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Analysis of Extracellular Polysaccharides Isolated From *Azospirillum brasilense* Wild Type and Mutant Strains

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Abstract

Bacteria of the genus *Azospirillum* occur in different ecological niches. Although strain differences are thought to be involved, little or no information is available to date. We have analyzed the cell bound and extracellular polysaccharides (EPS) of two *Azospirillum brasilense* strains: Sp7 (ATCC29145) and Sp245. High molecular weight (HMW) EPS of *A. brasilense* Sp7, isolated from solid medium, contains glucose, galactose, rhamnose, fucose and non-carbohydrate substituents. HMW EPS of *A. brasilense* Sp7, isolated from liquid medium, contains glucose, galactose, rhamnose, and fucose. HMW EPS of *A. brasilense* Sp245, isolated from solid medium, contains glucose, galactose and rhamnose. The difference of EPS between these two wild type stains could reflect the difference of hosts they colonize: Sp7 was isolated from *Digitaria* rhizosphere whereas Sp245 was isolated from surface sterilized wheat roots. The use of Tn5 induced mutants of *A. brasilense* Sp7 or of a streptomycin resistant derivative, 7030, allowed us to discriminate between cell bound and detached Calcofluor-binding polysaccharides.

Keywords: *Azospirillum brasilense*, extracellular polysaccharides, Calcofluor, fluorescence microscopy, proton nuclear magnetic resonance, mutant analysis

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Abbreviations: HMW: high molecular weight, CPS: capsular polysaccharides; EPS: extracellular polysaccharides, LPS: lipopolysaccharides, ^{1}H-NMR: proton nuclear magnetic resonance; PBS: phosphate buffered saline, Km: Kanamycine; Sm: streptomycine, LB: Luria-Bertani, rpm: revolutions per minute

1. Introduction

Azospirillum brasilense is a nitrogen fixing rhizosphere bacterium. Inoculation with *Azospirillum* results in plant growth promotion in field as well as *in vitro* conditions (Michiels et al., 1989a; Sumner, 1990; Bashan and Levanony, 1990). Several strains have been isolated from different plants: *A. brasilense* was isolated from roots of C-3 grasses and sugarcane (C-4) and *A. lipoferum* from roots of C-4 grasses (Baldani and Döbereiner, 1980; Lamm and Neyra, 1981). *A. brasilense* Sp7 was isolated from the rhizosphere of *Digitaria decumbens* and *A. brasilense* Sp245 from surface-sterilized wheat roots (Döbereiner and Day, 1976; Baldani et al., 1986).

An important factor in colonization of plant roots is the bacterial surface. Cyclic β 1–2 glucans are crucial for *Agrobacterium* and *Rhizobium* to form tumors and nodules respectively on plants (Puvanesarajah et al., 1985; Toro and Olivares, 1986; Geremia et al., 1987). Acidic EPS are necessary for nodule invasion by *Rhizobium meliloti* (Finan et al., 1985). For *Azospirillum*, the production of EPS has been demonstrated by the fluorescent dye Calcofluor, which detects β 1–3 and β 1–4 linkages in polysaccharides (Wood and Fulcher, 1978). Tn5-induced *A. brasilense* mutants with altered Calcofluor fluorescence have been isolated (Michiels et al., 1990). Genetic characterization of these mutants indicates that the biosynthesis of EPS is complex and also strictly regulated. Other mutants have been obtained by marker exchange of mutated *A. brasilense* Sp7 loci, isolated by genetic complementation of *R. meliloti ExoB* and *ExoC* mutants. The *A. brasilense ExoB* mutant colonies were more Calcofluor bright than the wild type colonies and the *A. brasilense ExoC* mutant did not show any difference in Calcofluor colony fluorescence compared to wild type. In *R. meliloti*, *ExoC* codes for a phosphoglucomutase (Uttaro et al., 1990). However, since the *ExoC* locus of *Azospirillum* does not fully complement the *R. meliloti ExoC* mutant (Michiels et al., 1989), no speculation can be made about the identity of the *A. brasilense ExoC* encoded gene product.

In this work the Calcofluor-binding material of *A. brasilense* Sp7 cells and mutants was examined by fluorescence microscopy. A preliminary glycosyl composition and structure analysis of the polysaccharides produced by *A. brasilense* wild type strains was made.

2. Materials and Methods

Strains and media

The bacteria strains examined are listed in Table 1. The azospirilla were maintained on BIV medium (BIII medium [Dazzo, 1982] with mannitol replaced by K-gluconate [12.9 g/l]) and solidified with 1.2% (w/v) purified agar. When necessary, Calcofluor white (Sigma, St. Louis) was added at 200 μg/ml.

Flocculation test

Flocculation of bacterial cultures is evaluated by visual inspection of settling flocs. Cultures were grown in liquid BIV medium for 120 hr on an orbital shaker (200 rpm).

Table 1. *A. brasilense* strains and mutants used in this study

Strains or mutants	Relevant properties	Reference and/or source
Sp7	wild type	ATCC 29145
Sp245	wild type	Baldani et al., 1986
7030	Sm^r derivative of Sp7	Franche and Elmerich, 1981
AB7001	*ExoC*::Tn5, Km^r	Michiels et al., 1990
AB7002	*ExoB*::Tn5, Km^r	Michiels et al., 1990
7030TN5-1	Cad^d, Km^r	Michiels et al., 1990
7030TN5-11	Cal^d, Km^r	Michiels et al., 1990
7030TN5-12	Cal^d, Km^r	Michiels et al., 1990
7030TN5-21	Cal^-, Km^r	Michiels et al., 1990
7030TN5-22	Cal^-, Km^r	Michiels et al., 1990
7030TN5-23	Cal^-, Km^r	Michiels et al., 1990
7030TN5-101	Cal^d, Km^r	L. Fourrie
7030TN5-102	Cal^d, Km^r	L. Fourrie
7030TN5-201	Cal^d, Km^r	P. Van Rhijn
7030TN5-202	mucoid and Cal^{++}, Km^r	P. Van Rhijn
7030TN5-203	Cal^d, Km^r	P. Van Rhijn

Cal^{++} increased Calcofluor fluorescence on LB* agar medium
Cal^d diminished Calcofluor fluorescence on LB* agar medium
Cal^- no Calcofluor fluorescence on LB* agar medium
LB* is LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$

EPS isolation and gel filtration chromatography

EPS was isolated according to Hollingsworth et al. (1984) from cells grown on solid BIV medium. EPS was isolated from cells grown in liquid BIV medium as follows: One liter was inoculated with 10^9 cells and shaken for 5 days at 30°C. The culture was centrifuged at 10000×g and EPS was precipitated from the supernatant with 2 volumes of cold ethanol. Subsequent procedure for purification was the same as for EPS isolation from BIV agar. EPS was dissolved in PBS and fractionated by gel filtration chromatography on a Superose HR12 prep grade column (Pharmacia, Uppsala, Sweden), with continuous monitoring of column effluents by UV absorption (214 nm) or differential refractometry using a R401 detector (Waters, Milford, MA, USA), and by total sugar determinations of collected fractions with the phenol-sulphuric acid test (Dubois et al., 1956). The fractions containing polysaccharides were pooled, extensively dialyzed against distilled, deionized water, concentrated with a flash evaporator and lyophilized.

^{1}H-NMR and glycosyl composition analysis of polysaccharides

^{1}H-NMR and glycosyl composition analysis of polysaccharides was done as described by Hollingsworth et al. (1984) using HP-5995C GC/MS and Varian VXR500 NMR instruments.

3. Results

Fluorescence microscopy

A. brasilense strains and mutants were grown on BIV agar medium with 200 μg/ml Calcofluor. The bacteria were incubated at 30°C. After 3 days growth, cells of the colonies were transferred to glass slides, mixed in Calcofluor solution (200 μg/ml) and examined through a Zeiss Research Photomicroscope equipped with a dichroic filterset. The wild type *A. brasilense* Sp7 and Sp245 show fluorescence both on the surface of cells and on material detached from the bacteria (Fig. 1). The latter material was fibrillar and resembled cellulose fibrils. Among the Tn5-induced mutants of Sp7 or 7030, variations exist in the intensity of fluorescence associated both with the cell surface and with the fibrillar material (Table 2 and Fig. 1).

Table 2. Flocculation in liquid BIV medium, Calcofluor phenotype on solid BIV medium and fluorescence-microscopy of *A. brasilense* Sp7 and mutants

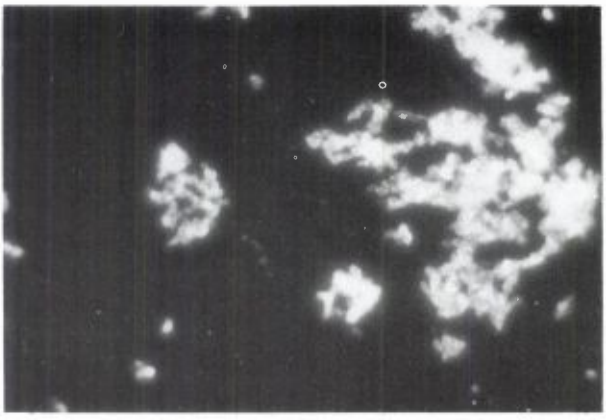
Strain or Mutant	Flocculation[a]	Cal phenotype[b]	Fluorescence cell bound[c]	Microscopy fibrillar material[d]
Sp7	+	+	Cal^+	Cal^+
ExoB	+	++	Cal^{+++}	Cal^+
ExoC	+	+	Cal^{++}	<, Cal^+
7030TN5-1	+	–	Cal^d	≪, Cal^+
7030TN5-11	<	dim	Cal^+	<, Cal^+
7030TN5-12	<	dim	Cal^d	Cal^+
7030TN5-21	–	–	Cal^-	–
7030TN5-22	–	–	Cal^-	–
7030TN5-23	–	–	Cal^-	–
7030TN5-101	–	–	Cal^-	–
7030TN5-102	<	dim	Cal^+	<, Cal^+
7030TN5-201	<	dim	Cal^d	<, Cal^+
7030TN5-202	+	+	Cal^d	++, Cal^+
7030TN5-203	+	dim	Cal^d	<, Cal^+

(a)
+ flocculation
< less flocculation than wild type

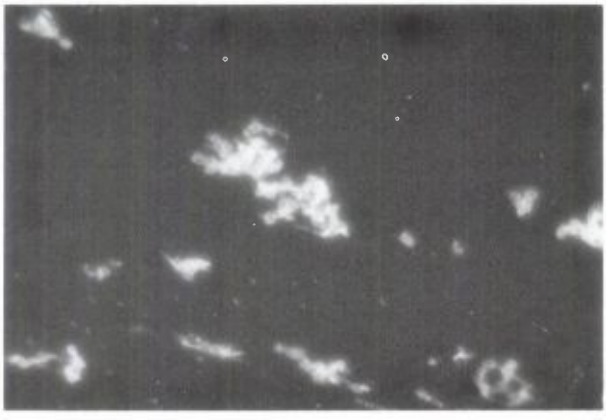
(b)
+ bright fluorescent colonies
dim dimmed fluorescent colonies
– dark fluorescent colonies
++ very bright fluorescent colonies

(c)
Cal^+ Calcofluor bright fluorescence
Cal^- Calcofluor dark fluorescence
Cal^{+++} Calcofluor very bright fluorescence
Cal^{++} Calcofluor more than wild type bright fluorescence

(d)
– no material present
Cal^+ Calcofluor bright fluorescent material, in equal amounts, compared to wild type
<, Cal^+ Calcofluor bright fluorescent material, in smaller amounts, compared to wild type
≪, Cal^+ Calcofluor bright fluorescent material, in very small amounts present
++, Cal^+ Calcofluor bright fluorescent material, in larger amounts compared to wild type



a)



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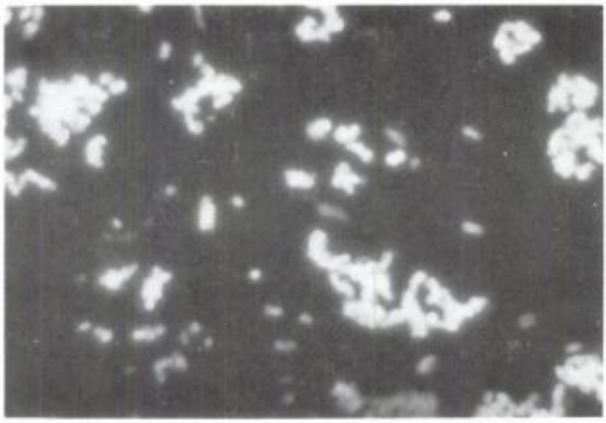
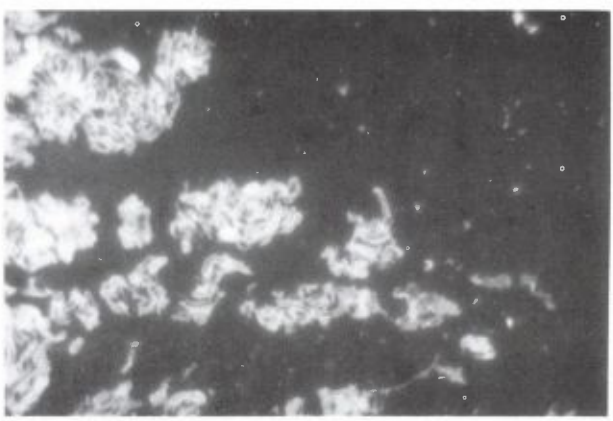


c)

d)



e)

Figure 1. Microscopic observation of Calcofluor fluorescence of *A. brasilense* Sp7 and mutant cells (Magnification 361×)
(a) Sp7
(b) Sp245
(c) Ab7002
(d) 7030 TN5-21
(e) 7030 TN5-202

Glycosyl composition

Alditol-acetates were prepared of a number of well-known sugars and their GC retention times and mass spectra were compared to the GC retention times and mass spectra of the alditol-acetates of the HMW fractions of Sp7 and Sp245, isolated from solid BIV medium and of the HMW fraction of Sp7, isolated from liquid BIV medium (Table 3). The ratio Galactose:Rhamnose:Fucose is 2:1:1 for EPS isolated from Sp7 from solid BIV medium, whereas this ratio for EPS isolated from liquid BIV medium is 1:1:1. More glucose is present in Sp7 EPS isolated from liquid than from solid BIV medium. This could be due to the production of a glucan in the liquid BIV medium. The polysaccharide of Sp7, isolated from the solid BIV medium, is different in glycosyl composition compared to the one of Sp245 also isolated from the same medium. A higher rhamnose content and no fucose were detected for EPS isolated from Sp245 but it can be noticed that that the high glucose content is a common feature for both strains.

^{1}H-NMR spectra of the different fractions

^{1}H-NMR spectra of the HMW fractions of EPS isolated from *A. brasilense* Sp7 and Sp245, grown on solid BIV agar, are represented in Fig. 2a&b. Both spectra exhibit strong resonances corresponding to the methyl groups of the deoxy-sugars (1.15–1.3 ppm). Additional signals at 1.3–1.4 and 2.0 ppm were tentatively assigned to methyl resonances of pyruvyl and acetyl substituents of the EPS, respectively. All the above mentioned signals, as well as the ones corresponding to anomeric protons and protons carried by carbons bearing non-carbohydrate substitutions (4.3–5.4 ppm), or sugar ring protons (3.3–4.2 ppm) displayed patterns clearly different for the Sp7 and Sp245 EPS. This supports the differences in glycosidic content of the two EPS and suggests additional differences in their non-carbohydrate substitutions patterns.

Table 3. Sugar composition in percentages of the high molecular weight fraction of EPS of *A. brasilense* strains

Sugar	Sp7		Sp245
	Liquid BIV medium	Solid BIV medium	Solid BIV medium
Glucose	67	44	60
Galactose	11	27	9
Rhamnose	13	14	31
Fucose	9	15	–

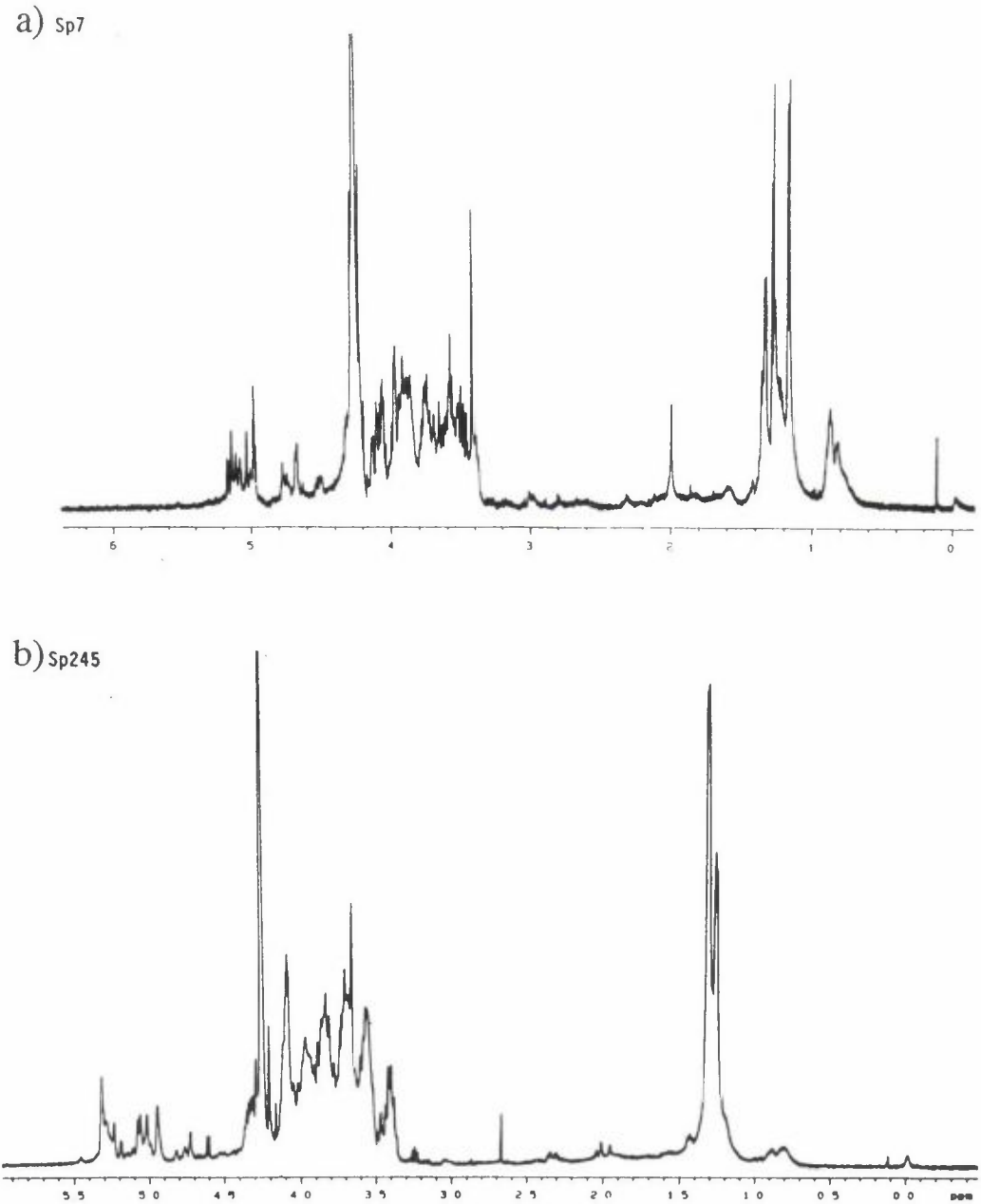


Figure 2. ^{1}H-NMR spectra of HMW EPS, isolated from solid BIV medium, recorded at 70°C. The peak at 4.25 ppm corresponds to HDO.
(a) *A. brasilense* Sp7
(b) *A. brasilense* Sp245

4. Discussion

This work shows that *A. brasilense* Sp7 produces cell-bound as well as extracellular Calcofluor-binding material. Cell bound Calcofluor fluorescence indicates the presence of β-linked glycoconjugates on the cell surface (Wood and Fulcher, 1978). These polymers are not liberated with the isolation techniques used for the study of EPS. The extracellular Calcofluor-binding material found in *A. brasilense* is fibrillar. Del Gallo et al. (1989) found similar material with microscopic observation of *A. brasilense* Sp7. Such a fibrillar, Calcofluor binding material has also been found in *Agrobacterium tumefaciens*, where this material appeared to be cellulose (Mathysse, 1983). The production of cellulose fibrils by *A. brasilense* has already been suggested by others (Bleakley et al., 1988; Sadisivan and Neyra, 1987; Del Gallo et al., 1989). Bacterial cellulose fibrils are often involved in flocculation (Deinema and Zevenhuizen, 1971; Napoli et al., 1975). In the present work we demonstrate that *A. brasilense* Cal^- mutants (7030TN5-21, 7030TN5-22, 7030TN5-23, and 7030TN5-101), which were shown to have lost the wild type ability to flocculate in liquid M9 (Michiels et al., 1990) and BIV medium (Table 2), are in fact deficient in the production of Calcofluor-binding extracellular fibrils. These findings support the hypothesis that the fibrillar material produced by *A. brasilense* is responsible for flocculation. Fibrillar material has also been observed in microscopic studies of attachment of *A. brasilense* cells to plant roots, and these fibrils are assumed to play a role in attachment (Patriquin, 1982; Umali-Garcia et al., 1980; Whallon et al., 1985). Recently, Michiels et al. (1991) demonstrated that all Cal^- mutants studied are indeed affected in the firm anchoring of the bacteria to the plant roots. The fluorescence microscopy reported here suggests that with some mutants, e.g. *ExoB*, *ExoC*, 7030TN5-1, 7030TN5-12, 7030TN5-21, 7030TN5-22, 7030TN5-23, 7030TN5-101, 7030TN5-201, 7030TN5-202 and 7030TN5-203, the Cal^+ cell bound glycoconjugates are altered or absent. The mutants obtained by the marker exchange of *A. brasilense* genes, which could genetically complement *R. meliloti ExoB* and *ExoC* mutants, show an increased cell bound Calcofluor fluorescence intensity. The biochemical mechanisms behind this are not clear. Chemical analysis of cell bound polysaccharides of *A. brasilense* should give more information. However, it cannot be excluded that polymers other than those that constitute the fibrillar material, are involved in flocculation, as would be suggested by the flocculation behaviour of mutant *ExoC*, TN5-1, TN5-12, TN5-202 and TN5-203 (Table 2).

The HMW fraction of *A. brasilense* Sp7 EPS contains a higher glucose content, when grown and isolated from liquid rather than solid BIV medium. This

is probably due to the production of a homoglucan that contains α1–4 linkages (P. De Troch, unpublished results).

The ratio of galactose, fucose and rhamnose in the HMW fractions of *A. brasilense* Sp7 EPS, isolated from both liquid and solid BIV medium is different, so more than one kind of EPS is produced by Sp7.

In an earlier study on the composition of LPS from *A. brasilense* and *A. lipoferum* strains, Choma et al. (1987) were unable to detect EPS and thus did not extract the cells with 0.5 M NaCl before extraction with hot phenol. In contrast, we were able to isolated EPS from the supernatant of shaken cultures of *A. brasilense* by ethanol precipitation. EPS can also be extracted from cells grown on the solid BIV medium with 0.5 M NaCl. Interestingly, they found partially the same sugars in the LPS of *A. brasilense* Sp7, as we find in the EPS, namely glucose, galactose, fucose and rhamnose.

The difference in sugar composition between *A. brasilense* Sp7 and Sp245 could possibly reflect a difference in interaction with the plant. The difference in plant association, i.e. *Digitaria* rhizosphere for Sp7 and wheat root interior for Sp245, forms an indication for differences in bacterial cell surface. In this study a first element in difference in surface between both bacteria is shown, namely the composition of the EPS.

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