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Characterization and Quantification of Exocellular Polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum**

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Abstract

Azospirilla produce different amount of capsular-(CPS) and exo-polysaccharides (EPS) depending on the strain and on the species considered. *A. lipoferum* and *A. brasilense* bind to a lectin similar to the lectin present on the root surface of several grasses, the Wheat Germ Agglutinin (WGA) with different intensity and in different amount depending on the culture age. The polysaccharides (CPS) of both strains are qualitatively similar in composition: glucose, galactose, rhamnose and putative N-acetylglucosamine, but varies quantitatively during the growth of the culture and among the strains.

Introduction

Nitrogen-fixing bacteria of the genus *Azospirillum* live associated with roots of grasses and other plants. Azospirilla include four species: *A. lipoferum*, *A. brasilense*, *A. amazonense* and *A. halopreferans*, having different nutritional and physiological properties. Even though Azospirilla are good nitrogen-fixers in laboratory conditions, the nitrogen contribution to plant growth and yield seems to be lower than expected and the increase repeatedly observed in growth and yield of inoculated plant seems to be induced by plant growth promoting substances produced by the bacterium, rather than by a nitrogen contribution.

Since 1979 (Tien et al.), it has been observed that *Azospirillum brasilense* produces in pure cultures large amount of auxines, small but biologically significant amounts of gibberellin and at least three cytokinin-like substances. Besides, Tien et al. observed that the morphology of pearl millet roots changed when plants in liquid culture were inoculated: the number of lateral roots was increased and all lateral roots were densely covered with root hairs.

*Reviewed

Root colonization by Azospirilla starts from the mucigel area (Del Gallo and Neyra, unpublished observations) and is likely mediated by an exchange of molecular signals similar to other plant-microbe interactions.

Okon and Kapulnik (1986) observed that *Azospirillum* is chemotactically attracted by a substance which is present in the root exudates and which is destroyed by trypsin. In previous works (Del Gallo and Neyra, 1988; Del Gallo et al., 1989) the production by Azospirilla of a dense glycocalyx which surrounds the cell containing polysaccharides has been described. These polysaccharides are likely to interact with lectins (mainly WGA-like) present on the surface of young roots and on the tip surface of older, but still active, roots (Mishkind et al., 1983; Raikhel et al., 1984).

In our study we will report the composition of the different polysaccharidic fractions excreted by two species of *Azospirillum* by HPLC analysis at various growth stages and the PS fractions lectin binding.

Material and Methods

Bacterial strains. A. brasilense Cd and *A. lipoferum* Col 5, kindly supplied by J. Döbereiner, were used.

Inoculum preparation and culture media. Strains were maintained at room temperature on Tryptic Soy Agar (TSA) or in OK-Gluconate agar (Martinez-Drets et al., 1984) plates. PS extraction and microscopical observations were carried out from cultures grown at 30°C in liquid OK-Gluconate medium enriched with 100 mg l^{-1} yeast extract.

Microscopical observations. A modification of the technique utilized by Bohlool and Schmidt (1974), described in a previous work (Del Gallo et al., 1989), for the assessment of lectin binding was used. Wheat Germ Agglutinin (WGA), Concanavalin A (Con A) and Soy Bean Lectin (SBL) labelled with Fluorescein isothiocyanate (FITC) where purchased from Sigma. A Leitz Dialux 20 microscope equipped with an incident light illumination and filter block I 2/3 was used.

HPLC analysis. A preliminary quantification of PS present in the different fractions was performed by the anthrone method using glucose as a standard (Dische, 1962). Samples were prepared as summarized in the scheme (Fig. 1) and analyzed on a Bio-Rad Aminex HPX-87C cation exchange column (300 × 7.8 mm) in the Calcium form at 85°C. The eluant was H_2O at a flow rate of 0.6 ml × min^{-1}. Polar sugars were analyzed on a Bio-Rad Aminex HPX-87H cation exchange column (300 × 7.8 mm) in the hydrogen form at 65°C. The eluant was 4 mM H_2SO_4 in water at a flow rate of 0.6 ml × min^{-1}. The sample size was 20 μl and the detection was by refractive index and UV light at 210 nm.

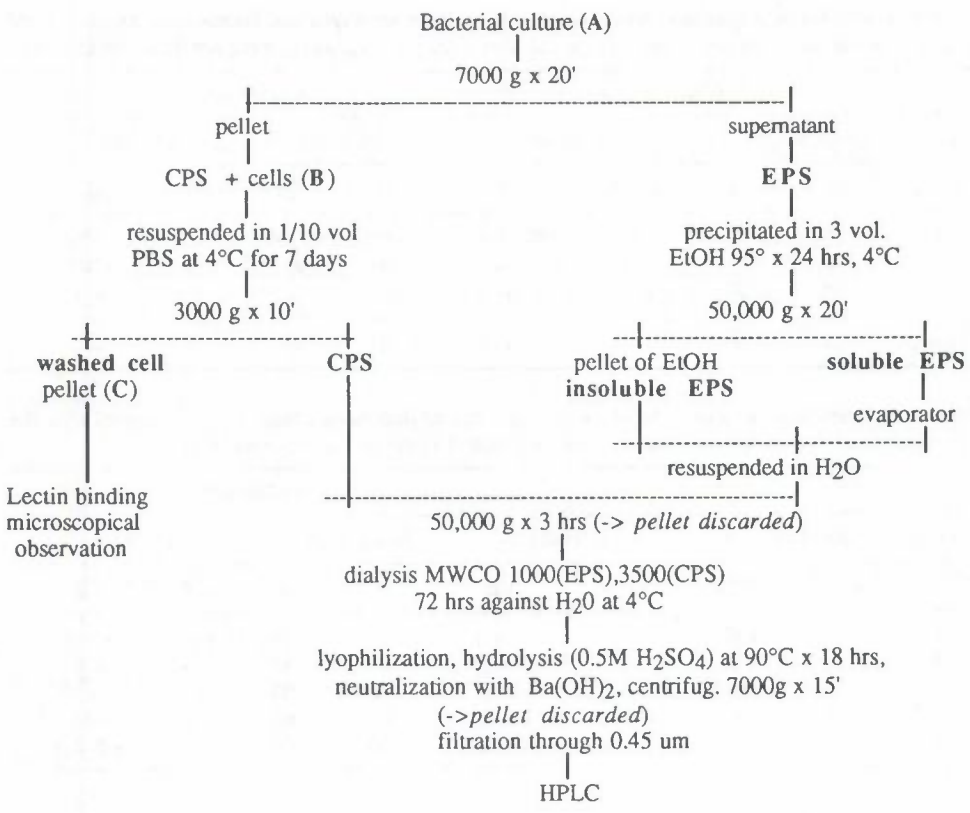

Figure 1. Sample preparation for the HPLC analysis, and for the lectin-binding microscopical observations (A, B and C).

Results

Microscopical analysis. From the results shown in Table 1 and 2 and in Fig. 2 (right) both strains are able to bind FITC-WGA and FITC-Con A but the amount of fluorescent cells changes with the species and with the age of the culture. Both strains considered increase affinity with WGA when the culture gets old, but Cd has a less intense fluorescence than Col 5. The Con A binding gave less stable result. The binding seems to be less stable in Col 5 whole culture, and cells seem to be more fluorescent in washed culture. In both strains, cells without EPS and CPS (C) do not bind lectins. Cells never bind to SBL.

Quantification of PS. Quantification of PS utilizing anthrone reagent (Fig. 2, left) shows a different pattern among the two strains. Cd produces more losely bind PS

Table 1. Wheat Germ Agglutinin (WGA) binding: % of fluorescent cells and fluorescence intensity. Cells stained after the solubilization of the capsule and after a second washing (C) gave not fluorescence at all

	Whole cells (A)				Washed cells (B)			
Days	Stained cells, %		Intensity		Stained cells, %		Intensity	
	Col 5	Cd	Col 5	Cd	Col 5	Cd	Col 5	Cd
1°	28	30	+	+	4.5	6.4	−+	++
2°	41	76	−+	+	100	40	+	+
3°	100	100	++++	++	100	40	++	++
4°	100	100	++++	+++	100	50	++	−+
5°	100	100	++	++	100	40	+	−+

Table 2. Concanavalin A binding: % of fluorescent cells and fluorescence intensity. Cells stained after the solubilization of the capsule and after a second washing (C) gave not fluorescence at all

	Whole cells (A)				Washed cells (B)			
Days	Stained cells, %		Intensity		Stained cells, %		Intensity	
	Col 5	Cd	Col 5	Cd	Col 5	Cd	Col 5	Cd
1°	50	100	+++	+++	100	40	+++	+++
2°	84	73	+	+	100	66	+	++
3°	100	83	++++	++	100	100	++++	+
4°	100	100	+++	++	100	100	+	++
5°	75	100	+++	++++	100	100	++	++++

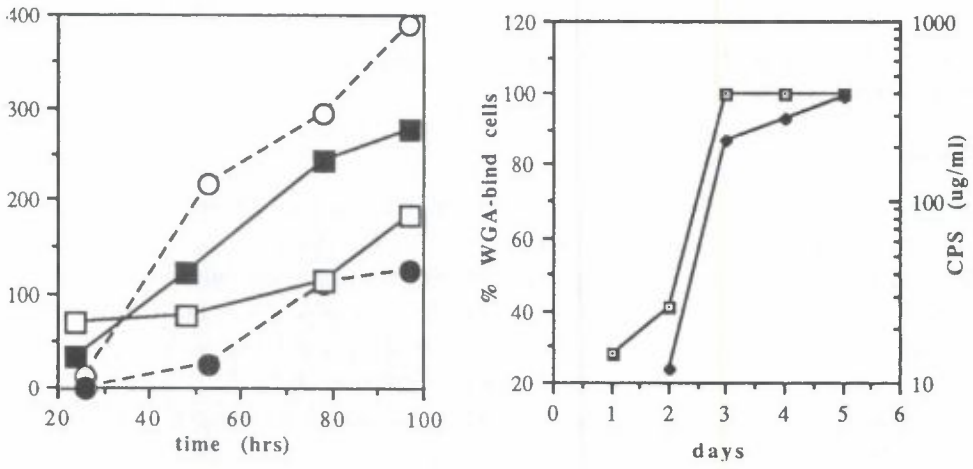

Figure 2. Production of EPS (■) and CPS (□) by *A. brasilense* Cd (–) and EPS (●) and CPS (○) of *A. lipoferum* Col 5 (– – –) at different growth stages (left). Production of CPS (◆) and % of FITC–WGA binding cells in Col 5 (⊡), (right).

Table 3. % of different sugars in Col 5 and in Cd CPS at different growth stages. *(nd = not detected separately)

	A. lipoferum Col 5				*A. brasilense* Cd			
Growth days	2	3	4	5	2	3	4	5
Galacturonic Acid	0	0	0	0	<1	<1	<1	3
GlcNAc (putative)	53	22	20	15	20	17	15	13
Glucose	4	16	40	50	31	32	34	38
Galactose + Rhamn.	43	62	40	35	49	50	51	48
Galactose	3	nd*	4	nd*	27	nd*	25	nd*
Rhamnose	40	nd*	36	nd*	22	nd*	26	nd*

(EPS) than CPS, Col 5 behaves in an opposite way, producing a larger capsule. Both strains PS increase during the growth of the culture.

HPLC analysis. CPS of both *A. lipoferum* and *A. brasilense* strains are qualitatively similar in composition (Table 3). In our cultural conditions they are constituted of glucose, galactose, rhamnose and N-Acetylglucosamine (GlcNAc). However, while the presence of the three sugars was confirmed on both HPLC columns utilized, the presence of GlcNAc was detected only by the Aminex-HPX87C. *A. brasilense* Cd CPS contain also a minimal amount of galacturonic acid.

Quantitative composition of the same monosaccharide, instead, changes with the culture age in the same strain and among the two strains. Even though the total amount of galactose + rhamnose is similar among the two strains and it is quite constant during the culture life, the proportions are different, 1:1 in Cd and 1:10 in Col 5. GlcNAc is present in greater amount in Col 5 than in Cd. Glucose increases in amount in both strains, more in Col 5 than in Cd.

Discussion

The fact that cells without capsule never bind lectin confirm that lectin receptors are located on the capsule which surrounds the bacteria.

During the growth of the culture there is a moment, at the beginning of the stationary phase, when lectin-binding seems to be more intense and involves a higher percentage of cells. In the late stationary phase, lectin-binding decreases or cells appear less fluorescent, except for Cd-Con A-binding.

It seems that, as previously reported (Del Gallo and Neyra, 1988), *A. lipoferum* Col 5 cells bind to FITC-WGA in a greater amount than *A. brasilense* Cd cells. It is possible to hypothesize that this is due to the larger amount of N-acetylglucosamine present in Col 5 CPS.

The low correlation between lectin binding and changes in the specific monosaccharide

composition in the same strain is probably due to the fact that root lectins bind in vivo to heteropolysaccharidic structures composed either by carbohydrate than by non-carbohydrate compounds. Abe et al. (1984) demonstrate that lectin-binding (trifoliin *A/Rhizobium trifolii* PS) is related with quantitative changes in monosaccharides and in non-carbohydrate residues with culture age, while the qualitative composition remains constant.

In particular, non-sugar constituents, like acetyl, piruvyl residues, etc., influence lectin-binding in high degree. For instance, in *Rhizobium japonicum*, (presently *Bradyrhizobium j.*) methylation of galactose – which remains quite constant during the culture growth – in CPS reduces affinity for Soy Bean Lectin (Mort and Bauer, 1980).

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