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Carbohydrate Research 339 (2004) 2045–2047
Carbohydrate RESEARCH

Note

The structure of the O-specific polysaccharide from *Ralstonia pickettii*

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Received 6 May 2004; accepted 19 May 2004
Available online 1 July 2004

Abstract—The following structure of the *Ralstonia pickettii* have been determined using NMR and chemical methods:

→ 4)-α-D-Rha-(1 → 4)-α-L-GalNAcA-(1 → 3)-β-D-BacNAc-(1 →

Keywords: *Ralstonia*; *Ralstonia pickettii*; LPS; O-Chain; Structure

The genus *Ralstonia* represents aerobic, nonfermentative Gram-negative bacteria. It was created to accommodate bacteria from diverse ecological niches previously classified as *Burkholderia* and *Alcaligenes*[1] with *Ralstonia pickettii* (ATCC 27511, formerly called *Pseudomonas pickettii* or *Burkholderia pickettii*) as a type strain. Recently it was proposed to split this genus into two—*Ralstonia* and *Wausteria*, with *R. pickettii* remaining in *Ralstonia* genus.[2] Some members of the genus are important plant pathogens.[3] *R. pickettii* is the most common human pathogen of its genus, although other strains were found recently in cystic fibrosis patients.[4] The virulence of *R. pickettii* is rather low, however it is able to cause bacteremia and nosocomial infections.[5–10] Sometimes it infects patients with indwelling intravenous devices[11] and was isolated from various other sources.[1,12–14] It is able to grow at a wide range of temperatures (15–42 °C) as well as in the saline solution and can pass through sterilization filters, thus contaminating sterilized solutions.[15] The bacterium was isolated also from chlorhexidine solutions used for disinfections.[6] It can degrade numerous toxic compounds.[16]

The knowledge of the structure of the O-antigenic component of lipopolysaccharide (LPS) is helpful in understanding of molecular details of bacterial biological activity. The structure of cell surface polysaccharide might be determinative factor to the bacterial ability to adhere to any wet surface for further growing and formation biofilm. This paper includes the results of structural analysis of the polysaccharide part of the *R. pickettii* LPS.

R. pickettii was isolated from purified water-storage bottle and identified with help of API 20 NE test (BioMerieux, France). Bacteria were cultivated at 37 °C on normal broth (BTL-Poland). The LPS was extracted by phenol–water procedure[17] and purified by ultracentrifugation. The polysaccharide O-chain was isolated from the LPS by conventional mild acid hydrolysis with 2% HOAc (100 °C, 2 h) followed by Sephadex G50 gel-filtration chromatography of the water soluble products, yielding a lipid-free high-molecular-mass (O-PS). However no core fraction was observed. GC analysis of alditol acetates prepared from the polysaccharide showed the presence of rhamnitol and small amount of glucitol, probably originating from an impurity.

Abbreviations: LPS, lipopolysaccharide; GalNA, 2-amino-2-deoxygalacturonic acid; BacN, 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine).

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0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.05.014

The set of 2D NMR experiments (COSY, TOCSY, NOESY, HSQC, HMBC) was recorded for the polysaccharide sample. The spectra were completely assigned (Fig. 1, Table 1) using Pronto program.[18] This led to the identification of spin systems of three monosaccharides, arbitrarily labeled A–C in decreasing order of their anomeric proton chemical shifts. Monosaccharides were identified on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts. Anomeric configurations were deduced from the $J_{1,2}$ coupling constants and chemical shifts of H-1 and C-1 signals. Position of C-6 signals of uronic acid was found from HMBC correlations to H-5 protons. Thus the residue A was identified as 2-acetamido-α-galactopyranosyluronic acid, residue B as α-rhamnopyranose, and the residue C as 2,4-diacetamido-2,4,6-trideoxy-β-glucopyranose. Connections between monosaccharides were identified on the basis of NOE and HMBC correlations. The NOE between protons A1 and C3, C1 and B4, B1 and A4 were observed, as well as corresponding HMBC correlations, in agreement with the presented monosaccharide sequence.

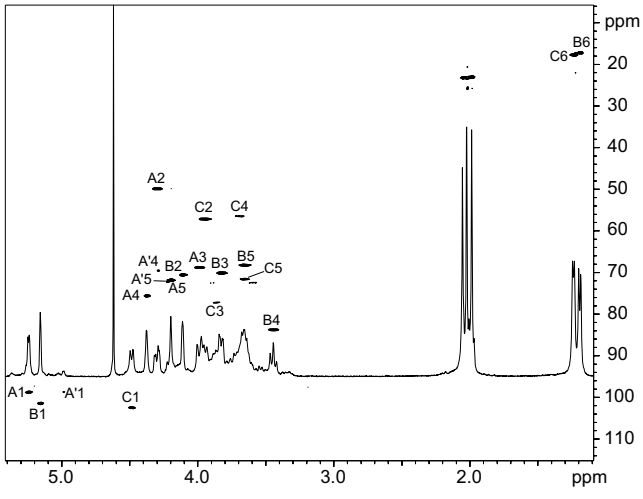

Figure 1. HSQC spectrum of *R. pickettii* O-specific polysaccharide.

B A C

→4)-α-D-Rha-(1→4)-α-L-GalNAcA-(1→3)-β-D-BacNAc-(1→

1

Minor signals in the NMR spectra of the polysaccharide were identified as belonging to the terminal residue of α-GalNAcA (residue A′ in Table 1). This may be the result of partial hydrolysis of Rha linkages during HOAc treatment of the LPS.

Treatment of the polysaccharide with 0.5 M HCl in MeOH at 70 °C followed by the separation of the products on reverse phase column gave a number of products, of which pure oligosaccharide **2**, methyl α-rhamnopyranoside, and a mixture of α-methyl glycosides of GalNAcA (**3**) and BacNAc (**4**).

A C

α-GalNAcA6OMe-(1→3)-α-BacNAc-(1→OMe

2

NMR spectra of compounds **2**–**4** were completely assigned (Table 1) and confirmed their structures.

Absolute configuration of Rha was determined by optical rotation measurement of methyl α-rhamnopyranoside, isolated from the products of the methanolysis of polysaccharide. The $[\alpha]_D$ value of +52 (*c* 0.1, water) was obtained, indicating D-configuration (lit. $[\alpha]_D$ +64.5 for D-isomer).[19]

For the determination of the absolute configuration of two other monosaccharides of repeating unit ^{13}C NMR chemical shifts were calculated for four possible combinations of the configurations—DDD, DLL, DDL, and DLD (assuming that rhamnose is always D) using data and method described in Ref. 20. The results

Table 1. NMR data for the polysaccharide (**1**) and its methanolysis products (**2**–**4**)

Unit, compound	Nucleus	1	2	3	4	5	6	1→OMe	6→OMe
GalNAcA A, **1**	^{1}H	5.24	4.30	3.99	4.37	4.20			
	^{13}C	98.7	49.9	68.9	75.6	71.8	173.8		
GalNAcA A′, **1**	^{1}H	5.24	4.22	3.85	4.29	4.22			
	^{13}C	98.7	49.9	68.1	69.4	71.9	175.7		
GalNAcA A, **2**	^{1}H	5.23	4.16	3.85	4.34	4.46			3.86
	^{13}C	98.4	50.1	67.7	70.4	72.2			54.3
GalNAcA A, **3**	^{1}H	5.24	4.18	3.86	4.34	4.47		3.48	3.86
	^{13}C	98.9	50.0	67.9	70.4	72.2		58.5	54.2
Rha B, **1**	^{1}H	5.16	4.11	3.82	3.44	3.66	1.19		
	^{13}C	101.4	70.5	70.0	83.8	68.3	17.3		
BacNAc C, **1**	^{1}H	4.49	3.95	3.87	3.69	3.66	1.24		
	^{13}C	102.5	57.2	77.3	56.5	71.6	17.8		
BacNAc C, **2**	^{1}H	4.64	4.14	3.92	3.71	3.85	1.20	3.37	
	^{13}C	99.2	55.5	74.8	56.6	67.7	17.9	56.5	
BacNAc C, **4**	^{1}H	4.76	4.00	3.73	3.65	3.84	1.22	3.39	
	^{13}C	99.2	55.3	70.1	58.4	68.0	18.0	56.4	

A′ in **1** is nonreducing terminal residue of GalNAcA. Acetate signals in **1**: CO 175.9, 174.9, 175.7; Me ($^{1}H/^{13}C$): 1.99/23.2, 2.02/23.3, 2.05/23.3 ppm. Acetate signals in **2**: Me ($^{1}H/^{13}C$): 1.97/23.3, 1.98/23.2, 2.03/23.4 ppm; in **3**: 2.05/23.3 ppm; in **4**: 1.97/23.4, 1.98/23.4 ppm.

showed best fit between calculated and experimental data for D-BacN and L-GalNA. This conclusion agrees with the observed optical rotation of the polysaccharide $[\alpha]_D$ −53 (*c* 1, water), which is to be expected qualitatively for the sum of two negative values contributed by α-L-GalNA and β-D-BacN, and one positive from α-D-Rha. The optical rotation of the disaccharide **2** was −16 (*c* 0.2, water) due to canceling of positive contribution of α-D-BacN and negative of α-L-GalNA; it would have large absolute value in case of identical configuration of both monosaccharide residues.

The structure of the *R. pickettii* polysaccharide includes a fragment:

→ 4)-α-L-GalNAcA-(1 → 3)-β-D-BacNAc-(1 →

which is present in other polysaccharides of Gram-negative bacteria, particularly in the O:3 serogroup of *Pseudomonas aeruginosa*,[21] which may lead to serological cross-reactivity.

1. Experimental

1.1. General methods

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer in D_2O soln at 40 °C for the polysaccharide and at 25 °C for mono- and oligosaccharides with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, and HMBC (optimized for a 5 Hz coupling constant).

For monosaccharide analysis, the polysaccharide (0.5 mg) was hydrolyzed (0.2 mL of 3 M TFA, 100 °C, 2 h), followed by evaporation to dryness under a stream of air. The residue was dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with concd HOAc (0.3 mL), dried, and MeOH (1 mL) was added. The mixture was dried twice with the addition of MeOH, and the residue was acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, and analyzed by GC on a HP1 capillary column (30 m × 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient of 170 (4 min) to 260 °C at 4 °C/min or on a Varian Saturn 2000 ion-trap GS–MS instrument in the same conditions.

Gel chromatography was carried out on Sephadex G-50 (2.5 m × 95 cm) and Sephadex-15 (1.6 m × 80 cm) columns using the pyridinium acetate buffer, pH 4.5 (4 mL pyridine and 10 mL HOAc in 1 L water) as eluent, monitored by a refractive index detector.

1.2. Preparation of the polysaccharide fragments

For the preparation of methyl α-rhamnopyranoside and compounds **2**–**4**, a sample (40 mg) was suspended in dry MeOH (2.0 mL), cooled in a dry ice/acetone bath, followed by addition of acetyl chloride (0.1 mL). The dissolved material was kept at 70 °C for 24 h, dried in an air stream, and the products were separated by HPLC with UV detection (220 nm) on a C18 column (Phenomenex Aqua, 10 × 250 mm) using a gradient of water to 90% MeCN. Fractions were also analyzed for the presence of sugars by charring spots on TLC plate after dipping in 5% H_2SO_4 in MeOH.

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