Bacterial cell-surface hydrophobicity, charge, and lectins as possible means for the initial attachment of *Azospirillum* spp. to surfaces

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Summary. For the initial stage of the attachment process of 10 Azospirillum *strains, we studied the involvement of bacterial cell-surface hydrophobicity, bacterial cell-surface charge, and the presence of bacterial cell-wall lectins.* Azospirillum *spp. has moderate cell-surface hydrophobicity and charge, lower than known values for human pathogens. The hydrophobicity and charge values were higher in solid than liquid media and could be modified by several external treatments. A similar cell-wall hydrophobic protein was isolated, and partially characterized, from two strains of* A. brasilense *and one strain of* A. lipoferum. *Cell-surface lectin-like proteins were screened in seven strains of* A. brasilense *and* A. lipoferum. *A particle agglutination assay, using latex beads coated with different neoglycoproteins showed the highest agglutination rate was with fucose and glucose, and lesser with mannose residues. Cell-wall proteins extracted from two* Azospirillum *spp. strains exhibited lectin-like activities. This study proposes: (i) cell-surface hydrophobicity and charge of* Azospirillum *spp. can be affected by external treatments of the bacterium cell, (ii) hydrophobicity and charge may play a role, perhaps small, in the primary adsorption of* Azospirillum *spp. to surfaces, and (iii) lectins are present in the cell-wall of* Azospirillum *spp.*

Introduction

The plant-growth-promoting bacterium (PGPB) *Azospirillum* is known to attach to surfaces whether alive, like roots, or to soil particles and synthetic surfaces. Attachment is a primary, major step in root colonization. It is manifested as multilayered-fibrilar material, commonly visible in photomicrographs, connecting the bacteria to the root and to each other. The primary adsorption phase is fast (reaches a maximum level within 2 h of incubation), weak, and probably governed by bacterial proteinaceous compounds (Bashan and levanony, 1988a). The second phase (anchoring) is stronger, takes several hours to form, irreversible, and mediated by polysaccharides creating a firm and permanent anchoring (De Troch and Vanderleyden 1996). The anchoring phase has a greater affinity for live roots than inert surfaces (Bashan and Holguin, 1993). Although the anchoring phase of this bacterium has been intensively studied, the initial stages of the attachment process are obscure. The *molecular* mechanism(s) responsible for bacterium-root attachment is unclear. Mediation by lectins is a possibility (Bashan and Levanony 1988b).

Few studies have addressed the cell-wall properties of *Azospirillum* sp. They have shown *A. brasilense* Cd can attach in large numbers to hydrophobic polystyrene surfaces (Bashan and Holguin, 1993) and that this strain is hydrophilic (Arunakumari *et al.* 1992). Cell-surface proteins and cell-surface hydrophobicity of *A. brasilense* were involved in the adhesion process (Dufrene and Rouxhet, 1996).

Cell-surface lectins of many bacteria, especially human pathogens, are known. In PGPB, bacterial cell-surface lectins interacting with polysaccharide residues on the bacterial surface. In *Rhizobium*-legume interactions, several legume lectins stimulate root infection (Vaneijsden *et al.* 1995). In *Azospirillum*-plant association, wheat germ agglutinin (WGA) revealed two putative receptors in the *A. lipoferum* capsular fraction (Karpati *et al.* 1995). WGA bound to *A. brasilense* Sp-245 was proposed as a signal molecule in wheat-*Azospirillum* association (Antonyuk *et al.* 1995). Rice embryo lectin specifically bound to *A. lipoferum* (Tabary *et al* 1984). The exocellular polysaccharides, a possible target for plant lectins, were characterized and quantified in both *A. brasilense* and *A. lipoferum* (Del Gallo and Haegi 1990). However, to the best of our knowledge, cell-surface lectins of *Azospirillum* spp. have not been reported.

The aims of this study were: 1) to measure the effect of bacterial culture conditions on cell-wall hydrophobicity and charges in 10 strains of *Azospirillum*; 2) to measure the effect of chemicals, temperatures, and enzymatic treatments on the possible changes in cell-surface charge and hydrophobicity; 3) to isolate and partially characterize a hydrophobic protein from three *Azospirillum* strains cell-wall surfaces; and 4) to record the presence of cell-surface lectins in seven strains of *Azospirillum* spp.

Materials and Methods

Bacterial strains, culture media and measurements of hydrophobicity and charge.

Ten *Azospirillum* strains were used: *A. brasilense* Sp-245, Cd, Sp-6; *A. lipoferum* 37, 378, [illegible]

Table 1. Cell-surface charge and cell-surface hydrophobicity of *Azospirillum* spp. in solid and liquid media

Strain	Δlog G*			
	Cell-surface charge		Cell-surface hydrophobicity	
	Solid medium (NA)	Liquid medium (NB)	Solid medium (NA)	Liquid medium (NB)
A. brasilense Sp-6	0.3157	0.001	0.3838	0.672
A. brasilense Sp-245	0.9919	0.2493	0.6817	0.0001
A. brasilense Cd	0.4389	0.001	0.5224	0.001
A. lipoferum 37	0.8937	0.001	0.988	0.0189
A. lipoferum 779	0.5211	0.3519	0.6849	0.5773
A. lipoferum JA 2	0.7377	0.3589	0.2882	1.060
A. lipoferum JA 4	0.1555	0.3035	0.3631	0.575
A. lipoferum 1842	0.1409	0.001	0.4481	0.001

* Δlog G is a calculated value for charge and hydrophobicity according to Johansson (1974).

The culture media used were: Nutrient Broth (NB), Luria-Bertani medium (LB) and N-free, malate minimal medium (OAB) (Bashan *et al.* 1993). Cell-surface charge and cell-surface hydrophobicity were evaluated and calculated using the aqueous two-polymer phase-partitioning assay of Johansson (1974).

Biochemical assays

1) Cell-surface proteins of *Azospirillum* spp. cells were extracted from cells growing on solid Nutrient Agar (NA) plates or from Nutrient Broth medium using the urea method described by Paulsson *et al.* (1992). 2) Hydrophobic interaction chromatography of the urea extraction of the cell-associated proteins from *A. brasilense* Cd and *A. lipoferum* 1842 cells was done on an Octyl Sepharose CL-4B (Sigma) column. The adsorbed proteins were eluted using a step gradient of decreasing $(NH_4)_2SO_4$ concentration (1.5, 1.0, 0.5, 0.25, and 0.1 M) and distilled water. The dialyzed fractions (in 0.01M ammonium bicarbonate) were employed for SDS-PAGE electrophoresis according to Laemmli (1970). Isoelectricfocusing of cell-associated protein samples was done on a gel according to Pharmacia fine chemicals (1982).

The presence of lectin-like proteins was determined by particle agglutination assays employing latex beads coated with neoglycoproteins and by western blot using neoglycoproteins labeled with horseradish peroxidase as a probe. 3) Preparation of neoglycoprotein-coated latex beads was done according to Amini *et al.* (1995). The prepared latex bead suspensions were mixed and incubated with the following neoglycoproteins (separately): BSA-fucosylamide, BSA-glucosamide, BSA-p-aminophenyl-N-acetyl-- D-glucosamine, BSA-p- aminophenyl-N- acetyl-- D-galactosamine, and BSA-D-mannopyranose. 4) Particle agglutination assay (PAA) was done as described by Naidu *et al.* (1988). 5) Particle-agglutination inhibition assay was done by bacterial cell suspension preincubated (separately) with arabinose, fucose, galactose, glucose, mannose, N-acetyl glucosamine, and N-acetyl galactosamine. Bacterial mixtures were employed in the PAA as described above. 6) Neoglycoprotein labeling with horseradish peroxidase (HRP) was done according to Harlow and Lane 1988 using the following neoglycoproteins: BSA-fucosylamide, BSA-BSA-cellobiosyl, and BSA-melibiosyl. 7) The separated proteins from SDS-PAGE and the 43 kDa hydrophobic protein from *A. lipoferum* 1842 were electrophoretically transferred to a immobilon-nitrocellulose membrane and were blotted according to Harlow and Lane (1988).

Results

Cell- surface charge (CSC) and cell-surface hydrophobicity (CSH).

Cell-surface charge and cell-surface hydrophobicity in solid and liquid media were evaluated in ten *Azospirillum* spp. strains. The *A. brasilense* strains were more homogeneous in their behavior than the *A. lipoferum* strains tested. *A. halopraeferens* and *A. irakense* did not show charge and hydrophobicity independent of the media used (their data are therefore, not shown).

The values of the cell-surface charge depended on the type of culture media used. When bacteria were grown on solid Nutrient Agar (NA) medium, four strains had CSC values lower than 0.5, and four strains had CSC values between 0.5 and 1. A. *brasilense* Sp-245 had the highest CSC value followed by *A. lipoferum* 37 and JA2 (Table 1). When bacteria were grown in liquid NB, CSC values were significantly lower. Only 4 strains had low positive CSC values of 0.2 to 0.3, while the rest had a negligible CSC (Table 1).

The CSH presented a pattern similar to that of CSC for bacteria grown in solid Nutrient Agar (NA). Of the cells grown on NA medium, four strains had high CSH, and four medium CSH (Table 1). In cells grown in liquid NB medium, the CSH differed from the CSH obtained using solid media. In liquid NB medium, four strains showed medium (>0.5) CSH, one had high CSH, and three strains had negligible CSH (Table 1).

Similar trends in results were obtained for LB and OAB media (data not shown).

Effect of temperature, chemical, and enzymatic treatments of Azospirillum *cells on their CSC and CSH.*

The CSC and CSH of 3 strains, *A. brasilense* Cd, *A. brasilense* Sp-245, and *A. lipoferum* 1842 grown on solid and liquid NB media were evaluated after using 11 different treatments. These treatments aimed to evaluate whether external treatment of the cells (as may occur in the soil after inoculation) can change the

le 2. External treatments of *Azospirillum* cells and their effect on cell-surface charge and cell-surface hydrophobicity.

Azospirillum strain and external treatment	Cell-surface charge				Cell-surface hydrophobicity			
	Increased		No effect		Increased		No effect	
	Solid medium	Liquid medium	Solid medium	Liquid medium	Solid medium	Liquid medium	Solid medium	Liquid medium
Heat								
A. brasilense Cd	100	40°C, 80°C	40°C-80°C	60°C, 100°C	-	-	40°C-100°C	40°C-100°C
A. brasilense Sp-245	-	60°C, 80°C, 100°C	40°C-100°C	40°C	40°C	40°C-100°C	60°C-80°C	-
A. lipoferum 1842	-	40°C, 80°C	40°C-80°C	100°C	-	40°C-100°C	40°C-100°C	-
Chemicals								
A. brasilense Cd	-	H_2SO_4, $NaBH_4$	all	$NaIO_4$	-	$NaIO_4$	all	H_2SO_4, $NaBH_4$
A. brasilense Sp-245	-	-	all	all	-	$NaIO_4$	all	H_2SO_4, $NaBH_4$
A. lipoferum 1842	$NaBH_4$	all	$NaIO_4$ H_2SO_4	-	-	all	all	-
Enzymes								
A. brasilense Cd	-	1, 3	2, 4	2, 4	3, 4	1	1, 2	2, 3, 4
A. brasilense Sp-245	2	2, 3, 4	3, 4	1	3, 4	2, 3, 4	1, 2	1
A. lipoferum 1842	3	2	4	1, 3, 4	3	2, 4	1, 2, 4	1, 3

t treatments: 40°C, 60°C, 80°C, 100°C.
mical treatments: $NaIO_4$, $NaBH_4$, H_2SO_4.
ymes: 1) Protease E, 2) Proteinase K, 3) Trypsin, 4) Chymotrypsin.
ll treatment of that kind.

CSC and CSH characteristics of the cells. The data in Table 2 demonstrate the resulting effects of these treatments on CSC and on CSH.

Isolation of a cell-surface hydrophobic protein.
Hydrophobic chromatography yielded 6 main peaks for *A. brasilense* Sp-245, Cd and *A. lipoferum* 1842. When the fractions of these chromatographies were separated in SDS-PAGE electrophoresis, one protein was constantly observed. The molecular weight of this protein was 43 kDa. Its isoelectricfocusing showed 3 bands, at pH 4.45, 4.7, and at 5.1.

Isolation of lectin-like proteins from cell surfaces.
The seven *Azospirillum* strains tested were agglutinated by the five neoglycoproteins to various degrees. Neoglycoproteins consisting of fucose and glucose residues had the highest agglutination rate followed by those containing mannose residues. Bovine-serum albumin (BSA) and the latex beads themselves, serving as controls, did not agglutinate *Azospirillum* spp. cells.

Variability in agglutination inhibition assays among the three selected strains tested (*A. brasilense* Sp-245, *A. lipoferum* 1842, and *A. lipoferum* 779) was common. With the exception of arabinose, which inhibited the agglutination of the five neoglycoproteins tested, no other monosaccharide inhibited agglutination completely. However, several monosaccharides almost completely inhibited the binding of three neoglycoproteins (BSA-p-aminophenyl-N-acetyl-- D-glucosamine, BSA-p-aminophenyl-N- acetyl-- D- galactosamine, and BSA-D-mannopyranose.) to *A. brasilense* Sp-245 and *A. lipoferum* 1842.

Blotting of cell-surface proteins from *A. lipoferum* 1842, *A. brasilense* Cd, and the hydrophobic protein of 43 kDa isolated from *A. lipoferum* 1842 showed that HRP-BSA-galactosamide bound strongly to the last, to a lesser degree to a protein of about 50 kDa of the cell-wall of *A. brasilense* Cd, and to several high molecular weight proteins. HRP-BSA-glucosamide bound to that hydrophobic 43 kDa protein, and in addition to two proteins of the cell-wall of *A. brasilense* Cd. HRP-BSA-mannopyranose and HRP-BSA-fucosylamide bound to the hydrophobic 43 kDa protein, and to an additional protein of *A. lipoferum* 1842. Two other neoglycoproteins, BSA-cellobiosyl and BSA-mellobiosyl were tested and no binding was observed.

Discussion

Azospirillum spp., in general, are not plant-specific bacteria. Numerous plant species are known to be colonized and affected by their inoculation (Bashan and Holguin 1995, 1997). This study concentrates on primary attachment variables. CSC and CSH properties of a Plant Growth-Promoting Bacteria (PGPB) like *Azospirillum*, and their possible involvement in the first step of the attachment process to roots might have an environmental impact. Physicochemical characteristics of the cell surface play a role in the nonspecific and specific attachment of bacteria to plant cell surfaces (Smit and Stacey, 1990). Bacterial surface hydrophobicity can be used to estimate the overall attachment potential of bacteria to soil particles (Stenström, 1989). Attachment data may have a practical use in assisting the selection of the most adequate PGPB strains for bacterial inoculants in future agriculture.

A general pattern of modifying cell-wall hydrophobicity and charge by external treatments of the cells has not been found. Our current in vitro study gives a clue that certain environmental variables may change the CSC and CSH of the cells, as many in vitro conditions did, and so facilitate attachment. However, this assumption needs to be further confirmed with plants.

Our study showed the genus *Azospirillum* has generally a low CSH value compared to human pathogenic bacteria such as *Vibrio cholerae*, known for their firm attachment. This may show the possibility of relatively low impact of cell-wall hydrophobicity and charge on the initial attachment by the bacteria. CSH variability in our study may be the outcome of the expression of varying amounts and types of polysaccharides, a variable number of fimbria per strain, the amount of capsular material (Del Gallo and Haegi 1990, Michiels *et al.* 1990), and cell pleomorphism (Bashan *et al.* 1991). All of these depend on the medium composition, growth stage, and agitation of the culture during growth (Lanm and Neyra, 1981; Madi *et al.* 1988).

Azospirillum spp. is applied to plants either in solid or liquid inoculants (Bashan and Holguin 1997). This study indicates the possibility the inoculant formulation may affect the initial adhesiveness capacity of the bacteria, being more hydrophobic and with a greater charge when grown on solid surfaces. With increased attachment of bacteria to the inoculant particles, this inoculant type may limit the release of the bacteria to the soil compared to liquid inoculant. On this point, future inoculant formulation research needs to be focused. Furthermore, knowledge of the charge and hydrophobicity of a particular strain to be used for inoculation might give a preliminary inductive clue about the prospects of success of the strain as inoculant.

Our agglutination data demonstrate *Azospirillum* spp. cells are interacting with several carbohydrate residues. Additionally, the neoglycoprotein-labelling experiments revealed the presence of several lectin-like proteins on the bacterium cell surface. Both data demonstrate lectins are present in *Azospirillum* spp. It is yet to be demonstrated if these lectins participate in the nonspecific recognition of root by *Azospirillum* spp. cells.

In summary, this study demonstrates that external physicochemical factors can change the cell-surface hydrophobicity and the cell-surface charge of 10 *Azospirillum* strains. Additionally, lectin-like proteins are present there. All this might have an impact on the initial attachment process of these bacteria to surfaces.

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References

Amini, H.R., Ascencio, F., Ldungh, Å., and Wadström, T. (1995). Particle agglutination assay for detection of albumin and IGg-binding cell surface components of

255-264.

Antonyuk, L.P., Fomina, O.R., Kalinina, A.V., Semenov, S., Nesmeyanova, M., and V.V. Ignatov. (1995). Wheat lectine possibly serves as a signal molecule in the *Azospirillum*-wheat association. In: *Azospirillum VI and Related Microorganisms Genetics-Physiology-Ecology*. NATO ASI series, vol. G37, Ed. I. Fendrick, M. del Gallo, J. Vanderleyden and M. de Zamaroczy; pp. 319-324. Springer-Verlag, Berlin, Heidelberg.

Arunakumari, A., Lamm, R.B., and Neyra-Estens, C.A. (1992). Changes in cell-surface properties of azospirilla in relation to cell pleomorphism and aggregation. *Symbiosis* 13, 291-305.

Bashan, Y., and Holguin, G. (1993). Anchoring of *Azospirillum brasilense* to hydrophobic polystyrene and wheat roots. *Journal of Geneneral Microbiology* 139, 379-385.

Bashan, Y., and Holguin,G. (1995). Inter-root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. *Microbial Ecology* 29, 269-281.

Bashan, Y., and Holguin, G. (1997). *Azospirillum*-plant relationships: environmental and physiological advances (1990- 1996). *Canadian Journal of Microbiology* 43, 103-121.

Bashan, Y. Holguin, G., and Lifshitz, R. (1993). Isolation and Characterization of plant growth-promoting rhizobacteria. In *Methods in Plant Molecular Biology and Biotechnology*. Ed. R. Glick, J.E. Thompson. pp. 331-345. CRC Press, Boca Raton, Fl.

Bashan, Y., and Levanony, H. (1988 a). Active attachment of *Azospirillum brasilense* Cd to quartz sand and to light-textured soil by protein bridging. *Journal of General Microbiology* 134, 2269-2279.

Bashan, Y., and Levanony, H. (1988 b). Migration, colonization and adsorption of *Azospirillum brasilense* to wheat roots. In *Lectin-Biology, Biochemistry, Clinical Biochemistry*. Eds. T.C. Bøg-Hansen and D.L.J. Freed. pp. 69-84; vol. 6. Sigma Chemical Company, St. Louis, Missouri. U.S.A.

Bashan, Y., Levanony, H., and Whitmoyer, R.E. (1991). Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense Cd. Journal of General Microbiology* 137, 187-196

Del Gallo, M., and Haegi, A. (1990). Characterization and quantification of exocellular polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum*. *Symbiosis* 9, 155-16

De Troch, P., and Vanderleyden, J. (1996). Surface properties and motility of *Rhizobium* and *Azospirillum* in relation to plant root attachment. *Microbial Ecology* 32, 149-169.

Dufrene, Y.F., and Rouxhet, P.G. (1996). Surface composition, surface properties, and adesiveness of *Azospirillum brasilense*-variation during growth. *Canadian Journal of Microbiology* 42, 548-556.

Harlow, E., and Lane. D. (1988). In *Antibodies. A Laboratory Manual*. Ed. E. Harlow and D. Lane. Cold Spring Harbor Laboratory. New York, USA.

Johansson, G. (1974). Partition of proteins and micro-organisms in aqueous biphasic systems. *Molecular and Cellular Biochemistry* 30, 169-180.

Karpati, E., Kiss, P., Afsshaarian, M., Voros, G., Del Gallo M., and Fendrik, I. (1995). Isolation of wheat germ agglutinin-receptor of *Azospirillum lipoferum*. In *Azospirillum VI and Related Microorganisms, Genetics-Physiology-Ecology*. Ed. I. Fendrick, M. del Gallo, J. Vanderleyden and M. de Zamaroczy. pp. 213-221. NATO ASI series; Vol. G37. Springer-Verlag, Berlin, Heidelberg.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lamm, R.B., and Neyra, C.A. (1981). Characterization and cyst production of azospirilla isolated from selected grasses growing in New Jersey and New York. *Canadian Journal of Microbiology* 27, 1320-1325.

Madi, L., Kessel, M., Sadovnik, E., and Henis, Y. (1988). Electron microscopic studies of aggregation and pellicle formation in *Azospirillum* spp. *Plant and Soil* 109, 115-121.

Michiels, K., Verreth, C., and Vanderleyden, J. (1990). *Azospirillum lipoferum* and *Azospirillum brasilense* surface polysaccharide mutant that are affected in floculation. *Journal of Applied Bacteriology* 69, 705-711.

Naidu, A.S., Paulsson, M., and Wadström, T. (1988). Particle agglutination assay for rapid detection of fibrinogen, and collagen receptors on *Staphylococcus aureus*. *Journal of Clinical Microbiology* 26, 1549-1554.

Paulsson, M., Liang, O.D., Ascencio, F., and Wadström, T. (1992). Vitronectin-binding srface proteins of *Staphylococcus aureus*. *Zentralblatt für Bakteriologie* 277, 54-64.

Pharmacia fine chemicals (1982). *Isoelectric focusing. Principles and methods*. pp. 45-64. Printed by Llungföretogen AB Örebro, Sweden

Smit, G., and Stacey, G. (1990). Adhesion of bacteria to plant cells: role of specific interactions versus hydrophobicity. In: *Microbial Cell-Surface Hydrophobicity*. Ed. M. Rosenberg, and R.J. Doyle. pp. 179-209. American Society for Microbiology, Washington, DC.

Stenström, T.A. (1989). Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. *Applied and Environmental Microbiology* 55, 142-147.

Tabary, F., Balandrean, J., and Bourrillon, R. (1984). Purification of the rice embryo lectin and its binding to nitrogen-fixing bacteria from rhizosphere of rice. *Biochemical and Biophysical Research Communications* 119, 549-555.

Vaneijsden, R.R., Diaz, C.L., Depater, B.S., and Kijne, J.W. (1995). Sugar-binding activity of pea (*Pisum sativum*) lectine is essential for heterologous infection of transgenic white clover hairy roots by *Rhizobium leguminosarum* biovar *viciae*. *Plant Molecular Biology* 29, 431-439.