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Note

Structures of two O-polysaccharides of the lipopolysaccharide of *Citrobacter youngae* PCM 1538 (serogroup O9)

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Abstract—Mild acid degradation of the lipopolysaccharide of *Citrobacter youngae* O9, strain PCM 1538 released a homopolysaccharide of 4-acetamido-4,6-dideoxy-D-mannose (D-Rha4NAc, *N*-acetyl-D-perosamine). Studies by methylation analysis and ^{1}H and ^{13}C NMR spectroscopy, using two-dimensional $^{1}H,^{1}H$ COSY, TOCSY, NOESY and H-detected $^{1}H,^{13}C$ HSQC experiments showed the presence of two structurally different polysaccharides consisting of the following units:

→ 2)-α-D-Rha*p*4NAc-(1 → and → 3)-α-D-Rha*p*4NAc-(1 → 3)-β-D-Rha*p*4NAc-(1 →

Keywords: *Citrobacter youngae*; O-Antigen; Lipopolysaccharide; Polysaccharide structure; 4-Acetamido-4,6-dideoxy-D-mannose

Bacteria of the genus *Citrobacter* are inhabitants of the intestinal tract and are present in sewage, surface waters and in food contaminated with faecal material. In the immunocompromised hosts they may cause outbreaks of febrile gastro-enteritis, opportunistic infections, including urinary and respiratory tract infections.[1] *Citrobacter* strains are serologically heterogeneous and are classified into more than 40 O-serogroups based on the structures of the O-polysaccharide chains (O-antigens) of the lipopolysaccharides (LPSs).[2] Structural and immunochemical studies of the O-polysaccharides of *Citrobacter* aim at creation of the molecular basis for classification of *Citrobacter* strains and substantiation of their serological cross-reactivity with other bacteria.[3]

Citrobacter serogroup O9 includes two strains, *Citrobacter gillenii* PCM 1537 and *Citrobacter youngae* PCM 1538. Recently, we have studied the O-antigen of *C. gillenii* O9, strain PCM 1537 and found it to consist of two structurally different polysaccharides, both being homopolymers of 4-acetamido-4,6-dideoxy-D-mannose (D-Rha4NAc).[4] It was reasonable to suggest that the O-antigen of the serologically related strain, *C. youngae* PCM 1538, has a polysaccharide (or polysaccharides) that is identical or very similar to those of *C. gillenii* PCM 1537. In this work we studied the O-antigen of *C. youngae* PCM 1538 and showed that its LPS has also two O-polysaccharides, one of which is identical to one of the polysaccharides of *C. gillenii* PCM 1537 and the other is different.

A high-molecular-mass polysaccharide (PS) was isolated by mild acid degradation of the LPS of *C. youngae* PCM 1538 followed by fractionation of the carbohydrate portion by GPC on TSK HW-60S. Sugar analysis of the PS revealed a 4-amino-4,6-dideoxyhexose as the single monosaccharide constituent, which was identified as 4-amino-4,6-dideoxy-D-mannose (D-Rha4N, D-perosamine) using GLC of the alditol

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acetates and acetylated (*S*)-2-butyl glycosides in comparison with the corresponding authentic samples from the LPS of *C. gillenii* PCM 1537.[4] In addition, the N-acetylated derivative of Rha4N was obtained by acid hydrolysis of the PS followed by N-acetylation and characterized by ^{1}H and ^{13}C NMR spectra (Tables 1 and 2).

Methylation analysis of the PS revealed the presence of 4,6-dideoxy-3-*O*-methyl-4-(*N*-methyl)acetamidomannose and 4,6-dideoxy-2-*O*-methyl-4-(*N*-methyl)acetamidomannose in the ratio ~1:2.8, which were identified by GLC–MS of the partially methylated alditol acetates. The electron impact mass spectra of both compounds were identical to those reported previously.[4]

These data indicate that the PS is linear and contains 2-substituted and 3-substituted residues of D-perosamine. Further studies indicated that the PS includes two polysaccharides with the same sugar composition but different structures.

The ^{13}C NMR spectrum of the PS (Fig. 1) contained signals for three different Rha4NAc residues, including signals for anomeric carbons (C-1) at δ 98.1–103.3, nitrogen-bearing carbons (C-4) at δ 52.3–54.3, CH_3–C groups (C-6) at δ 18.0 and *N*-acetyl groups at δ 23.3–23.7 (CH_3) and 175.7–176.0 (CO). Accordingly, the ^{1}H NMR spectrum of the PS contained, inter alia, major signals for anomeric protons (H-1) at δ 4.63–5.13, CH_3–C groups (H-6) at δ 1.16–1.24 and *N*-acetyl groups at δ 2.04. The spectrum contained also a number of minor signals, which could belong to terminal monosaccharides of the polysaccharide chain or/and to components of the LPS core.

The 2D COSY and TOCSY spectra of the PS revealed spin systems for three different Rha4NAc residues, which were designated as units **A**–**C** in order of decreasing chemical shifts. The COSY spectrum allowed differentiation between protons within each spin system (Table 1). The ^{13}C NMR spectrum of the PS (Table 2) was assigned using an H-detected $^{1}H,^{13}C$ HSQC experiment. A relatively low-field position at δ 75.8–80.7 of the signals for C-3 of units **B** and **C** and C-2 of unit **A** demonstrated the modes of substitution of the monosaccharides (compare the position at δ 69.4–72.3 of the signals for nonlinked C-2 and C-3 in the free monosaccharide, Table 2).

In the NOESY spectrum of the PS, no interresidue cross-peak was observed for unit **A** but intraresidue H-1,H-2 and H-1,H-5 cross-peaks at δ 5.13/4.12 and 5.13/3.81, respectively (Fig. 2). The latter is typical of α-(1→2)-linked monosaccharides having the *manno* configuration,[5] including Rha4NAc. Hence, residues **A** are α-(1→2)-linked and thus form a homopolymer PS1 (Fig. 3). The NOESY spectrum showed an intraresidue **B** H-1,H-2 cross-peak at δ 4.93/3.93 and an interresidue

Table 1. ^{1}H NMR data (δ, ppm; *J*, Hz)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
PS1						
→2)-α-D-Rha*p*4NAc-(1→ (**A**)	5.13	4.12	4.03	3.88	3.81	1.16
PS2						
→3)-α-D-Rha*p*4NAc-(1→ (**B**)	4.93	3.93	4.07	3.91	3.91	1.19
→3)-β-D-Rha*p*4NAc-(1→ (**C**)	4.63	4.02	3.68	3.84	3.47	1.24
Monosaccharide						
α-D-Rha*p*4NAc	5.16	3.93	3.88	3.83	3.93	1.18
	$J_{1,2}$ 1.5	$J_{2,3}$ 2.8	$J_{3,4}$ 9.8	$J_{4,5}$ 9.8	$J_{5,6}$ 6.1	
β-D-Rha*p*4NAc	4.84	3.95	3.68	3.75	3.48	1.21
	$J_{1,2} \leqslant 1$	$J_{2,3}$ 3.0	$J_{3,4}$ 10.8	$J_{4,5}$ 10.8	$J_{5,6}$ 6.2	

An additional chemical shift for the *N*-acetyl groups in the PS and the monosaccharide is δ 2.04.

Table 2. ^{13}C NMR data (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
PS1						
→2)-α-D-Rha*p*4NAc-(1→ (**A**)	101.7	78.2	69.0	54.3	69.7	18.0
PS2						
→3)-α-D-Rha*p*4NAc-(1→ (**B**)	103.3	68.2	75.8	52.3	69.5	18.0
→3)-β-D-Rha*p*4NAc-(1→ (**C**)	98.1	71.3	80.7	53.1	71.9	18.0
Monosaccharide						
α-D-Rha*p*4NAc	95.2	71.1	69.4	54.4	68.6	18.2
β-D-Rha*p*4NAc	94.8	71.8	72.3	54.1	72.4	18.1

Additional chemical shifts for the *N*-acetyl groups in the PS are δ 23.3–23.7 (CH_3) and 175.7–176.0 (CO) and in the monosaccharide δ 23.5 (CH_3) and 176.0 and 176.1 (CO of the α- and β-anomers, respectively).

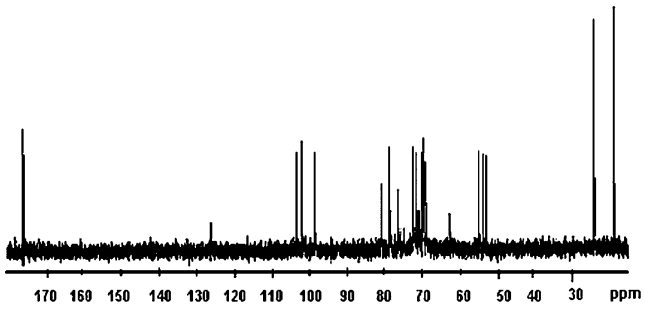

Figure 1. ^{13}C NMR spectrum of the polysaccharide of. *C. youngae* PCM 1538.

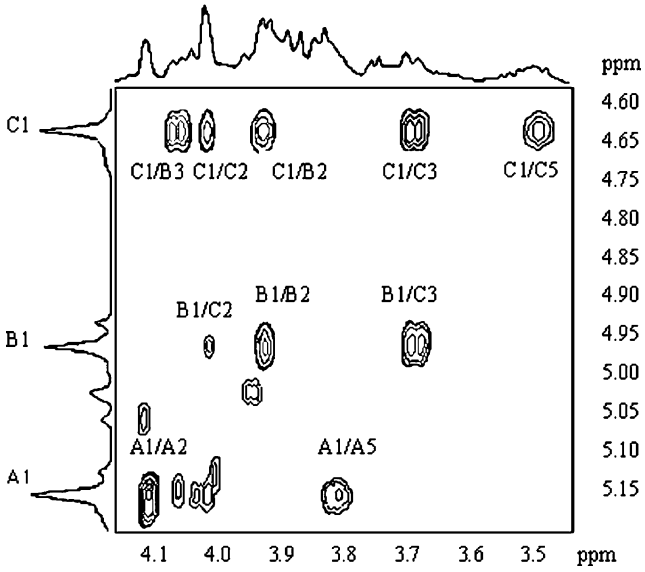

Figure 2. Part of a NOESY spectrum of the polysaccharide of *C. youngae* PCM 1538. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. Arabic numerals refer to protons in sugar residues denoted as shown in Table 1.

B H-1, **C** H-3 cross-peak at δ 4.93/3.68, thus indicating an α-(1 → 3) linkage between units **B** and **C**. At the **C** H-1 coordinate (δ 4.63), there were intraresidue cross-peaks with H-2, H-3 and H-5 at δ 4.02, 3.68 and 3.47, and interresidue cross-peaks with H-2 and H-3 of unit **B** at δ 3.93 and 4.07, respectively. These data, along with the ^{13}C NMR chemical shifts and methylation analysis data (see above), indicated a β-(1 → 3) linkage between units **C** and **B**. Therefore, another homopolymer of Rha4NAc with a →3)-α-D-Rha*p*4NAc-(1 → 3)-β-D-Rha*p*4NAc-(1 → disaccharide repeating unit was identified (PS2, Fig. 3).

In conclusion, the O-antigen of *C. youngae* PCM 1538 consists of two structurally different polysaccharides. One of them (PS1) is an α-(1 → 2)-linked homopolymer of Rha4NAc, which has been earlier identified in *C. gillenii* PCM 1537 from the same *Citrobacter* serogroup O9 (Fig. 3).[4] The other polysaccharide (PS2) is composed of regularly alternating α-(1 → 3)- and β-(1 → 3)-linked Rha4NAc residues and is a new homopolymer of this monosaccharide. These data and results of serological studies, which will be published elsewhere, show the expediency of the division of serogroup O9 into two subgroups. It remains unknown whether in *C. youngae* PCM 1538 the different Rha4NAc polymers build up two separate polysaccharides, as in *C. gillenii* PCM 1537,[4] or blocks within the same polysaccharide chain. An attempt to separate the PS to PS1 and PS2 will be made to solve this problem.

1. Experimental

1.1. Bacterial strain, isolation and degradation of LPS

Citrobacter gillenii O9 (strain PCM 1538) was obtained from the collection of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The LPS was isolated by phenol–water extraction and purified by ultracentrifugation.[6] The yield of the LPS was 4.8% of dry bacterial mass.

A portion of the LPS (150 mg) was heated with 2% acetic acid (4.5 mL) for 1.6 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column of TSK HW-60S (1.6 × 90 cm) in aq pyridinium

C. youngae PCM 1538 (this work)

→2)-α-D-Rha*p*4NAc-(1→ PS1
A

→3)-α-D-Rha*p*4NAc-(1→3)-β-D-Rha*p*4NAc-(1→ PS2
B **C**

C. gillenii PCM 1537 (Ref. 4)

→ 2)-α-D-Rha*p*4NAc-(1→

→3)-α-D-Rha*p*4NAc-(1→2)-α-D-Rha*p*4NAc-(1→2)-α-D-Rha*p*4NAc-(1→3)-α-D-Rha*p*4NAc2Ac-(1→

Figure 3. Structures of the O-polysaccharides of *Citrobacter* O9.

acetate buffer (4 mL pyridine and 10 mL HOAc in 1 L water) with monitoring using a Knauer differential refractometer. The yield of the PS was 36% of the LPS weight.

1.2. Chemical methods

For sugar analysis, the PS (0.5 mg) was hydrolysed with 1 M HCl for 30 min at 80 °C, reduced with $NaBH_4$, peracetylated, and the alditol acetates derived were analysed by GLC on a Hewlett-Packard 5880 chromatograph (USA) equipped with a capillary column of HP-1 stationary phase, using a temperature program of 180–290 °C at 10 °C min^{-1}. For determination of the absolute configuration,[7] the LPS (0.5 mg) was subjected to 2-butanolysis [300 μL (*S*)-2-butanol and 20 μL AcCl, 100 °C, 3 h], the products were acetylated and analysed by GLC as above.

For isolation of Rha4NAc, the PS (7 mg) was hydrolysed with 10 M HCl for 30 min at 80 °C, after evaporation the product was decolourised with charcoal in water, N-acetylated with acetanhydride in satd aq $NaHCO_3$ (0 °C, 1 h), deionised with an IRA-120 (H^+-form) cation-exchange resin, evaporated and the residue coevaporated with MeOH three times.

Methylation of the PS (0.5 mg) was performed according to the Hakomori procedure,[8] the products were recovered using a Sep-Pak cartridge, hydrolysed with 10 M HCl for 30 min at 80 °C, and the partially methylated alditol acetates derived were analysed by GLC–MS using a Hewlett-Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and temperature program of 150–270 °C at 8 °C min^{-1}.

1.3. NMR spectroscopy

A solution of a PS sample in D_2O was freeze-dried twice and the sample was dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded using a Bruker DRX-500 spectrometer at 50 °C. 2D NMR experiments were performed using standard Bruker software. Mixing time of 150 and 200 ms was used in TOCSY and NOESY experiments, respectively.

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