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The structure of the polysaccharide O-chain of the LPS from *Acinetobacter baumannii* strain ATCC 17961

Leann L. MacLean, Malcolm B. Perry, Wangxue Chen, Evgeny Vinogradov *

Institute for Biological Sciences, National Research Council Canada, 100 Sussex Dr., Ottawa, ON, Canada K1A 0R6

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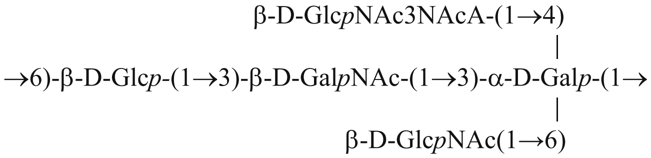
ABSTRACT

The gram-negative bacterium *Acinetobacter baumannii* strain ATCC17961 has been used by several laboratories in mouse models of respiratory *A. baumannii* infection, and a study of the role of its lipopolysaccharide in the pathogenicity is of interest. The structure of the O-deacylated polysaccharide O-chain component of its LPS has been determined by 2D NMR spectroscopy and mass spectrometry methods, and by the structural identification of oligosaccharides obtained by sequential application of the Smith degradation of the O-antigen. The O-chain was determined to be a polymer of a branched pentasaccharide repeating unit composed of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, D-glucose, and D-galactose, and has the following structure:



$$\begin{array}{r@{}l}
 & \beta\text{-D-Glc}p\text{NAc3NAcA-}(1\to4)\\
 & \qquad\qquad\qquad\qquad\quad |\\
\to6)\text{-}\beta\text{-D-Glc}p\text{-}(1\to3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\to3)\text{-} & \alpha\text{-D-Gal}p\text{-}(1\to\\
 & \qquad\qquad\qquad\qquad\quad |\\
 & \beta\text{-D-Glc}p\text{NAc}(1\to6)
\end{array}$$

1. Introduction

Acinetobacter baumannii is an ubiquitous gram-negative bacterium that can survive for prolonged periods in the environment. During the last decade, this organism has emerged as a major cause of both nosocomial and community-associated infections worldwide.[1] Moreover, infections with *A. baumannii* have become increasingly difficult to treat because of the rapid development of resistance to multiple antibiotics.[2] Despite its clinical importance, little is known about the virulence, mechanism of antibiotic resistance, or persistence strategies of this pathogen. It is possible that the lipopolysaccharide (LPS) plays an important role in the virulence of *A. baumannii*, as has been shown for many other gram-negative bacteria. Therefore, further structural characterization and biological function studies of *A. baumannii* LPS are necessary.

Herein, we describe the structure of the O-polysaccharide (O-PS) of the LPS from *A. baumannii* strain ATCC 17961 as the first step to elucidate the molecular mechanisms of *A. baumannii* virulence. This strain, identified initially as *Herellea vaginicola*, belongs to genomic species 2, in which serotyping was not performed. It has been recently used by several laboratories[3,4] in the studies of pathogenesis and immunity to respiratory *A. baumannii* infection in mouse models.

2. Results and discussion

Fermenter grown cells of *A. baumannii* (ATCC 17961) were extracted by a modified hot aqueous phenol procedure, and the LPS was obtained in 3.9% yield (based on dry cell mass) as a precipitated gel on ultracentrifugation of the concentrated water phase extract. Polysaccharide obtained after mild acid hydrolysis of the LPS showed very poor NMR spectra and was not suitable for further analysis. Hydrazinolysis of the native LPS resulted in a high molecular mass O-PS having $[\alpha]_D$ −5 (*c* 0.61, water) that was subsequently used for 2D NMR and chemical analysis. The question of whether this polysaccharide was acylated in the LPS remained open.

The O-PS composition was determined by GLC–MS of derived alditol acetates that identified Glc, Gal, GlcN, and GalN in a molar ratio of 1:0.8:1:0.7. The characterization of the sugar units was established from the identity of the individual paper chromatographically

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Abbreviations: LPS, lipopolysaccharide; HMBC, heteronuclear multiple bond connectivity; GalNAc, 2-acetamido-2-deoxy-D-galactose; GlcNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-glucuronic acid.

* Corresponding author. Tel.: +1 613 990 0832; fax: +1 613 952 9092.
E-mail address: evguenii.vinogradov@nrc.ca (E. Vinogradov).

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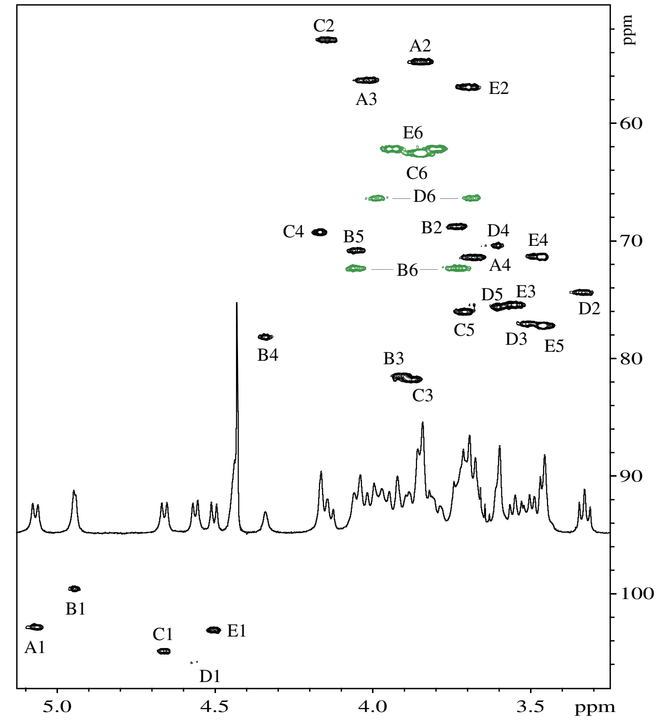

Figure 1. High orifice voltage MS/MS spectrum of the *A. baumannii* ATCC 17961 polysaccharide. Ions are $[M-H_2O+H]^+$.

separated samples with those of reference ^1H NMR spectra. The D-configuration of all the component sugars was established by GLC–MS of their derived acetylated (*R*)-2-butyl glycosides and by the determination of their specific optical rotations.

NMR spectra of the polysaccharide (COSY, TOCSY, NOESY, ^1H–^{13}C HSQC, and ^1H–^{13}C HMBC) contained signals that demonstrated the presence of five monosaccharides in roughly equal amounts (Fig. 1) with typical signal patterns of β-Glc*p*, β-Glc*p*N, β-Gal*p*N, and α-Gal*p* for four of the component glycoses. The putative five-component sugars were designated **A** to **E** in the order of decreasing anomeric proton chemical shifts. The signals of one additional monosaccharide (residue **A**) having an apparent β-*gluco* configuration were present, but the expected signals H/C-5 and C-6 were not observed. Residue **A** was subsequently identified as 2,3-diamino-2,3-dideoxy-glucuronic acid. The O-PS required high temperature conditions for NMR spectra acquisition and resolution. At 40 °C, many signals were missing; however, at 60 °C most of the signals appeared with the exception of H/C-5 of residue **A**. Higher temperatures were not attempted because of spectrometer limitations. The HSQC spectrum of the O-PS contained four CH_2OH signals assigned to the residues **B** to **E**. Residue A had two aminogroups at positions 2 and 3 (^{13}C NMR signals at 54.7 and 56.3 ppm). The 1D ^{13}C NMR spectrum of the O-PS contained five CO signals around 175 ppm which agreed with the presence of 4 *N*-acetyl groups and one uronic acid (residue A). The sequence of the monosaccharides was determined from NOE correlations between protons **A**1 and **B**4, **B**1 and **D**4, **D**1 and **C**3, **C**1 and **B**3, and **E**1 and **B**6 as presented in Chart 1. Corresponding HMBC correlations from H-1 to the transglycosidic carbon of each monosaccharide were also observed (Table 1).

Treatment of the polysaccharide with anhydrous HF gave oligosaccharide **1** mixed with its α-glycosyl fluoride, which was isolated by gel chromatography. The product was heated in water to hydrolyze glycosyl fluoride, and oligosaccharide **1** was additionally purified by reverse-phase chromatography. It was then analyzed by NMR, which confirmed its structure expected from the analysis of the whole polysaccharide. All NMR signals of the residue A were observed, confirming that it was 2,3-diacetamido-2,3-dideoxy-β-glucuronic acid (β-GlcNAc3NAcA). Its absolute D-configuration was subsequently identified from the results of GC–MS analysis of the acetylated (*S*)-2-butyl glycosides of 2,3-diacetamido-2,3-dideoxy-D-glucose, derived by the reduction of the methyl ester

β-D-Glc*p*NAc3NAcA-(1→4)
|
→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→ **O-PS**
|
β-D-Glc*p*NAc-(1→6)

β-D-Glc*p*NAc3NAcA-(1→4)-α,β-D-Gal **1** (HF)
A **B**

A
β-D-Glc*p*NAc3NAcA-(1→4)
|
β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→1)-glycerol **2** (first $NaIO_4$)
C **B**

A **B**
β-D-Glc*p*NAc3NAcA-(1→4)-α-D-Gal*p*-(1→glycol **3** (second $NaIO_4$)

A
β-D-Glc*p*NAc3NAcA-(1→2)-D-threitol **4** (third $NaIO_4$)

Chart 1.

Table 1
HMBC correlations from H-1 in monosaccharide units (Chart 1)

Unit	H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6a	H/C 6b
β-GlcNAc3NAcA **A**, O-PS	5.07	3.84	4.02	3.68			
	102.8	*54.7*	*56.3*	*71.3*		*175.4*	
α-Gal **B**, O-PS	4.94	3.73	3.91	4.34	4.05	3.72	4.05
	99.5	*68.7*	*81.4*	*78.1*	*70.7*	*72.3*	
β-GalNAc **C**, O-PS	4.66	4.14	3.87	4.16	3.71	3.85	3.85
	104.8	*52.8*	*81.7*	*69.2*	*76.0*	*62.5*	
β-Glc **D**, O-PS	4.56	3.33	3.51	3.60	3.60	3.68	3.99
	105.8	*74.3*	*77.0*	*70.4*	*75.5*	*66.3*	
β-GlcNAc,**E** O-PS	4.50	3.69	3.55	3.46	3.46	3.80	3.94
	103.0	*56.9*	*75.4*	*71.3*	*77.1*	*62.1*	
β-GlcNAc3NAcA **A**, **1**	4.83/4.84	3.82	4.01	3.63	3.85		
	103.3	*55.1*	*56.1*	*71.3*	*77.5*	*176.7*	
α-Gal **B**, **1**	5.21	3.66	3.97	4.17	4.08	3.64	3.80
	93.6	*69.9*	*70.3*	*77.7*	*71.0*	*61.6*	
β-Gal **B**, **1**	4.57	3.35	3.75	4.11	3.70	3.68	3.80
	97.7	*73.3*	*73.9*	*76.8*	*75.3*	*61.5*	

Polysaccharide (O-PS) spectra were recorded at 60 °C, oligosaccharide (OS **HF-1**) at 30 °C in D_2O with acetone standard. *N*-Acetate signals H/C: PS, 175.4; 2.01–2.05/23.3–23.8 ppm; OS: 176.3; 2.01/23.2. Italic, ^{13}C chemical shifts.

of the parent uronic acid. Further confirmation of the D-configuration of **A** was indicated from the observed negative specific optical rotation found for oligosaccharide **4** (see below).

Three steps of Smith degradation resulted in the products **2**–**4**, as outlined in Chart 1. They all were analyzed chemically and by NMR (Table 2). Results were fully consistent with the structures shown in Chart 1. Interestingly, H/C-5 signal was not visible in the HSQC spectrum of oligosaccharide **2**, but it appeared in the spectra of **3** and **4**.

Oligosaccharide **4**, obtained after a final Smith degradation, was composed of β-GlcNAc3NAcA and threitol. Its optical rotation value $[\alpha]_D$ −54.8 (*c* 0.4, water) gives further indication that the diaminoglucuronic acid residue **A** has the D-configuration.

Polysaccharide structure was analyzed by mass spectrometry employing high orifice voltage (Fig. 2). Signals of all possible oligosaccharides obtained through the fission of glycoside linkages within the repeating unit (repeating unit *m/z* 989.7) were observed along with the ions of some fragment including sugars from two repeating units.

Structures of the O-PSs from several *Acinetobacter* strains have been reported previously.[5–22] They show no close structural similarities between each other, thus reflecting the extensive O-antigenic variability of this bacterium.

3. Experimental

3.1. LPS preparation

Cells of *A. baumannii* ATCC 17961, NRCC 6416 (285 g) in 50% (wt/vol) aq phenol (200 mL) were stirred for 15 min at 70 °C, and the following dilution with water (200 mL) the cells were dialyzed against running water until they were free from phenol. The concentrated dialyzed residue, cleared of cell debris by low speed centrifugation, was treated sequentially with RNase, DNase, and proteinase K (37 °C, 3 h each). The digest was next subjected to ultracentrifugation (105,000g, 4 °C, 12 h), and the precipitated gel was dissolved in water and lyophilized to give LPS in 1.2 g yield.

3.2. Hydrazinolysis

LPS and O-PS (100 mg) were treated with anhyd hydrazine (4–5 mL) at 37 °C for 2 h, then added dropwise to cold acetone and the precipitated O-deacetylated product was collected by low speed centrifugation, dissolved in water, lyophilized and taken up in 0.02 M PyAc buffer (4 mL) for column fractionation on Sephadex G-50. Subsequent analyses were made on both products that provided essentially the same results for O-PS analysis.

Table 2
NMR data for oligosaccharides **2**–**4**

Unit, compound	H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6a	H/C 6b
GlcNAc3NAcA **A**, **2**	5.12 (8.3)	3.84	4.06	3.64	–	–	–
	102.5	*54.6*	*55.8*	*70.8*	–	*172.6*	–
Gal **B**, **2**	4.90 (3.8)	3.71	3.93	4.38	3.94	3.73	3.67
	99.8	*68.3*	*81.3*	*77.0*	*71.4*	*61.7*	–
GalNAc **C**, **2**	4.56	3.99	3.72	3.95	3.68	3.8	3.85
	105.2	*52.8*	*72.3*	*69.0*	*76.3*	*62.4*	–
Glycerol, **2**	3.58/3.74	3.94	3.65				
	69.6	*71.3*	*63.8*				
GlcNAc3NAcA **A**, **3**	4.90 (8.5)	3.84	4.05	3.72	4.19	–	–
	103.5	*54.8*	*55.8*	*70.8*	*76.7*	*176.0*	–
Gal **B**, **3**	4.94 (3.7)	3.67	4.00	4.14	3.97	3.76	–
	99.7	*69.8*	*70.5*	*78.5*	*71.5*	*62.8*	
Glycol, **3**	4.35	3.85					
	68.4	*60.5*					
GlcNAc3NAcA **A**, **4**	5.00 (8.6)	3.96	4.20	3.87	4.34	–	
	101.3	*53.8*	*54.8*	*69.7*	*75.7*	*170.4*	
Threitol, **4**	3.88/3.79	3.98	3.94	3.84/3.78			
	60.8	*79.7*	*70.7*	*62.4*			

Spectra were recorded at 30 °C in D_2O with acetone standard. Italic, ^{13}C chemical shifts. Coupling constants $J_{1,2}$ in Hz are given in parentheses.

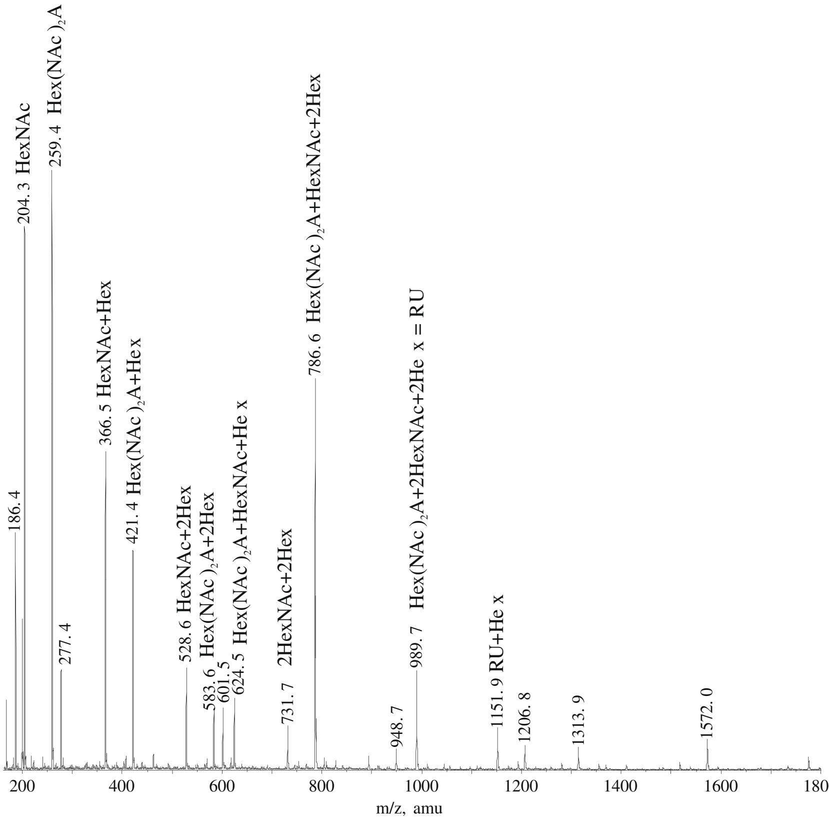

Figure 2. ^{1}H–^{13}C HSQC correlation spectrum of the *A. baumannii* ATCC 17961 O-polysaccharide.

3.3. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded using Varian Inova 500 MHz and 400 MHz spectrometers for samples in D_2O solutions at 60 °C for the polysaccharide and at 30 °C for the oligosaccharides with acetone as internal reference (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, and HMBC (100 ms long range transfer delay).

3.4. Mass spectrometry

MS experiments were performed on a Q-star quadrupole/time-of-flight or an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada). For pseudo MS/MS analysis, precursor ions were generated with an orifice voltage of +120 V and mass spectra were acquired with nitrogen in the RF-only quadrupole collision cell.

3.5. Monosaccharide analysis

The polysaccharide (0.5 mg) was hydrolyzed (1 mL of 3 M TFA, 120 °C, 2 h) and evaporated to dryness under a stream of air. The residue was dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with acetic acid (0.3 mL), dried, and methanol (1 mL) was added. The mixture was dried twice with the addition of methanol, and the residue was acetylated with acetic anhydride (0.5 mL, 100 °C, 30 min), dried, and analyzed by GLC on a HP1 capillary column (30 m × 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient of 170 (4 min) to 260 °C at 4 °C/min.

For the identification and determination of the configuration of the 2,3-diacetamido-2,3-dideoxyhexuronic component of the O-PS and oligosaccharide products, the GLC–MS analysis of the derived 2-(*S*)-butyl 4,6-di-*O*-acetyl-2,3-diacetamido-2,3-dideoxyhexopyranosides derivatives was done using the following steps: (1) methanolysis of O-PS or oligosaccharide (2–3 mg, 10:1 MeOH–AcCl (0.5 mL), 90 °C, 2 h); (2) evaporation to dryness and acetylation (1:1 pyridine–Ac_2O 90 °C, 30 min); (3) evaporation to dryness, dissolution in EtOH (0.5 mL), and reduction with $NaBH_4$ (20 mg in 0.5 mL water at 60 °C, 3 h); (4) neutralization (AcOH), concentration to dryness, and codistillation from MeOH (3 × 3 mL); (5) butanolysis with 2-(*S*)-butanol (0.5 mL + AcCl (0.06 mL) at 90 °C, 2 h; (6) evaporation to dryness and acetylation (1:1 pyridine–Ac_2O, 90 °C, 1 h) followed by evaporation and GLC–MS analysis. Standards were prepared from synthetic methyl 2,3-diacetamido-2,3-dideoxy-α-D-glucopyranoside with (*S*)- and (*R*)-2-butanol.

3.6. Determination of the absolute configuration of the monosaccharides

O-PS (1 mg) was treated with 10:1 2-(*S*)-butanol–AcCl (0.25 mL, 2 h, 85 °C), dried under a stream of air, acetylated, and analyzed by GC in comparison with authentic standards, prepared from respective monosaccharides with 2-(*S*)-butanol.

3.7. Gel and paper chromatography

Performed on Sephadex G-50 (2.5 × 80 cm) or Sephadex G-15 (1.6 × 80 cm) columns using 0.05 M pyridinium acetate buffer (pH 4.5) as the mobile phase. The column eluents were monitored using a Waters 402 refractometer. Paper chromatography was performed on water washed Whatman 3 MM paper using 10:3:3 butanol–pyridine–water as the mobile phase, and detection was made using the silver nitrate spray reagent.

3.8. Hydrogen fluoride solvolysis

The polysaccharide (10 mg) was dissolved in anhyd HF (~5 mL) and kept at 25 °C for 2 h, and HF was evaporated on a plastic Petri dish. The residue was dissolved in water and separated by gel chromatography on Sephadex G-15 column. Oligosaccharide products were heated in water (2 h, 100 °C) and separated on C18 reverse phase column (25 × 0.9 cm, Aqua, Phenomenex) in 0.1% TFA. Fractions were dried and analyzed by NMR, and fractions containing the oligosaccharide **1** were combined and analyzed by 2D NMR.

3.9. Smith degradation

Samples of O-PS and oligosaccharides were oxidized by periodate, reduced ($NaBH_4$), and hydrolyzed (3% AcOH, 100 °C, 2 h), and chromatographically pure products were obtained by Sephadex G-15 column chromatography as described.[23]

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