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Note

The structure of the polysaccharide isolated from *Acinetobacter baumannii* strain LAC-4

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ABSTRACT

The structure of the surface polysaccharide from a hypervirulent for mice *Acinetobacter baumannii* strain LAC-4 was studied. The polysaccharide was built of trisaccharide repeating units containing α-L-fucosamine, α-D-glucosamine, and α-8-epi-legionaminic acid. The structure interpretation was based mostly on NMR data. Polysaccharide was obtained using a procedure of LPS O-chain preparation, although whether it is an LPS O-chain or capsular polysaccharide remained unclear.

Acinetobacter baumannii has emerged as a major cause of hospital- and community-acquired infections worldwide. Infections have also become increasingly difficult to treat because of the rapid development of antibiotic resistance by the pathogen. *A. baumannii* causes bacterial pneumonia that may lead to severe and often fatal bacteremia.[1] Strains of this bacterial species have demonstrated varying abilities to cause bacteremia in both human patients and animal models, suggesting the existence of specific virulence factors.[2–4] Therefore, further identification of virulence factors and understanding of their mechanisms of action in the pathogenesis of *A. baumannii* infection are essential for the development of novel therapeutics and vaccines.

We have recently identified a clinical strain of *A. baumannii*, LAC-4, which shows significantly enhanced virulence in conventional mice such as BALB/c and C57BL/6 strains.[4] The LAC-4 strain was one of 20 clinical isolates obtained from nosocomial outbreaks in several hospitals of Los Angeles County, CA in late 1990s and early 2000s.[5] The LAC-4 possesses a unique genetic profile compared to other clinical outbreak isolates and, like the rest of the strains examined, was found to be multidrug resistant.[5] When it is intranasally administered, LAC-4 rapidly replicates in the lungs, then spreads to local draining lymph nodes, spleen, and blood, and eventually kills mice within 48 h. The infection induced by this isolate reliably reproduces the most relevant features of human pulmonary *A. baumannii* infection and this is a valuable model for identifying novel virulence factors and potential disease pathogenesis.

Polysaccharides surrounding bacterial cells play an important role in the virulence of many other gram-negative bacteria including *A. baumannii*. Therefore, further structural characterization and biological function studies of polysaccharide from *A. baumannii* LAC-4 may provide some insights on the reason why this strain is hypervirulent in mice. Herein, as the first step to elucidate the molecular mechanisms of the hypervirulence of LAC-4, we characterized the structure of the polysaccharide prepared by the procedure of the LPS O-chain isolation from *A. baumannii* strain LAC-4.

Bacterial cells were treated with phenol–water extraction[6] and LPS (or LPS together with capsular polysaccharide) was purified by ultracentrifugation. The LPS gave no ladder-like picture on PAGE with silver staining, which may indicate the absence of the O-chain but also can be caused by unknown factors. *Acinetobacter* LPSs were reported to poorly stain with silver reagent for unknown reasons.[7] Polysaccharide was obtained from ultracentrifuge precipitated LPS, which may indicate that it was a part of the LPS molecule. Solution of the material before acid or base hydrolysis showed no resolved ^{1}H NMR spectra, indicating that it might be LPS.

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Mild acid hydrolysis of the LPS led to depolymerization of the polysaccharide chain due to partial cleavage of the linkages of the nonulosonic acid. Combination of size-exclusion and anion-exchange chromatography led to isolation of the trisaccharide, representing a repeating unit (RU) of the polysaccharide, dimer of the RU, and higher oligomers. Non-depolymerized polysaccharide was also obtained by O-deacylation of the LPS.

NMR analysis of the RU and RU-dimer led to the identification of the α-FucNAc (2-amino-2,6-dideoxy-L-galactose, unit A), α-D-GlcNAc (B), and 5,7-diacetamido-3,5,7,9-tetradeoxy-nonulosonic acid (C). L-FucN and D-GlcN were also identified by GC of alditol acetates and their absolute configuration was determined by GC of acetylated 2-butyl glycosides obtained with optically pure 2-butanol.

The configuration of the pyranose ring of the nonulosonic acid residue C was identical to that of neuraminic acid and legionaminic acid, which followed from the observation of all large (8–11 Hz) vicinal coupling constants between ring protons (except for the non-informative small $J_{3e,4}$). The configuration of C-7 and C-8 was determined by comparison of the NMR chemical shifts of H/C-6,7,8,9 with published data for similar fragments of the polysaccharides from *Pseudomonas aeruginosa* O12,[8] *Providencia stuartii* O20,[9] and synthetic isomers of 5,7-diacetamido-3,5,7,9-tetradeoxy-nonulosonic acids.[10,11] Thus it was identified as 8-epi-legionaminic acid (L-*glycero*-D-*galacto*, 8eLeg). The 8eLeg had different anomeric configurations at the reducing end of the PS fragments and inside the polymeric chain. Large difference in the position of H3e and H3a signals demonstrated by the residue inside the polymeric chain indicated α-configuration; in the reducing form dominant was β-anomer with H3e signal shifted closer to H3a (Table 1 and Fig. 1).[10,11]

The sequence of the monosaccharides was determined using ROESY (A1/B3, B1/C8) and HMBC (A1/B3, B1/C8, A3/C2) data, indicating substitutions as shown in the Scheme 1.

Several polysaccharides with similar structures were described. *P. stuartii* O20 polysaccharide contained identical fragment α-D-GlcNAc-(1-8)-α-8eLeg.[9] The closest similarity shows the O-chain of the LPS from *P. aeruginosa* O12,[8] which differs from *A. baumannii* LAC-4 by the replacement of *N*-acetyl group at N-2 of α-D-FucN by the amidino group, and absence of OH at C-6 of GlcNAc (GlcNAc replaced with QuiNAc). This similarity provides an interesting subject for the study of biosynthesis, horizontal gene transfer, and virulence mechanism.

There are contradictory data regarding the presence of the polysaccharide O-chains in *A. baumannii* LPS. *Acinetobacter* LPSs normally do not show O-polysaccharide bands on PAGE after staining with periodate–silver nitrate reagent and for this reason it was believed they contain no O-chain, although sometimes a ladder-like band pattern can be obtained with the standard silver staining[12] or with immunostaining.[7] Several *A. baumannii* polysaccharide structures were reported as LPS O-chains.[13–29]

Recently it was shown that *A. baumannii* does not have the WaaL gene, required for the linkage of the O-polysaccharide to the LPS core.[30] However there is still a possibility that the polysaccharide can be connected to the LPS core by a yet unidentified enzyme. Usually *A. baumannii* polysaccharides are obtained together with LPS and can be precipitated in an ultracentrifuge. Polysaccharides require acid or base treatment to produce real (non-colloid) solutions; without one of these treatments they do not show resolved sugar signals in the NMR spectra, probably due to their connection to a lipid. Some *A. baumannii* polysaccharides were obtained without acid or base treatment, they did not sediment in the ultracentrifuge, and could not be immunostained on electrophoresis with anti-lipid A antibodies, indicating that they are capsular polysaccharides and not a part of the LPS.[31] Existing observations provide indirect evidences on the nature of the polysaccharides. Definite answers could be obtained by the structural characterization of the reducing end of the polysaccharide, which have not been achieved yet.

1. Experimental

1.1. LPS isolation

Cells were precipitated from the growth medium by low speed centrifugation, washed with water twice. Cells (200 g wet weight) were stirred with 400 mL of 45% phenol for 20 min at 70 °C, cooled to room temperature and dialyzed against running water for 3 days. The content of dialysis bag was mixed with 10% (v/v) of acetic acid, stirred for 20 min and the precipitate removed by centrifugation. The solution was dialyzed for another 3 days, concentrated to 200 mL, and subjected to ultracentrifugation at 120,000*g* for 3 h. The precipitate was dissolved in water and dried to give LPS (1.8 g).

LPS was hydrolyzed with 2% AcOH (100 °C, 1 h), the precipitate removed, and solution separated on Bio-gel P6 column. RU, di-RU, and longer fractions were collected, RU and di-RU purified by anion-exchange chromatography.

1.2. NMR spectroscopy

NMR experiments were carried out on a Varian INOVA 500 MHz (^{1}H) spectrometer with 3 mm gradient probe at 25–50 °C with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C), using standard pulse sequences gCOSY (gradient COrrelation SpectroscopY), TOCSY (Total Correlation Spectroscopy) (mixing time 120 ms), ROESY (Rotating frame Nuclear Overhauser Effect

Table 1
NMR data (δ, ppm; Varian INOVA 500 MHz) for the polysaccharide and repeating unit of *A. baumannii* LAC-4 polysaccharide

Sugar	H/C 1	H/C 2 (H3a)	H/C 3 (H3e)	H/C 4	H/C 5	H/C 6	H/C 7	H/C 8	H/C 9
A, FucNAc	5.00	4.17	3.93	3.99	4.38	1.20			
PS	98.4	49.1	75.0	72.5	67.2	16.9			
B, GlcNAc	4.87	4.03	3.63	3.63	3.53	3.86			
PS	94.4	54.5	77.0	68.9	73.4	61.4			
C, α-8eLeg		1.64	2.63	3.53	3.68	4.07	3.92	3.73	1.31
PS		104.6	42.4	69.2	53.8	73.5	54.6	73.1	14.8
A, FucNAc	4.96	4.10	3.88	3.80	4.40	1.20			
RU	98.6	50.8	69.2	72.6	68.0	16.9			
B, GlcNAc	4.87	4.03	3.65	3.55	3.58	3.78; 3.84			
RU	94.5	54.7	77.1	69.4	73.7	61.8			
C, β-8eLeg		1.84	2.26	3.92	3.74	4.09	3.97	3.81	1.12
RU		97.6	41.0	68.7	54.2	71.7	54.2	73.0	15.4

PS spectra were measured on LPS-HY (50 °C). NAc signals in PS: 1.96:23.3; 1.96:23.3; 2.02:23.5; 2.05:23.5 ppm. RU NAc: 1.96:23.1; 1.99:23.5; 2.02:23.7; 2.04:23.7 ppm.