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Structural characterization of an acidic exoheteropolysaccharide produced by the nitrogen-fixing bacterium *Burkholderia tropica*

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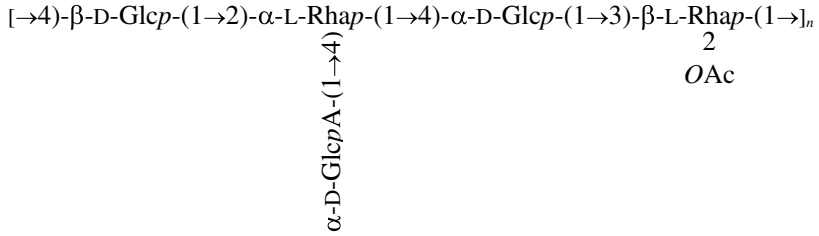
Abstract

An acidic exopolysaccharide (EPS) produced by the diazotrophic bacterium *Burkholderia tropica*, strain Ppe8, was isolated from the culture supernatant of bacteria grown in a synthetic liquid medium containing mannitol and glutamate. Monosaccharide composition showed Rha, Glc and GlcA in a 2.0:2.0:1.0 molar ratio, respectively. Further structural characterization was performed by a combination of NMR, mass spectrometry and chemical methods. Partial acid hydrolysis of EPS provided a mixture of acidic oligosaccharides that were characterized by ESI-MS, giving rise to ions with *m/z* 193 (GlcA-H)$^-$, 339 (GlcA,Rha-H)$^-$, 501 (GlcA,Rha,Glc-H)$^-$, 647 (GlcA,Rha$_2$,Glc,-H)$^-$, 809 (GlcA,Rha$_2$,Glc$_2$,-H)$^-$ and 851 (GlcA,Rha$_2$,Glc$_2$,*O*Ac-H)$^-$. Carboxyreduced EPS (EPS-CR) had Glc and Rha in a 3:2 ratio, present as D- and L-enantiomers, respectively. Methylation and NMR analysis of EPS and EPS-CR showed a main chain containing 2,4-di-*O*-Rha*p*, 3-*O*-Rha*p* and 4-*O*-Glc*p*. A GlcA side chain unit was found in the acidic EPS, substituting O-4 of α-L-Rha*p* units. This was observed as a non-reducing end unit of glucopyranose in the EPS-CR. Acetyl esters occured at O-2 of β-L-Rha*p* units. From the combined results herein, we determined the structure of the exocellular polysaccharide produced by *B. tropica*, Ppe8, as being a pentasaccharide repeating unit as shown:



Keywords: *Burkholderia*; EPS; Endophytic; Diazotrophic; Exopolysaccharide

1. Introduction

Microbial exopolysaccharides (EPS) are found ubiquitously in nature and occur in several soil bacteria. They can influence soil stability and the physical properties of soil aggregates and since their associated microenvironments and mechanical characteristics are in continuous evolution, a wide range of effects can occur, including influence of crop yields (Burdman, Jurkevitch, Schwartsburd, & Okon, 1999). The highly hydrated layer of EPS that surrounds the bacteria offers a safe environmental protection

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against desiccation and the mechanical removal from the soil (Romantschuk, 1992; Whitfield, 1988). The conformational characteristics of EPS affect the binding of useful and/or toxic molecules available to its cells (Costerton, Geesy, & Cheng, 1987).

As far as biological activity is concerned, EPS produced by nitrogen-fixing bacteria have been reported to serve as molecular signals during plant invasion. In the case of the *Rhizobiaceae*, numerous EPS structures are produced and, in the case of legume hosts that form indeterminate nodules, are required for invasion of the host cells and the formation of functional N_2-fixing nodules (Carlson, Reuhs, Forsberg, Kannenberg, & Goldberg, 1999). A specific example is that of *Sinorhizobium meliloti* and its host, alfalfa. *Sinorhizobium meliloti* can produce two EPS, namely a succinoglycan and a galactoglucan (González, Reuhs, & Walker, 1996) Bacterial invasion and formation of functional N_2-fixing nodules require these EPS (Leigh, Signer, & Walker, 1985), although their function is not yet clear. Non-nodulating, free-living diazotrophs such as *Azospirillum* spp., have also been reported to produce EPS (Burdman, Jurkevitch, Soria-Díaz, Serrano, & Okon, 2000), which are involved in attachment to grass root cells (Steenhoudt & Vanderleyden, 2000).

To date, approximately 30 different species are recognized as belonging to the genus *Burkholderia* (Coenye & Vandamme, 2003). For an extended period, the ability for nitrogen fixation in *Burkholderia* was recognized only for *B. vietnamiensis*, which was isolated from the rhizosphere of rice plants (Gillis et al., 1995). Recently, endophytic species of *Burkholderia* have been isolated from a variety of plants, and were found to be nitrogen-fixing (Caballero-Mellado, Martínez-Aguilar, Paredes-Valdez, & Estrada-De Los Santos, 2004; Estrada et al., 2002), and some of which were found to produce exopolysaccharides. *B. brasiliensis* synthesizes two EPS (Mattos, Jones, Heise, Previato, & Mendonça-Previato, 2001), one of which is a linear *O*-acetylated EPS with the pentasaccharide repeating unit: →4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*[2*O*Ac]-(1→4)-β-D-Glc*p*-(1→. Rare sugar units were found in the EPS produced by *B. caribensis*, strain MWAP71 and *B. pseudomallei*. The former produces an extracellular polysaccharide containing D-Glc*p*, L-6dTal*p* (6-deoxy-L-talose), Kdo*p* (3-deoxy-2-manno-2-octulosonic acid) and *O*-acetyl substituents (Vanhaverbeke, Heyraud, Achouak, & Heulin, 2001). *B. pseudomallei* also produced a Kdo-containing polysaccharide (Steinmetz et al., 2000) which was characterized as a tetrasaccharide repeating group of →3)-β-D-Gal*p*[2*O*Ac]-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→5)-β-Kdo*p*-(2→. *Burkholderia cepacia* has been described as the type species for the genus (Yabuuchi et al., 1992). It was originally described as a plant pathogen (Burkholder, 1950), and has become an important opportunistic pathogen in patients with cystic fibrosis. *B. cepacia* was reported to produce a complex EPS containing an unusual tri-*O*-substituted α-Glc*p*A residue (Linker, Evans, & Impallomeni, 2001), which confers interesting rheological properties (Sist et al., 2003). A second EPS with the disaccharide repeating group →3)-β-D-Glc*p*-(1→3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p* has also been described (Cérantola, Marty, & Montrozier, 1996). The involvement of these EPS in the interactions of clinical isolates of *B. cepacia* (Govan & Deretic, 1996) and *B. pseudomallei* (Steinmetz, Rhode, & Brennek, 1995) with their host cells has been suggested.

The nitrogen-fixing bacterium *Burkholderia tropica*, strain Ppe8, is an endophyte isolated from sugar cane (Reis et al., 2004), which showed an amplified 16S rDNA restriction pattern identical to that of pineapple isolates (Cruz et al., 2001). When grown in a liquid synthetic medium containing mannitol and glutamate, *B. tropica* Ppe8 produced copious amounts of a 300-kDa acidic exopolysaccharide containing Glc, Rha and GlcA in a molar ratio of 2:2:1, respectively (Serrato et al., 2006). We now report the complete structural characterization of the EPS produced by *B. tropica* Ppe8, using chemical, spectrometric and spectroscopic techniques.

2. Experimental

2.1. Bacterial strain and growth conditions

Burkholderia tropica, strain Ppe8 (=ATCC BAA-831(T)=LMG 22274(T)=DSM 15359(T), was furnished by the *Empresa Brasileira de Pesquisa Agropecuária* (EMBRAPA), Seropédica, Brazil, and was maintained on 50% aq. glycerol at −20 °C. Cells were grown in 2 L Erlenmeyer flasks containing 500 mL of synthetic JMV medium (Baldani et al., 1996) containing mannitol (5.0 g L^{-1}) and glutamate (2.0 g L^{-1}). Batch cultures were maintained at 30 °C for 72 h under constant orbital shaking (120 rpm).

2.2. Purification methodology

After cell removal by centrifugation, the medium was concentrated and added to excess cold ethanol (3 v/v). The resulting precipitate was recovered by centrifugation (8000*g*, 10 min) the pellet re-solubilized in water, and dialyzed for 48 h against dH_2O in a 14 kDa molecular weight cut-off (MWCO) membrane. Retained material was then purified by anion-exchange chromatography using a Q-Sepharose fast-flow column (170 × 15 mm id) coupled to a FPLC system. A continuous NaCl gradient was used up to 1 M.

The acidic EPS was submitted to partial hydrolysis with M CF_3CO_2H at 100 °C for 1 h in order to obtain oligosaccharides. After freeze-drying, the material was dissolved in water and added to excess cold ethanol. The resulting precipitate was removed by centrifugation and the supernatant containing the mixture of oligosaccharides was freeze-dried, diluted in dH_2O (5.0 mg mL^{-1}) and loaded onto a BioGel P-2 column (120 × 10 mm id) and eluted with degassed dH_2O.

All fractions obtained were analyzed for their carbohydrate content using the PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Carboxyreduction of acidic EPS

A portion of the purified acidic EPS was carboxyreduced in order to reduce uronic acid units to their neutral analogues. To 10 mg of EPS were added 10 mL of 200 mM 2-*N*-morpholine-ethanosulfonic acid (MES buffer) at pH 4.75. The material was kept under constant stirring for 2 h with further additions (10 mL) of *N*-cyclohexyl-*N*′-[β-(methyl-morpholine)ethyl]-carbodiimide *p*-toluenesulfonate. The solution was then neutralized with 2 M TRIS buffer pH 7.0 and $NaBH_4$ was added up to 2 M. The reaction was left overnight and the pH then adjusted to 5.0 with HCl (Taylor & Conrad, 1972). The solution was dialyzed for 48 h (14 kDa MWCO membrane) and freeze-dried. The carboxyreduction process was repeated twice to guarantee the complete reduction of all uronic acid units. The resulting product (EPS-CR) was then analyzed by infrared spectroscopy coupled to Fourier transform (FT-IR), performed with a Bomem Hartman (MB-Series) spectrophotometer using 100 mg KBr disks containing 2% of material.

2.4. Derivatization methodology and GC–MS analysis

Monosaccharide composition of each sample was determined by GC–MS as trimethylsilylated methyl glycosides (per-*O*-TMS-glycosides), which were obtained after methanolysis (1 M HCl in anhydrous methanol; 18 h at 80 °C) followed by trimethylsilylation using TRI-SIL reagent (Pierce) for 20 min at 80 °C (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). The resulting methyl per-*O*-trimethylsilylglycosides were then analyzed by GC–MS using a DB-1 capillary column (Hewlett–Packard HP 5890 GC interfaced to a 5970 MSD). The temperature program used for GC–MS analysis was: 50–160 °C (20 °C min^{-1}), hold for 2 min, increase to 200 °C at a rate of 2 °C min^{-1} and then to 250 °C at 10 °C min^{-1}. Neutral monosaccharides were also analyzed as their alditol acetate derivatives after hydrolysis (1 M TFA, 8 h at 100 °C), overnight reduction with $NaBH_4$ and acetylation of the material (Wolfrom & Thompson, 1963a, 1963b). GC–MS conditions were the same as described above.

Glycosyl linkage analysis was performed by the preparation of partially methylated alditol acetates (PMAA) according to Ciucanu and Kerek (Ciucanu & Kerek, 1984). The resulting PMAA derivatives were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, with a DB-225 capillary column. Analyses were carried out from 50 to 220 °C at 40 °C min^{-1}, then maintaining the temperature (30 min). The products were identified by their typical retention times and electron impact profiles (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

The enantiomeric configuration of monosaccharides forming the acidic EPS and EPS-CR was determined by their 2-octyl-glycoside acetate derivatives as described by Leontein, Lindberg, and Lönngren (1978) except that (−)-2-octyl-glycoside was used and that the column used was DB-23.

2.5. NMR spectroscopy

NMR spectra were obtained using a 400 MHz Bruker DRX Avance spectrometer. Samples were deuterium exchanged by repeated freeze-drying from 99.8% D_2O (Aldrich) and then solubilized in 350 μL D_2O (100% D; Cambridge Isotope Laboratories) and transferred to 5 mm tubes. Analyses were carried out at 70 °C, acetone being used as chemical shift reference for ^1H (δ 2.22) and ^{13}C (δ 31.5). Several 1D and 2D NMR experiments were done to assign the chemical shifts of constituent sugars. $^1H—^1H$ COSY and TOCSY, both collected with 64 pulses of 1024×256 data points ($t1 \times t2$) and mixing time of 80 ms. Carbon chemical shifts were assigned by comparing COSY and TOCSY with 1D ^{13}C NMR and 2D heteronuclear multiple quantum coherence (HMQC) experiment. $^1J_{C—H}$ coupling constants were determined from the proton couples ^{13}C NMR experiment.

2.6. ESI-MS of oligosaccharides

The oligosaccharide mixture obtained after partial hydrolysis of the acidic EPS was dissolved in MeOH:H_2O (1:1 v/v) (30 μg/mL) and analyzed using a Micromass Quattro LC spectrometer by direct injection. Analysis was carried out on negative mode using a single quadrupole with a mass window of *m*/*z* 150–950. The parameters used for the acquisition of ions were: capillary 5 kV, cone 200 V and ion energy 1.9 V.

3. Results

3.1. EPS purification and carboxyreduction

Burkholderia tropica, strain Ppe8, was grown for 72 h in liquid JMV medium until its exponential phase was reached ($OD_{600} \sim 8.0$). The medium was freed of cells by centrifugation and the resulting supernatant was concentrated, treated with excess cold ethanol and the resulting precipitate dialyzed. The product was then applied to a Q-Sepharose column eluted with a gradient of NaCl up to 1 M. A single carbohydrate-containing fraction was eluted at 520 mM NaCl, providing purified acidic EPS. The amount of material obtained after purification corresponded to approximately 850 mg L^{-1} of liquid culture. A portion of the EPS was submitted to carboxyreduction to give neutral EPS-CR. Reduction was complete when FT-IR analysis showed the absence of a band corresponding to carboxyl groups at 1740 cm^{-1}. The yield of the material recovered as neutral EPS-CR was approximately 95%.

3.2. Monosaccharide composition and enantiomeric characterization

Both native EPS and EPS-CR had their monosaccharide composition determined by their per-*O*-TMS-methyl glycosides. The acidic EPS contained Rha, Glc and GlcA in an approximate molar ratio of 2.0:2.0:1.0, while carboxyreduced EPS (EPS-CR) had Rha and Glc in a 1.0:1.5 molar ratio, respectively. Neutral sugars were also analyzed as their alditol acetate derivatives. As acidic units are not represented on GC–MS of alditol acetate derivatives, both the acidic EPS and EPS-CR gave rise to Rha and Glc, although EPS-CR showed a higher amount of Glc, resulting from the reduction of the GlcA unit of the acidic EPS to its corresponding neutral analogue. Results on the monosaccharide composition are summarized on Table 1. The hydrolyzate of EPS and EPS-CR were also derivatized to form (−)-2-octyl glycoside acetates, which were then submitted to GC–MS analysis. The chromatogram profiles were very similar for both samples, each containing eight peaks with retention times and fragmentation profiles corresponding to L-rhamnose and D-glucose. Since the chromatogram for EPS-CR showed only (−)-2-octyl-D-glucosyl acetate derivatives, it was assumed that the glucuronic acid unit present on the native EPS structure was present as the D-enantiomer.

3.3. Linkage analysis and NMR results for EPS and EPS-CR

Methylation analysis of acidic EPS (Table 1) showed the presence of 3-*O*- and 2,4-di-*O*-substituted Rha*p* and 4-*O*-substituted Glc*p* units. The molar ratio found for the PMAA derivatives was 1.0:1.1:2.0 for 2,4-Me_2-Rha*p*, 3-Me-Rha*p* and 2,3,6-Me_3-Glc*p*, respectively. PMAA derivatives of neutral EPS-CR revealed the presence of a non-reducing end unit of Glc*p*A, not shown for EPS, as a derivative of 2,3,4,6-Me_4-Glc*p*, as well as 2,4-Me_2- and 3-Me-Rha*p*, and 2,3,6.Me_3-Glc*p* derivatives in a molar ratio of 1.2:1.1:1.0:2.2, respectively.

The ^{13}C NMR spectrum of EPS (Fig. 1) contained four signals int the anomeric region at δ 103.0 (E1), 99.0 (D1), 97.9 (C1 + B1) and 94.6 (A1). The CH_3-6 of rhamnosyl units was at δ 16.7 and those at δ 175.4 and 173.4 were from GlcA $-CO_2$ (A6) and acetyl substituents $-CO_2CH_3$, respectively, the latter also giving rise to a CO_2CH_3 signal at δ 20.3. Two $-CH_2OH$-6 signals of Glc*p* units were present at δ 60.9 (C6) and 60.7 (E6), suggesting the absence of O-6 substituted Glc*p* units, also shown by methylation analysis. The ^{13}C/^{1}H-coupled spectrum of EPS had C-1 doublets centered at δ 94.6, 97.9, 99.0 and 103.0. Coupling constants of each pair-signals were attributed to α-D-Glc*p*A ($J_{C\text{-}1,H\text{-}1} = 170$ Hz), α-D-Glc*p* ($J_{C\text{-}1,H\text{-}1} = 170$ Hz), α-L-Rha*p* ($J_{C\text{-}1,H\text{-}1} = 160$ Hz), β-L-Rha*p* ($J_{C\text{-}1,H\text{-}1} = 168$ Hz) and β-D-Glc*p* ($J_{C\text{-}1,H\text{-}1} = 160$ Hz) units.

The ^{13}C NMR spectrum of EPS-CR had five anomeric signals at δ 103.2 (E′1), 100.7 (B′1), 98.3 (D′1), 98.2 (C′1) and δ 95.7 (A′1), corresponding to β-D-Glc*p*, α-L-Rha*p*, β-L-Rha*p* and two α-D-Glc*p* units, respectively, the last being the corresponding to non-reducing from the terminal Glc*p*A units, found in the acidic EPS. Three CH_2OH-6 signals arose at δ 61.4 (E′6), 61.1 (C′6) and 60.7 (A′6), the extra one arising from the carboxyreduced Glc*p*A units. CH_3-6 of rhamnosyl units appeared at δ 17.1 (B′6 + D′6). High frequency signals of uronic acids were absent, as were those of *O*-acetyl substituents, due to de-*O*-acetylation during carboxyreduction. The signal that arose at δ 81.1 was assigned to the *O*-substituted C-3 of a rhamnopyranosyl units.

Fig. 2 shows the ^{1}H NMR anomeric region for both EPS and EPS-CR. The acidic EPS gave rise to four anomeric signals, and the integrated relative area suggested that the one at δ 5.02 might be from two overlapping signals (A1 + D1) attributed to the terminal α-D-Glc*p*A and 3-*O*-[2-*O*Ac]-β-L-Rha*p* units, which was confirmed by 2D experiments. The other signals were assigned as 4-*O*-β-D-Glc*p* (δ 4.68), 2,4-*O*-α-L-Rha*p* (δ 4.88) and 4-*O*-α-D-Glc*p* (δ 4.96). Five anomeric signals appeared in the ^{1}H NMR spectrum of EPS-CR. Integration areas were determined and the signals found at δ 4.61 (E′1), 4.73 (B′1), 4.94 (D′1), 4.97 (C′1) and 5.03 (A′1) were attributed to 4-*O*-β-

Table 1
Monosaccharide composition and linkage analysis for the acidic EPS and its carboxyreduced product (EPS-CR)

	Derivatives						Linkage
	per-*O*-TMS[a]		Alditol acetate[b]		PMAA[c]		
	EPS	EPS-CR	EPS	EPS-CR	EPS	EPS-CR	
Rha	1.9	1.0	1.0	1.0	1.0	1.0	→3)-Rha*p*-(1→[A]
Glc	2.0	1.5	1.0	1.5	1.1	1.1	→2,4)-Rha*p*-(1→[B]
GlcA	1.0	–	–	–	2.0	2.2	→4)-Glc*p*-(1→[C]
					–	1.2	*t*-Glc*p*[D]

Linkages shown are abbreviations of: [A]1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol, [B]1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-rhamnitol, [C]1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol, [D]1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol.
Values are shown as relative molar contents.
[a] Monossacarides were analyzed as per-*O*-trimethylsilyl-methyl glycoside derivatives obtained after methanolysis and TRI-SIL treatment.
[b] Alditol acetate derivatives were formed after hydrolysis, overnight reduction with $NaBH_4$ and acetylation.
[c] Partially *O*-methylated alditol acetates obtained after methylation, followed by methanolysis, hydrolysis, reduction with $NaBD_4$ and acetylation.

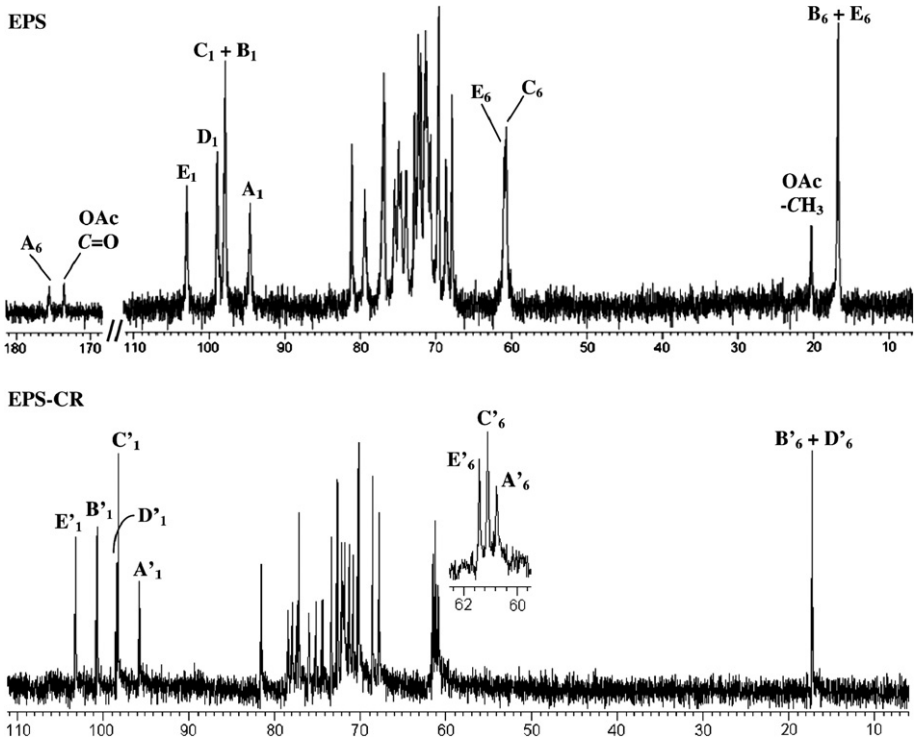


Fig. 1. ^{13}C NMR spectrum of acidic EPS and neutral EPS-CR. Monosaccharide units for the acidic EPS are represented as: A (*t*-α-D-GlcA*p*), B (→2,4-α-L-Rha*p*), C (→4-α-D-Glc*p*), D (→3-[2-*O*Ac]-β-L-Rha*p*), E (→4-β-D-Glc*p*). EPS-CR monosaccharide units are represented as: A′ (*t*-α-D-Glc*p*), B′ (→2,4-α-L-Rha*p*), C′ (→4-α-D-Glc*p*), D′ (→3-β-L-Rha*p*), E′ (→4-β-D-Glc*p*). Scale is shown in δ (ppm).

D-Glc*p*, 2,4-*O*-α-L-Rha*p*, 3-*O*-β-L-Rha*p*, 4-*O*-α-D-Glc*p* and *t*-α-D-Glc*p* units, respectively. The signal at δ 4.61 in both spectra (E1 and E′1) showed a $J_{H1,H2} = 7.05$ Hz confirming it to be from β-D-Glc*p* units. A down-field signal at δ 5.59 arose from EPS, and was attributed to H-2 of 3-*O*-β-L-Rha*p* units with an acetyl substituent (—CH_3 at δ 2.15) at C-2. This signal was not present in the EPS-CR spectrum, since the acetyl groups are removed after carboxyreduction. The area of this signal in comparison to that obtained for H-1 of β-L-Rha*p* units was 0.8:1.0, showing that acetyl substituents were present at approximately 80% of C-2 nuclei of the β-L-Rha*p* units.

When EPS was submitted to HMQC analysis, the spectrum indicated that it had five distinct anomeric carbons, with overlapping signals in both the ^{13}C (δ 97.9) and ^{1}H (δ 5.02) spectra. A ^{1}H/^{13}C correlation was found at δ 5.59/68.1 corresponding to H2/C2 of the β-L-Rha*p* units with its *O*-acetyl substituent. The methyl group of *O*-acetyl was detected by correlation of δ 2.15/20.3. The *O*-substituted C-3 signal of the β-L-Rha*p* units was assigned to that with δ 4.16/81.1, corresponding to the H3/C3.

The TOCSY spectrum of EPS (Fig. 3) had several correlations for anomeric protons from each unit. The H-1 signals of β-L-Rha*p* and α-D-Glc*p*A were each at δ 5.02. The 3-*O*-β-L-[2-*O*Ac]-Rha*p* showed correlations of H1/H2 (δ 5.02/5.59), H2/H2 (δ 5.59/5.59), H2/H3 (δ 5.59/4.16), H2/H4 (δ 5.59/3.87) and H2/H5 (δ 5.59/3.49). These correlations confirmed the position of the *O*-acetyl groups linked to C-2 of the β-L-Rha*p* units. Correlations for →4)-β-D-Glc*p*-(1→ were observed at δ 4.62/4.62 (H1/H1), δ 4.62/3.28 (H1/H2), δ 4.62/3.41 (H1/H3) and δ 4.62/3.62 (H1/H4). Correlation of the →4)-α-D-Glc*p*-(1→ residue corresponded to H1/H2 at δ 4.96/3.74, H1/H3 at δ 4.96/3.45 and H1/H4 at δ 4.96/3.54. The di-*O*-substituted α-L-Rha*p* units (2,4-di-*O*-α-L-Rha*p*) showed couplings of H1/H2 and H1/H4 at δ 4.88/4.18 and δ 4.88/3.99, respectively. Vicinal ^{1}H/^{1}H correlations were also confirmed by COSY.

HMQC and TOCSY correlations for EPS-CR were also determined. In respect to the chemical shifts observed due to the carboxyreduction process and removal of acetyl ester substituents, the main difference in correlations observed were for the β-L-Rha*p* units, which showed a H1/H2 coupling at δ 4.94/3.50, as well as others from H1/H3 and H1/H4, found at δ 4.94/3.38 and δ 4.94/3.62, respectively. Table 2 summarizes all assignments found for the acidic EPS and EPS-CR after HMQC, COSY and TOCSY analyses.

3.4. Structural analysis of oligosaccharides obtained from the acidic EPS

After partial hydrolysis of EPS, the resulting oligosaccharides were isolated by size exclusion chromatography on a BioGel-P2 column. Four fractions were obtained (I–IV), fraction I being the later eluting fraction and fraction IV being the early eluting fraction. Monosaccharide compositions of each fraction was determined by GC–MS as

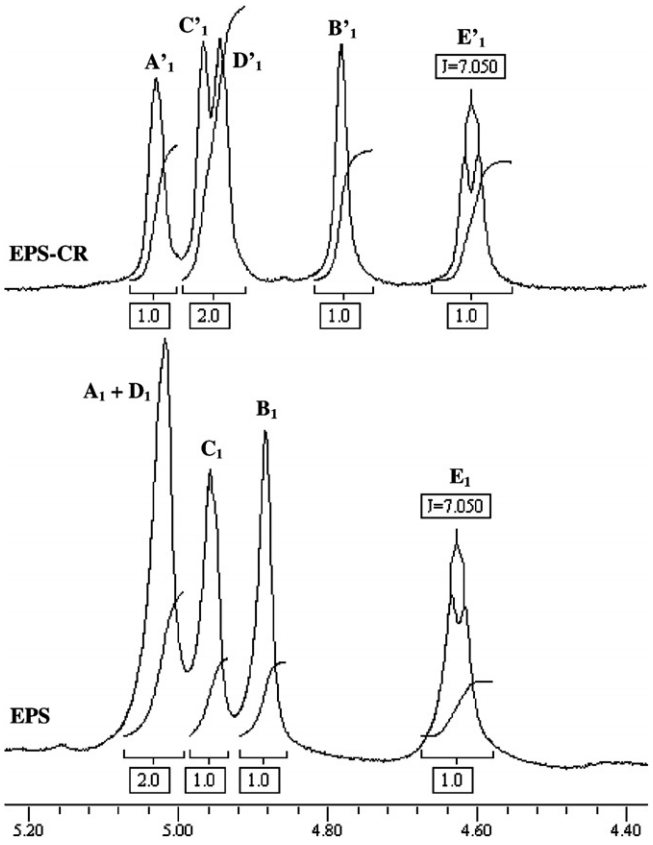


Fig. 2. Partial ^{1}H NMR spectra of EPS and EPS-CR. Anomeric proton signals for the acidic EPS are as: A_1 (*t*-α-D-GlcA*p*), B_1 (→2,4-α-L-Rha*p*), C_1 (→4-α-D-Glc*p*), D_1 (→3-[2-*O*Ac]-β-L-Rha*p*), E_1 (→4-β-D-Glc*p*). EPS-CR anomeric proton signals are represented as: A'_1 (*t*-α-D-Glc*p*), B'_1 (→2,4-α-L-Rha*p*), C'_1 (→4-α-D-Glc*p*), D'_1 (→3-β-L-Rha*p*), E'_1 (→4-β-D-Glc*p*). Integration areas are shown below each signal. Coupling constant (*J*) for E_1 and E'_1 are shown in Hz above their corresponding signals. Scale is shown in δ (ppm).

per-*O*-TMS methyl glycoside derivatives. Methylation analyses were also performed to determined the structure of their glycosidic linkage (Table 3). All fractions were analyzed by ^{1}H NMR and HMQC, although, assignments of all proton and carbon resonances was possible only for fraction I (Table 4).

^{1}H NMR analysis of fraction I showed a characteristic rhamnosyl —CH_3-6 signal at δ 1.35. Reducing signals of L-Rha*p* units were found at δ 5.18 and δ 4.90, from α and β anomers, respectively. The assignment of all protons and carbons was determined by HMQC analysis. The glucuronosyl residue was shown to be α-D-GlcA*p* linked to O-4 of the reducing rhamnosyl unit (H4/C4 at δ 3.94/76.6), which was confirmed by methylation analysis that showed a single 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol derivative (unit B). The structure of the acidic disaccharide found in fraction I is shown below:

A **B**

(I) α-D-Glc*p*A-(1→4)-L-Rha*p*

The proton spectrum of fraction II suggested the presence of a trisaccharide as H-1 signals were found at δ 5.19, 4.96 and 5.06/4.92, the last arising from the α and β configuration of →4)-D-Glc*p* units (C), as also shown by methylation analysis. A characteristic signal at δ 1.29 was attributed to H-6 of rhamnosyl units (B). Based on the PMAA derivatives found and on HMQC analysis, the structure of the oligosaccharide obtained on fraction II was:

A **B** **C**

(II) α-D-Glc*p*A-(1→4)-α-L-Rha*p*-(1→4)-D-Glc*p*

Both fractions III and IV had rhamnosyl units as reducing ends, as shown by their H-1 signals at δ 5.09/4.92, arising from α and β forms, respectively. However, as determined by PMAA derivatives and by the anomeric signals found for each sample, fraction III was a tetrasaccharide and fraction IV a pentasaccharide. HMQC of both fractions had H6/C6 coupling of two rhamnosyl units at δ 1.31/17.1 (B) and δ 1.29/16.8 (D), respectively. Fraction IV also had β-D-Glc*p* units (E) linked to O-2 of di-*O*-substituted rhamnosyl residues (B). The corresponding ^{1}H/^{13}C

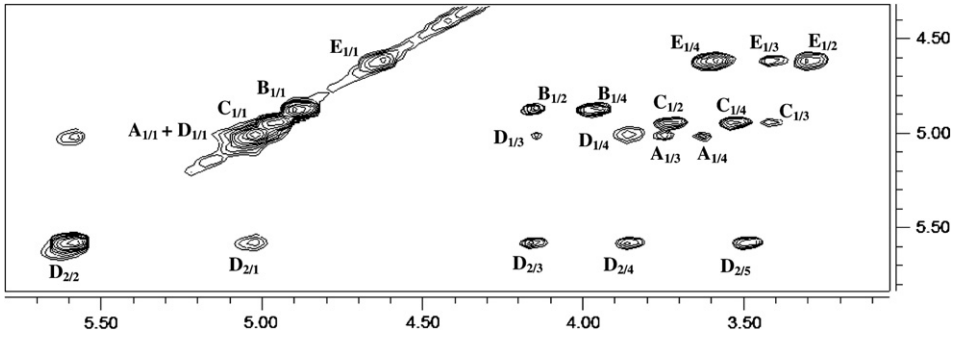


Fig. 3. Partial TOCSY spectrum of EPS showing anomeric region and ^{1}H/^{1}H correlation signals of residues: A (*t*-α-D-GlcA*p*), B (→2,4-α-L-Rha*p*), C (→4-α-D-Glc*p*), D (→3-[2-*O*Ac]-β-L-Rha*p*), E (→4-β-D-Glc*p*). EPS-CR monosaccharide units are represented as: A′ (*t*-α-D-Glc*p*), B′ (→2,4-α-L-Rha*p*), C′ (→4-α-D-Glc*p*), D′ (→3-β-L-Rha*p*), E′ (→4-β-D-Glc*p*). Scale is shown in δ (ppm).

Table 2
^{1}H and ^{13}C assignments for the acidic EPS and EPS-CR, acquired after mono- and bidimensional NMR analyses

Unit		EPS												
		H_1	H_2	H_3	H_4	H_5	H_6	C_1	C_2	C_3	C_4	C_5	C_6	C=O[a]
A	α-D-GlcA*p*-(1→	5.02	3.55	3.77	3.63	3.69	–	94.6	75.6	74.2	76.6	73.1	175.5	–
B	→2,4)-α-L-Rha*p*-(1→	4.88	4.18	3.58	3.99	3.88	4.07	97.9	74.0	74.5	78.1	72.3	16.7	–
C	→4)-α-D-Glc*p*-(1→	4.96	3.74	3.5	3.54	3.55	3.76	97.9	72.1	71.8	79.5	72.9	60.7	–
D	→3)-[2-*O*Ac]-β-L-Rha*p*-(1→	5.02	5.59	4.16	3.87	3.49	1.32	99.0	68.1	81.1	nd	70.8	16.7	173.4
E	→4)-β-D-Glc*p*-(1→	4.62	3.28	3.41	3.62	3.45	3.71	103.0	73.8	75.5	78.3	nd	60.9	–
		EPS-CR												
A′	α-D-Glc*p*-(1→	5.03	3.54	3.70	3.75	3.62	3.74	95.7	77.4	74.6	nd	74.1	60.7	–
B′	→2,4)-α-L-Rha*p*-(1→	4.73	4.21	3.59	3.92	3.35	1.26	100.7	73.8	75.5	78.3	70.2	17.1	–
C′	→4)-α-D-Glc*p*-(1→	4.97	3.77	3.49	3.48	3.58	3.67	98.2	67.9	67.1	79.1	nd	61.1	–
D′	→3)-β-L-Rha*p*-(1→	4.94	3.50	4.22	3.72	3.42	1.30	98.3	79.9	81.4	nd	69.6	17.1	–
E′	→4)-β-D-Glc*p*-(1→	4.61	3.28	3.38	3.62	3.46	3.72	103.2	73.9	nd	78.5	nd	61.4	–

Values are shown in δ (ppm).
nd, not determined.
[a] Carbonyl group of the acetyl substituent. ^{1}H and ^{13}C for methyl group of acetyl were found at δ 2.15 and δ 20.3, respectively.

Table 3
Monosaccharide composition and linkage analysis of oligosaccharides obtained from partial hydrolysis of the acidic EPS

	Derivatives								Linkage
	per-*O*-TMS[a]				PMAA[b]				
	I	II	III	IV	I	II	III	IV	
Rha	1.0	1.0	1.9	1.9	–	–	1.0	1.0	→3)-Rha*p*-(1→^{A}
Glc	–	1.1	1.0	2.0	1.0	1.0	1.0	–	→4)-Rha*p*-(1→^{B}
GlcA	1.1	1.0	1.0	1.0	–	1.1	1.1	2.1	→4)-Glc*p*-(1→^{C}
					–	–	–	1.1	→2,4)-Rha*p*-(1→^{D}

Linkages shown are abbreviations of: ^{A}1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol, ^{B}1,4,5-tri-*O*-acetyl-3-*O*-methyl-rhamnitol, ^{C}1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol, ^{D}1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-rhamnitol. Values are those of as relative molar contents.
[a] Monossacarides were analyzed as per-*O*-trimethylsilyl-methyl glycoside derivatives obtained after methanolysis and TRI-SIL treatment.
[b] Partially *O*-methylated alditol acetates obtained after methylation, followed by methanolysis, hydrolysis, reduction with $NaBD_4$ and acetylation.

Table 4
^{1}H and ^{13}C NMR assignments for the acidic disaccharide (fraction I) obtained on partial hydrolysis of EPS

Unit	C or H	^{1}H	^{13}C
α-D-GlcA*p*	1	5.13	96.6
	2	3.68	72.1
	3	4.25	72.9
	4	3.96	69.5
	5	3.58	73.0
	6	–	–
L-Rha*p*	1	5.18/4.90[a]	94.7/94.2[a]
	2	4.19	68.8
	3	3.88	73.8
	4	3.94	76.6
	5	3.48	73.0
	6	1.35	17.2

[a] α/β configurations of L-Rha*p* reducing units.

coupling found in fraction IV at δ 4.29/72.1 confirms the linkage, together with the formation of 1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-rhamnitol derivative, formed on methylation analysis. Structures found for the oligosaccharides of fractions III and IV are as shown:

A B C D
(**III**) α-D-Glc*p*A-(1→4)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→3)-L-Rha*p*

E B C D
(**IV**) β-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→3)-L-Rha*p*

A α-D-Glc*p*A-(1→4)

The results for these oligosaccharides were consistent with negative mode ESI-MS examination for the mixture obtained on partial hydrolysis of EPS (Fig. 4). Six main molecular ions were formed, namely M1 to M6. Based on the mass difference between two adjacent ions, as well as on the methylation and NMR analysis of the purified oligosaccharides, each *m/z* signal was assigned as follows. M5 was at *m/z* 809.2 and was determined as being the pentasaccharide present in fraction IV, arising from $[GlcA,Glc_2,Rha_2\text{-}H]^-$. The ion that appeared at *m/z* 851.3

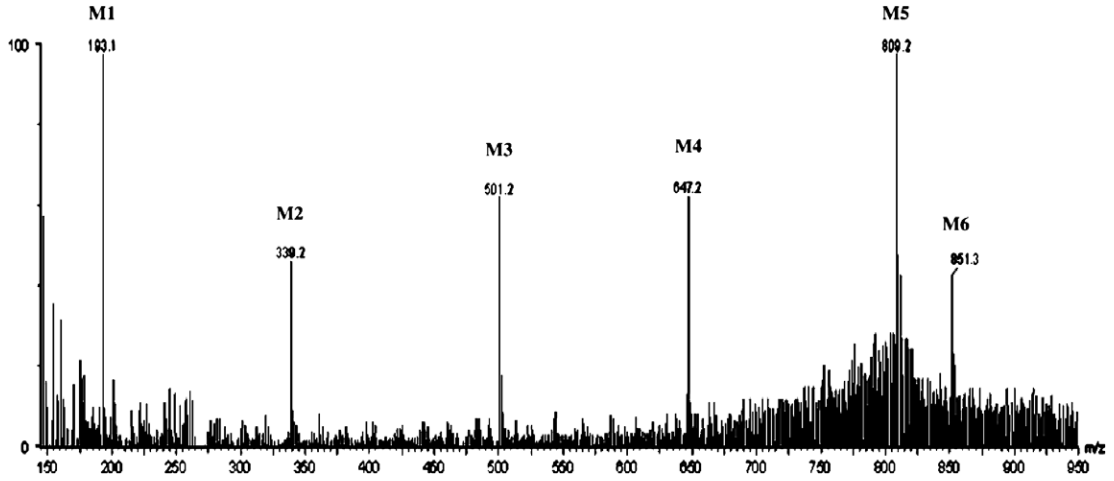
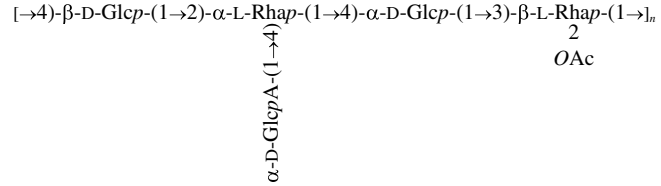


Fig. 4. Negative mode ESI-MS of oligosaccharide mixture obtained on partial hydrolysis of EPS. Signals are named: M1 [GlcA-H]$^-$, M2 [GlcA,Rha-H]$^-$, M3 [GlcA,Glc,Rha-H]$^-$, M4 [GlcA,Glc,Rha$_2$-H]$^-$, M5 [GlcA,Glc$_2$,Rha$_2$-H]$^-$, M6 [GlcA,Glc$_2$,Rha$_2$,*O*Ac-H]$^-$.

(M6) had a 42 mass unit greater than that at M5, characteristic of an acetyl substituent, and was thus assigned as [GlcA,Glc$_2$,Rha$_2$,*O*Ac-H]$^-$. Ions found at *m/z* 647.2 (M4), 501.2 (M3) and 339.2 (M2) corresponded to the oligosaccharides present in fractions III, II and I, respectively, and are thus as [GlcA,Glc,Rha$_2$-H]$^-$, [GlcA,Glc,Rha-H]$^-$ and [GlcA,Rha-H]$^-$. An ion at *m/z* 193.1, corresponding to [GlcA-H]$^-$ was present, which might have been either hydrolyzed during the process, or formed as a daughter ion from one of the oligosaccharides during ionization.

The pentasaccharide isolated in fraction IV, assigned on ESI-MS analysis as the ion arising at *m/z* 809.2 (M5), together with its corresponding acetylated form (M6), served to show the repeating unit of the acidic EPS. The reducing units of oligosaccharides III and IV, assigned as 3-*O*-β-L-Rha*p* (D), is also that which bears the *O*-acetyl substituent, as determined by the NMR assignments, and thus is present as a →3)-[2-*O*Ac]-β-L-Rha*p* unit.

The results shown herein can indicate that the extracellular polysaccharide produced by *B. tropica*, strain Ppe8, is formed by a branched pentasaccharide repeating unit with the following structure:



4. Discussion

Exopolysaccharides are important signaling molecules during plant–bacteria association. Nitrogen-fixing bacteria of the family *Rhizobiaceae* are widely known to have EPS as key factors during nodulation and nitrogen fixation in leguminous plants. Endophytic bacteria, which do not produce specialized structures in plant tissues, have also been observed to produce EPS that is involved in plant invasion. We have now shown the structure of an acidic heteroexopolysaccharide produced by a strain of *B. tropica*, an endophytic diazotroph. This structure is different from those found for other species of the genus *Burkholderia*. However, one may note some similarities between the EPS biosynthesized by all species of *Burkholderia*, suggesting that the structure is somewhat fairly conserved and probably important for the association with grasses. The process by which *B. tropica* invades and colonizes plant tissues is still unknown. The branched structure bearing α-D-glucuronopyranosyl units may be directly involved in the symbiosis of this bacterium with its plant hosts. These non-reducing acidic units that substitute α-L-rhamnosyl units in the main chain of its EPS, seem to be ideal points of recognition by lectins present in plant root cells. Also, the *O*-acetyl substituents present in the β-L-rhamnosyl units may be involved in the symbiotic process. We believe that the fine structural characterization of bacterial EPSs is a first step towards understanding the importance and function of these molecules for plant–bacteria relationships.

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