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Structural studies of an exopolysaccharide produced by *Gluconacetobacter diazotrophicus* Pal5

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ABSTRACT

Gluconacetobacter diazotrophicus is a nitrogen-fixing bacterium that has been found colonizing several plants. This acid-tolerant bacterium produces phytohormones that promote plant growth and is also able to grow in high-sugar concentrations. It has been demonstrated that exopolysaccharides (EPS), which are produced by strain Pal5 of *G. diazotrophicus*, play an important role in plant infection. We have investigated the structure of the EPS, which was produced by a strain of Pal5 grown in liquid medium containing mannitol as the sole carbon source. The results reveal an EPS with Glc, Gal, Man in a molar ratio of 6:3:1, respectively. NMR spectroscopy and chemical derivatization have revealed that the EPS structure has 4-*O*-substituted units of β-glucose, 3-*O*-substituted units of β-galactose and 2-*O*-substituted units of α-mannose. Glucose and galactose units linked at C6 were also found. The structure proposed herein is different from EPS produced by other species of *Gluconacetobacter* published to date.

1. Introduction

Gluconacetobacter diazotrophicus corrig. (Gillis et al., 1989) Yamada, Hoshino, & Ishikawa, 1998, comb. nov., is an endophyte, nitrogen-fixing bacterium found to colonize plants of the family Poaceae, such as sugar-cane (Cavalcante & Döbereiner, 1988), rice (Muthukumarasamy et al., 2007) and elephant grass (Videira et al., 2012); this organism can also be found inhabiting other plants, such as pineapple (Tapia-Hernández, Bustillos-Cristales, Jiménez-Salgado, Caballero-Mellado, & Fuentes-Ramirez, 2000), sweet potato (Paula, Reis, & Dobereiner, 1991) and coffee (Jiménez-Salgado et al., 1997). The isolation of *G. diazotrophicus* has been recently reported from the rhizosphere and phyllosphere of several desert plants (Hanna et al., 2012). In addition to its ability to fix N_2, this acid-tolerant bacterium also tolerates high concentrations of glucose (Kesters, Lisdiyanti, Komagata, & Swings, 2006) and is able to promote plant growth and biomass gain induced by auxin production (Sevilla, Burris, Gunapala, & Kennedy, 2001). *G. diazotrophicus* has other interesting physiological properties, such as zinc solubilization, and it also plays a role in the control of phytopathogens (Saravanan, Madhaiyan, & Thagaraju, 2007). Additionally, the presence of *G. diazotrophicus* post-infection shows no signs of harm or pathogenicity toward the host plant (Rouws et al., 2010).

Bacteria of the genus *Gluconacetobacter* are shown to produce large amounts of exopolysaccharides (EPS). In *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*), bacterial cellulose is biosynthesized from sugars and organic acids (Ruka, Simon, & Dean, 2012). In addition to insoluble cellulose, *G. xylinus* is also able to produce a water soluble EPS named acetan, which has a backbone of $[\rightarrow 4)$-β-D-Glc*p*-$(1\rightarrow]_n$ branched with a penta- or tetrasaccharide containing Man, Glc, Rha and GlcA (Couso, Ielpi, & Dankert, 1987, Jansson, Lindberg, Wimalasiri, & Dankert, 1993). Other species of *Gluconacetobacter* have also been reported to produce EPS, such as *Gluconacetobacter hansenii* (Jung, Park, & Chang, 2005), *Gluconacetobacter swingsii* (Castro et al., 2011), *Gluconacetobacter sacchari* (Trovatti, Serafim, Freire, Silvestre, & Neto, 2011) and *Gluconacetobacter intermedius* (Yang, Jia, Xing, Chen, & Lu, 2013). A nitrogen-fixing bacterial strain of *Gluconacetobacter kombuchae* sp. nov., a latter heterotypic synonym of *Gluconacetobacter hansenii* (Cleenwerck, De Wachter, González, De Vuyst, & De Vos, 2009), was found to produce cellulose even in nitrogen-free culture media (Dutta & Gachhui, 2007). Within the species *G. diazotrophicus*, strain Pal5 has been reported to have an enhanced EPS production when grown using mannitol as the carbon source (Meneses, Rouws, Simões-Araújo, Vidal, & Baldani, 2011).

Diazotrophic bacteria from the family Rhizobiaceae are studied for their ability to form nodules in leguminous plants in a highly specific symbiotic relationship, in which EPS play a key signaling

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role for the efficiency of colonization (Dazzo et al., 1984; Halverson & Stacey, 1986). Specifically, *Synorhizobium meliloti* may produce two EPS, which are a succinoglycan and a galactoglucan (Becker et al., 2002); these compounds are required for bacterial invasion and the formation of functional N_2-fixing nodules in alfalfa (Jones, 2012). However, non-nodulating, associative diazotrophs, such as *Azospirillum* spp., have also been reported to produce EPS (Burdman, Jurkevich, Soria-Díaz, Serrano, & Okon, 2000), which, in these cases, are involved in the attachment to plant root cells (Steenhoudt & Vanderleyden, 2000). The importance of EPS during the *Azospirillum*-wheat interaction has been demonstrated (Skvortsov & Ignatov, 1998). Reports show that strain Sp7 of *Azospirillum brasilense* is not found inside plant tissues, while the EPS-producing strain Sp245 is found endophytically in host plants (Assmus et al., 1995; Hartmann et al., 1995). Many other reports detail the production of EPS by associative and endophytic diazotrophs (Mattos, Jones, Heise, Previato, & Mendonça-Previato, 2001; Serrato et al., 2008; Skvortsov & Ignatov, 1998), although their function remains unclear. One of the proposed roles for EPS function is the molecular communication between plants and microbes during interaction (Leigh & Coplin, 1992). Although EPS may create a favorable microenvironment for bacteria by acting as a barrier against plant defenses and abiotic factors, it seems that there is more to EPS function than simply physical protection (Halverson & Stacey, 1986). EPS-deficient mutants of *Erwinia stewartii* and *Xanthomonas axopodis* do not exhibit any of the typical symptoms when infecting their respective plant hosts (Denny, 1995). Disruption of the genes involved in the biosynthesis of dTDP-rhamnose in *A. brasilense* ceases EPS production and impairs colonization (Jofré, Lagares, & Mori, 2004). In *Xanthomonas campestris*, a mutation in the gene *gumD*, which codes for a glycosil transferase involved in the biosynthesis of xanthan, drastically diminishes colonization (Chou et al., 1997). During the sequencing of the *G. diazotrophicus* Pal5 genome (Bertalan et al., 2009), fifteen genes related to the biosynthesis of polymeric carbohydrate substances (EPS and LPS) were found, including a gene homologous to *gumD*. It has been reported that the integrity of *gumD* is crucial for EPS production in *G. diazotrophicus* Pal5 and that root colonization is highly affected after disruption of this gene. It has also been shown that colonization of rice plants was restored when isolated EPS from the original strain was added to the plant growth solution (Meneses et al., 2011).

Because *G. diazotrophicus* shows interesting ecological and physiological characteristics, in addition to demonstrating the potential to promote plant growth, we have investigated the chemical and structural characteristics of the EPS produced by strain Pal5^T when grown on LGI medium, which contains mannitol as the sole carbon source The results demonstrate that the EPS produced by *G. diazotrophicus* Pal5 is different from the others reported in related species of *Gluconacetobacter* and insight to the role of these polymeric substances on plant–microbe interactions may be gained by its study.

2. Materials and methods

2.1. Strains, culture medium and growth conditions

Strain Pal5 of *G. diazotrophicus* (BR 11281 = ATCC 49037) was obtained from the Biological Resource Center at Embrapa Agrobiologia (Seropédica, RJ, Brazil) and grown in Dygs liquid medium (Rodrigues-Neto, Malavolta, & Victor, 1986) as pre-inoculum (5.0 mL). Cells were centrifuged and washed three times with 0.9% NaCl, suspended and inoculated into 1 L Erlenmeyer flasks containing 250 mL modified liquid LGI medium with 20.0 g L^{-1} of mannitol as the carbon source (Meneses et al., 2011). Cultures were kept at 30 °C in a rotary shaker (200 rpm) for 120 h. After cell removal, EPS in the supernatant was precipitated with cold ethanol (3:1, v/v) and kept overnight at −20 °C. It was recovered by centrifugation, dialyzed (MWCO 6 kDa) and freeze-dried (Serrato et al., 2006).

2.2. HPSEC-MALLS analysis

The homogeneity of the EPS was determined using a high performance steric exclusion chromatograph (HPSEC) with four Ultrahydrogel (Waters) columns (2000; 500; 250; 120) used in series; this system was coupled to a refractive index (RI) and a Wyatt Technology Dawn-F Multi Angle Laser Light Scattering (MALLS) apparatus. EPS solution (1.0 mg mL^{-1}) was prepared in the same eluent used to run the analyses (100 mM $NaNO_2$ containing 0.02% NaN_3) and filtered through a 0.22 μm nitrocellulose membrane (Millipore) before injection. Differential refractive index increments of the solvent–solute solution (dn/dc) were determined using a differential refractometer (Waters model 2410) with five concentrations of EPS (between 0.1 and 1.0 mg mL^{-1}). Data were collected and analyzed with the Wyatt Technology ASTRA 4.70.07 software. Light scattering (LS) signal was detected simultaneously at 11 scattering angles (θ), which ranged from 35° to 132°. All experiments were carried out at 25 °C with flow rate of 0.6 mL min^{-1}.

2.3. Partial hydrolysis of exopolysaccharides

Freeze-dried samples of EPS (10 mg) were hydrolyzed in 50 mM TFA at 100 °C; oligosaccharide formation was monitored over time by silica thin layer chromatography, which used 1-propanol:H_2O (6:1, v/v) as the mobile phase. Plates were developed with 0.25% orcinol solution in 5% H_2SO_4:EtOH (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). Oligosaccharide fractions were submitted to HPLC analysis coupled to an IR detector and an automated fraction collector. The stationary phase was a Rezex-RSO Oligosaccharides™ (Phenomenex) column eluted with deionized water (0.3 mL min^{-1}) and resolution was achieved at 75 °C. Glucose, maltose, maltotriose, maltotetraose and maltopentaose (Sigma–Aldrich) were used as standards (1.0 mg mL^{-1}).

2.4. Chemical derivatization and GC–MS analyses

The monosaccharide composition of the samples was determined by GC–MS as trimethylsilylated methyl glycosides (per-O-TMS-glycosides); these derivatives were obtained by methanolysis (1 M HCl in anhydrous methanol; 18 h at 80 °C) followed by trimethylsilylation using Tri-Sil reagent (Pierce Chemicals) for 20 min at 80 °C (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). Per-O-TMS-glycosides were recovered after partitioning with hexane–water and collecting the organic phase before injection. Monosaccharides were analyzed as their alditol acetate derivatives after hydrolysis (1 M TFA, 8 h, 100 °C), overnight reduction with $NaBH_4$ (Wolfrom & Thompson, 1963a) and acetylation with pyridine-Ac_2O (1:1, v/v), which was heated for 1 h at 120 °C (Wolfrom & Thompson, 1963b). Alditol acetate derivatives were recovered after addition of 1.0 mL of $CHCl_3$ and exhaustive partition washes with 5% copper sulfate solution to remove pyridine.

For the linkage analysis of monosaccharide units, freeze-dried samples were fully methylated with iodomethane (CH_3I) after solubilization in dimethyl sulfoxide (DMSO) with an excess of NaOH (Ciucanu & Kerek, 1984). Permethylated EPS were exhaustively dialyzed (MWCO 6 kDa) against water and permethylated oligosaccharides were recovered after a partition with $CHCl_3$:H_2O (1:1, v/v) and collection of the organic phase. Samples were then submitted to reduction and acetylation as described above, except that $NaBD_4$ (Sigma–Aldrich) was used as reduction agent, to form permethylated alditol acetate monosaccharide derivatives (PMAA) (Gorin & Iacomini, 1984).

GC–MS analyses were performed using a gas chromatograph (Varian Saturn 2000R), equipped with a Ross injector and DB-1 or DB-225 columns (30 m × 0.25 mm). Injector and flame ionization detector (FID) temperatures were kept at 260 °C. The temperature program was set to 100 °C to 140 °C (5 °C min^{-1}), then heated to 240 °C (4 °C min^{-1}) and held for 10 min with He as carrier gas (1.0 mL min^{-1}). Electron impact (EI) spectra were obtained with an ion trap mass spectrometer (Finnigan 810 R12) at 70 eV, 200 °C and mass range of 30–650 *m/z*. Post-run analysis was performed with Saturn Workstation 5.1 software.

2.5. Electrospray ionization mass spectrometry (ESI-MS) analyses

Oligosaccharide samples (100 μg/mL) were solubilized in MeOH:H_2O (2:1, v/v) containing 5 mM NaCl and submitted to positive ion mode ESI-MS investigation, which was performed on a triple quadrupole instrument (Waters Quattro LC) with N_2 as nebulizer and desolvation gas. Samples were injected directly into the ESI-MS source with a syringe infusion pump (10 μL min^{-1}). Ions were obtained via capillary at 3.4 kV and cone at 74 V.

2.6. Nuclear magnetic resonance (NMR) analyses

Monodimensional ^{13}C NMR spectra were obtained using a 400 and 600 MHz Bruker Avance III spectrometer equipped with 5.0 mm inverse probes, BBI and QXI, respectively. Samples were deuterium exchanged by repeated freeze-drying after dilution with 99.8% D_2O (Sigma–Aldrich); the samples were then solubilized in 350 μL of 99.996% D_2O (Cambridge Isotope Laboratories) and transferred to quartz tubes (i.d. 5 mm × 150 mm). Heteronuclear ($^1H/^{13}C$) single quantum coherence (HSQC) spectra were obtained with spectral width of 2.25 and 13.9 kHz, respectively and in datasets (t1 × t2) of 256 × 1024 with 16 or 32 scans. All chemical shifts are expressed in ppm (δ) relative to standard ^1H and ^{13}C signals of acetone, which are δ 2.22 and δ 31.5, respectively. Data were acquired and manipulated using the Bruker TopSpin software v.3.1.

3. Results and discussion

3.1. EPS homogeneity and molecular mass

G. diazotrophicus, strain Pal5, was grown for 5 days in 250 mL of modified LGI liquid medium containing 20 g L^{-1} of mannitol as the carbon source, as previously described. The medium was centrifuged and the supernatant was treated with an excess of cold ethanol; the resultant precipitate was dialyzed exhaustively against tap water. The material was then freeze-dried, which yielded 85 mg of EPS or an approximate production of 340 mg L^{-1} of cultivated medium. The EPS showed poor solubility in water after freeze-drying and concentrations above 5.0 mg mL^{-1} formed a viscous solution with precipitated material that could not be dissolved, even after mild heating. These observations suggested a very high molecular weight polymer, which was then analyzed by HPSEC-MALLS to determine its homogeneity and molecular mass. A single peak at 37.4 min was found in the chromatogram after injection of 1.0 mg mL^{-1} EPS solution (Supplementary Material). With the homogeneity of the sample determined, five concentrations of EPS (0.1–1.0 mg L^{-1}) were used to run a dn/dc analysis. The results showed that the molar mass found for the EPS produced by *G. diazotrophicus* Pal5 was 8.720×10^5 g mol^{-1} with a polydispersivity (Mw/Mn) of 1.611 ± 0.299.

3.2. Monosaccharide composition and glycosidic linkage analysis

After a series of reactions to form either alditol acetate or per-O-methyl-glycoside derivatives, the monosaccharide composition of the EPS produced by *G. diazotrophicus* Pal5 was determined. The GC–MS analysis of alditol acetate derivatives gave rise to Glc, Gal and Man in a molar ratio of 6:3:1, respectively. To determine the presence of acidic monosaccharides, per-O-trimethylsilyl-glycoside derivatives were also analyzed by GC–MS; however, the chromatograms and MS fragmentation profiles showed no signs of carboxylated units. In fact, the results obtained with the per-O-TMS derivatives confirmed the same composition of Glc:Gal:Man (6:3:1) previously found for the alditol acetate derivatives. PMAA were also analyzed by GC–MS and revealed the presence of the derivative 2,3,4,6-Me_4-1,5-*O*Ac-glucitol (25%), which indicates a terminal of glucose in the structure. Glucose units were also found as 4-*O*-Glc*p* (25%) and 6-*O*-Glc*p* (13%), indicated by their derivatives 2,3,6-Me_3-1,4,5-*O*Ac-glucitol and 2,3,4-Me_3-1,5,6-*O*Ac-glucitol, respectively. A disubstituted unit of galactose (2,4-Me_2-1,3,5,6-*O*Ac-galactitol) was also found in the same amount, which indicates that this saccharide is the branching unit connected to the *t*-Glc residue. The only mannose derivative found was 3,4,6-Me_3-1,2,5-*O*Ac-mannitol (12%), which corresponds to a 2-*O*-Man unit. Table 1 shows a summary of the PMAA found for the EPS of *G. diazotrophicus*, Pal5. The ^{13}C NMR spectrum of the EPS gave rise to five anomeric signals, which were assigned to the corresponding monosaccharide units obtained from the PMAA derivatives as 2-*O*-α-Man*p* (100.1 ppm), *t*-β-Glc*p* (101.8 ppm), 6-*O*-β-Glc*p* (102.4 ppm), 4-*O*-β-Glc*p* (102.7 ppm) and 3,6-*O*-β-Gal*p* (103.1 ppm). Linkage signals were also determined for each unit. The signal found at 78.9 ppm was assigned to C2 of mannose units. The C4 and C6 linkages of the monosubstituted glucose units were found at 80.5 and 69.9 ppm, respectively. Di-substituted galactose residues exhibited linkage signals at 67.4 ppm (C6) and 80.8 ppm (C3). Unsubstituted C6 from all hexoses could be observed as three high field signals found at 60.1, 60.5 and 61.0 ppm (Fig. 1).

Table 1
Partially methylated alditol acetate (PMAA) derivatives obtained from the EPS produced by *Gluconacetobacter diazotrophicus*, Pal5.

PMAA derivatives[a]	R_t (min)[b]	Glycosidic linkage	Mol%
2,4-Me_2-1,3,5,6-OAc-galactitol	22.3	3,6-Galp	24.8
2,3,4-Me_3-1,5,6-OAc-glucitol	13.0	6-Glcp	12.6
2,3,6-Me_3-1,4,5-OAc-glucitol	14.4	4-Glcp	25.1
3,4,6-Me_3-1,2,5-OAc-mannitol	12.7	2-Manp	12.3
2,3,4,6-Me_4-1,5-OAc-glucitol	10.1	t-Glcp	25.2

[a] PMAA obtained after methylation with CH_3I (methyl iodine) in 30% NaOH–DMSO, hydrolysis, reduction with $NaBD_4$ and acetylation.
[b] Column DB-225; temp. 215 °C.

3.3. Partial hydrolysis of the EPS and ESI-MS analysis

Due to its high viscosity, the EPS produced by *G. diazotrophicus* Pal5 was submitted to partial hydrolysis with 50 mM TFA to obtain more soluble oligomers. The reaction was monitored by TLC to verify the oligosaccharide formation. After 3 h of hydrolysis, free monosaccharides started to arise in the TLC profiles, which were developed with orcinol as described; the EPS was submitted to a 2 h-hydrolysis to produce oligosaccharides for further analyses. The resulting oligosaccharide pool was submitted to ESI-MS analysis by direct injection into the ionization chamber. The positive mode spectrum revealed the presence of ions at *m/z* 851.3 attributed to a hexose-based pentasaccharide ($[Hex_5+Na]^+$) and sequential mass differences (Δ 162 *m/z*) found in other ions unveiled the presence of oligossacarides with different degrees of polymerization, which were a tetrasaccharide at *m/z* 689.4 ($[Hex_4+Na]^+$), a trisaccharide at *m/z* 526.9 ($[Hex_3+Na]^+$) and a disaccharide at *m/z* 365.0 ($[Hex_2+Na]^+$). Fig. 2 shows the spectrum for the oligosaccharides that arose from the partially hydrolyzed EPS. ESI-MS results validate the monosaccharide composition measured previously to

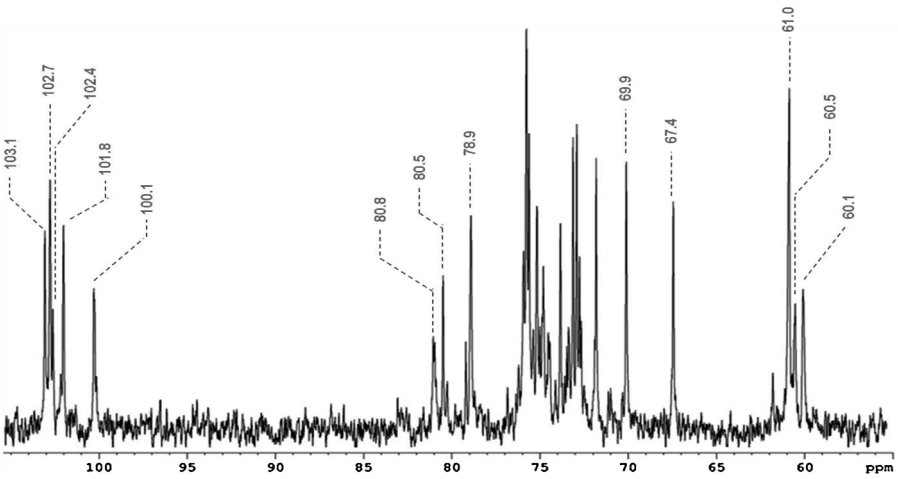

Fig. 1. ^{13}C-NMR spectrum of the EPS produced by *Gluconacetobacter diazotrophicus* Pal5. Chemical shifts are shown as δ (ppm).

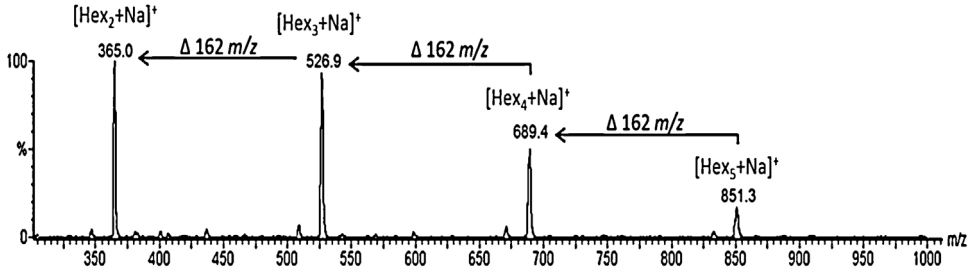

Fig. 2. Positive ion mode ESI-MS spectrum of the oligosaccharide pool obtained from the partially hydrolized EPS, which was produced by *Gluconacetobacter diazotrophicus* Pal5.

identify the hexoses in the EPS structure. The 2 h-hydrolyzate was then submitted to fractionation by HPLC, as described. The resulting chromatogram showed a very intense peak at 42.28 min, which was attributed to a pentasaccharide (DP = 5) by comparison to the retention times of oligosaccharide standards. It was also possible to observe other oligosaccharides with different degrees of polymerization in small amounts, with the exception of a higher mass oligomer (DP > 8) eluted on V_o (Fig. 3). The pentasaccharide was recovered via automated collection and submitted to chemical and structural analysis. Alditol acetate derivatives showed a monosaccharide composition of Glc:Gal:Man in a molar ratio of 3:1:1, respectively. Four PMAA derivatives were found after GC–MS analysis for linkage determination. The derivative, 2,3,6-Me$_3$-1,4,5-OAc-glucitol (40%), indicates the presence of two C4-substituted residues of glucose. A terminal non-reducing end of Glc*p* was also found (20%) as 2,3,4,6-Me$_4$-1,5-OAc-glucitol. Residues of Man*p* (2-O-substituted) and Gla*p* (3-O-substituted) were also found as their respective PMAA derivatives 3,4,6-Me$_3$-1,2,5-OAc-mannitol (20%) and 2,4,6-Me$_2$-1,3,5-OAc-galactitol (20%). However, substitutions on C6 were not found for any of the derivatives, which suggests that the partial hydrolysis process could remove the labile C6-linked monosaccharides. Monosaccharide composition and PMAA derivatives found for the isolated oligosaccharide corroborate the structure of a linear pentasaccharide. 2D-NMR analyses were performed to further elucidate structure in detail. The HSQC-TOCSY spectrum (Fig. 4) shows direct $J_{C/H}$ correlation signals at 100.9/5.32, which were attributed to C1/H1 of the 2-*O*-α-Man*p* unit. C2/H2 correlation signals for this same residue were found at 79.3/4.44. A glucose-based reducing-end was assigned by the presence of two correlation signals found at 93.0/5.30 (α-anomer) and 96.9/4.73 (β-anomer). Anomeric signals of 4-O-substituted units of glucose were assigned at 102.3/4.52, while glycosidic linkage signals (C4/H4) were found at 78.6/3.74 (reducing terminal) and 80.1/3.58. Signals found at 101.8/4.56 were attributed to C1/H1 of a β-Glc*p* non-reducing terminal unit. The 3-*O*-substituted galactopyranosyl unit showed anomeric correlations at 103.7/4.67 and linkage signals

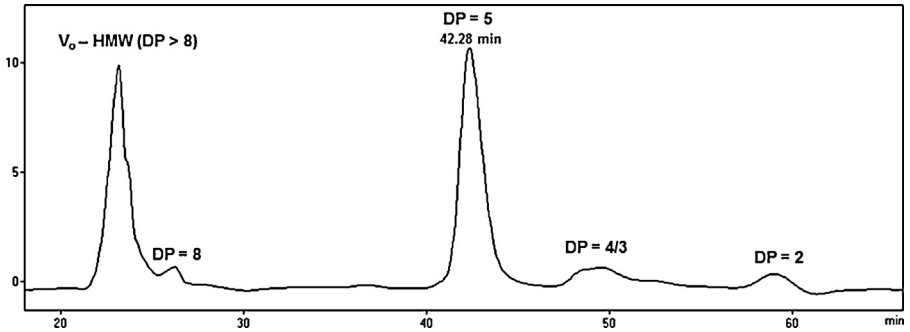

Fig. 3. HPLC profile of oligosaccharides obtained after 2 h-partial hydrolysis of the EPS, which was produced by *Gluconacetobacter diazotrophicus* Pal5. Column Rezex-RSO Oligosaccharides^{TM} (0.3 mL min^{-1}; 75 °C).

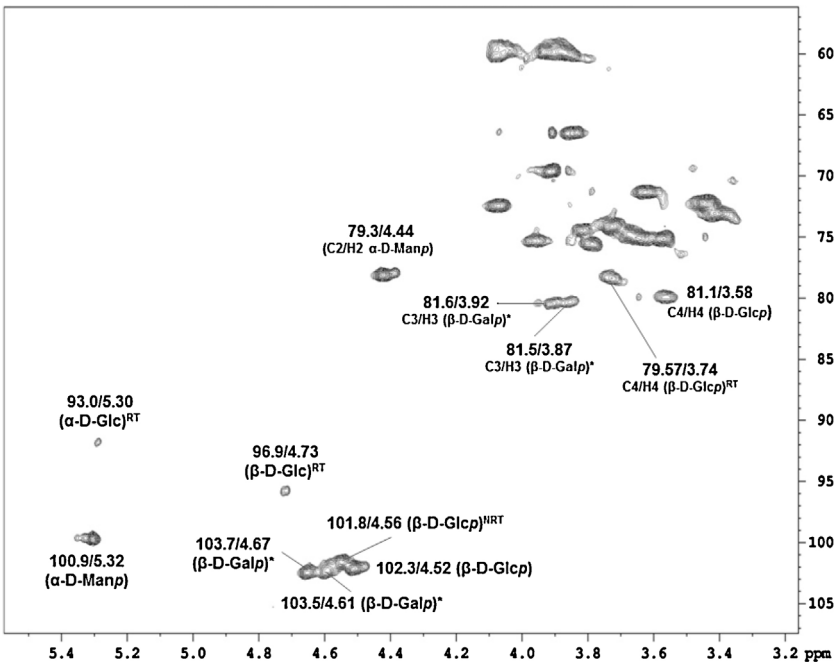

Fig. 4. HSQC spectrum of the pentasaccharide obtained after partial hydrolysis of the EPS, which was produced by *Gluconacetobacter diazotrophicus* Pal5. (RT) reducing terminal; (NRT) non-reducing terminal; (*) mutarotation effect from RT on chemical shift of galactopyranosyl residues.

were found at 81.5/3.87 (C3/H3). The effect of the mutarotation from the glucopyranosyl reducing end seemed to affect the chemical shifts of the galactopyranosyl residue; signals at 103.5/4.61 (C1/H1) and 81.6/3.92 (C3/H3) may also be observed for that unit, which suggests that these are neighboring monosaccharides in the structure. Anomeric configuration of all units was also confirmed by a proton coupled HSQC experiment and determination of J_{CH} coupling constants (Fig. 5). As expected from the ^{13}C and ^{1}H chemical shifts previously observed, the alpha anomeric configuration for the mannopyranosyl residue was confirmed (J_{C1H1} = 174.1 Hz) and all other monosaccharide units present in the structure showed beta anomeric configurations (J_{C1H1} < 164.0 Hz). The coupling constants for the alpha and beta configurations on the reducing unit of glucose were also determined to be 170.8 and 157.8 Hz, respectively. Fig. 6 shows the inter-residual correlations between anomeric protons and nearby protons, which gives information about the monosaccharide sequence. A characteristic anomeric signal from the 2-linked-α-mannose unit showed a correlation with the H3 from the galactopyranosyl residue (δ 5.31/3.87). Additionally, the signal attributed to H2 of mannose (δ 4.44) had a strong correlation with δ 4.52, which was assigned to the anomeric proton of a 4-linked-β-glucopyranose unit. This unit shows a H4 chemical shift at δ 3.58, which was correlated with the anomeric proton from the non-reducing glucopyranose terminus (δ 4.56). A strong correlation was also found between the anomeric proton of the 3-O-substituted galactopyranosyl unit and the H4 of the reducing terminal glucose (δ 4.61/3.74). We have inferred that the pentasaccharide isolated has the following structure: β-Glc*p*-(1 → 4)-β-Glc*p*-(1 → 2)-α-Man*p*-(1 → 3)-β-Gal*p*-(1 → 4)-Glc-ol.

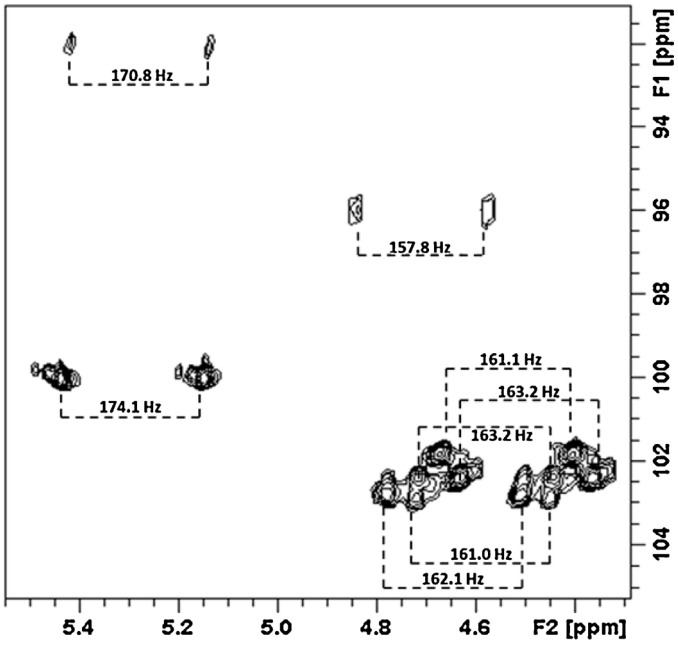

Fig. 5. Coupled HSQC spectrum (anomeric region) of the pentasaccharide isolated after partial hydrolysis of the EPS, which was produced by *Gluconacetobacter diazotrophicus* Pal5. Coupling constants ($J_{C;H/H}$) are shown in Hz.

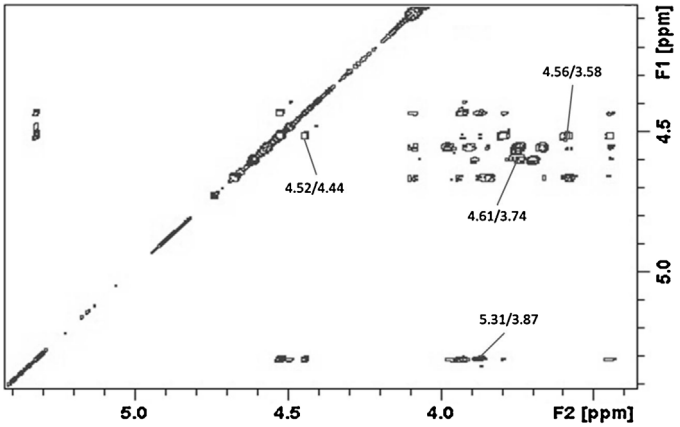

Fig. 6. NOESY spectrum of the pentasaccharide isolated after partial hydrolysis of the EPS, which was produced by *Gluconacetobacter diazotrophicus* Pal5.

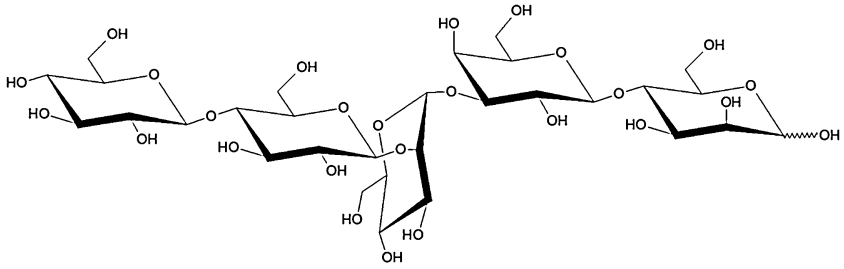

Fig. 7. Proposed structure of the pentasaccharide isolated after partial hydrolysis of the EPS produced by *Gluconacetobacter diazotrophicus*, strain Pal5 (explicit hydrogens not shown).

4. Conclusions

In this work we describe the chemical characteristics of an exopolysaccharide produced by *G. diazotrophicus*, strain Pal5, when grown in a liquid medium containing mannitol as the sole carbon source. Based on chemical derivatization and spectroscopic analysis, we have demonstrated that the EPS structure lacks deoxyhexoses and acidic units, which were characteristics of acetan, an EPS previously described for other species of *Gluconacetobacter*. The presence of galactopyranosyl residues on the EPS produced by strain Pal5 is also a major difference when comparing to acetan. Some structural similarities, however, may be found between acetan and the EPS described here. Both polymers show 4-O-substituted glucopyranosyl residues in appreciable amounts. Another structural resemblance with acetan is the presence of a 2-O-linked-α-mannose unit. In contrast to acetan, the EPS produced by strain Pal5 of *G. diazotrophicus* has poor solubility in water. This may be explained by the lack of acidic units and the large number of beta-anomeric configurations in the structure. Partial hydrolysis performed to aid in the study showed that C6-linked units were more labile and therefore were the cleaving point in the structure, which yielded a pentasaccharide whose proposed structure is shown in Fig. 7.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.07.025.

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