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Note

The structure of the *Citrobacter freundii* O8a,8b O-specific polysaccharide containing D-xylofuranose

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Strains of a serologically heterogeneous enterobacterial species *Citrobacter freundii* are subdivided into at least 42 O-serogroups; a few of them, including serogroup O8, are complex. We continue our study of *Citrobacter* O-antigens (Ref [1] and references therein) and now report the structure of the O-specific polysaccharide chain of *C. freundii* subgroup O8a,8b lipopolysaccharide.

Lipopolysaccharide was isolated from dried bacterial cells by extraction with aqueous phenol [2] and degraded with dilute acetic acid. After removal of a lipid precipitate, the water-soluble portion was separated by gel chromatography on Sephadex G-50 to give the O-specific polysaccharide.

Hydrolysis of the polysaccharide with 2 M CF_3CO_2H revealed rhamnose and xylose identified by GLC as alditol acetates in the ratio 3.6:1. The monosaccharides were separated by anion-exchange chromatography in borate buffer, and on the basis of optical rotation, it was concluded that both rhamnose and xylose are D.

The ^{13}C NMR spectrum of the polysaccharide contained signals for four anomeric carbons at 98.0, 101.5, 102.7, and 103.4 ppm, three methyl groups of 6-deoxy sugars (C-6 of rhamnose) at 17.7, 18.0, and 18.2 ppm, one hydroxymethyl group (C-5 of xylose) at 61.7 ppm, and other sugar carbons in the region 68.5–79.6 ppm. The ^1H NMR spectrum of the polysaccharide (Fig. 1a) contained signals for four anomeric protons at 4.80, 5.14, 5.25

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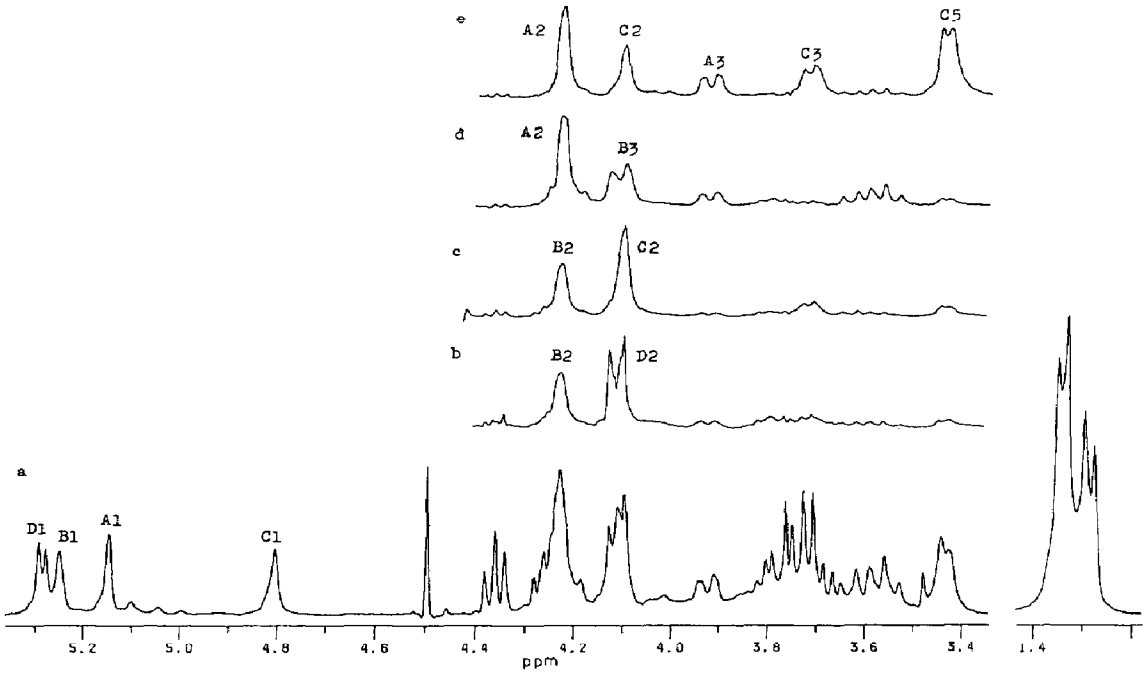


Fig. 1. 250-MHz spectrum of the O-specific polysaccharide of *C. freundii* O8a,8b (a) and NOE spectra with pre-irradiation of H-1 of (b) α-Xyl*f* (unit **D**), (c) α-Rha*p* (unit **B**), (d) α-Rha*p* (unit **A**), (e) β-Rha*p* (unit **C**). Arabic numerals refer to the protons in the sugar residues denoted by the letters.

(all broadened singlet-like signals), and 5.28 ppm (d, $J_{1,2}$ 4.5 Hz), three methyl groups of 6-deoxy sugars (H-6 of rhamnose) at 1.28 (d, $J_{5,6}$ 6 Hz) and 1.34 ppm (superposition of two doublets), and other signals in the region 3.4–4.4 ppm.

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing three residues of D-rhamnose (units **A**–**C**) and one residue of D-xylose (unit **D**).

Methylation analysis of the polysaccharide resulted in identification of 2,3,5-tri-*O*-methylxylose, 2,4-di-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 4-*O*-methylrhamnose identified by GLC as alditol acetates in almost equal amounts. These data showed that the polysaccharide is branched, a xylofuranose residue is the terminal sugar of the side chain, and all residues of rhamnose are pyranosidic, one of them being at the branching point.

The ^{1}H NMR spectrum of the polysaccharide (Fig. 1a) was completely assigned using 2D shift-correlated spectroscopy (COSY, Table 1). The coupling constants determined from this spectrum showed that all rhamnose residues are pyranoses and the xylose residue is an α-linked furanose (cf. the published data [3]).

The following interresidue proton contacts were revealed from the NOE spectra with selective, sequential pre-irradiation of anomeric protons (Figs. 1b–e): H-1 **D**–H-2 **A** or H-2 **B**, H-1 **B**–H-2 **C**, H-1 **A**–H-3 **B**, and H-1 **C**–H-2 **A** and H-3 **A**. Similar results were obtained when rotating-frame NOE spectroscopy (ROESY) was applied. These data showed that xylofuranose (unit **D**) is attached as a monosaccharide side chain and three rhamnose residues are in the main chain and form the fragment **A**-(1→3)-**B**-(1→2)-**C**-(1→. It remains unknown whether unit **C** is linked to unit **A** at position 2 or 3 and whether unit **A** or **B**, having the same position of resonance of H-2, is the site of attachment of the side chain (unit **D**).

Table 1
^{1}H NMR data for the O-specific polysaccharide of *C. freundii* O8a,8b (δ in ppm, J in Hz)

	H-1	H-2	H-3	H-4	H-5	H-6
→3)-α-D-Rha*p*-(1→ (unit **A**)						
δ	5.14	4.23	3.92	3.56	3.79	1.34
J	$J_{1,2}$ ≤2	$J_{2,3}$ 2.7	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6}$ 5.6	
→3)-α-D-Rha*p*-(1→ (unit **B**) 2↑						
δ	5.25	4.23	4.11	3.61	4.21	1.28
J	$J_{1,2}$ ≤2	$J_{2,3}$ ~3	$J_{3,4}$ ~10	$J_{4,5}$ ~10	$J_{5,6}$ 6	
→2)-β-D-Rha*p*-(1→ (unit **C**)						
δ	4.80	4.11	3.71	3.44	3.43	1.34
J	$J_{1,2}$ ≤2	$J_{2,3}$ ~3	$J_{3,4}$ ~10			
α-D-Xyl*f*-(1→ (unit **D**) [a]						
δ	5.28	4.11	4.36	4.25	3.77 (H-5a) 3.69 (H-5b)	
J	$J_{1,2}$ 4.5 (4.4)	$J_{2,3}$ ~6 (5.5)	$J_{3,4}$ ~6 (5.8)	$J_{4,5a}$ 3.6 (3.5)	$J_{4,5b}$ 6.1 (5.7)	$J_{5a,5b}$ 12.3 (12.1)

[a] Data from Ref. [3] are given in parentheses.

With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of the polysaccharide was assigned using heteronuclear ^{13}C,^{1}H shift-correlated spectroscopy (Table 2).

The values of the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants determined from the gated-decoupling ^{13}C NMR spectrum of the polysaccharide showed [4] that units **A** and **B** are α-linked ($^1J_{C\text{-}1,H\text{-}1}$ 171 and 174 Hz, respectively) and unit **C** is β-linked ($^1J_{C\text{-}1,H\text{-}1}$ 157 Hz).

As judged by the chemical shift 78.5 ppm for C-3 of unit **A**, this unit is substituted by unit **C** at position 3, in agreement with the methylation data (see above). That pre-irradiation of H-1 of unit **C** causes NOE's on H-2 and H-3 of unit **A** is typical of β-(1→3)-linked D-rhamnose disaccharides, in which H-1 of the glycosylating sugar is in close proximity to both H-2 and H-3 of the glycosylated sugar [5].

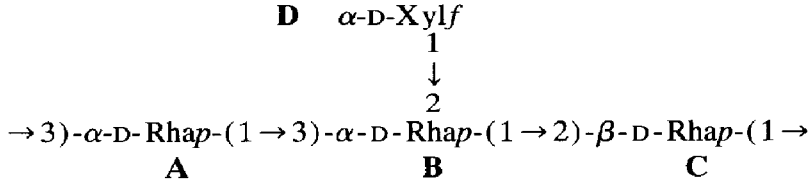
Table 2
^{13}C NMR chemical shifts for the O-specific polysaccharide of *C. freundii* O8a,8b (δ in ppm)

Unit	C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-D-Rha*p*-(1→ (unit **A**)	102.7	68.5	78.5	71.6	70.6	18.2 [a]
→3)-α-D-Rha*p*-(1→ (unit **B**) 2↑	101.5	78.5	77.6	73.3	70.2	18.0 [a]
→2)-β-D-Rha*p*-(1→ (unit **C**)	98.0	78.8	74.5	73.8	73.6	17.7 [a]
α-D-Xyl*f*-(1→ (unit **D**)	103.4	78.8	76.2	79.6	61.7	

[a] Assignment could be interchanged.

The position of the signal for C-2 of the 2-unsubstituted rhamnose residue at 68.5 ppm indicated that it belongs to unit **A** and, hence, the side chain is attached to unit **B**. In fact, if the branching point is unit **A**, C-2 of 2-unsubstituted unit **B** would resonate near 71.5 ppm [6] (this difference is caused by a strong spatial contact between H-1′ and H-2 in β-(1→3)-linked D-rhamnose disaccharides, like **C**→**A**, and the absence of such contact in α-(1→3)-linked disaccharides, like **A**→**B**) [6,7].

Therefore, on the basis of these data, the following structure of the *C. freundii* O8a,8b O-specific polysaccharide was established:



Xylose has been reported [8] to be a component of three strains of *Citrobacter* O-antigens including O8a,8b. To the best of our knowledge, xylofuranose is found for the first time in bacterial polysaccharides.

1. Experimental

General methods.—Gel-permeation chromatography was performed on a column (70×3 cm) of Sephadex G-50 in pyridine–acetate buffer (pH 5.5) and monitored with a Knauer differential refractometer. Anion-exchange chromatography was performed on a column (20×0.6 cm) of Durrum DA×4 in 0.5 M sodium borate buffer (pH 9.0) at 55°C and monitored by the orcinol–H_2SO_4 reaction. GLC was carried out using a Hewlett–Packard 5890 instrument equipped with a glass capillary column (25 m×0.2 mm) of Ultra 1 stationary phase. GLC–MS was performed with a Varian MAT 311 instrument.

The ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-300 spectrometer in D_20 at 60°C using acetone as internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). Standard Bruker software was used for running two-dimensional spectra. Optical rotations were measured with a Jasco DIP 360 polarimeter at 25°C in water.

C. freundii O8a,8b, strain 64/57 was obtained from the National Collection of Reference Strains (Institute of Hygiene, Prague). Growth of bacteria [1], isolation of lipopolysaccharide [2] and O-specific polysaccharide [1], $[\alpha]_D$ +72.2° (*c* 1), were performed as described.

For sugar analysis, the polysaccharide (1 mg) was hydrolyzed with 2 M CF_3CO_2H for 1 h at 120°C, sugars were conventionally converted into alditol acetates and analyzed by GLC. For preparing monosaccharides, the polysaccharide (25 mg) was hydrolyzed as above, D-rhamnose, $[\alpha]_D$ −7.2° (*c* 1.3), cf. data [8] $[\alpha]_D$ −6.13° (H_2O), and D-xylose, $[\alpha]_D$ +13.5° (*c* 0.43), cf. data [9] $[\alpha]_D$ +18.8° (H_2O), were isolated by anion-exchange chromatography.

Methylation analysis of the polysaccharide (2 mg) was performed by a published method [10].

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