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Structure of a novel exopolysaccharide produced by *Burkholderia vietnamiensis*, a cystic fibrosis opportunistic pathogen

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ABSTRACT

Burkholderia vietnamiensis belongs to the *Burkholderia cepacia* complex and is an opportunistic pathogen for cystic fibrosis patients. As many other *Burkholderia* species, it has a mucoide phenotype, producing abundant exopolysaccharide. In general, polysaccharides contribute to bacterial survival in a hostile environment, are recognised as virulence factors and as important components in biofilm formation. The primary structure of the exopolysaccharide produced by *B. vietnamiensis* LMG 10929 was determined mainly by use of 1D and 2D NMR spectroscopy and ESI mass spectrometry. The polymer consists of the trisaccharidic backbone 3)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-α-L-Fuc*p*-(1→ with the side chain α-D-Glc*p*-(1→4)-α-D-GlcA*p*-(1→3)-α-L-Fuc*p*-(1→ linked to C-3 of the α-D-Glc*p* residue. The polysaccharide also bears acetyl substituents on about 20% of its repeating units and on at least two different positions. The presence of fucose residues is a novel structural feature among the exopolysaccharides produced by species of the *B. cepacia* complex.

1. Introduction

Burkholderia vietnamiensis is one of the closely related seventeen species which constitute the *Burkholderia cepacia* complex (BCC) (Vanlaere et al., 2009), a group of Gram-negative opportunistic pathogens for cystic fibrosis (CF) and chronic granulomatous patients, and immunocompromised individuals. *B. vietnamiensis* is the third most prevalent species of the BCC infecting CF patients (Menard et al., 2007), the first two being *Burkholderia cenocepacia* and *Burkholderia multivorans*. What makes it particularly interesting is its nitrogen fixing ability which connects it strongly with environmental reservoirs. Therefore, it can be considered an excellent model to investigate the problems of community acquired strains and their adaptation to CF lung (Menard et al., 2007). CF is a common autosomal recessive disorder with a prevalence of 1 in 2000–3000 newborns in the European Union and 1 in 3500 in USA (http://www.who.int/genomics/public/geneticdiseases/en/index2.html#CF). Mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) are the cause of the disease. This protein regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs. Different organs are affected in CF, but the most compromised are the lungs, where the production of abnormally thick mucus favours the establishment of bacterial infections, which decrease life quality and in the end lead the patient to death. Several virulence factors have been recognised in BCC microorganisms: extracellular and cell surface polysaccharides, systems that regulate gene expression, secretion systems, metabolic and nutrient acquisition pathways, molecules required for resistance to host antimicrobial compounds, and several proteins with unknown functions (Loutet & Valvano, 2010). Regarding extracellular polysaccharides (EPS), investigation of BCC species revealed that six different polymers were produced by clinical as well as environmental isolates. The most common EPS is cepacian, a highly branched, highly acetylated, negatively charged polysaccharide (Cèrantola, Lemassu-Jacquier, & Montrozier, 1999; Cescutti et al., 2000). Cepacian forms double stranded aggregates in dilute aqueous solutions and a polymer network in more concentrated systems (Herasimenka et al., 2008; Sist et al., 2003). Therefore, it may account for the structural and mechanical properties of the hydrophilic matrix surrounding bacterial cell and colonies. A second EPS is composed of three galactose residues, one 3-deoxy-D-manno-oct-2-ulosonic acid, a residue commonly found in the core region of lipopolysaccharides, and one acetyl group (Cescutti et al., 2003), and it is identical to the capsular polysaccharide produced by *Burkholderia pseudomallei* (Masoud, Ho, Schollardt, & Perry, 1997; Nimtz et al., 1997). A third polymer contains one glucose and one galactose residues, the latter stoichiometrically substituted with a pyruvyl group (Cèrantola, Marty, & Montrozier, 1996; Herasimenka et al., 2007). Then, three other neutral polysaccharides were synthesised by single strains always in mixture with one of the three EPS reported

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above: levan, constituted of 6 linked β-D-Fru*f* units (Herasimenka et al., 2007), dextran (Conway, Chu, Bylund, Altman, & Speert, 2004), and a 1 → 2-linked glucan with occasional branching at position 3 (Chung, Altman, Beveridge, & Speert, 2003).

Within the framework of characterising the EPS produced by different *Burkholderia* species belonging to the complex, preliminary NMR studies indicated that *B. vietnamiensis* LMG 10929 was able to biosynthesize an EPS different from the ones described above. Therefore, the structural determination of this EPS was accomplished mainly by use of NMR and mass spectrometry, and it is hereafter described.

2. Experimental

2.1. Bacterial strain and growth conditions

B. vietnamiensis LMG 10929 was purchased from BCCM™ bacteria collection and it is a reference strain from the panel of *B. cepacia* complex strains (Mahenthiralingam et al., 2000). Bacteria were grown for 16 h in 5 mL of LB broth. Aliquots of 100 μL were spread on YEM medium agar plates (20 g mannitol, 2 g yeast extract, 15 g agar per litre) and incubated at 30 °C for 4 days. The cells were collected with a 0.9% NaCl solution and after addition of sodium azide were gently stirred at 4 °C for about 2 h. The bacterial cells suspension was centrifuged at 28,000 × *g* for 30 min at 4 °C. The supernatant was precipitated in 4 volumes of cold isopropanol, dissolved in water, dialysed first against 0.1 M NaCl, and then against water. After neutralising and filtering the polysaccharide solution, the absence of proteins and nucleic acids was verified by UV spectroscopy. The EPS was recovered by lyophilisation and stored at 4 °C.

2.2. General procedures

Analytical GLC was performed on a Perkin–Elmer Autosystem XL gas chromatograph equipped with a flame ionisation detector and an SP2330 capillary column (Supelco, 30 m), using He as carrier gas. The following temperature programmes were used: for alditol acetates, 200–245 °C at 4 °C/min; for partially methylated alditol acetates, 150–250 °C at 4 °C/min. Separation of the trimethylsilylated (+)-2-butyl glycosides was obtained on a HP1 column (Hewlett–Packard, 50 m), using the following temperature programme: 135–240 °C at 1 °C/min. GLC–MS analyses were carried out on an Agilent Technologies 7890A gas chromatograph coupled to an Agilent Technologies 5975 C VL MSD. Hydrolysis of the samples were performed in the following conditions; native EPS: 2 M trifluoroacetic acid (TFA) for 2 h at 125 °C; carboxyl reduced EPS: 2 M TFA for 1 h at 125 °C. Methanolysis was conducted for 16 h at 85 °C with 2 M HCl in methanol for the native EPS, and with 1 M HCl in methanol for the carboxyl reduced EPS. Alditol acetates were prepared as already described (Albersheim, Nevins, English, & Karr, 1967), permethylation of the carboxyl reduced EPS was achieved following the protocol by Harris (Harris, Henry, Blakeney, & Stone, 1984), while oligosaccharides were permethylated using the protocol by Dell (Dell, 1990). Determination of the absolute configuration of the sugar residues was performed as described (Gerwig, Kamerling, & Vliegenthart, 1978). De-O-acetylation was achieved treating a sample of EPS with 0.01 M NaOH at 22 °C for 5 h.

High performance size exclusion chromatography (HP-SEC) was performed on a Agilent Technologies, 1200 series HPLC equipped with three columns in series (Tosoh Bioscience, TSKgel G3000PW, G5000PW and G6000PW, i.d. 7.5 mm, length 30 cm) kept at 40 °C with a thermostat (Waters Millipore). Calibration of the chromatographic system was performed using pullulans standards (Polymer Laboratories, Germany and Sigma for pullulan with MM = 1,600,000). Elution was performed with 0.15 M NaCl, with a flow rate of 0.4 mL/min and monitored using a refractive index detector (Knauer, Labservice Analytica), interfaced with a computer via Agilent software. Purified *B. vietnamiensis* EPS was named **BV-EPS**.

2.3. Carboxyl reduction

BV-EPS (11.5 mg) was dissolved in 6 mL of H_2O and treated as previously described (Taylor & Conrad, 1972), using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. For reduction of carboxyl groups, solid $NaBD_4$ was slowly added to the solution to reach 2 M final concentration. After 2 h, addition of 50% aqueous acetic acid destroyed the excess of $NaBD_4$. The solution was dialysed and lyophilised to obtain 7.6 mg of carboxyl reduced EPS (**BV-EPS-R**).

2.4. Smith degradation

A sample of de-O-acetylated EPS (**BV-EPS-deOAc**) was subjected to Smith degradation following the original protocol (Goldstein, Hay, Lewis, & Smith, 1965). Briefly, 44 mg of **BV-EPS-deOAc** were dissolved in 5.7 mM $NaIO_4$, 40 mM $MgCl_2$ and stirred for 7 days in the dark at 4 °C. Magnesium salt was added to aid the oxidation of GlcA. After addition of glycerol to react with excess of periodate, reduction of the aldehyde groups was achieved by incubation with $NaBH_4$ at room temperature for 16 h. Excess of borohydride was destroyed with 50% aqueous acetic acid and the solution was dialysed. The recovered material was hydrolysed with 0.1 M TFA for 7 days at room temperature, dialysed and 9 mg were recovered by lyophilisation. The sample was named **BV-EPS-S**.

2.5. Production of oligosaccharides for ESI-MS

In order to choose the best conditions for the production of oligosaccharides, **BV-EPS** was hydrolysed with 0.1 and 0.5 M TFA at 80 °C, and 0.5 and 1.0 M TFA at 100 °C for 1, 2 and 4 h. The 12 hydrolysates were then subjected to TLC on a silica gel on aluminium foil (Fluka) using CH_3OH:$CHCl_3$:H_2O = 20:20:7 as eluent; detection of sugars was achieved by spraying with a solution of *p*-anisaldehyde/sulphuric acid/ethanol (1:1:18, by vol.) followed by heating at 110 °C for 5–10 min. The samples hydrolysed with 0.5 M TFA for 2 h at 100 °C and for 1 h at 80 °C were the most promising in term of size and number of oligosaccharides produced and therefore were analysed by ESI-MS in their native state and also after reduction with $NaBD_4$, followed by permethylation.

2.6. Production of oligosaccharides for NMR spectroscopy

Following the results obtained by ESI-MS of the partial hydrolysates, an aliquot of 52 mg were hydrolysed with 0.5 M TFA at 100 °C for 2 h, while about 57 mg of BV-EPS was treated with 0.5 M TFA at 80 °C for 1 h. The acid was then removed by rotovaporation under reduced pressure and each sample was dissolved in 3.8 mL of 0.05 M ammonium acetate. After centrifugation to remove insoluble particles they were separated on a Bio Gel P2 column (1.6 cm i.d. × 90 cm), loading 1.9 mL of each sample at a time, using 0.05 M ammonium acetate as eluent, and a flow rate of 6 mL/h. Fractions were collected at 15 min intervals. Elution was monitored using a refractive index detector (WGE Dr. Bures, Lab-Service Analitica), which was connected to a paper recorder and interfaced with a computer via PicoLog software. Fractions to be analysed by NMR spectroscopy were evaporated to dryness under reduced pressure several times in order to remove ammonium acetate.

2.7. NMR spectroscopy

The molecular mass of EPS solutions (1 g/L) was decreased by treatment with a Branson sonifier equipped with a microtip at 2.8 Å. Samples were cooled in an ice bath and sonicated using 5 bursts of 1 min each, separated by 1 min intervals. They were subsequently exchanged three times with 99.9% D_2O by lyophilisation and finally dissolved in 0.7 mL 99.96% D_2O. Spectra were recorded on a 500 MHz VARIAN spectrometer operating at 50 °C for native EPS and at 25 °C all the other samples. ^{13}C NMR spectrum was acquired overnight at 25 °C on deacetylated **BV-EPS**. 1D TOCSY of **BV-EPS-S** were recorded at 50 °C using 200 ms of spin-lock time. 2D experiments were performed using standard VARIAN pulse sequences and pulsed field gradients for coherence selection when appropriate. HSQC spectra were recorded using 140 Hz one bond Jxh constant. TOCSY spectra were acquired using 100 ms spin-lock time, while ROESY spectra were acquired with 400 ms mixing time and 2.0 s relaxation time. NOESY experiments were recorded with 200 ms mixing time and 1.5 s relaxation time. Chemical shifts are expressed in ppm using acetone as internal reference (2.225 ppm for ^1H and 31.07 ppm for ^{13}C). NMR spectra were processed using MestreNova software.

2.8. ESI mass spectrometry

ESI mass spectra were recorded on a Bruker Esquire 4000 ion trap mass spectrometer connected to a syringe pump for the injection of the samples. The instrument was calibrated using a tune mixture provided by Bruker. Samples were dissolved in 50% aqueous methanol–11 mM NH_4OAc at an appropriate concentration and injected at 180 μL/h. Detection was always performed in the positive ion mode.

3. Results and Discussion

3.1. Bacteria growth and exopolysaccharide production

B. vietnamiensis LMG 10929 was grown on solid YEM medium and the EPS was harvested and purified with a yield of about 35 mg per Petri dish. ^1H NMR spectroscopy showed resonances in the anomeric region belonging to cepacian (Cescutti et al., 2000) as well as signals indicating a novel EPS (Fig. 1a). Methyl groups of acetyl substituents were detected at 2.3–2.1 ppm, while three signals belonging to 6-deoxy sugars resonated at 1.4–1.1 ppm, clearly suggesting two other 6-deoxy monosaccharides, besides the one rhamnose residue present in the mixture and belonging to cepacian. Composition analysis as alditol acetates showed Rha, Fuc, Man, Gal and Glc in the molar ratios: 0.31:0.23:0.08:1.00:1.00, respectively, further confirming the presence of an unknown EPS in addition to cepacian. The two polysaccharides were completely separated by HP-SEC using three columns in series, as revealed by composition and NMR analysis. The novel EPS was named **BV-EPS**. According to calibration performed with pullulan standards, HP-SEC analysis showed that **BV-EPS** has a MM higher than 1×10^3 kDa, while that of cepacian was about 40 kDa.

When a second batch of *B. vietnamiensis* LMG 10929 was grown in the same experimental conditions, pure **BV-EPS** was obtained, with no cepacian being synthesised. In this study, HP-SEC purified **BV-EPS** or pure **BV-EPS,** as obtained from the second batch culture, were used.

3.2. Composition of BV-EPS

Composition analysis as alditol acetate derivatives revealed Fuc and Glc in the molar ratio 1.00:1.75, and after carboxyl reduction the ratio changed to 1.00:2.39, suggesting the presence of glucuronic acid.

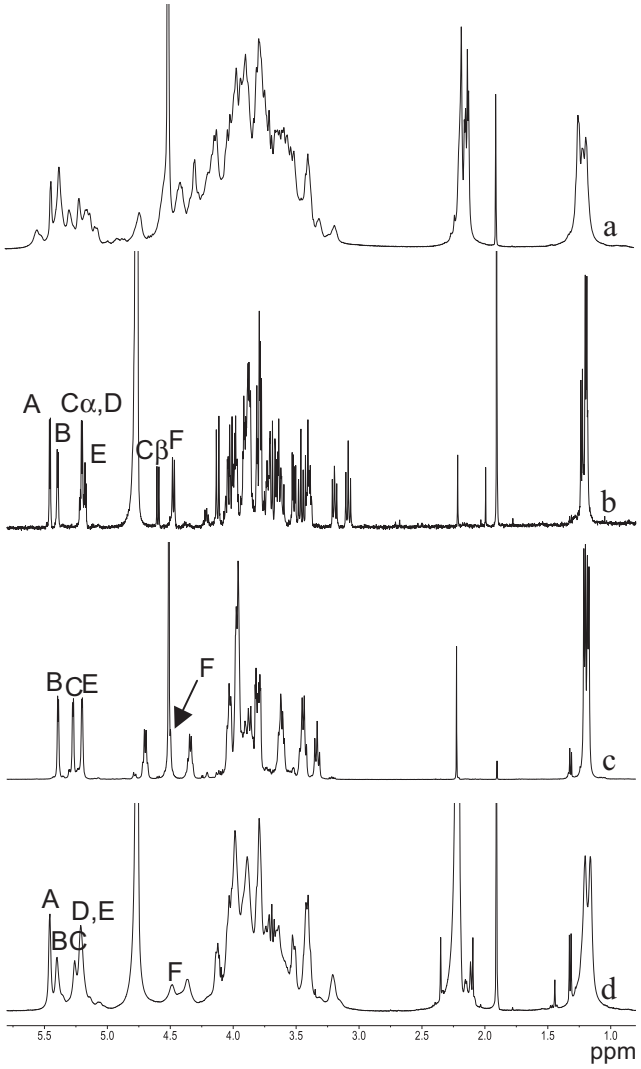


Fig. 1. ^1H NMR spectra of the mixture of EPS produced by *B. vietnamiensis* LMG 10929 (a), the hexasaccharide corresponding to the repeating unit of **BV-EPS** (b), **BV-EPS** after Smith degradation (c), native **BV-EPS** added with acetone (d), recorded at 25 °C (b and d) and 50 °C (a and c).

Linkage analysis was performed both on the native **BV-EPS** and after carboxyl reduction (**BV-EPS-R**) and the results are reported in Table 1. The data showed that **BV-EPS** is a branched, negatively charged polysaccharide constituted of two 3-linked Fuc, one t-Glc, one 3-linked Glc, one 3,4-linked Glc and one 4-linked GlcA. Since the ratio of 3-Fuc increased after carboxyl reduction, it is likely that GlcA is linked to one Fuc residue.

Table 1
Determination of glycosidic linkages in the samples **BV-EPS** and **BV-EPS-R**.

Linked residue[a]	RRT[b]	**BV-EPS**	**BV-EPS-R**
t-Glc	0.83	0.65	0.98
3-Fuc	0.87	0.88	1.40
3-Glc	1.00	1.00	1.00
4-$GlcD_2$ [c]	1.09	–	0.66
3,4-Glc	1.18	1.15	0.87

[a] Position of glycosidic linkages.
[b] Relative retention time.
[c] C6 di-deuterated according to GC–MS.

Table 2
Composition and sequence of oligosaccharides obtained by partial hydrolysis with 0.5 M TFA for 2 h at 100 °C and for 1 h at 80 °C.

Ion (u)[a]	Composition	Ion (u)	Sequence
0.5 M TFA 2 h 100 °C			R(D)M[b]
		446.3	[Glc-Fuc-ol–H_2O]
349.2	Hex, dHex	464.3	Glc-Fuc-ol
363.2	HexA (H^+)[c], dHex	478.4	GlcA-Fuc-ol
365.2	Hex, Hex	494.3	Glc-Glc-ol
385.1	HexA (Na^+)[d], dHex	–	GlcA-Fuc-ol
511.2	Hex, Hex, dHex	668.4	Glc-Glc-Fuc-ol
0.5 M TFA 1 h 80 °C			
819.2	3 Hex, 2 Fuc	1046.7	
833.3	2 Hex, 1 HexA (H^+), 2 Fuc	1060.7	
855.3	2 Hex, 1 HexA (Na^+), 2 Fuc	–	
981.4	4 Hex, 2 Fuc	1250.9	Glc-Glc-Fuc-Glc-Glc-Fuc-ol
995.4	3 Hex, 1 HexA (H^+), 2 Fuc	1264.9	Glc-GlcA-Fuc-Glc-(Glc)-Fuc-ol or Glc-GlcA-Fuc-Glc-Glc-Fuc-ol
1017.4	3 Hex, 1 HexA (Na^+), 2 Fuc	–	

[a] All ions are sodium adducts.
[b] Carbonyl reduced with $NaBD_4$, followed by permethylation.
[c] Protonated form.
[d] Sodium salt.

Determination of the absolute configuration was performed on **BV-EPS-R** and showed that the glucuronic acid as well as all the glucose residues are in the D absolute configuration, while the fucose residues are in the L absolute configuration.

3.3. Partial hydrolysis of BV-EPS and characterisation of the obtained oligosaccharides

BV-EPS was subjected to partial hydrolysis with 0.5 M TFA for 2 h at 100 °C. ESI-MS of the sample showed mainly disaccharides and a trisaccharide (Table 2). After reduction with $NaBD_4$ to label the reducing end, followed by permethylation, mass spectra showed the expected ions (Figure S3a in supplementary data file), and MS^2 gave sequence information identifying the following oligosaccharides: Glc-Fuc-ol, GlcA-Fuc-ol, Glc-Glc-ol, Glc-Glc-Fuc-ol. With the aim of obtaining a reasonable amount of the trisaccharide Glc-Glc-Fuc, to be subjected to NMR spectroscopy investigation, 52 mg of **BV-EPS** were treated with 0.5 M TFA for 2 h at 100 °C. Separation of the products was achieved by size exclusion chromatography on a Bio Gel P2 column and ESI-MS (data not shown) led to the identification of the fraction containing the desired trisaccharide. A second partial hydrolysis was performed with 0.5 M TFA for 1 h at 80 °C and ESI-MS of the products showed mainly pentasaccharides and hexasaccharides (Figure S3b in supplementary data file, Table 2). In particular, two different hexasaccharides were detected, one being composed of four Hex and two dHex, and the other containing three Hex, one HexA and two dHex, the latter corresponding to the composition of repeating unit of **BV-EPS** (**BV-RU**). The sample was reduced at the reducing end with $NaBD_4$ and permethylated with CH_3I. ESI-MS analysis showed the corresponding reduced and permethylated species (Table 2). MS^2 and MS^3 of the parent ion at 1250.9 u, having the composition four Hex and two dHex, as well as of its daughter ion at 654.5 u, respectively, resulted mainly in fragmentation from the non reducing end, giving rise to C type ions (Domon & Costello, 1988). The data indicated a linear hexasaccharide with the sequence: Glc-Glc-Fuc-Glc-Glc-Fuc-ol.

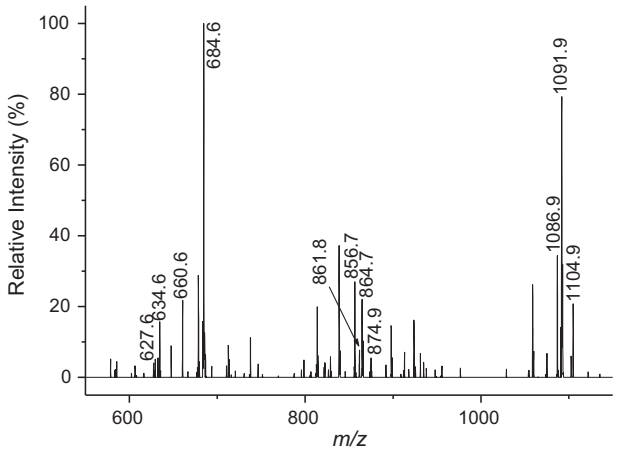


Fig. 2. MS^2 of the parent ion at 1322.2 u corresponding to the sodiated adduct of $NaBD_4$ reduced and perdeuteriomethylated **BV-RU**.

The same type of analysis was performed on the parent ion at 1264.9 u, composed of three Hex, one HexA and two dHex residues. However, since the difference in unified atomic mass unit (u) between a hexose and a hexuronic acid is identical to the difference between a free hydroxyl and a methyl ether, some uncertainty arose in the fragmentation spectra. In particular, fragmentation was compatible both with a linear Glc-GlcA-Fuc-Glc-Glc-Fuc-ol and a branched structure Glc-GlcA-Fuc-(Glc)-Glc-Fuc-ol (Table 2).

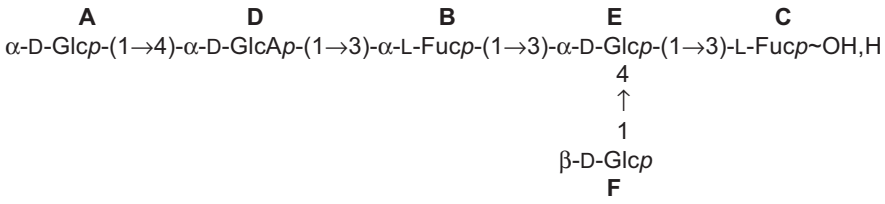
The same partial hydrolysis was repeated on 57 mg of **BV-EPS**, and the products mixture was separated on a Bio Gel P2 column. Most of the sample eluted in the V_0 of the column, but there were also four small but very well separated peaks (data not shown). The fractions at the top of each peak were subjected to ESI-MS, MS^2 and MS^3 analysis which revealed their identity as follows: fr 68: Glc; fr 53: Glc-GlcA-Fuc; fr 43: hexasaccharide with composition corresponding to **BV-RU**; fr 37: oligosaccharides higher than the repeating unit. The peak containing the trisaccharide and that one containing the hexasaccharide repeating unit were subjected to detailed NMR spectroscopy investigation.

Once NMR analyses were completed, in order to overcome the difficulties in the ESI-MS spectra as explained above, the hexasaccharide was reduced with $NaBD_4$ and permethylated with CD_3I. ESI-MS showed the corresponding parent ion at 1322.2 u, while MS^2 gave fragments arising from both reducing and non-reducing end, thus establishing the branched nature of **BV-RU** (Fig. 2, Table 3).

Table 3
Assignment of ions generated upon fragmentation of $NaBD_4$ reduced and perdeuteriomethylated hexasaccharide corresponding to the repeating unit of **BV-EPS**.

Observed u	Fragment ions	Ion type[a]
627.6	[GlcA-Fuc-Glc-OH−H_2O+Na]$^+$	B
634.6	[Fuc-Glc-Fuc-ol+Na]$^+$	Y
660.6	[Glc-GlcA-Fuc-OH−H_2O+Na]$^+$	B
678.5	[Glc-GlcA-Fuc-OH+Na]$^+$	B
684.6	[Glc-Glc-Fuc-ol+Na]$^+$	Y
856.7	[Glc-GlcA-Fuc-Glc-OH−H_2O+Na]$^+$ [GlcA-Fuc-[Glc]-Glc-OH−H_2O+Na]$^+$	B
861.8	[GlcA-Fuc-Glc-Fuc-ol+Na]$^+$	Y
864.7	[Fuc-[Glc]-Glc-Fuc-ol+Na]$^+$	Y
874.9	[Glc-GlcA-Fuc-Glc-OH+Na]$^+$ [GlcA-Fuc-[Glc]-Glc-OH+Na]$^+$	B
1086.9	[Glc-GlcA-Fuc-[Glc]-Glc-OH−H2O+Na]$^+$	B
1091.9	[GlcA-Fuc-[Glc]-Glc-Fuc-ol+Na]$^+$	Y
1104.9	[Glc-GlcA-Fuc-[Glc]-Glc-OH+Na]$^+$	B

[a] According to the fragmentation proposed by Domon and Costello (1988).



Scheme 1. Structure of the oligosaccharide **BV-RU**.

3.4. NMR spectroscopy of Glc-GlcA-Fuc and Glc-Glc-Fuc trisaccharides

Detailed 1D and 2D NMR spectroscopy analysis was performed on these two compounds, obtaining the complete chemical shift assignment of each spin system in the two trisaccharides. These assignments were very useful for the determination of the chemical shifts in the hexasaccharide, in **BV-EPS-S** and **BV-EPS**. NMR spectra together with chemical shifts data are reported in the supplementary data file.

3.5. NMR spectroscopy of the hexasaccharide Glc-GlcA-Fuc-(Glc)-Glc-Fuc corresponding to the repeating unit of BV-EPS

The ^{1}H NMR spectrum of the hexasaccharide repeating unit, **BV-RU**, showed α-anomeric proton signals at 5.46 (**A**), 5.39 (**B**), 5.22–5.17 (**Cα**, **D**, **E**) ppm and two β-anomeric proton signals at 4.60 (**Cβ**) and 4.47 (**F**) ppm (Fig. 1b). The nomenclature of the peaks followed that one used for **BV-EPS** (see Section 3.7). Integration of the peak areas gave the following respective values: 1.17:1.00:2.50:0.46:1.09. After inspection of the HSQC plot, the signals at 5.22–5.17 ppm were attributed to two, still unassigned, anomeric protons and to the α-anomeric proton of Fuc (**Cα**) at the reducing end, while the resonance at 4.60 ppm was assigned to the β-anomeric proton of the reducing Fuc (**Cβ**), since ESI-MS data showed a d-Hex at the reducing end. In the 1.25–1.15 ppm region, three partially overlapping signals, having an integration value of 6.70, were attributed to the methyl groups of two Fuc residues, one being in-chain and another at the reducing end. Assignments of H-6's to in-chain and reducing end Fuc was based on intensity of the signals, comparison with chemical shifts of the trisaccharide Glc-Glc-Fuc (see supplementary data) and with reference values (Bock & Thøgersen, 1982). COSY and TOCSY experiments led to the attribution of most of the proton resonances, while the HSQC plot (Fig. 3) gave the respective ^{13}C signals. In particular, H-1 to H-4 of the two Fuc residues were determined from the 2D homonuclear

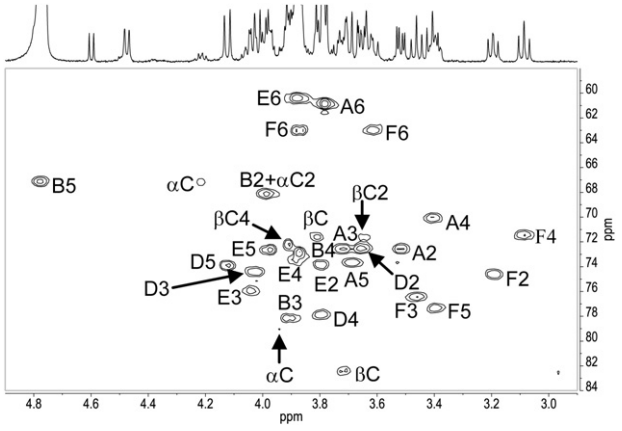


Fig. 3. Expansion of the HSQC plot of **BV-RU** recorded at 25 °C.

spectra, while H-6 and H-5 of residue **C** were assigned after comparison with the chemical shifts of the trisaccharide Glc-Glc-Fuc, since the reducing end in both samples has a very similar electronic environment. The other couple of resonances attributed to H-6 and H-5 was assigned to the other Fuc residue (**B**). The complete assignment of all spin systems (Table 4), apart from the α-Fuc reducing end, was achieved comparing the HSQC plot of **BV-RU** with those of the two trisaccharides (see supplementary data). ROESY experiments showed H-1 to H-2 intra-residue connectivities for **A**, **B**, **C**α, **D**, and **E** residues; in addition, **C**β**1** to **C**β**5**, **F1** to **F3** and **F5** connectivities were observed. The same experiment gave also the following inter-residues cross peaks: **A1** to **D4**; **B1** to **E3**, **D1** to **B3**; **E1** to **C3**β, and **F1** to **E4**, thus leading to the complete definition of **BV-RU** saccharidic sequence, in agreement with data obtained by ESI-MS.

In conclusion, NMR data together with composition and MS analysis established that **BV-RU** oligosaccharide has the structure reported in Scheme 1.

3.6. NMR spectroscopy of the Smith degraded BV-EPS

The ^{1}H NMR spectrum of **BV-EPS-S** (Fig. 1c) showed three main α- and one β-anomeric signals at 5.40 (**B**), 5.28(**C**), 5.19 (**D** or **E**) and 4.50 (**F**) ppm, respectively, and each peak had an integration value of one. Two doublets at 1.21 and 1.17 ppm were attributed to two methyl groups of fucose residues, since each of them integrated for three protons. The nomenclature of the peaks followed that one given to **BV-EPS** (see Section 3.7). Comparison of the ^{1}H NMR spectrum of **BV-EPS-S** with those of **BV-RU** and **BV-EPS** evidenced the absence of residue **A,** corresponding to terminal non reducing glucose, and either of residue **D** or **E**. Discrimination between these two residues was achieved only after assigning completely the spin system characterised by H-1 at 5.19 ppm in **BV-EPS-S**. The chemical shifts indicated that residue **D**, attributed to GlcA in **BV-RU**, was absent. Therefore, periodate oxidised the expected monosaccharides, t-Glc and 4-linked GlcA, maintaining the polymeric structure, and confirming that the uronic acid is in the side chain.

COSY and TOCSY experiments assigned most of the proton signals. In particular, since H-4 is equatorial in fucose, both $J_{H\text{-}3/H\text{-}4}$ and $J_{H\text{-}4/H\text{-}5}$ are small resulting in an inefficient magnetisation transfer in the fucose ring. In fact, only H-1 to H-3 and H-6 to H-5 were determined for the each fucose residue. The complete spin system assignment of the 6-deoxy residues was achieved by performing 1D TOCSY with selective excitation of H-5's of each fucose residue (4.72 and 4.34 ppm); in this way clear correlations of each H-5 with the respective H-6, H-4 and H-1 were detected, thus leading to assignment of the complete spin system for each fucose residue. Inspection of the HSQC plot gave the corresponding carbon atoms assignment and the chemical shifts for **BV-EPS-S** are reported in Table 5. All the data collected showed that **BV-EPS-S** has the structure reported in Scheme 2.

3.7. NMR spectroscopy of BV-EPS

The ^{1}H NMR spectrum of **BV-EPS** recorded at 50 °C (Figure S4 in supplementary data file) showed four resonances in

Table 4
^{1}H and ^{13}C chemical shift (ppm)[a] of *B. vietnamiemnsis* exopolysaccharide repeating unit after partial hydrolysis and separation on Bio Gel P2.

		1	2	3	4	5	6
A	^{1}H	5.46	3.52	3.72	3.40	3.69	3.78
α-D--**Glc*p*-(1**	^{13}C	99.44	72.57	72.57	70.08	73.67	60.84
B	^{1}H	5.39	3.99	3.91	3.87	4.78	1.20
3)-α-L-**Fuc*p*-(1**	^{13}C	99.56	68.14	78.13	72.85	67.12	16.05
C(	^{1}H	5.22	3.97	nd[b]	nd	4.22	1.19
3)-α-L-**Fuc*p***	^{13}C	93.03	72.65	nd	nd	67.14	16.02
D	^{1}H	5.20	3.65	4.03	3.80	4.12	
4)-α-D--**GlcA*p*-(1**	^{13}C	101.45	72.50	74.40	77.86	73.88	
E	^{1}H	5.17	3.80	4.04	3.88	3.97	3.88
3,4)-α-D--**Glc*p*-(1**	^{13}C	101.54	73.84	75.98	73.55	72.66	60.37
Cβ	^{1}H	4.60	3.64	3.71	3.91	3.81	1.23
3)-β-L-**Fuc*p***	^{13}C	96.89	71.63	82.41	72.24	71.62	16.24
F	^{1}H	4.47	3.19	3.46	3.09	3.41	3.62, 3.88
β-D--**Glc*p*-(1**	^{13}C	102.09	74.62	76.43	71.46	77.35	62.97

[a] ^{1}H and ^{13}C chemical shift are given in ppm relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).
[b] nd, not determined.

Table 5
^{1}H and ^{13}C chemical shift (ppm)[a] of *B. vietnamiemnsis* exopolysaccharide after Smith degradation.

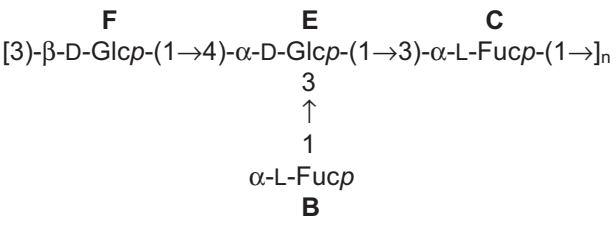
		1	2	3	4	5	6
B	^{1}H	5.40	3.81	3.98	3.78	4.72	1.21
3)-α-L-**Fuc*p*-(1**	^{13}C	99.58	68.99	79.01	72.77	67.29	16.26
C	^{1}H	5.29	4.02	3.98	3.97	4.34	1.17
3)-α-L-**Fuc*p*-(1**	^{13}C	100.24	68.29	79.01	70.21	67.70	16.16
E	^{1}H	5.19	3.80	4.05	3.87	3.97	3.89
3,4)-α-D--**Glc*p*-(1**	^{13}C	101.37	74.01	75.95	74.10	72.68	60.47
F	^{1}H	4.50	3.44	3.61	3.32	3.45	3.60, 3.96
3)-β-D--**Glc*p*-(1**	^{13}C	102.22	75.16	83.00	69.73	77.02	62.64

[a] ^{1}H and ^{13}C chemical shift are given in ppm relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).

the α anomeric region at 5.43 (**A**), 5.38 (**B**), 5.24 (**C**), 5.20 (**D**, **E**) ppm with integration values of 1.0, 1.08, 0.66 and 1.70, respectively, the latter clearly suggesting overlapping of two signals. Methyl groups at 1.50–1.00 ppm integrated for six protons and indicated two 6-deoxy monosaccharides; two further resonances at 2.14 and 2.11 ppm accounted for a total of 20% of acetyl groups distributed at least on two different positions. Another anomeric signal resonating at 4.50 (**F**) ppm, and indicative of a β configuration, was detected in the ^{1}H NMR spectrum recorded at 25 °C (Fig. 1d), thus confirming a repeating unit composed of six monosaccharides, two of which are 6-deoxy sugars.

In order to eliminate signals arising from acetylation, a sample of **BV-EPS** was de-O-acetylated (**BV-EPS-deOAc**) and subjected to NMR spectroscopy. In general, NMR spectra of **BV-EPS-deOAc** at 25 °C were characterised by poor resolution, but precipitation of the polysaccharide occurred when they were recorded at 50 °C. Nevertheless, the ^{13}C NMR spectrum of this sample recorded at 25 °C showed enough resolution to detect the carbonyl group of the glucuronic acid at 176.26 ppm. Because of the limited solubility of this sample, only **BV-EPS** was investigated, disregarding the low amount of acetyl substitution.

COSY and TOCSY experiments of **BV-EPS** showed extensive overlapping of signals, as expected, since two spin systems are fucose and four have the gluco configuration. Again, as experienced with the sample **BV-EPS-S** for the fucose residues, only H-1 to H-3 and H-6 to H-5 were revealed in these 2D homonuclear experiments; their complete assignment followed from comparison with NMR data of **BV-EPS-S,** since the electronic environments of these residues is very similar in the two polysaccharides. Inspection of the HSQC plot (Fig. 4) gave the corresponding carbon atoms. Complete assignment of the chemical shifts of each spin system (Table 6) was



Scheme 2. Structure of the polysaccharide **BV-EPS-S** repeating unit.

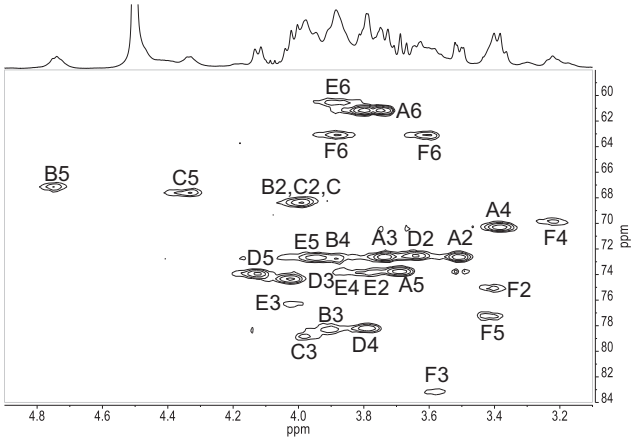
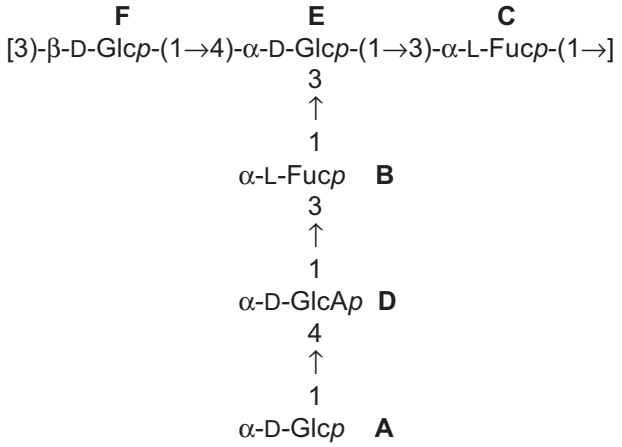


Fig. 4. Expansion of the HSQC plot of **BV-EPS** recorded at 50 °C.

Table 6
^{1}H and ^{13}C chemical shift (ppm)[a] of *B. vietnamiemnsis* exopolysaccharide.

		1	2	3	4	5	6
A	^{1}H	5.43	3.51	3.74	3.39	3.69	3.75, 3.80
α-D--**Glc*p*-(1**	^{13}C	99.37	72.59	72.64	70.27	73.74	61.17
B	^{1}H	5.38	3.99	3.90	3.88	4.74	1.19
3)-α-L-**Fuc*p*-(1**	^{13}C	99.68	68.34	78.28	72.76	67.09	16.00
C	^{1}H	5.24	3.99	3.98	3.99	4.34	1.16
3)-α-L-**Fuc*p*-(1**	^{13}C	100.20	68.34	78.85	68.34	67.63	16.00
D	^{1}H	5.20	3.64	4.02	3.79	4.12	176.26
4)-α-D--**GlcA*p*-(1**	^{13}C	101.31	72.54	74.33	78.19	73.92	
E	^{1}H	5.20	3.80	4.02	3.87	3.95	3.88
3,4)-α-D--**Glc*p*-(1**	^{13}C	101.31	73.86	76.31	73.81	72.70	60.56
F	^{1}H	4.47	3.40	3.58	3.22	3.42	3.61, 3.88
3)-β-D--**Glc*p*-(1**	^{13}C	102.05	75.08	83.17	69.85	77.25	63.10

[a] ^{1}H and ^{13}C chemical shift are given in ppm relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).



Scheme 3. Structure of the polysaccharide **BV-EPS** repeating unit.

achieved also resorting to the data obtained for the two trisaccharides (see supplementary data), **BV-RU** and **BV-EPS-S**.

NOESY experiments confirmed the assignments of 6-deoxy sugars: in fact the signal at 1.21 ppm (H-6 of **B**), showed correlations with its H-5 at 4.74 ppm and H-4 at 3.89 ppm; similarly, the signal at 1.16 ppm (H-6 of **C**), correlated with its H-5 at 4.34 ppm and with H-4 at 3.98 ppm. Moreover, NOESY plot showed also useful inter-residues connectivities: **A1** to **D4**, **B1** to **E3**, **C1** to **F3**, **D1** to **B3**, **E1** to **C3**, and **F1** to **E4**. All the data collected defined the primary structure for **BV-EPS** reported in Scheme 3.

4. Conclusions

B. vietnamiensis LMG 10929 was shown to produce a novel EPS, either alone or in mixture with cepacian. The primary structure of this EPS was established mainly resorting to NMR spectroscopy of the native EPS, as well as of the polysaccharide after Smith degradation and of oligosaccharides obtained by mild acid hydrolysis. ESI-MS was very useful for the determination of the sequence of the oligosaccharides and for a rapid and precise screening of the chromatographic fractions.

BV-EPS is the seventh different EPS produced by bacteria of the BCC, revealing a rather high biosynthetic capacity of this genus. This EPS is the first one produced by bacteria of the BCC to contain fucose residues; in general fucose is not as common as other monosaccharides in bacterial EPS, while it is produced by various species of brown algae, composing a sulfated ester polysaccharide known as fucoidan, and it is a constituent of mammal glycoproteins. However, among bacterial EPS containing fucose residues, colanic acid (Garegg, Lindberg, Onn, & Sutherland, 1971) produced by several Enterobacteriaceae, and three other EPS, share structural similarities with **BV-EPS**: the polysaccharide from Pseudomonas strain 1.15 isolated from freshwater biofilm (Cescutti, Toffanin, Pollesello, & Sutherland, 1999), the EPS produced by *Erwinia chrysanthemi* Ech6 (Yang, Gray, & Montgomery, 1994), a Gram-negative phytopathogen, and the one synthesised by *Enterobacter amnigenus* (Cescutti et al., 2005), a bacterium isolated from sugar beets. These three EPS together with colanic acid are all O-acetylated, branched polymers and have a repeating unit constituted of six sugar residues, two of which are fucose and one is glucuronic acid. Three residues constitute the backbone and three form the side chain. A feature common to all these EPS, but absent in **BV-EPS**, is the substitution of the terminal non reducing end of the side chain with a pyruvyl group which adds another negative charge to the polysaccharides.

The latter structural characteristic is interesting and should be commented together with the observation that deacetylated **BV-EPS** exhibited marked hydrophobic properties. In fact, as mentioned in the NMR structural characterisation paragraph, deacetylation produced poor NMR resolution at 25 °C and precipitation at higher temperatures. Evidently, the presence of several 6-deoxysugars introduces inter-chain hydrophobic interaction eventually leading to polymer precipitation. To avoid this, bacteria introduce acetyl groups and pyruvyl substituents in the other above described polysaccharides. All these substituents exhibit polar characteristics thus enhancing water solubility. It is also possible that the residual hydrophobicity might stimulate some polymer aggregation contributing to the stability of the polysaccharidic matrix around bacterial cell and colonies. In fact, experimental observation in the course of this study showed that **BV-EPS** water solutions are very viscous both when alone or in mixture with cepacian.

Whether this EPS plays some role in infection maintenance and biofilm formation needs to be exploited; however, a polymer with similar structure, colonic acid, is required for establishing the complex three-dimensional structure of an *Escherichia coli* biofilm (Danese, Pratt, & Kolter, 2000).

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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.2013.01.047.

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