both *A. lipoferum* strains, the inhibition affected mainly agglutination in the presence of N-acetyl-β-D-galactosamine and N-acetyl-β-D-glucosamine (Fig l b, c).

Blotting of cell-surface proteins from *A. lipoferum* 1842 and the partially purified hydrophobic protein of 43

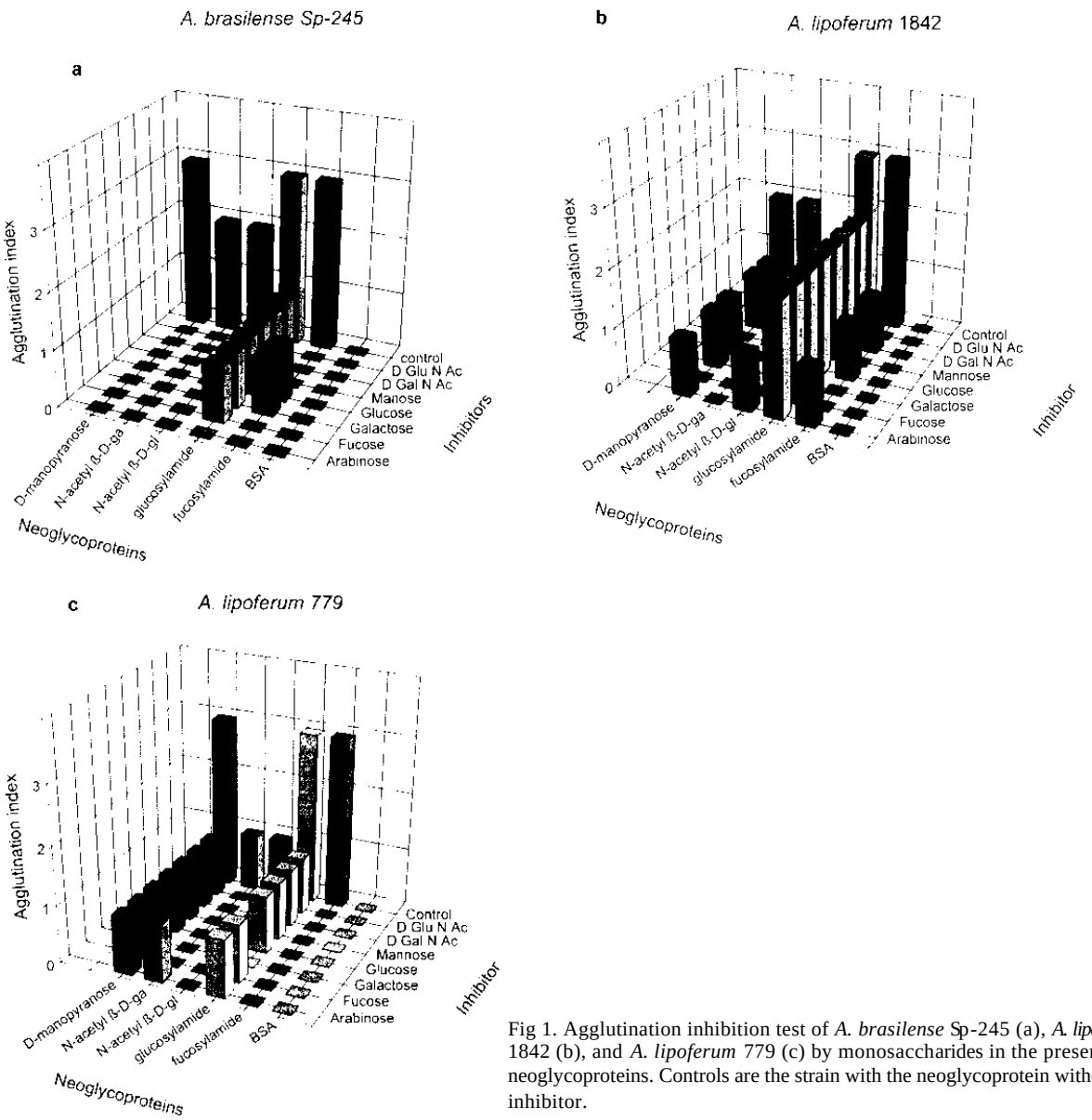

Fig 1. Agglutination inhibition test of *A. brasilense* Sp-245 (a), *A. lipoferum* 1842 (b), and *A. lipoferum* 779 (c) by monosaccharides in the presence of neoglycoproteins. Controls are the strain with the neoglycoprotein without an inhibitor.

kDa previously isolated from *A. lipoferum* 1842 [6] showed that HRP-BSA-galactosamide binds strongly to an immobilized protein of 43 kDa from *A. lipoferum* (Fig 2A, lane 2), and it bound to a lesser degree to proteins of different molecular masses (28, 39, 45, 48, 66, 68, 70 kDa) from the total extract of A. lipoferum (Fig 2A, lane 1). It also bound a protein of about 50 kDa from the cell wall of A. brasilense Cd (data not shown). HRP-BSA-glucosamide binds to a 43-kDa protein from *A. lipoferum* 1842 and to five additional proteins of approximately 38, 45, 66, 68, and 70 kDa, in addition to two proteins (33 and 47 kDa) from the cell wall of *A. brasilense* Cd (data not shown). HRP-BSA-mannopyranoside also binds to a 43-kDa protein (Fig 2C, lane 2) and to an additional 10 proteins from 15 to 50 kDa of *A. lipoferum* 1842 (Fig 2C, lane 1). HRP-BSA-fucosylamide binds strongly to the same proteins as HRP-BSA-mannopyranoside (Fig 2D, lane 2). BSA-cellobiosyl and BSA-melibiosyl were tested, and no binding was observed (data not shown).

Our data demonstrate (i) *Azospirillum* spp. cells can interact with several carbohydrate residues and (*ii*) the neoglycoprotein-labeling experiments revealed the pres-

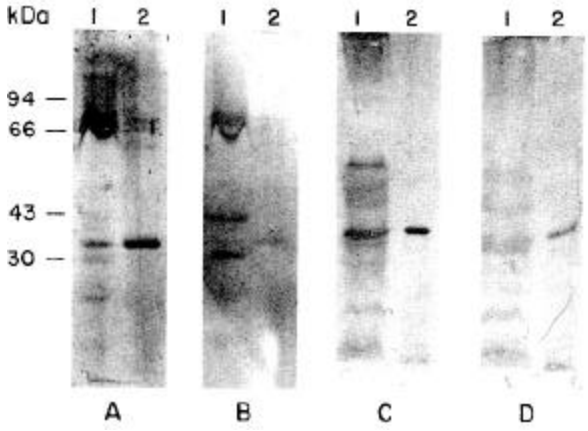

Fig 2. Western Blot analysis of *A. lipoferum* 1842 cell-wall proteins with affinity for HRP-labeled neoglycoproteins. (1) Protein extract of cell wall of *A. lipoferum* 1842. (2) Partially purified 43-kDa hydrophobic protein of cell wall of *A. lipoferum* 1842. (A) HRP-BSA-galactosamide. (B) HRP-BSA-glucosamide. (C) HRP-BSA-mannopyranoside. (D) HRP-BSA-fucosylamide.

ence of several lectin-like proteins on the bacterium cell-wall surface. It is yet to be demonstrated whether these lectins participate in the nonspecific recognition of root surfaces by *Azospirillum* spp. cells.

ACKNOWLEDGMENTS

We thank Mrs. Maria de Jesus Romero for excellent technical assistance and Dr. Ellis Glazier for constructive English corrections. We thank the following scientists for donating strains of *Azospirillum*: Drs. J. Döbereiner, E. Ruckdäschel, W. Klingmüler, and V.L.D. Baldani. Dr. Yoav Bashan participated in this study in memory of the late Mr. Avner Bashan from Israel. This study was supported by two grants (#3541-A and #87149) from Consejo Nacional de Ciencia y Tecnologia (CONACyT), Mexico

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