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Structure of a surface polysaccharide from *Acinetobacter baumannii* strain 214

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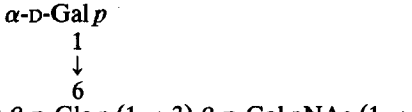
Abstract

A polysaccharide containing D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-galactose was obtained from an aqueous phenol extract of isolated cell walls from *Acinetobacter baumannii* strain 214. By means of NMR studies and chemical degradations, the repeating unit of the polymer was identified as a branched trisaccharide of the structure shown.



1. Introduction

Acinetobacter species are of both clinical and industrial interest [1], and their taxonomy has recently undergone major revision [2–5]. Whereas a single species (*Acinetobacter calcoaceticus*) was defined by Juni [6] in 1984, at least 18 genospecies are now recognised. Most strains associated with nosocomial colonisation and infection belong to *Acinetobacter baumannii* and the similar genospecies 3 and 13. The growing clinical importance of the organisms has encouraged the development of many methods for the differentiation of individual species and strains [3,5,7–10], including serotyping of the heat-stable antigens for *A. baumannii* [11] and genospecies 3 [12]. It is not known whether these correspond to the classical O antigens (lipopolysaccharide side-chains) or to other surface polymers.

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Little is known of the surface polysaccharides of *Acinetobacter* species, although many strains elaborate capsules [13] or other [14,15] exopolysaccharides. The organisms also have a reputation for producing R-type lipopolysaccharides (LPSs) with unusual structural features. For example, the LPS from strain NCTC 10305 (now classified as *Acinetobacter haemolyticus*) contains 3-deoxy-D-*lyxo*-hept-2-ulosaric acid [16] and D-*glycero*-D-*talo*-oct-2-ulosonic acid [17] as well as 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). The LPS is also unusual in containing phosphorylated glucose but no aldoheptose [17]. Some strains of *Acinetobacter* do appear to produce S-type LPS {visualised as a typical ladder pattern after resolution by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) [15,18,19]}. However, no structural studies of a putative side-chain have been described. Here we report the results of such a study on a polymer from a clinical isolate of *A. baumannii*.

2. Results and discussion

The LPS from *A. baumannii* strain 214 was mainly (91%) recovered from the aqueous phase when defatted cell walls were treated with hot aqueous phenol (total yield, 46%). The phosphorus content was low (0.25%), and the neutral glycoses (28%) consisted mainly of glucose and galactose (both ~ 12%), together with a little rhamnose (~ 2%) and a trace of mannose; no aldoheptose was detected. Both 2-amino-2-deoxyglucose (the major hexosamine) and 2-amino-2-deoxygalactose were also components of the LPS. After SDS-PAGE, only a fast-moving band (no ladder) was detected, consistent with R-type LPS. On being warmed in 1% acetic acid, the LPS gave an almost clear solution which slowly turned brown and slightly turbid on further heating (100°C, 2.5 h); only a little solid could be removed by centrifugation (10000 rpm, 15 min). After freeze-drying, the water-soluble products were separated by chromatography on Sephadex G-25 or G-50, to give a polymeric fraction (38% of the whole LPS).

The polymeric material consisted mainly of glucose, galactose, and 2-amino-2-deoxygalactose (each as the D isomer), with the hexoses being in equimolecular proportions; a little rhamnose was also present. The ^{1}H NMR spectrum of the polymer contained three major anomeric signals (each 1 H) at δ 4.98 (unresolved), 4.78 ($J_{1,2}$ 7.6 Hz), and 4.52 ($J_{1,2}$ 7.7 Hz) and a methyl singlet for an *N*-acetyl group at δ 2.03, *inter alia*, indicating a trisaccharide repeating unit. This inference was supported by the ^{13}C NMR spectrum (Fig. 1), which contained 19 major signals (that with δ 61.89 corresponding to 2 C), including anomeric signals at δ 105.13, 102.67, and 98.91, and acetamido signals at δ 175.81, 52.42, and 23.19. The NMR data also point to the presence of one α-pyranosyl and two β-pyranosyl residues in the repeating unit. By means of COSY and C–H correlation spectroscopy, it was shown that the signals with δ_C 102.67 and δ_H 4.78 were derived from a 2-acetamido-2-deoxy-β-galactopyranosyl residue.

Methylation analysis showed that the polymer was branched: the products derived from an unsubstituted galactopyranosyl residue, a 3,6-disubstituted glu-

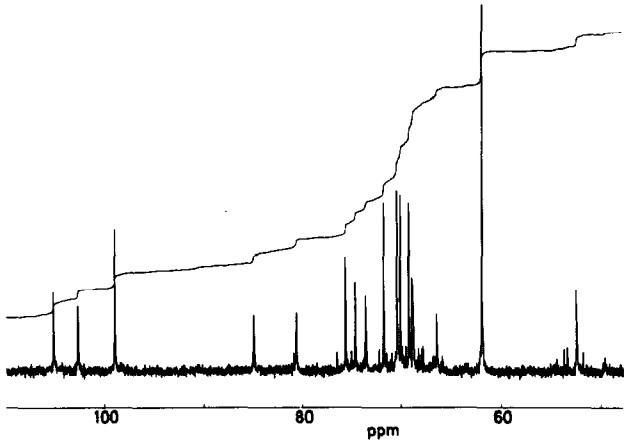
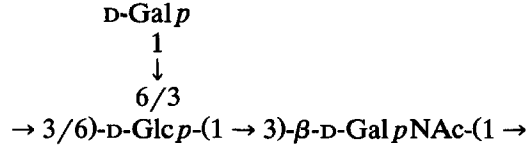


Fig. 1. ^{13}C NMR spectrum of the native polymer. The spectrum for the sample in D_2O was recorded at 100.61 MHz and 40°C with acetone (δ 31.07) as the internal reference. In addition to the signals shown, the spectrum contained others at δ 175.81 and 23.19.

copyranosyl residue, and a 3-substituted 2-acetamido-2-deoxygalactopyranosyl residue were identified by GLC (ratios of peak areas, 0.90 : 1.00 : 0.31) and by MS. Thus, the partial structure **1** could provisionally be assigned to the repeating unit.



1

Attempts to elucidate the positions of substitution at the branch-point glucose residue by carrying out a Smith degradation were unsuccessful, as the glycan obtained was highly insoluble in water. Instead, the polymer was degraded by successive *N*-deacetylation, deamination, and reduction, to give a dihexosyl-2,5-anhydroalditol (DA). Evidence for the molecular size of DA was provided by the presence in the ^{13}C NMR spectrum (Fig. 2) of 18 discrete signals, and by FABMS of the permethylated derivative [pseudomolecular ions with *m/z* 651 $(M + Na)^+$ and 667 $(M + K)^+$]. EIMS of the permethylated derivative gave the expected [20] peaks with *m/z* 219 (aA_1), 187 (aA_2), 155 (aA_3), 189 (cA_1), 157 (cA_2), 249 (bcJ_1), 393 (bcA_1), 361 (bcA_2), and 453 ($abcJ_1$). The NMR spectra of DA contained the following anomeric signals: δ_H 5.00 ($J_{1,2}$ 2.1 Hz) and 4.56 ($J_{1,2}$ 7.9 Hz), δ_C 103.94 and 99.08, i.e., one α and one β, confirming the β configuration of the 2-acetamido-2-deoxygalactopyranosyl residue in the parent polymer. As expected, acid hydrolysis of DA gave glucose, galactose, and 2,5-anhydrotalitol. Methylation analysis of DA gave the products derived from 3-substituted 2,5-anhydrotalitol, unsubstituted galactopyranosyl, and 6-substituted glucopyranosyl

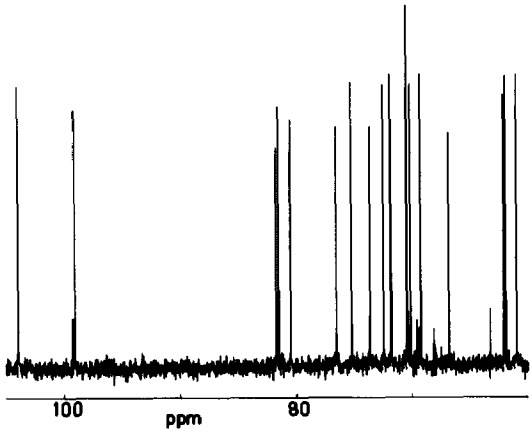


Fig. 2. ^{13}C NMR spectrum of the reduced deamination product (DA). Operating conditions were those given in the legend to Fig. 1.

residues (ratios of peak areas in GLC of the products, 0.54:1.00:1.04). Similar analysis of DA-d_1 (prepared by treatment of the deamination product with $NaBD_4$ in place of $NaBH_4$) gave consistent data [21]. These results confirmed direct galactosyl substitution of the branch-point glucose residue, and showed that both residues in the main chain of the original polymer were 3-substituted.

The anomeric configurations of the glucose and galactose residues were determined after partial acid hydrolysis (0.1 M trifluoroacetic acid, 100°C, 2 h) of the parent polymer. The hydrolysate was initially resolved by HPLC (HPX-87P), giving monosaccharides (mainly galactose) and two major oligosaccharide fractions. The fraction OS1 was reduced ($NaBH_4$), and shown to contain a hexosylhexitol by MS of the permethylated derivative [22]; the peaks with m/z 219 (aA_1), 187 (aA_2), 155 (aA_3), 235 (bA_1), 203 (bA_2), 171 (bA_3), and 295 (abJ_1) were characteristic of the two moieties, while the peak with m/z 177 was diagnostic for 6-substitution of the hexitol. The anomeric region of the ^1H NMR spectrum of oligosaccharide OS2 was rather complex but indicative of a trisaccharide; major signals were present at δ 5.05 (1 H, unresolved), 5.29 (~ 0.5 H, $J_{1,2}$ ~ 3 Hz), 4.78 (~ 0.5 H, $J_{1,2}$ ~ 8 Hz), 4.63 (~ 0.5 H, $J_{1,2}$ ~ 8 Hz), and 4.57 (~ 0.5 H, $J_{1,2}$ ~ 8 Hz). The spectrum of the reduction product (OS2R) was much simpler, containing anomeric signals (each 1 H) at δ 5.06 ($J_{1,2}$ 3.8 Hz) and 4.63 ($J_{1,2}$ 7.8 Hz) and a methyl singlet at δ 2.10. These data point to OS2R being a trisaccharide-alditol containing one GalNAc residue, an α-hexopyranosyl residue at the nonreducing end, and an internal β-linked residue. Splitting of the anomeric signal (δ ~ 4.6) for the latter residue in OS2 is attributable to the presence of both pyranose anomers of the adjacent reducing residue. A further sample of partial hydrolysate was fractionated by gel permeation HPLC (TSKgel G-Oligo-PW). When the reduced ($NaBH_4$) oligomeric fraction was treated with α-D-galactosidase (but not with β-D-galactosidase), galactose was released, showing that structure **2** could be assigned to the repeating

Table 1
NMR data [a] for the polymer

Atom	Residue		
	α-Gal*p*	β-Glc*p*	β-Gal*p*NAc
1 C	98.91	105.13	102.67
H	4.98	4.52	4.78
2 C	69.23	73.57	52.42
H	~3.82	3.41	4.02
3 C	70.40	84.95	80.63
H	~3.82	3.64	3.93
4 C	70.04	68.90	68.81
H	4.00	3.59	4.12
5 C	71.77	74.65	75.65
H	3.90	~3.63	~3.72
6 C	61.89	66.45	61.89
H	[b]	~3.76	
6′ H		~3.95	

[a] Values for chemical shifts relative to acetone (δ_H 2.22; δ_C 31.07); acetyl signals were also present at δ 175.81 and 23.19. [b] Not determined.

unit in the parent polymer. Further interpretation of the NMR data for the polymer (Table 1) supported this conclusion.

α-D-Gal*p*
1
↓
6
→ 3)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 →
2

The origin of the polymer (excluded from Sephadex G-50) described is uncertain. Although it was isolated from an aqueous phenol extract of the cell walls, the failure to detect S-type LPS by SDS-PAGE means that the polymer may not be an O-specific LPS side-chain but a separate surface glycan. A neutral glycan lacking a hydrophobic anchor would not be expected to enter the gel and migrate during electrophoresis. However, the possibility of a misleading SDS-PAGE result cannot be discounted; the presence in the polymeric material of some rhamnose (not accounted for in this study) could be explained by an attached core oligosaccharide, as rhamnose is a core component [23] in '*Acinetobacter calcoaceticus*' NCTC 10303 (= ATCC 17904), which is now assigned to *A. baumannii* [2]. Rhamnose is also a component of the capsular polysaccharide from another *Acinetobacter* strain [13].

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS. — *A. baumannii* strain 214 was a clinical isolate originally received as *A. anitratus*, many strains of which have since been reclassified as *A. baumannii*; this assignment was confirmed for strain 214. The culture 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. Cell walls were prepared by mechanical disintegration of the cells (122 g wet weight), followed by treatment with trypsin and RNase and repeated washing [24]. Lipids were extracted from the freeze-dried cell walls (3.29 g) by stirring with 2:1 $CHCl_3$–MeOH at room temperature for 2 h, and LPS was extracted from the insoluble residue by the aqueous phenol method [24] [yields, 1.28 g (aqueous phase) and 0.12 g (phenol phase)]. Both products had similar monosaccharide compositions, but further work was confined to the LPS from the aqueous phase. The water-soluble products from mild acid hydrolysis (aq 1% AcOH, 100°C, 2.5 h) of the LPS were fractionated by chromatography on Sephadex G-25 or G-50 in pyridine–AcOH buffer (pH 5.4). Elution profiles were monitored for total carbohydrate (phenol–H_2SO_4 method) and for phosphorus [24].

General methods.—NMR spectra for samples in D_2O were recorded with either a Bruker WH-400 spectrometer (native polymer and the product from *N*-deacetylation–deamination–reduction) or a Jeol JNM-GX270 spectrometer (other products). ^{13}C NMR spectra were usually obtained at 40°C with acetone (δ 31.07) or 1,4-dioxane (δ 67.40) as the internal reference. ^{1}H NMR spectra were recorded at 70°C: those for the polymer and product DA were obtained at 400 MHz with acetone (δ 2.22) as the internal reference, and those for oligomeric products OS1, OS2, and OS2R at 270 MHz; chemical shifts were obtained by reference to the HOD signal (δ 4.37). 2D-NMR spectra (C–H correlation, COSY, and relayed COSY) were obtained by using standard pulse sequences.

GLC analysis of (methylated) alditol acetates was carried out with a Carlo Erba Mega 5160 chromatograph fitted with a fused-silica capillary column (25 m) of BP1 or BP10 (SGE). GLC–MS was performed with a Finnigan 1020B instrument, and FABMS with an AutoSpec instrument (VG). Most HPLC separations utilised Gilson equipment and columns of TSKgel G-Oligo-PW (Anachem) or HPX-87P (Bio-Rad). A Dionex DX-300 HPLC system with a CarboPac PA100 column was used to monitor the enzymic release of galactose from oligosaccharides. PC and high-voltage electrophoresis (HVE) were carried out on Whatman No. 1 paper with solvent *A*, 13:5:4 EtOAc–pyridine–water and buffer *B*, 5:2:43 pyridine–AcOH–water (pH 5.3), respectively. SDS-PAGE analysis of isolated LPS utilised a resolving gel containing acrylamide (12.5%), silver staining of the fractions [25], and S-type LPS from *Xanthomonas maltophilia* [26] as a control.

Sugar composition.—Hydrolysis conditions used were 2 M HCl at 105°C for 2 h (for neutral sugars), and 6.1 M HCl at 105°C for 4 h (for amino sugars). The aldohexoses were identified by PC (solvent *A*), GLC of the alditol acetates, and HPLC (HPX-87P). After separation of the hexoses by HPLC, enzymic assays to confirm their identities and establish their configurations were carried out by using

D-glucose oxidase (EC 1.1.2.4) or D-galactose oxidase (EC 1.1.3.9), in conjunction with peroxidase (EC 1.11.1.7). Galactosamine was identified by HVE (buffer *B*), GLC of the alditol acetate, conversion into 2,5-anhydrotalitol by deamination [27] and reduction (monitored by GLC and GLC–MS of the tetra-acetate), cation-exchange chromatography [28], HPLC of the *N*-acetyl derivative (HPX-87P), modifications of the Elson–Morgan reaction [29], and the ^{1}H NMR spectrum of the hydrochloride. The D configuration was established by using the combination of D-galactose oxidase and peroxidase [30].

Degradative methods.—Methylation analyses were carried out by standard procedures [31–33]. *N*-Deacetylation [31] of the reduced ($NaBH_4$) polymer was carried out for 16 h at 85°C under N_2, and the product was isolated by chromatography on Sephadex G-15 and deaminated [31]. After reduction ($NaBH_4$ or $NaBD_4$), the deamination product was purified by chromatography on Sephadex G-15 and examined by NMR spectroscopy, sugar and methylation analyses, and by both FABMS and EIMS of the permethylated product.

Partial hydrolysis of the polymer was carried out with 0.1 M CF_3CO_2H at 100°C for 2 h. After repeated drying, the products were fractionated by HPLC (HPX-87P or TSKgel G-Oligo-PW). Oligomeric products were reduced ($NaBH_4$) to destroy any galactose contaminant, then samples were treated separately with α-D-galactosidase (EC 3.2.1.22) and β-D-galactosidase (EC 3.2.1.23), both from Sigma, in 0.1 M phosphate buffer (pH 7.2) at 37°C. The release of galactose was monitored by HPLC (Dionex).

Acknowledgments

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