Symbiosis, 13 (1992) 207–216
Balaban, Philadelphia/Rehovot

Characterization of Capsular Antigens in *Acetobacter diazotrophicus*

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Received October 20, 1991; Accepted April 1, 1992

Abstract

The capsular polysaccharides from PAL_3 and PAL_5 strains of *Acetobacter diazotrophicus*, isolated from sugarcane roots, were extracted and partially characterized. Polyclonal antisera against PAL_3 and PAL_5 cells, with a titer of 1:256, were used to characterize immunochemically their capsular material. Immunodiffusion tests with the PAL_3 antiserum showed antigenic differences between the capsular polysaccharide preparations of PAL_3 and PAL_5 strains inspite of their common cellular antigens. The SDS-PAGE profiles of the capsules were different. The capsular polysaccharide obtained from the PAL_5 strain contains 80% of mannose while that of the PAL_3 strain is a heteropolysaccharide containing glucose, mannose, rhamnose, fucose, arabinose, and galactose in a molar ration of 3.3: 1.8:1.5:1.3:1:1.

Keywords: *Acetobacter diazotrophicus*, sugarcane, capsular polysaccharide, immunochemistry

Abbreviations: CPS = capsular polysaccharide, SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis, PBS = phosphate buffered saline, TMPBS = milk-tween-phosphate buffered saline

1. Introduction

Cell surface structure of nitrogen-fixing bacteria is important for taxonomical relationship (Carlson et al., 1978) and in the process of plant-bacteria interaction. The association between the nitrogen-fixing rhizobial microsymbionts and root hairs of the host plant is a well studied plant-microbe interaction (Dazzo, 1984). The nitrogen fixers are gram negative bacteria which have, on their surface, carbohydrate containing molecules that are important in the process of cellular recognition (Roche et al., 1991). Characterization of the interaction between the carbohydrate receptor, located in the bacterial cell wall, and a glycoprotein isolated from seeds and seedlings, has been described with trifoliin obtained from clover and *Rhizobium trifolii* cells (Dazzo and Hollingsworth, 1984; Bhuvaneswari et al., 1977; Bohlool and Schmidt, 1974; Stacey et al., 1980). This was similar to the attachment of *Azospirillum brasilense* sp_7 to root hairs of grasses (Bashan and Levanony, 1986; Umali-Garcia et al., 1980). The sugar composition and the biological effect of the exopolysaccharide, lipopolysaccharide and capsular polysaccharide have been studied in *Azospirillum* spp. (Levanony and Bashan, 1989; Choma et al., 1987) as well as in *Rhizobium* cells (Carlson et al., 1978; Sanders et al., 1978). Virtually nothing is known concerning the surface components of the nitrogen-fixing bacterium *Saccharobacter nitrocaptans*, recently isolated from roots and stems of sugarcane (Cavalcante and Döbereiner, 1988) and taxonomically reclassified as a new species within the genus *Acetobacter*, being named *Acetobacter diazotrophicus* (Gillis et al., 1989). Although it has not been isolated from the sugarcane rhizosphere, its attachment to plant roots must have occurred during a stage in the development of the symbiosis process. The characterization of its surface structure could elucidate its biological function.

We have now isolated and characterized chemically and immunochemically the capsular polysaccharides of two different strains of *A. diazotrophicus*, PAL_3 and PAL_5. The sugar composition, antigenic determinant(s), antibody titers and cross-reactivity of these two strains were compared.

2. Materials and Methods

Bacterial strains and growth conditions

Acetobacter diazotrophicus PAL_5 strain (ATCC 49037) was used as type strain (Gillis et al., 1989). That of PAL_3 was obtained from the EMBRAPA/CNPBS culture collection (RJ-Brazil). Both PAL_3 and PAL_5 were isolated from roots of sugarcane cultivated in a field, at Alagoas State, Brazil. For serological studies, PR_2 (ATCC 49039), PR_4 and PR_{14} strains, acquired from the

EMBRAPA/CNPBS culture collection were used. Cultures were maintained on agar plates with a medium containing in g/liter: peptone 1, yeast extract 0.5, beef extract 1, NaCl 2, and sucrose 20. For capsular polysaccharide isolation, growth was performed on ten liters of a liquid LGIM medium (Stephan et al., 1991) modified for carbon and nitrogen sources, using D-mannitol 20 g/l and L-glutamic acid 2 g/l, respectively. The cultures were grown for 70 hr at 28°C and 120 rpm. Cells for inocula, in the proportion of 2% (v/v), were previously grown under the same conditions. The bacteria were harvested in the late exponential phase by centrifugation at 12,000×g for 15 min and at 4°C, yielding a net weight of 3.7 g/l for PAL_5 strain and 5.9 g/l for PAL_3 strain.

Capsular polysaccharide (CPS) isolation

CPS was prepared as described by Bhagwat and Thomas (1984).

Analytical methods

Neutral sugars were assayed by the phenol-H_2SO_4 (Dubois et al., 1956) and protein was assayed by the Bradford method (1976). For detection and analysis of component monosaccharide, the CPS was hydrolyzed with 3 M TFA at 100°C for 3 hr. Following evaporation of the hydrolysate, the residue was successively reduced with aqueous $NaBH_4$ and acetylated to yield alditol acetates, which were analysed by gas-liquid chromatography (GLC) by the method of Sawardeker et al. (1965). Products were identified by their characteristic retention times. The analyses were carried out with a Hewlett-Packard chromatograph 5840 fitted with a capillary column-OV 225 (25 m ×0.3 mm). The column was programmed from 170°C (20 min) to 200°C at 7°C/min.

SDS-PAGE

SDS-PAGE of CPS, containing approximately 40 μg neutral sugar, was carried out using a continuous buffer system with 12% running gel and 4% stacking gel according to Laemmli (1970). Proteins of 92 to 14 KDa (Bio Rad) were used as molecular weight markers. Electrophoresis was carried out at 25 mA for 5 hr. The gels were silver stained for polysaccharide (Hitchcock and Brown, 1983).

Antisera

Anti-*A. diazotrophicus* sera were obtained by immunizing rabbits with 1 mg of acetone-dried cells (Lloyd and Travassos, 1975) suspended in 0.5 ml of PBS

and injected intravenously three times a week for a period of one month. The animals were bled 7 days after the last intravenous injection.

Serological methods

Ouchterlony immunodiffusion tests on agarose gel plates (Ouchterlony, 1962) were performed at 4°C overnight. The gels were stained with Coomassie Blue R-250 (Garvey et al., 1977), and the periodic acid-Schiff reagent according to Fairbanks et al. (1971). For agglutination reactions, 5×10^7 cells of *A. diazotrophicus* were suspended to 50 μl of PBS and added to microtiter plates containing serial dilution of PAL_3 and PAL_5 antisera or preimmune serum. After 1 hr at 37°C and 30 min at room temperature with occasional shaking, the antisera titration was quantitated by measuring the relative number of cells which had clumped compared with the cells that were dispersed.

The Immunological detection of bound CPS to nitrocellulose

Immunoblotting of capsular polysaccharide was carried out using SDS-PAGE that was transferred to nitrocellulose sheets (0.2 μm pore size), for 1 hr, at room temperature (120 mA), as described by Towbin et al. (1979). The nitrocellulose sheets were soaked in Milk-Tween-phosphate buffered saline (TMPBS): 0.1% Tween 20, 50 mM Phosphate buffer, 0.5 g/l NaCl and 5% unfatted milk. The sheets were washed three times in TMPBS after incubation for at least 60 min with antisera to PAL_3 and PAL_5 cells or with preimmune serum, respectively. The sera were diluted 1:100 in TMPBS. Bound antibodies were visualized after incubation for 1 hr with goat anti-rabbit serum diluted 1:1000 in TMPBS, followed by 3 washes in TMPBS, and reaction with 3,3′-diaminobenzidine.

3. Results

Isolation and chemical analysis of A. diazotrophicus *CPS*

The methodology for extraction of capsular polysaccharide of the recently isolated *A. diazotrophicus* was based on that used for *Rhizobium* sp. (Bhagwat and Thomas, 1984). The efficiency of this extraction procedure is now shown by the low protein and high carbohydrate content (Table 1) of *A. diazotrophicus* extracts.

Crude CPS from two strains of *A. diazotrophicus* (PAL_3-CPS and PAL_5-CPS), were obtained from PBS washed cells. The dry weight of each CPS preparation isolated from 10 l culture medium was of 264 mg and 210 mg for

Table 1. Chemical analysis (%) of *Acetobacter diazotrophicus* capsular polysaccharides

Analysis	Strains	
	PAL_3	PAL_5
Protein	0.8	1.5
Total neutral sugar	13.5	25.3
Rhamnose[a]	15.4	0
Fucose[a]	13.4	0
Arabinose[a]	9.9	0
Mannose[a]	18.4	77
Galactose[a]	9.9	16
Glucose[a]	32.8	8.6

[a] The sugar composition is given as per cent of total sugar, determined by GLC.

strains PAL_3 and PAL_5, respectively. Their protein contents were from 0.8 to 1.35%, as quantified by the Bradford method (1976). The total neutral carbohydrates in the CPS of strain PAL_5 was almost twice that found in that of PAL_3. Qualitative differences in the carbohydrate components of the CPS of PAL_3 and PAL_5 strains were also observed (Table 1). While mannose was the major monosaccharide found in the CPS of PAL_5, that of PAL_3 contained glucose, mannose, rhamnose, fucose, arabinose and galactose in a molar ratio of 3.3: 1.8: 1.5: 1.3: 1: 1.

On SDS-PAGE, PAL_3-CPS gave a large diffuse band of apparent MW from 43 to 92 KDa, detected by silver staining specific for carbohydrate (Hitchcock and Brown, 1983), while PAL_5-CPS showed two diffuse bands, one running around 92 KDa and another from 42 to 68 KDa (Fig. 1).

Immunochemical analyses

The results of the agglutination titer of *A. diazotrophicus* cells with polyclonal antibodies are summarized in Table 2. The antisera against both acetone-dried PAL_3 and PAL_5 cells showed an agglutination titer of 1:256, while no cross-reaction was observed with heterologous strains, including PR_2, PR_4 and PR_{14}. Also, Ouchterlony double diffusion plates were used to test the reactivity of PAL_3 and PAL_5 antisera. Both gave a strong precipitin line with homologous cells, although PAL_3-antiserum gave a cross-reaction with PAL_5-cells. Antigenic differences related with capsular molecules were observed, inspite of the presence of common cellular antigens (Fig. 2). A precipitin reaction line was not seen when the PAL_3-antiserum was diffused against PAL_5-CPS

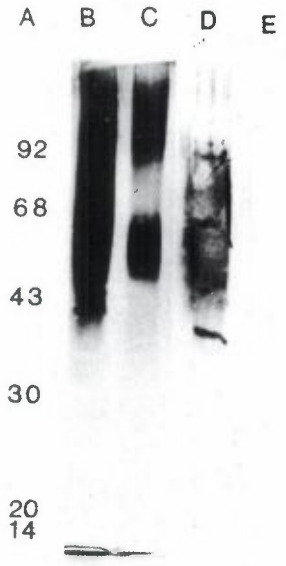

Figure 1. SDS-PAGE pattern and immunoblot of *Acetobacter diazotrophicus* capsular polysaccharides: lane A is a molecular weight marker; lane B is a silver stained PAL_3-CPS; lane C is a silver stained PAL_5-CPS: lane D is a PAL_3-CPS immunostained with PAL_3-antiserum; lane E is a PAL_5-CPS immunostained with PAL_3 antiserum.

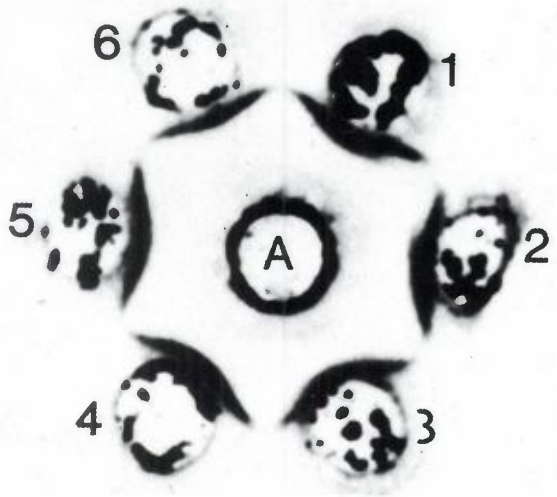

Figure 2. Immunodiffusion reaction. Undiluted PAL_3-antiserum (A) was diffused against acetone-dried cells of PAL_3-strain (1 and 3); PAL_5-strain (4 and 6), PAL_3-CPS (5), and PAL_5-CPS (2). Suspensions of acetone-dried cells were used in the concentration of 100 mg/ml (Carlson et al., 1978). The plate was stained with Coomassie Blue.

Table 2. Agglutination test of polyclonal antisera against *Acetobacter diazotrophicus* cells with homologous strains and cross-reactions with heterologous strains

Antisera	Strains				
	PAL_3	PAL_5	PR_2	PR_{14}	PR_4
PAL_3	1/256	neg	1/4	neg	1/2
PAL_5	neg	1/256	neg	neg	1/2

neg = negative response

(Fig. 2, well 2). this may be significant since these two strains have a different capsular monosaccharide composition (Table 1). The PAL_3-CPS showed a precipitin band from 10 to 50 μg of total neutral sugar, tested with undiluted PAL_3-antiserum (Fig. 3). The carbohydrate nature of this preparation was demonstrated by staining the plate with the periodic acid-Schiff reagent (Fig. 3).

Electrophoresis and immunoblotting

In immunoblotting of SDS-PAGE gels of the PAL_3-CPS antigen, a large diffuse band appeared within a glycan positive (Fig. 1B) and immunopositive (Fig. 1D) region of 43 to 92 KDa markers. No cross-reaction with PAL_5-CPS was observed on immunostaining, using the PAL_3-antiserum (Fig. 1E).

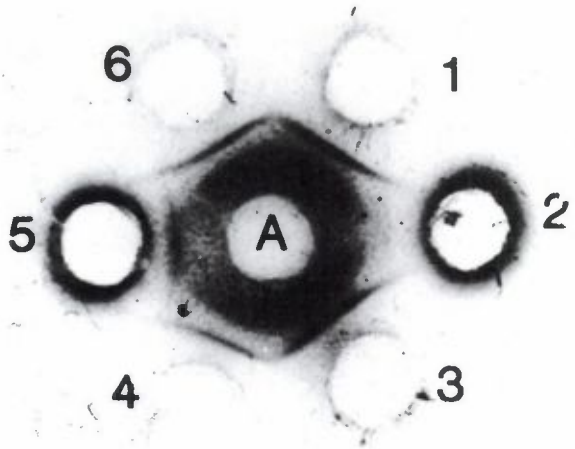

Figure 3. Periodic acid-Shiff reagent stained immunodiffusion plate. Undiluted PAL_3-antiserum was diffused against PAL_3-CPS in the following concentration: (1) 50 μg; (2) 40 μg; (3) 30 μg; (4) 20 μg; (5) 15 μg; (6) 10 μg.

4. Discussion

Chemical and immunochemical analyses of the capsular material of nitrogen-fixing PAL_3 and PAL_5 strains of *A. diazotrophicus*, isolated from roots of sugarcane cultivated in Brazil, was carried out on capsular polysaccharides isolated via the extraction procedure described for *Rhizobium* sp. (Bhagwat and Thomas, 1984).

When agglutination tests were performed with PAL_3 and PAL_5-antisera and whole heterologous cells (PAL_3, PAL_5, PR_2, PR_4 and PR_{14}), no cross-reaction was obtained, indicating the possibility of using this agglutination reaction for strain screening. In Ouchterlony double diffusion tests, PAL_3-antiserum reacted with PAL_3-cells, PAL_5-cells and PAL_3-CPS but not with PAL_5-CPS, indicating that *A. diazotrophicus*, PAL_3-strain, has immunochemical determinants that are quite different from those of PAL_5-CPS. The antigenicity and glycan composition of PAL_3 and PAL_5-CPS were also demonstrated. The PAL_5-CPS showed no antigenicity with the homologous and heterologous antisera (results not shown). The lack of antigenicity with PAL_5-CPS could be explained by the presence of 80% of mannose in this fraction. A high mannose content has also been described in the exopolysaccharide of cowpea rhizobia (Hollingsworth et al., 1985). The specificity of PAL_3 polyclonal antiserum to its capsular antigen was also observed.

Differences between infective and non infective strains of *Rhizobium* have been attributed to specific antigens in the infective strain (Dazzo and Hubbell, 1975). The participation of CPS in the infection process has been demonstrated for the legume-*Rhizobium* symbiosis (Dazzo et al., 1982; Bagwat and Thomas, 1984). As we considered that CPS could also participate in the process of sugarcane infection, we used a meristematic clone of sugarcane (cultivar NA-5679), grown under sterile conditions, in order to study the infection process of these newly isolated bacteria. Preliminary results demonstrated that after 5 days of interaction the PAL_3 strain is able to infect the plant through the root system of seedlings, cultivated in a synthetic liquid medium.

Since the PAL_3 and PAL_5 strains showed different capsular material, as demonstrated by immunodiffusion, SDS-PAGE, immunoblotting and monosaccharide composition; further studies with these strains could add data about the possible role of the CPS in the infective process.

Acknowledgements

This research was supported by grants from the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (RHAE and PADCT); Financiadora

de Estudos e Projetos (FINEP), Fundação de Amparo a Pesquisa do Rio de Janeiro, Fundação Banco do Brasil (FBB), and CEPG-UFRJ. The authors are indebted to Mr. Orlando Augusto Agrellos Filho, Mrs. Lucy Jacinto do Nascimento, Mr. Jose Carlos Firmino for their technical assistance, and to Mr. Arlindo de Souza Penteado for typing the manuscript.

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