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Carbohydrate Research 339 (2004) 321–325
Carbohydrate RESEARCH

Structure of the O-polysaccharide of *Citrobacter youngae* O1 containing an α-D-ribofuranosyl group

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Received 18 June 2003; accepted 9 October 2003

Abstract—The lipopolysaccharide of *Citrobacter youngae* O1, strain PCM 1492 was degraded with acid or alkali under mild conditions, and the resultant polysaccharide was isolated by GPC and studied by sugar and methylation analyses and ^{1}H and ^{13}C NMR spectroscopies, including 2D COSY, TOCSY, NOESY and ^{1}H, ^{13}C HSQC experiments. The following structure of the branched tetrasaccharide repeating unit of the O-polysaccharide was established:

α-D-Rib*f*
1
↓
4
→4)-α-D-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→

where substitution with the α-D-Rib*f* group is nonstoichiometric. This group occurs rarely in bacterial polysaccharides and is easily cleaved under mild acidic conditions. Studies with polyclonal rabbit antisera against whole cells of *C. youngae* PCM 1492 and PCM 1506 showed the serological identity of the lipopolysaccharides of *C. youngae* PCM 1492, PCM 1493 and PCM 1506, which are classified in serogroup O1.

Keywords: Enterobacteria; O-antigen; Lipopolysaccharide; Bacterial polysaccharide structure; α-D-Ribofuranose; Serological classification; *Citrobacter youngae*

1. Introduction

Strains of the genus *Citrobacter* from the family Enterobacteriaceae are widespread in the environment, including soil, sewage and food, and are normal inhabitants of animal and human intestinal tract.[1] *Citrobacter* is an opportunistic human pathogen causing urinary and respiratory tract infections.[2] Incidents of meningitis, brain abscesses and neonatal sepsis caused by *Citrobacter* have also been reported.[3,4] At present, the genus *Citrobacter* is divided into 11 species.[5,6]

The first O-antigen-based classification scheme of serologically heterogeneous *Citrobacter* strains was established by West and Edwards in 1954[7] and has been revised recently.[8] Strains of *Citrobacter* are classified into 42 O-serogroups,[9,10] and many from them were found to be related serologically to other enteric bacteria, such as *Escherichia coli*,[11,12] *Hafnia alvei*,[13–15] *Salmonella*[16] and others.[9,16,17]

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doi:10.1016/j.carres.2003.10.008

The O-antigens of *Citrobacter*, which represent the O-polysaccharide chains of the lipopolysaccharides, belong to at least 20 chemotypes.[17] Structures of about 30 O-polysaccharides in various *Citrobacter* serogroups have been established, and the chemical basis of the serological cross-reactivity of a number of strains with other bacteria has been elucidated.[18] Strains of several serogroups, for example, O27, O4 and O36, are differentiated based on the lipopolysaccharide core structures, whereas their O-polysaccharides are structurally identical.[19,20]

Now we report on the structure of the O-polysaccharide of *Citrobacter youngae* PCM 1492 (former *Citrobacter freundii* PCM 1492), which belongs to serogroup O1. Strains of this serogroup are among those which are the most frequently isolated from children patients with enteritis,[9] and from patients with etiologically obscure dysentery. A relationship of the O-antigens of *C. freundii* O1a,1b,1c and *E. coli* O9 has been reported.[9]

2. Results and discussion

The lipopolysaccharide was isolated from dried bacteria by phenol–water extraction. Hydrolysis of the lipopolysaccharide with dilute acetic acid followed by centrifugation gave a carbohydrate-containing supernatant and a lipid A sediment. The material present in the supernatant was fractionated by GPC on Sephadex G50 to give a high-molecular-mass polysaccharide (PS-1) in a yield of 45% of the lipopolysaccharide weight.

Sugar analysis of PS-1 using GLC–MS of the alditol acetates revealed the presence of mannose, rhamnose and ribose in the molar ratios 2.1:1.0:0.5. The D configuration of the monosaccharides was determined by GLC of the acetylated (+)-2-octyl glycosides. Methylation analysis revealed the presence of terminal ribofuranose, 3-substituted Man, 4-substituted Rha and Man and 3,4-disubstituted Man in the ratios 0.25:0.91:1:0.74:0.48.

The ^{13}C NMR spectrum of PS-1 showed a heterogeneity, which could be accounted for the presence of ribose in a nonstoichiometric amount. Therefore, the lipopolysaccharide was deacylated by alkaline treatment but the resulting polysaccharide (PS-2) possessed the same type of structural heterogeneity (Fig. 1). Partial hydrolysis of PS-2 with 48% HF resulted in a regular linear polysaccharide (PS-3) that lacked ribose. The ^{13}C NMR spectrum of PS-3 represented a part of the spectrum of PS-2, and, hence, the heterogeneity of PS-1 and PS-2 is associated with incomplete substitution with lateral ribose residues.

For structural analysis, ^{1}H and ^{13}C NMR spectra of the PS-2 and PS-3 were assigned using 2D COSY, TOCSY, NOESY and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 1 and 2). The spin systems for the monosaccharides were identified by ^{3}J coupling constant values and the ^{1}H and ^{13}C NMR chemical shifts compared with the data of the corresponding monosaccharides.[21–25] The configurations of the glycosidic linkages of mannose (Man^{I} and Man^{II}) and rhamnose residues were determined by the H-5 and C-5 chemical shifts, which were close to the values of the monosaccharides β-Man*p* and α-Rha*p* and different from those of the corresponding anomers.[22,23] The configurations were confirmed by intraresidue correlations (H-1,H-2 for α-Rha; H-1,H-3 and H-1,H-5 for β-Man^{I} and β-Man^{II}), which were revealed by a NOESY experiment.

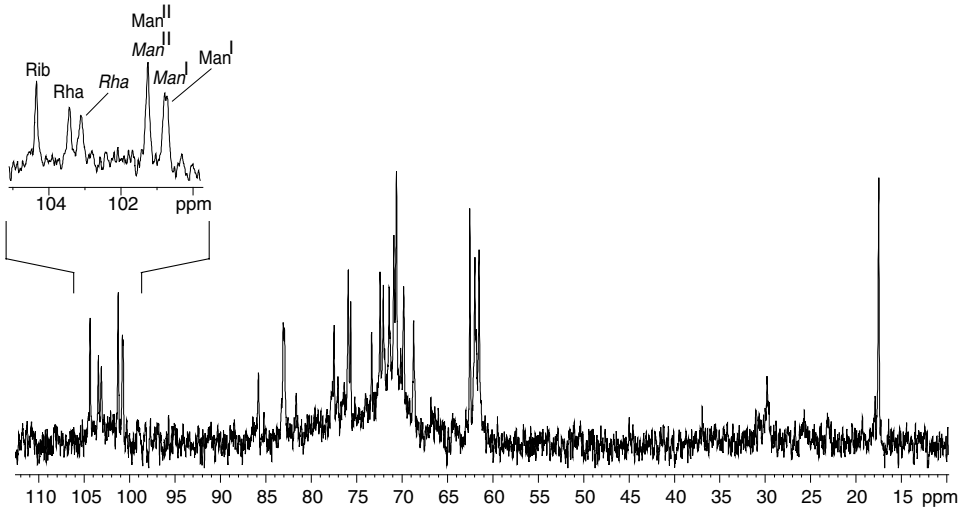

Figure 1. ^{13}C NMR spectrum of the polysaccharide (PS-2) obtained by alkaline hydrolysis of the lipopolysaccharide of *C. youngae* PCM 1492. Annotations for the signals of the anomeric protons in the sub-spectrum that correspond to PS-3 are italicised.

Table 1. 500 MHz ^{1}H NMR data of the polysaccharides from *C. youngae* O1 (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5(5a)	H-6a(5b)	H-6b
Polysaccharide (PS-2)[a]							
→4)-α-D-Rha*p*-(1→	5.06	4.13	3.97	3.67	3.96	1.32	
→3,4)-β-D-Man*p*^{I}-(1→	4.77	4.11	3.90	3.79	3.58	3.77	3.96
→4)-β-D-Man*p*^{II}-(1→	4.81	4.15	3.83	3.83	3.56	3.76	3.91
α-D-Rib*f*-(1→	5.32	4.15	4.04	4.12	3.65	3.73	
Deribosylated polysaccharide (PS-3)							
→4)-α-D-Rha*p*-(1→	5.05	4.13	3.98	3.66	3.95	1.32	
→3)-β-D-Man*p*^{I}-(1→	4.76	4.11	3.72	3.68	3.48	3.75	3.95
→4)-β-D-Man*p*^{II}-(1→	4.81	4.14	3.82	3.84	3.55	3.77	3.91

[a]Given are the data of the major tetrasaccharide repeating units.

Table 2. 125 MHz ^{13}C NMR data of the polysaccharides from *C. youngae* O1 (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide (PS-2)[a]						
→4)-α-D-Rha*p*-(1→	103.3	70.7	69.9	83.1	68.7	17.5
→3,4)-β-D-Man*p*^{I}-(1→	100.8	71.4	83.0	73.3	75.6	62.0
→4)-β-D-Man*p*^{II}-(1→	101.3	70.9	72.4	77.5	75.9	61.5
α-D-Rib*f*-(1→	104.4	72.0	70.6	85.8	62.5	
Deribosylated polysaccharide (PS-3)						
→4)-α-D-Rha*p*-(1→	103.0	70.6	69.8	83.1	68.6	17.6
→3)-β-D-Man*p*^{I}-(1→	100.9	71.3	81.7	66.8	77.1	61.9
→4)-β-D-Man*p*^{II}-(1→	101.3	70.9	72.5	77.5	75.9	61.5

[a]Given are the data of the major tetrasaccharide repeating units.

The α configuration of ribofuranose was deduced from the C-1 chemical shift of δ 104.4 (C-1 of β-ribofuranose would resonate near δ 110[24]).

The glycosylation pattern of Man and Rha was established by the downfield displacement of the signals for the linkage carbons by 6–11 ppm, as compared with their positions in the corresponding nonsubstituted monosaccharides.[22,23] The terminal position of Rib was confirmed by the C-2–C-5 chemical shifts, which were close to those in the nonsubstituted α-ribofuranose.[24] The NOESY spectrum of PS-2 showed interresidue correlations between the following anomeric protons and protons at the linkage carbon atoms: Rha H-1,Man^{I} H-3; Man^{I} H-1,Man^{II} H-4; Man^{II} H-1,Rha H-4; Rib H-1, Man^{I} H-4. These data, in combination with the methylation analysis and ^{13}C NMR chemical shift data, defined the monosaccharide sequence in the repeating unit. Therefore, the O-polysaccharide of *C. youngae* PCM 1492 from serogroup O1 has the following structure:

α-D-Rib*f*
1
↓
4
→4)-α-D-Rha*p*-(1→3)-β-D-Man*p*^{I}-(1→4)-β-D-Man*p*^{II}-(1→

where substitution with the α-D-Rib*f* group is nonstoichiometric.

Like the majority of the other *Citrobacter* O-antigens, the O-polysaccharide of *C. youngae* PCM 1492 is neutral. It contains α-D-ribofuranose, which, to the best of our knowledge, has been hitherto found only once in bacterial polysaccharides.[26] As opposite to the more common β-ribofuranosyl group, the α-ribofuranosyl group is easily cleaved under mild acidic conditions to give a linear main-chain polysaccharide.

Keleti et al. ascribed serotype O1a,1b,1c to chemotype VIII, which is characterised by the presence of the common sugars, galactosamine and rhamnose,[17] whereas ribose was not taken into account in the chemotyping, being considered as a constituent of nucleic acids rather than the lipopolysaccharide. In contrast, galactosamine is not included in the O-polysaccharide and may derive from the core of the lipopolysaccharide, whose structure remains to be established. The O-polysaccharide studied and those of some other *Citrobacter* strains include D-rhamnose, which is widespread in O-antigens of phytopathogenic bacteria, such as *Pseudomonas syringae* and *Xanthomonas campestris*, but is uncommon in enterobacterial lipopolysaccharides beyond the genus *Citrobacter*.[27,28]

In immunoblotting, the lipopolysaccharide of three serogroup O1 strains, *C. youngae* PCM 1492 (Be 1/50,

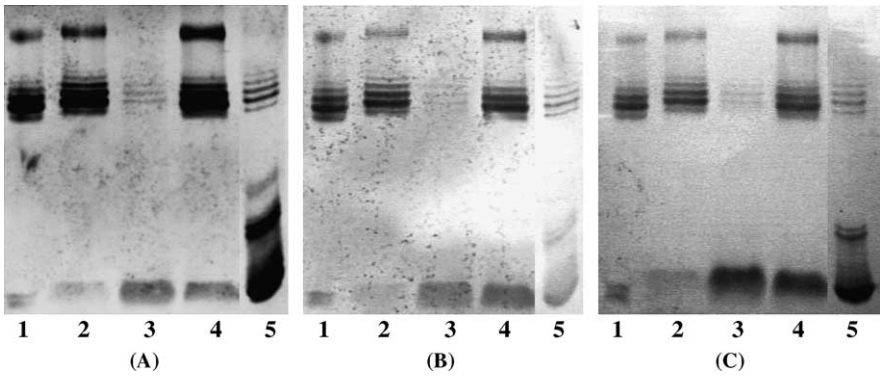

Figure 2. SDS-PAGE electrophoresis (A) and immunoblotting with *C. youngae* PCM 1492 (B) and *C. youngae* PCM 1506 (C) antisera of the lipopolysaccharides from *C. youngae* PCM 1492 (lane 1) and PCM 1493 (lane 5) isolated by the phenol–water method and of the lipopolysaccharides from *C. youngae* PCM 1506 (lane 2), PCM 1493 (lane 3) and PCM 1492 (lane 4) extracted by the proteinase K method.

Na1A), PCM 1493 (Be 2/50, Ala 20) and PCM 1506 (Be 33/57), reacted with anti-*C. youngae* PCM 1492 and anti-*C. youngae* PCM 1506 sera (Fig. 2). In the case of *C. youngae* PCM 1493, staining of the long-chain S-type LPS species was faint. This could be accounted by low proportion and poor extraction of these species by the proteinase K method compared to the other strains under the same experimental conditions and to LPS 1493 extracted by the phenol–water method. The immunoblotting data suggest that all these strains have the O-antigen of the same structure and, hence, *Citrobacter* serogroup O1 is serologically homogenous.

3. Experimental

3.1. Miscellaneous methods

Immunoblotting[29] was carried out with anti-*C. youngae* PCM 1492 and anti-*C. youngae* PCM 1506 sera obtained as described.[30] GPC was carried out on columns of Sephadex G50 (60 × 2.6 cm) in pyridinium acetate buffer pH 4.5 or a column (80 × 1.6 cm) of TSK HW-40 in aq 1% AcOH and monitored using a Knauer differential refractometer (Germany). GLC–MS was performed with a Hewlett-Packard 5971A instrument equipped with an HP-1 glass capillary column (12 m × 0.2 mm) using a temperature program of 150–270 °C at 8 °C min^{-1}.

3.2. Bacterial strains and isolation of the lipopolysaccharide

Strains of *C. youngae* O1a,1b,1c:1,2 (*C. freundii* PCM 1506, IHE Be 33/57, P. R. Edwards strain, 'Na1A'), *C. youngae* O1a,1b,1c:1 (*C. freundii* PCM 1492, IHE Be 1/50, CIP 55.13, M. W. Wright strain 'Na1A') and *C. youngae* O1:8,9 (*C. freundii* PCM 1493, IHE Be 2/50, M. W. Wright strain 'Ala 20')[8,9,17] were obtained from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Strains PCM 1492 and PCM 1493 were cultivated in a liquid medium[31] and the lipopolysaccharides of these strains were isolated by phenol–water extraction[32,33] in a yield of 9.3% and 5.1% of dried cells weight, respectively. For immunoblotting, the lipopolysaccharides of strains PCM 1492, PCM 1493 and PCM 1506 were isolated by the proteinase K-treatment method.[34]

3.3. Mild acid and alkaline degradations of the lipopolysaccharide

The lipopolysaccharide of strain PCM 1492 (185 mg) was hydrolysed with 1% AcOH (100 °C, 1 h), and the carbohydrate portion was fractionated by GPC on a column (60 × 2.5 cm) of Sephadex G50 in 0.05 M pyridinium acetate buffer pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water) and the elution was monitored using a Knauer differential refractometer. A high-molecular-mass polysaccharide (PS-1) was obtained in a yield of 45% of the lipopolysaccharide weight.

The lipopolysaccharide (100 mg) was treated with 0.16 M NaOH (100 °C, 1 h) and a high-molecular-mass polysaccharide (PS-2, 61% mg) was isolated by GPC on Sephadex G50.

PS-1 (22 mg) was treated with aq 48% HF (4 °C, 48 h), the reagent was flushed out using a trap with solid NaOH for absorption and a modified polysaccharide (PS-3, 45% yield) was isolated by GPC on a column (90 × 2.5 cm) of TSK HW-40 in aq 1% AcOH.

3.4. Sugar analysis

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), monosaccharides were analysed using a Technicon sugar analyser on a column (15 × 0.4 cm) in 0.5 M sodium borate buffer pH 7.7 at 65 °C. The abso-

lute configurations of Rha, Man and Rib were determined by GLC of the acetylated glycosides with (+)-octan-2-ol as described[35] on a Hewlett-Packard 5880 instrument with a DB-5 capillary column using a temperature gradient of 160 °C (3 min)–250 °C at 10 °C min^{-1}.

3.5. Methylation analysis

Methylation of the polysaccharide was carried out with methyl iodide in dimethyl sulfoxide in the presence of solid base according to Gunnarsson,[36] and methylated products were hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Partially methylated monosaccharides derived were converted into the alditol acetates and analysed by GLC–MS on a Hewlett-Packard 5890 chromatograph equipped with a DB5 fused-silica capillary column and combined with a NERMAG R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min)–250 °C at 3 °C min^{-1}.

3.6. NMR spectroscopy

Prior to measurements, the samples were lyophilised twice from D_2O. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX500 spectrometer for solutions in D_2O at 50 °C. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45 ppm) as reference. A mixing time of 150 and 200 ms was used in TOCSY and ROESY experiments, respectively.

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