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Note

Determination of the O-specific polysaccharide structure in the lipopolysaccharide of *Ochrobactrum anthropi* LMG 3331

Julián Velasco [a,b], Hermann Moll [b], Evgeny V. Vinogradov [b], Ignacio Moriyón [a], Ulrich Zähringer [b,*]

[a] *Dpto. Microbiología, Universidad de Navarra, 31080, Pamplona, Spain*

[b] *Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, D-23845 Borstel, Germany*

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Ochrobactrum anthropi is a recently defined species [1] formerly included in the heterogeneous CDC group Vd. This organism has been isolated with increasing frequency in blood cultures from immunocompromised patients and from environmental and hospital water sources [2]. More recently, *O. anthropi* has been described as an endo-symbiont of nematodes [3], suggesting that the original habitat could be the soil, and this could explain the unpredictable multiple antibiotic resistance of the isolates [4]. On the basis of the 16S rRNA sequence, *O. anthropi* has been included in the α-2 subgroup of the class *Proteobacteria*, showing the greatest relatedness with important pathogens like *Brucella* and the *Rochalimaea–Bartonella* group [5]. Since the cell-surface lipopolysaccharides (LPSs) are of critical importance in pathogenicity [6] a comparison of the LPSs of *Brucella* and *O. anthropi* could contribute to a better understanding of the biology of these bacteria. The LPS of *Brucella* has been partially characterised [7] but there are no data on the LPS of *O. anthropi*. Here we report the structure of the O-chain polysaccharide of the LPS of *O. anthropi* LMG 3331 (type strain). It was found to consist of a hitherto unknown, linear, repeating disaccharide unit of the following structure: $\rightarrow$ 3)-α-D-Glc*p*NAc-(1 $\rightarrow$ 2)-α-L-Rha*p*-(1 $\rightarrow$.

The polysaccharide (PS) was isolated from the LPS after acid hydrolysis and

* Corresponding author.

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Table 1
^{13}C NMR data of the O-specific chain isolated from lipopolysaccharide of *O. anthropi* LMG 3331 (δ in ppm, 90.6 MHz, 323 K, D_2O, relative to acetone δ 31.45)

Residue	C-1	C-2	C-3	C-4	C-5	C-6	*Me*CO	Me*C*O
→ 3)-α-D-Glc*p*NAc-(1 →	97.0	54.7	80.9	69.9	73.6	62.0	23.8	175.6
→ 2)-α-L-Rha*p*-(1 →	99.2	77.2	71.0	73.8	70.8	18.3		

subsequent gel permeation chromatography. Sugar analysis of the PS was done by GLC of the alditol acetates [hydrolysis (0.1 M HCl, 48 h, 100 °C), reduction ($NaBH_4$), and peracetylation (Ac_2O–pyridine)]. Besides smaller amounts of core sugar components (data not shown), rhamnitol and glucosaminitol were identified as the main sugar components of the PS. Methylation analysis [methylation (CH_3I), hydrolysis (4 M trifluoroacetic acid, 100 °C, 4 h), reduction ($NaBH_4$), and peracetylation] revealed the presence of 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol and 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol, indicating a linear polymer consisting of two pyranoid sugars, that is, a 3-substituted Glc*p*NAc and a 2-substituted Rha*p*. In addition, small amounts (< 10%) of 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylrhamnitol indicated heterogeneity with some substitution of the Rha residue by GlcNAc at positions 3 and 4, respectively. Besides the methylated monosaccharide-alditols, disaccharide derivatives were also obtained, among which 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucopyranosyl-(1 → 2)-1,5-di-*O*-acetyl-3,4-di-*O*-methylrhamnitol predominated (> 90%). The substitution pattern of the other disaccharides could not be clearly assigned because of the partial degradation of methylated rhamnitol observed under the hydrolytic conditions tested (2 to 8 M trifluoroacetic acid, 1 to 4 h at 100 °C). Therefore, the accurate establishment of the heterogeneous substitution in the rhamnose sugar residue could not be achieved by these experiments.

The ^{13}C NMR spectrum of the polysaccharide contained a major series of 12 signals and many small signals (< 10% by intensity) further supporting the above-mentioned heterogeneity of the disaccharide substitution pattern. The major signals were assigned using 2D COSY and ^{13}C,^1H COSY (Tables 1 and 2), and, based on the vicinal proton coupling constant values, corresponded to Rha and GlcNAc residues [8]. The ^1H NMR

Table 2
^1H NMR data of the O-specific chain isolated from lipopolysaccharide of *O. anthropi* LMG 3331 (δ in ppm, *J* in Hz, 360 MHz, 323 K, D_2O, relative to acetone δ 2.23)

Residue	H-1	H-2	H-3	H-4	H-5	H-6	MeCO
→ 3)-α-D-Glc*p*NAc-(1 →	4.85	4.07	3.83	3.60	4.00	3.81	2.06
	$J_{1,2}$ 3.5	$J_{2,3}$ 10.3	$J_{3,4}$ 3.5	$J_{4,5}$ 9.4	[a]	[a]	
→ 2)-α-L-Rha*p*-(1 →	4.96	3.82	3.86	3.55	4.00	1.27	
	$J_{1,2}$ < 1	[a]	$J_{3,4}$ 9.4	$J_{4,5}$ 9.4	$J_{4,5}$ 3.8		

[a] Non-resolved multiplet.

spectrum contained two signals of anomeric protons at δ 4.85 and 4.96 ($J_{1,2}$ 3.5 and < 1 Hz) assigned to H-1 of GlcNAc and Rha, respectively, thus indicating GlcNAc to be α-linked. Other characteristic signals of the polysaccharide were the *N*-acetyl group signal at δ 2.06, a signal of the methyl group of 6-deoxyhexose at δ 1.27, and the signals of sugar ring protons at δ 3.5–4.3. Analysis of the ^{13}C NMR spectrum using a computer data base search [9] was consistent with the following structure for the repeating unit of the *O. anthropi* polysaccharide: → 3)-α-D-Glc*p*NAc-(1 → 2)-α-L-Rha*p*-(1 → . In particular, this ^{13}C NMR data base analysis revealed that Rha and GlcNAc had the opposite absolute configuration. GLC analysis of the acetylated (*R*)-2-butyl and (*R*)-2-octyl glycosides showed the absolute configuration to be L for Rha and D for GlcNAc, thus finally confirming the above structure. The 2D COSY and $^{13}C,^{1}H$ COSY spectra of the polysaccharide contained peaks corresponding to low-intensity signals in the 1D ^{1}H NMR spectrum. The observed positions of these signals and the results of methylation analysis suggested the presence of small amounts of two more different types of linkage in the polymer, which were not assigned completely.

1. Experimental

Bacterial strains and LPS.—*O. anthropi* LMG 3331 was grown in tripticase soy broth supplemented with 3% of D-glucose at 37 °C. The culture medium was removed by tangential flow filtration and cells washed with phosphate-buffered saline (pH 7.2). The LPS was obtained from the water phase of a phenol–water extract as described by Leong et al. [10], and digested with nucleases and proteinase K [11]. Phospholipids were removed by a four-fold extraction with $CHCl_3$–MeOH (2:1 v/v). LPS (505 mg) was hydrolysed with 2% AcOH (100 °C, 3 h), and the soluble material was freeze-dried (yield, 380 mg) and subjected to gel permeation chromatography. The high-molecular-weight fraction contained the O-specific polysaccharide (final yield, 140 mg).

Gel chromatography.—GPC was performed on a column (45 cm × 2.4 cm) of Sephadex G-50 (Pharmacia), using water–pyridine–AcOH (1000:4:10, by vol, pH 4.5). The eluent was monitored using a Knauer differential refractometer.

Methylation analysis.—The polysaccharide was methylated [12], hydrolysed with 4 M CF_3CO_2H at 100 °C for 4 h, reduced ($NaBH_4$), and acetylated with Ac_2O (85 °C, 20 min) followed by Ac_2O–pyridine (1:2, by vol) (85 °C, 30 min).

GLC and GLC–MS.—GLC was performed on a Hewlett–Packard 5890 series II instrument equipped with a fused-silica capillary column HP-5® (30 m), and operated at 150 °C for 3 min, then raised to 320 °C at 5 °C/min and held for 30 min at the final temperature. Helium was used as carrier gas (0.1 MPa). GLC–MS was carried out with a Hewlett–Packard Model 5989A mass spectrometer. EI mass spectra were recorded at 70 eV and CI mass spectra were obtained with ammonia as the reactant gas. The ion-source temperature was 200 °C. The absolute configurations of the sugars were determined by GLC with acetylated (*R*)-2-butyl glycosides [13] or (*R*)-2-octyl glycosides [14].

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were recorded for solutions in D_2O at pD 7 with a Bruker AM-360 spectrometer operating at 323 K (internal standard

acetone, δ_H 2.23 and δ_C 31.45). Standard Bruker software was used to perform 2D homonuclear COSY (COSYHG) and $^{13}C,^1H$ COSY heteronuclear COSY (XHCORRD) experiments.

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