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Note

Structure of a surface polysaccharide from *Acinetobacter baumannii* O16

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Acinetobacter species are free-living, non-fermentative, Gram-negative bacteria, some of which have become important as opportunistic pathogens, often characterised by multiple drug resistance [1]. Of the various genospecies currently recognised, *Acinetobacter baumannii* is the one most commonly associated with nosocomial colonisation and infection, and many typing methods have been evaluated for the epidemiological monitoring of clinical isolates [2–4]. Typing of strains by their heat-stable antigens [assumed to be the O-specific side-chains of lipopolysaccharide (LPS)] has not been used extensively, but 34 O serogroups have been recognised [5]. During the course of a systematic study of the surface polysaccharides of *A. baumannii*, we have confirmed the presence of O-specific polymers in LPS extracts from the reference strains [6] for serogroups O2 [7], O5 [8], O10 [9], and O11 [10], as well as strain 214 (a clinical isolate) [11]. In each case the polymer had a branched, oligosaccharide repeating-unit incorporating at least one amino sugar residue, features also found in polymers isolated from other O serogroups [1]. Here we report the structure of the O16 polymer, which probably also occurs as a minor polysaccharide in the O11 reference strain [10].

1. Results and discussion

The LPS from the reference strain for *A. baumannii* O16 (341 mg) was hydrolysed (1% acetic acid, 100 °C, 2 h) and the water-soluble products were eluted from a

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[1] S.R. Haseley and S.G. Wilkinson, unpublished results.

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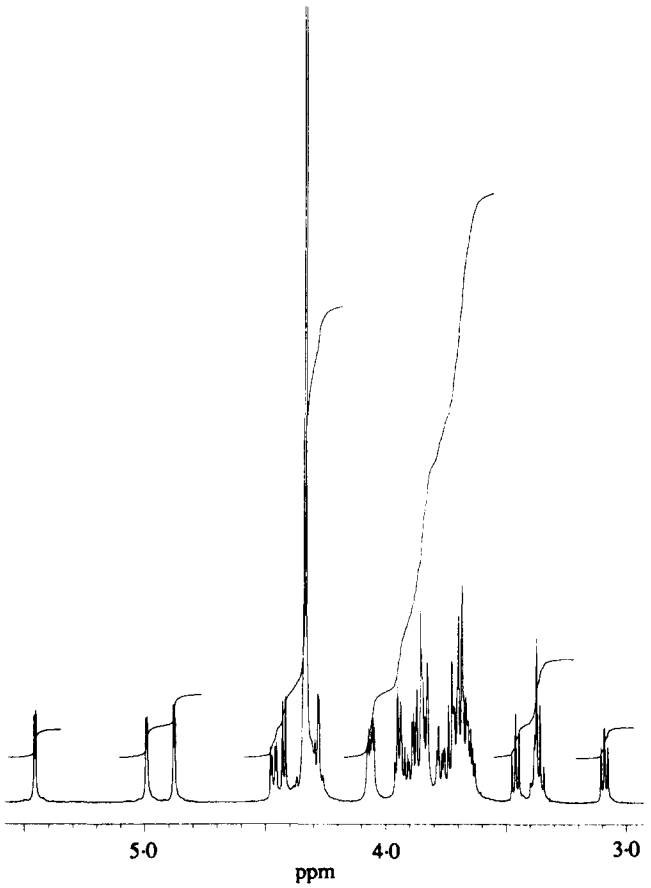


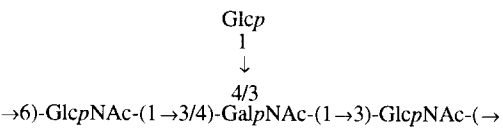
Fig. 1. ^{1}H NMR spectrum of the O16 polymer. The spectrum for the sample in D_2O was recorded at 600 MHz and 70 °C with acetone (δ 2.22) as the internal reference. In addition to the signals shown, the spectrum contained those for three *N*-acetyl groups at δ 2.06, 2.05, and 2.01.

Sephadex G-50 column to yield a major polymeric fraction (133 mg) and minor oligomeric and monomeric fractions. The oligomeric fraction contained phosphorus ($\sim$ 2%), glucose, and 2-amino-2-deoxyglucose, but no heptose, consistent with its identification as uncapped core oligosaccharide from the LPS [12]. Monosaccharide analyses of the polymeric fraction showed the presence of glucose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose in the approximate ratios 1:2:1. Glucose was identified as the D isomer by the reaction with D-glucose oxidase in the presence of peroxidase, while the amino sugars were both identified as the D isomers by GLC of their but-2-yl glycoside acetates.

The ^{1}H NMR spectrum of the polysaccharide (Fig. 1) contained signals (each 1 H) for anomeric protons at δ 5.46 ($J_{1,2}$ 3.6 Hz), 4.99 ($J_{1,2}$ 3.2 Hz), 4.88 ($J_{1,2}$ 3.4 Hz), and 4.42 ($J_{1,2}$ 7.9 Hz), pointing to a tetrasaccharide repeating-unit with one β- and three

α-pyranoside residues. Also present in the spectrum were three methyl singlets at δ 2.06, 2.05, and 2.01, consistent with the presence of three acetamido sugars. The ^{13}C NMR spectrum contained 27 signals of which three probably represented two carbons each. Diagnostic signals included those for four anomeric carbons [δ 105.04, 97.24, and 97.20 (2 C)], three acetyl groups [δ 174.56, 174.34, 173.95, 22.24 (2 C), and 21.80], three representing carbon bound to nitrogen (δ 54.14, 52.13, and 48.88), and three (each 1 C) representing hydroxymethyl groups (δ 60.88, 60.33, and 59.75). These data confirmed that the repeating-unit was constructed from residues of Hex*p*NAc (3) and Glc*p* (1), and showed that one residue was 6-substituted.

Methylation analysis of the polymer revealed four products (relative peak areas on GLC of the methylated alditol acetates, 1.00:0.51:0.74:0.53). The products were identified, both by comparison with reference compounds (GLC) and by MS, as the derivatives from unsubstituted Glc, 3-substituted GlcNAc, 6-substituted GlcNAc, and 3,4-disubstituted GalNAc, respectively. Unexpectedly, Smith degradation gave a rather complex mixture of products (HPLC), but methylation analysis confirmed the loss of Glc and the 6-substituted GlcNAc. Products obtained from the oligomeric fraction were the derivatives of unsubstituted GalNAc, 4-substituted GalNAc, and 3-substituted GlcNAc (relative peak areas on GLC, 0.56:0.53:1.00), indicating incomplete cleavage during the hydrolytic step of the degradation. Although a classical Smith-degradation product could not be isolated, it was apparent that the parent polymer had a branched repeating-unit with the partial structure **1**. This structure was confirmed and completed by the use of COSY, relayed COSY, HMQC, and NOESY experiments.



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For the purposes of spectral interpretation, the monosaccharide residues were designated **A**–**D**, in order of decreasing chemical shift for the anomeric protons. From the COSY and relayed COSY spectra, the complete spin system for **D** could be identified. Because of signal overlap and missing cross-peaks, those for **A** and **C** could initially be traced only from H-1 to H-4, and that for **B** only to H-3. The proton assignments and those for the corresponding carbons (obtained from an HMQC spectrum, Fig. 2) are included in Table 1. Even with only a partial interpretation of the spectra, the identities of residues **A**–**D** were clear-cut. The β-linked residue **D** was identified as Glc*p* from the chemical shift for C-2 (δ 73.19, inconsistent with carbon bound to nitrogen) and the general agreement with literature data for an unsubstituted β-Glc*p* residue [13]. Similarly, residue **A** was identified as α-Gal*p*NAc from the relatively high-field location [13] for C-2 (δ 48.88), consistent also with an upfield shift resulting from glycosylation at O-3. Evidence for substitution at both positions 3 and 4 of residue **A**, as expected from structure **1**, was provided by the large downfield shifts for both carbons compared with those in the free monosaccharide [13]. Residue **C** was similarly identified as 3-sub-

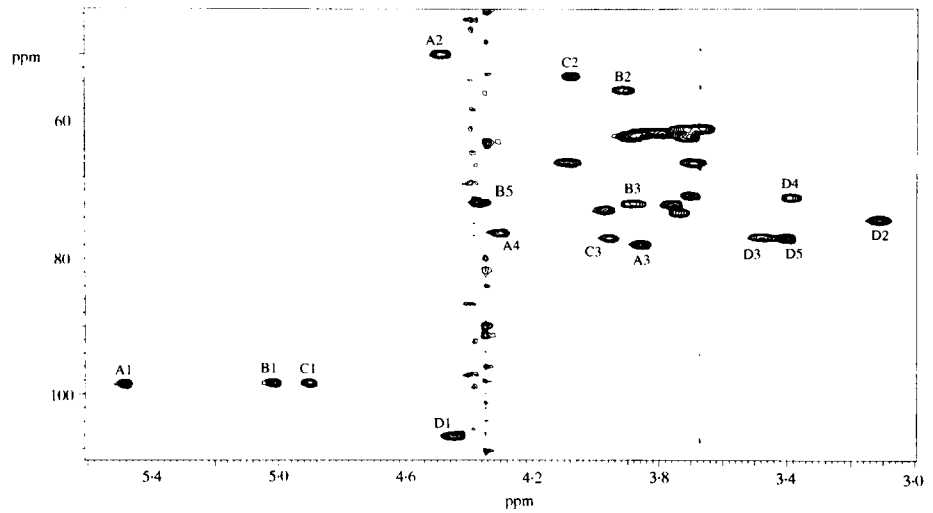


Fig. 2. HMQC spectrum of the O16 polymer. The spectrum for the sample in D_2O was recorded at 70 °C with a Varian DXR600S spectrometer and acetone (δ_H 2.22, δ_C 31.07) as internal reference.

stituted α-Glc*p*NAc from the chemical shift for C-3, and hence **B** was the 6-substituted α-Glc*p*NAc. The latter inference made possible the assignments for C-6 and H-6a/H-6b of **B** (from the HMQC spectrum) and thence for H-5 (from the proton correlation

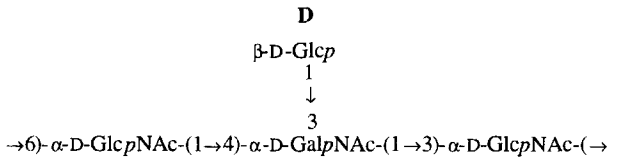
Table 1
NMR data [a] for the O16 polymer

Atom		Residue			
		→ 3,4)-α-GalNAc-(1 → **A**	→ 6)-α-GlcNAc-(1 → **B**	→ 3)-α-GlcNAc-(1 → **C**	β-Glc-(1 → **D**
1	H	5.46	4.99	4.88	4.42
	C	97.34	97.20	97.20	105.04
2	H	4.47	3.90	4.06	3.09
	C	48.88	54.14	52.13	73.19
3	H	3.83	~ 3.86	3.94	3.46
	C	76.83	~ 70.8	~ 75.9	75.71
4	H	4.28	3.68	3.72	3.36
	C	75.17	~ 69.6	72.10	69.84
5 [b]	H	–	4.34	–	~ 3.38
	C	–	~ 70.7	–	75.71
6 [c]	H	~ 3.8	4.07; 3.66	~ 3.7; ~ 3.6	~ 3.85; ~ 3.70
	C	60.33	64.71	59.75	60.88

[a] Values for chemical shift relative to acetone (δ_H 2.22; δ_C 31.07). The *N*-acetyl signals had δ_H 2.06, 2.05, and 2.01.
[b] Pairs of values for **A**5 and **C**5 appear to be δ_H 3.73, δ_C 70.92 and δ_H 3.95, δ_C 71.77.
[c] Sets of assignments for **A**6 and **C**6 may be interchanged.

spectra). The assignments shown (Table 1) for other signals of residues **A**, **B**, and **C** are provisional but are in general agreement with the chemical shifts expected.

The lateral glucosyl substituent was located, and the sequence of residues in the main chain of **1** was confirmed, from a NOESY spectrum for the polymer. This showed an inter-residue contact between H-1 of **D** and H-3 of **A**, as well as the intra-residue contacts with H-3 and H-5 (as expected for a β-pyranose residue). The large downfield shift for C-3 of **A** and the absence of an NOE effect between H-1 of **D** and H-4 of **A** are as expected for β-D-glucosylation [14,15]. For the α-linked residues (**A**, **B**, and **C**), intra-residue contacts with H-2 and the following inter-residue contacts were also observed: H-1 of **A** with H-3 of **C**, H-1 of **B** with H-4 of **A**, and H-1 of **C** with H-6a/H-6b of **B**. The structure of the repeating-unit for the O16 polymer can therefore be finalised as **2**.



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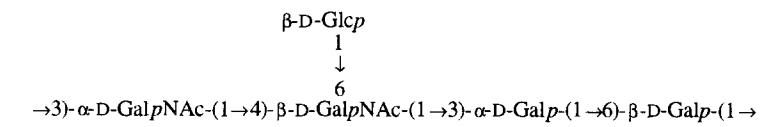
In its general features, the repeating-unit of the O16 polymer resembles those of other O antigens of *A. baumannii* so far characterised (a branched structure, the presence of common hexoses and hexosamines, often with the D-*gluco* or D-*galacto* configuration) [7–11]. In fact, it is likely that the O16 polymer is a minor component of the LPS from *A. baumannii* O11 [10]. All minor signals detectable in the NMR spectra for the polymeric fraction from the O11 strain [10] could be attributed to the presence of the O16 polymer (with only small, systematic differences of ~ 0.02 ppm (δ_H) and ~ 1.0 ppm (δ_C), attributable to differences in recording parameters). The major O11 polymer has a branched, pentasaccharide repeating-unit (**3**).



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2. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—The reference strain for *A. baumannii* O16 [6] was grown in Nutrient Broth no. 2 (Oxoid, 20 L) for 24 h at

30 °C with aeration at 20 L min^{-1} and stirring at 300 rpm. The cells (162 g wet weight) were collected by continuous centrifugation (Sharples) and disintegrated mechanically (Dyno Mill KDL). LPS (0.99 g) was extracted from purified, defatted cell walls (4.35 g) by the aqueous phenol method as in previous studies [11], and was recovered from the aqueous phase. The water-soluble products obtained on mild hydrolysis (aq 1% AcOH, 100 °C, 2 h) of the LPS (341 mg) were eluted from Sephadex G-50 to give a polymeric fraction (133 mg), oligomeric material (14 mg), and low-M_r products (14 mg).

General methods.—NMR spectra (^1H and ^{13}C) for the O16 polymer in D_2O were recorded with a Jeol JNM-GX270 or a Varian DXR600S spectrophotometer. The NMR data reported were obtained at 70 °C (^1H) or 40 °C (^{13}C) with acetone (δ_H 2.22, δ_C 31.07) as internal reference. Two-dimensional spectra (COSY, relayed COSY, HMQC, and NOESY) were obtained by using standard pulse sequences. Descending PC was carried out with 5:5:1:3 EtOAc–pyridine–HOAc–H_2O as the solvent and alkaline $AgNO_3$, ninhydrin, and aniline hydrogen oxalate as detection reagents. Amino sugars were also examined by TLC on Silica Gel 60 F_{254} (E. Merck) with 3:1:1 Bu^nOH–HCO_2H–EtOH as the solvent, and by high-voltage electrophoresis (HVE) in 2:3:20:180 pyridine–HCO_2H–AcOH–H_2O (pH 2.8). HPAEC utilised a CarboPac PA100 column in a Dionex DX-300 instrument; monosaccharides were eluted with 0.1 M NaOH. HPLC of monosaccharides was also carried on an HPX-87P column (Bio-Rad) in Gilson equipment. A TSKgel G-Oligo-PW column (Anachem) was used for gel permeation HPLC. GLC of (methylated) alditol acetates was carried out with a Carlo Erba Mega 5160 chromatograph fitted with a fused-silica column (25 m) of BP1 (SGE). A Finnigan 1020B instrument was used for GLC–MS.

Determination of monosaccharide composition.—Hydrolysis conditions used were 2 M HCl at 105 °C for 2 h (for neutral sugars), 6.1 M HCl at 105 °C for 4 h (for amino sugars), and 1 M trifluoroacetic acid at 98 °C for 16 h (for all sugars [16]). Products were identified by PC, TLC, HVE, HPAEC, and HPLC, and by GLC of the alditol acetates. Glucose was identified as the D isomer by enzymic assay using D-glucose oxidase (EC 1.1.2.4) and peroxidase (EC 1.11.1.7). The D configuration of amino sugars was determined by the method of Leontein et al. [17], but using butan-2-ol [the racemate and the (*S*) isomer] in place of octan-2-ol.

Degradative methods.—Methylation analyses, monitored by GLC and MS, were carried out by standard procedures [18–20]. For Smith degradation, the O16 polymer (30 mg) was treated with 0.05 M $NaIO_4$ for 7 days at 4 °C in the dark. After the addition of ethylene glycol, reduction ($NaBH_4$), acidification (2 M AcOH), and dialysis, the solution was freeze-dried. The product was treated with 1 M trifluoroacetic acid at room temperature for 16 h and fractionated either by HPLC (TSKgel G-Oligo-PW) or by chromatography on Sephadex G-15, monitoring the eluate for NH groups [21].

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