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Structural analysis of the exopolysaccharide from *Burkholderia caribensis* strain MWAP71

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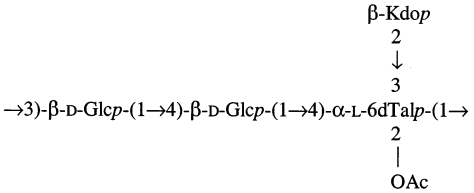
Abstract

Burkholderia caribensis strain MWAP71 was isolated from rhizosphere soil microaggregates in Martinique. The extracellular polysaccharide produced by this strain was found to be composed of D-glucose (D-Glc), 6-deoxy-L-talose (L-6dTal), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), and an *O*-acetyl group in a molar ratio of 2:1:1:1. The primary structure of the polysaccharide was shown by sugar analysis, electrospray mass spectrometry, partial acid hydrolysis and 1-D and 2-D NMR spectroscopy to consist of a tetrasaccharide repeating unit having the following structure:



Keywords: Exopolysaccharides; Structural analysis; NMR spectroscopy; Kdo; Soil bacteria

1. Introduction

Soil structure has a strong impact on a range of processes influencing crop yield. The basic units of soil structure, named aggregates, comprise solid material and pores. These aggregates determine the mechanical and physical properties of soil such as retention and exchange of water, aeration, and temperature.[1] Aggregate formation is an important factor controlling germination and root growth.[2] Formation of stable aggregates strongly depends on both the nature and the content of organic matter; unstable aggregates generally have a lower content of organic matter than do stable ones. Plant roots contribute to soil organic material, and thereby to soil aggregate stability and it is generally believed that the microbial contribution to soil aggregation is due to the production of exopolysaccharides (EPS).[3]

Up to now relatively little attention has been paid to the influence of microorganisms,

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particularly EPS-producing rhizobacteria, on the aggregation of root-adhering soil (RAS).[4,5] Recently Heulin and coworkers demonstrated that the EPS produced by *Paenibacillus polymyxa*,[6] *Pantoea agglomerans*[4] and *Rhizobium* sp.[7] are implicated in the aggregation of RAS on wheat or sunflower.

The strain MWAP71 of *Burkholderia caribensis* was isolated from a fraction of a vertisol in the south-east of the island of Martinique in the French West Indies.[8] Vertisols are characterised by a very high clay content. In these soils, microaggregation results mainly from the fact that bacterial colonies embedded in their exopolysaccharides (EPS) are covered with clay particules.[9] These bacteria are selected for their EPS production and found to be responsible for the formation of microaggregates in this soil. The determination of the structure of the EPS is therefore an important step in understanding the mechanism of soil aggregation.

2. Results and discussion

Sugar composition of the polysaccharide.—*B. caribensis* strain MWAP71 produces only one EPS. The polysaccharide (PS) extracted from liquid culture of this strain (see Section 3) was subjected to total acid hydrolysis with trifluoroacetic acid (TFA). Quantitative analysis of neutral sugars by cation-exchange high performance liquid chromatography (HPLC) showed the presence of glucose and another neutral sugar in the ratio 2:1.

Partial acid hydrolysis.—In order to elucidate the structure of the polysaccharide, mild hydrolysis with TFA was performed and resulted in a degraded polysaccharide (DPS), several oligosaccharides and an acidic sugar cleaved from PS. This mixture was fractionated on Bio-Gel P-2, yielding fractions I to IV.

Analysis of acidic sugar.—The acidic sugar isolated by partial acid hydrolysis seemed to be an unknown carbohydrate but we suspected that it could have been degraded by the hydrolysis. Consequently, milder acid hydrolysis with acetic acid was carried out. The compound thus obtained was separated from degraded material and studied by NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra showed a mixture of two acidic sugars, including signals identical to those of 3-deoxy-D-*manno*-octulosonic acid (Kdo).[10,11] This mixture and Kdo were also studied by HPLC–ESMS in acid conditions (0.1% TFA, see Section 3). The positive-ion ESMS spectra of these samples were identical and both showed quasimolecular ion peaks which were due to attachment of Na^+, $[M + Na]^+$ and $[M + 2Na - H]^+$, at m/z 260.8 and 282.8. In addition, both mass spectra showed two fragment ions at m/z 220.7 and 242.8 corresponding probably to ions $[M + H - H_2O]^+$ and $[M + Na - H_2O]^+$. These data confirmed that Kdo was present in EPS and that the acidic sugar was a degradation product of Kdo, probably a bicyclic compound. The D configuration of Kdo was confirmed by GLC of acetylated (*R*)-2-butyl glycosides.[12,13]

Analysis of Fraction I.—Fraction I was a mixture of two monosaccharides, which were separated by liquid chromatography and identified as glucose and 6-deoxy-talose, according to the 1-D ^{1}H NMR spectra which were identical to those described previously.[14,15] The $[\alpha]_D$ values demonstrated that the glucose and the 6-deoxy-talose sugars were D and L, respectively.

Analysis of Fraction II.—Fraction II was separated by liquid chromatography to afford two disaccharides which will be referred to as **IIa** and **IIb**.

The disaccharide **IIa** was studied by NMR spectroscopy. Three anomeric signals were observed in its 1-D ^{1}H NMR spectrum (at 40 °C in D_2O); two of them at δ 4.82 and 5.27 were assigned to the β- and α-6dTal*p* forms (residue **A** $^{3}J_{1,2} < 2$ Hz) of the reducing sugar according to the chemical shifts.[14,15] The signal at δ 4.50 was assigned to the anomeric proton of the β-Glc*p* residue (**B** $^{3}J_{1,2}$ 7.7 Hz). The complete proton and carbon assignments for these two residues were based on 1-D TOCSY, COSY and HMQC experiments.

In the 2-D ^{13}C–^{1}H HMBC spectrum of disaccharide **IIa**, intraresidual two- and three-bond ^{13}C–^{1}H couplings can be observed, as well as interresidual three-bond connectivities over the glycosidic linkages. The β-Glc*p*-(1 → 4)-6dTal*p* linkage was determined from a

cross-peak between C-1 of glucose (δ 103.5) and H-4 of 6dTal ($\delta_{H\text{-}4\alpha}$ 4.05 and $\delta_{H\text{-}4\beta}$ 3.99). Hence the structure of **IIa** was assigned as follows:

β-D-Glc*p*-(1→4)-L-6dTal*p*
B A

The second disaccharide **IIb** was shown by NMR spectroscopy to be cellobiose, demonstrating a β-(1→4) linkage between the two glucose units in the polysaccharide. Since there are only two glucose residues, the reducing end corresponds to residue **B**, and the other glucose residue was called **C**:

β-D-Glc*p*-(1→4)-D-Glc*p*
C B

Analysis of Fraction III.—Fraction III contained a trisaccharide referred to as **III** in the following.

In the 1-D ^{1}H NMR spectrum of **III** at 40 °C, four anomeric signals were detected. The two signals at δ 4.82 and 5.25 were assigned to the β- and α-forms of the 6dTal residue (unit **A**, $^3J_{1,2} < 2$ Hz), the two signals at δ 4.50 and 4.55 to two β-Glc residues (residue **B** $^3J_{1,2}$ 7.7 Hz, residue **C** $^3J_{1,2}$ 8 Hz). The structure of this trisaccharide could be directly deduced from the structures **IIa** and **IIb**. The complete proton assignments for residues **A**, **B**, **C** presented in Table 1, are based on 1-D TOCSY and COSY experiments.

The ^{13}C NMR spectrum of **III** contained four signals in the anomeric region. Signals at δ 94.3 and 94.9 were assigned to the β- and α-forms of residue **A**, signals at δ 103.3 to residue **B** (as in disaccharide **IIa**) and the signal at δ 102.9 to residue **C**. The 2-D ^{13}C–^{1}H HMQC spectrum of **III** allowed complete assignment of the ^{13}C NMR spectrum (Table 1). Hence **III** has the following structure:

β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-L-6dTal*p*
C B A

Analysis of Fraction IV.—Fraction IV contained the degraded polysaccharide (DPS).

The 75 MHz ^{13}C NMR spectrum of DPS (at 30 °C in D_2O) contained three signals in the anomeric region (at δ 102.0, 102.7 and 103.3). This spectrum was very similar to that of **III**, with the exception of the signals resulting from the α–β equilibrium at the anomeric centre of the reducing sugar. This indicated that the polysaccharide main chain was a trisaccharide repeating unit composed of **A**, **B** and **C** residues. The linkage of residue **C** in DPS was shown to be an **A**(1→3)**C** linkage according to the **C** C-3 chemical shift (δ 82.4 to be compared to δ 75.9 in **III**). For the **B** and **C** residues, the C-1, H-1 coupling constants observed in the HMQC experiment, without ^{13}C decoupling, were relatively small (< 165 Hz) confirming the β configuration of the anomeric carbons.[16] The C-1, H-1 coupling constant of 170 Hz for residue **A** indicated an α-anomeric configuration. These data suggested that DPS has the following trisaccharide repeating unit:

→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-α-L-6dTal*p*-(1→
C B A

Table 1
^{1}H and ^{13}C NMR chemical shifts (in ppm) of the trisaccharide **III** in D_2O at 40 °C

Residue	δ (^{1}H)/δ (^{13}C)						
	1	2	3	4	5	6a	6b
A β	4.82 (<2)	3.84 (3)	3.85 (3)	3.98 (<2)	3.80 (6.5)	1.39	
	94.3	71.1	68.5	79.3	71.4	16.0	
α	5.25 (<2)	3.77 (3)	4.01 (3)	4.04 (<2)	4.30 (6.7)	1.36	
	94.9	70.2	65.4	80.3	66.8	16.0	
B	4.50 (8)	3.50 (8.6)	3.69 (8.5)	3.73 (8.3)	3.64 (4.8)	3.88 (<2)	4.01 (12.4)
	103.3	73.5	74.3	78.8	75.1	60.2	
C	4.55 (8)	3.35 (9.2)	3.55 (9.5)	3.45 (7.7)	3.53 (5.2)	3.77 (1.9)	3.95 (12.3)
	102.9	73.5	75.9	69.8	76.5	60.9	

Coupling constants ($^3J_{H,H}$ in Hz) are given in brackets. **A**: →4)-L-6dTal*p*; **B**: →4)-β-D-Glc*p*-(1→; **C**: β-D-Glc*p*-(1→.

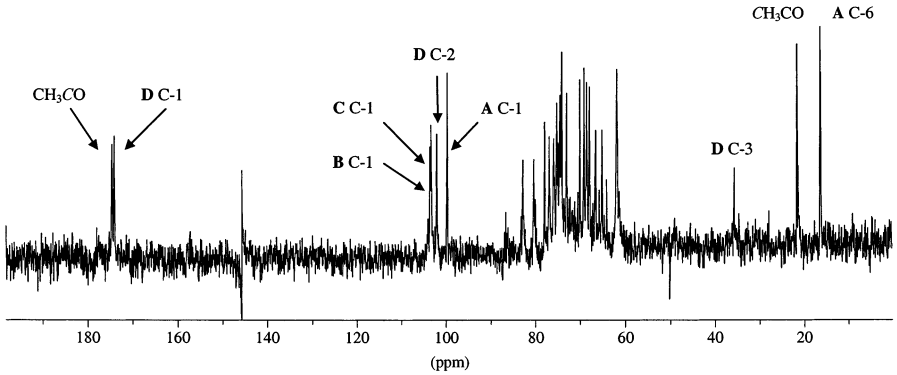


Fig. 1. 75 MHz ^{13}C NMR spectrum of crude polysaccharide in D_2O at 79 °C

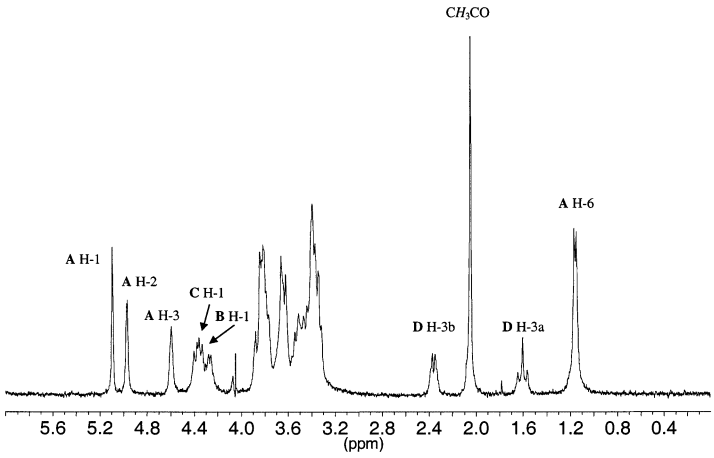


Fig. 2. 500 MHz ^1H NMR spectrum of crude polysaccharide in D_2O at 79 °C

NMR spectroscopy of PS.—When compared to the spectrum of DPS, the ^{13}C NMR spectrum of PS (Fig. 1) contained ten additional signals. Two of them at δ 21.7 and 174.6 are characteristic of an *O*-acetyl group (OAc). Integration of the signal at δ 2.18 in 1-D ^1H NMR spectrum of PS (Fig. 2) indicated one OAc group per repeating unit. The four signals at δ 35.8, 65.2, 102.1 and 174.1 were characteristic of a methylene group of a deoxy unit (CCH_2C), a hydroxymethyl group ($HOCH_2$), a non-protonated carbon (C-2 of a keto sugar), and a carboxyl group, respectively, according to their chemical shifts and the DEPT (Distortionless Enhancement by Polarisation Transfer) 135° ^{13}C NMR spectrum of PS.

These data were consistent with the presence of a 3-deoxy-octulosonic acid residue, most likely, the well-known 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Since Kdo has a highly acid-labile glycosidic linkage, it was completely cleaved from PS by mild hydrolysis with 1% acetic acid. The fact that depolymerisation of the polymeric chain did not occur indicated that this monosaccharide was a side chain sugar residue.

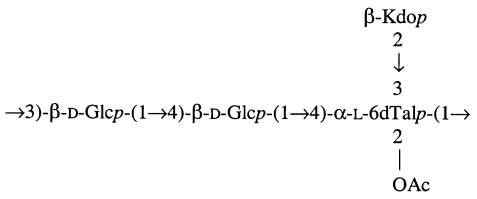
Finally, the ^{13}C chemical shifts for the 3-deoxy-octulosonic acid residue in PS were similar to those of sodium (methyl 3-deoxy-β-D-*manno*-octulosid)onate,[11] confirming that the fourth sugar constituent of PS is a β-linked Kdo residue.

The presence in the 2-D HMBC spectrum of a cross-peak between H-3 of 6dTal and C-2 of Kdo at δ 4.73/102.1 indicated that Kdo is attached at position 3 of the 6dTal residue.

Comparison of the ^1H NMR spectra of DPS and PS showed a large α-effect of *O*-acetylation for the H-2 signal of 6dTal, which was shifted from δ 3.93 to 5.09, reflecting the presence of an OAc group at O-2 of the 6dTal unit. This was confirmed by a significant β-effect of *O*-acetylation[17] for the C-1 signal of

6dTal, which was shifted from δ 102.0 in the ^{13}C NMR spectrum of DPS to δ 99.8 in the ^{13}C NMR spectrum of PS. The ^{1}H and ^{13}C data for PS have been collected in Tables 2 and 3, respectively.

It can be concluded that the exopolysaccharide from *Burkholderia caribensis* strain MWAP71 is composed of a branched tetrasaccharide-repeating unit with the following structure:



3. Experimental

Production and purification of the polysaccharide.—EPS-producing bacteria were isolated and grown as described previously.[8] The yield of this production was about 3g/L. The culture supernatant solution of *B. caribensis* strain MWAP71 has been filtered through a 0.8 μm filter to eliminate proteins and cellular fragments. The crude polysaccharide (PS) was obtained as a 60% (v/v) isopropanol water precipitate from the filtered solution.

Monosaccharides analysis.—The polysaccharide was hydrolysed in 4 M TFA at 100 °C for 4 h. The TFA was removed by repeated evaporation with water. Quantitative HPLC of monosaccharides was performed on a Waters HPLC system with RI detection, using a CHO-682 cation exchange column (300 × 7.8 mm Interchim, France) eluted with water at 85 °C.

Partial acid hydrolysis.—The native polysaccharide (100 mg) was hydrolysed in 1 M TFA for 1 h at 100 °C. After evaporation of TFA, oligosaccharide fractions were eluted from a Bio-Gel P-2 column (2 m × 1.5 cm) with water at 60 °C and at a flow rate of 40 mL/h. The fractions were analysed by reverse phase HPLC with a Interchrom CP5C18-25F apolar column (250 × 4.6 mm Interchim,

Table 2
^{1}H NMR chemical shifts [a] of the *B. caribensis* strain MWAP71 polysaccharide in D_2O at 79 °C

Residue	Chemical shifts (ppm)							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
A	5.22	5.09	4.73	3.89	4.40	1.28	-	
B	4.46	3.46	3.66	3.60	3.57	3.76	3.99	
C	4.51	3.47	3.60	3.52	3.53	3.76	3.93	
	H-3a	H-3b	H-4	H-5	H-6	H-7	H-8a	H-8b
D	1.73	2.48	3.77	3.93	3.50	3.96	3.77	3.99

A: →3,4)-L-6dTal*p*2Ac-(1→; **B**: →4)-β-D-Glc*p*-(1→; **C**: →3)-β-D-Glc*p*-(1→; **D**: β-Kdo*p*-(2→.
[a] Additional signals: δ 2.18 (CH_3CO).

Table 3
^{13}C NMR chemical shifts [a] of the *B. caribensis* strain MWAP71 polysaccharide in D_2O at 79 °C

Residue	Chemical shifts (ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
A	99.8	73.1	68.1	78.0	68.7	16.4		
B	103.6	74.6	75.3	80.4	75.9	61.9		
C	103.4	74.2	82.8	69.2	76.9	61.9		
D	174.1	102.1	35.8	68.5	66.7	74.2	70.1	65.2

A: →3,4)-L-6dTal*p*2Ac-(1→; **B**: →4)-β-D-Glc*p*-(1→; **C**: →3)-β-D-Glc*p*-(1→; **D**: β-Kdo*p*-(2→.
[a] Additional signals: δ 21.7 (CH_3CO) and 174.6 (CH_3CO).

France) eluted with water at 40 °C with a flow rate of 0.4 mL/min.

Analysis of the acidic sugar.—Native polysaccharide was hydrolysed under milder conditions; 1% AcOH for 30 min at 100 °C. AcOH was removed by successive freeze drying. Separation from degraded material was performed using a liquid chromatograph equipped with a Biorad HPX-87H column (350×7.5 mm) eluted with 0.1% TFA at rt with a flow rate of 0.6 mL/min.

Absolute configuration of the acidic sugar.—The absolute configuration of this monosaccharide of the native polysaccharide was determined using the procedure described by Gerwig et al.[12,13] Separation of acetylated derivatives was performed using a SP-2380 capillary column ($30\ m \times 0.53$ mm) with a temperature program increasing from 195 °C (3 min) to 225 °C at 2.5 °C/min.

High performance liquid chromatography and electrospray mass spectrometry.—HPLC–ESMS analyses were carried out on a Micromass Quattro 2 mass spectrometer, coupled to a liquid chromatograph equipped with a Biorad HPX-87H column (350×7.5 mm) eluted with 0.1% TFA at rt with a flow rate of 0.6 mL/min.

NMR spectroscopy.—The samples were dissolved in D_2O. 1-D ^1H and 2-D NMR spectra of acidic sugar, **IIa**, **IIb** and **III** were recorded at 40 °C, and at 79 °C for the polymer, with a Varian Unity Plus 500 spectrometer (500 MHz). Proton-decoupled 75.470 MHz ^{13}C NMR spectra were recorded with an AC 300 Bruker instrument (300 MHz) equipped with a 5 mm $^{13}C/^1H$ dual probe. ^1H spectra were obtained with a spectral width of 2000 Hz and a pulse length of 12.7 μs (90°). Eight scans were accumulated with an acquisition time of 2 s. Data were zerofilled to 32 K and suppression of HOD signal was achieved by a presaturation for 0.4 s.

1-D TOCSY spectra were recorded using a selective (26 Hz) soft pulse sequence with an eburp 1-256 shape, a pulse length of 174 ms and arrayed selective frequency. The mixing time was from 5 to 75 ms. The spectrum was collected with 8–120 transients.

gCOSY Experiments were carried out with the standard pulse sequence. The time-domain data set was of 2 K and 16 pulses were performed for each of the 256 experiments. The time-domain data sets were multiplied with a phase shifted sine bell. After zerofilling and Fourier transform, data sets of 4×4 K were obtained.

2-D $^{13}C-^1H$ HMQC and HMBC experiments with inverse detection were carried out at a ^1H frequency of 499.836 MHz (125.696 MHz for ^{13}C) with a spectra width of 2000 and 26,000 Hz for ^1H and ^{13}C, respectively, using the standard pulse sequences. The time-domain data sets were multiplied with a phase shifted sine bell and after zerofilling and Fourier transformation, data sets of 4×4 K were obtained.

^{13}C spectra were obtained with a spectral width of 15,000 Hz and a recycle delay of 0.8 s. The data were collected in 16 K data sets and before Fourier transformation exponential multiplication was applied.

^1H NMR chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) and ^{13}C NMR chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm).

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