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Characterisation of the core part of the lipopolysaccharide O-antigen of *Francisella novicida* (U112)

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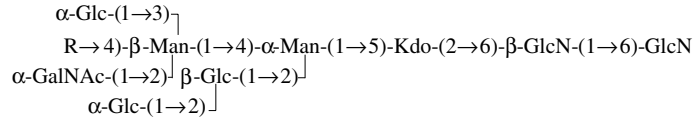
Abstract—*Francisella novicida* (U112), a close relative of the highly virulent bacterium *F. tularensis*, is known to produce a lipopolysaccharide that is significantly different in biological properties from the LPS of *F. tularensis*. Here we present the results of the structural analysis of the *F. novicida* LPS core part, which is found to be similar to that of *F. tularensis*, differing only by one additional α-Glc residue:



where R is an O-chain, linked via a β-bacillosamine (2,4-diamino-2,4,6-trideoxyglucose) residue. The lipid part of *F. novicida* LPS contains no phosphate substituent and apparently has a free reducing end, a feature also noted in *F. tularensis* LPS.

Keywords: Francisella; *Francisella novicida*; LPS; Core structure

1. Introduction

Francisella novicida (often referred to as *F. tularensis* biovar *novicida*) was originally isolated in 1951 and since then six additional human isolates have been reported.[1–4] Interest in the pathogenicity of *F. novicida* has intensified because of its close relationship with *F. tularensis*, a highly infectious and virulent intracellular bacterial pathogen of humans and animals that is considered to be a potential bioterrorism agent.[5] *F. novicida* is widely used as a model of *F. tularensis* for genetic work.

Clinically, *F. tularensis* subspecies *tularensis* (type A *F. tularensis*) followed by subspecies *holarctica* (type B *F. tularensis*) are the most virulent, with infections by the latter being the most common.[6] The lipopolysaccharides (LPS) of types A and B *F. tularensis* appear to be identical, and show very low biological activity, about 1000-fold less than that of *E. coli* LPS.[7,8] In contrast, the LPS of *F. novicida* is immunochemically distinct[9] and biologically more active.[9,10] Thus, *F. novicida* LPS stimulated a very modest in vitro proliferation of mouse splenocytes at high doses, but *F. tularensis* LVS (live vaccine strain) LPS did not. Murine bone marrow macrophages treated in vitro with *F. novicida* LPS produced IL12 and TNF-alpha, but did not produce detectable interferon-gamma, IL10, or nitric oxide; in contrast, murine macrophages treated with *F. tularensis* LVS LPS produced none of these mediators.

Recently, we reported the structure of the *F. novicida* LPS O-specific polysaccharide moiety.[11] Here we present

Abbreviations: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-octulosonic acid; BacN or QuiN4N, 2,4-diamino-2,4,6-trideoxyglucose; GalNA, galactosaminuronic acid; GalNAcAN, *N*-acetylgalactosaminuronamide; Δ-GalNA, 2,4-dideoxy-2-amino-β-L-*threo*-hex-4-eno-pyranoside; *P*, phosphate; HPAEC, high-performance anion-exchange chromatography.

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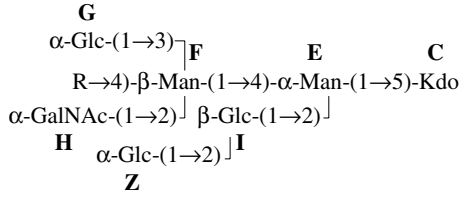
the results of the structural analysis of the core part of this LPS.

2. Results

The LPS of *F. novicida* yielded on mild acetic acid hydrolysis, apart from the polysaccharide fraction, several oligosaccharide products representing the unsubstituted core and the core with one and two repeating units (compounds **1**–**3**), respectively. The products were isolated by gel-chromatography and anion-exchange chromatography (which was important for the separation from β-glucan[12]) and analysed by NMR spectroscopy (Figs. 1 and 2) and ESIMS. Complete assignment of the 2D NMR spectra (Table 1) led to the determination of the structures presented in Chart 1. Monosaccharides were identified on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts. Anomeric configurations were deduced from the $J_{1,2}$ coupling constants and chemical shifts of H-1, C-1 and C-5 signals. The β-configuration of the mannose residue **F** was confirmed by the observation of strong intraresidual NOE between H-1 and H-3, H-1 and H-5. Connections between the monosaccharide residues were identified on the basis of NOE and HMBC correlations. The following NOEs were observed in product **3**: E1C5, E1C7, E1I1, F1E4, F1E6, I1E2, G1F2, G1F3, H1F2, H1G5, L1F4, L1F6, J1L3, K1J4, M1K4, L′1M4, J′1L′3, K′1J′4, M′1K′4. Respective correlations, where applicable, were also observed in the smaller oligosaccharides. All expected transglycoside HMBC correlations were identified, except for those discussed below.

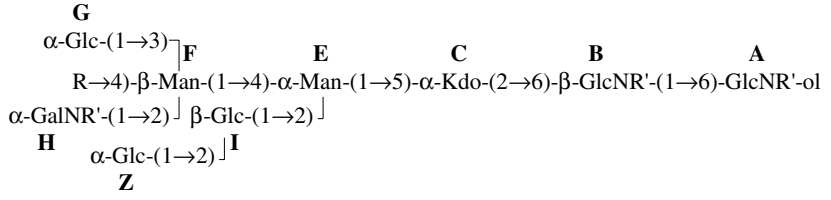


1 R = H

2 R = α-GalNAcAN-(1→4)-α-GalNAcAN-(1→4)-α-GalNAcAN-(1→3)-β-QuiNAc4NAc-(1→

3 R = α-GalNAcAN-(1→4)-α-GalNAcAN-(1→4)-α-GalNAcAN-(1→3)-α-QuiNAc4NAc-(1→

→4)-α-GalNAcAN-(1→4)-α-GalNAcAN-(1→4)-α-GalNAcAN-(1→3)-β-QuiNAc4NAc-(1→



4, R = H, R' = H

5, R = Δ-GalNA-(1→3)-β-QuiN4NAc-(1→, R' = H

6, R = Δ-GalNA-(1→3)-β-QuiNAc4NAc-(1→, R' = H

7, R = Δ-GalNAcA-(1→3)-β-QuiNAc4NAc-(1→, R' = Ac

β-GlcN-(1→6)-α-GlcN1*P* **8**

Chart 1.

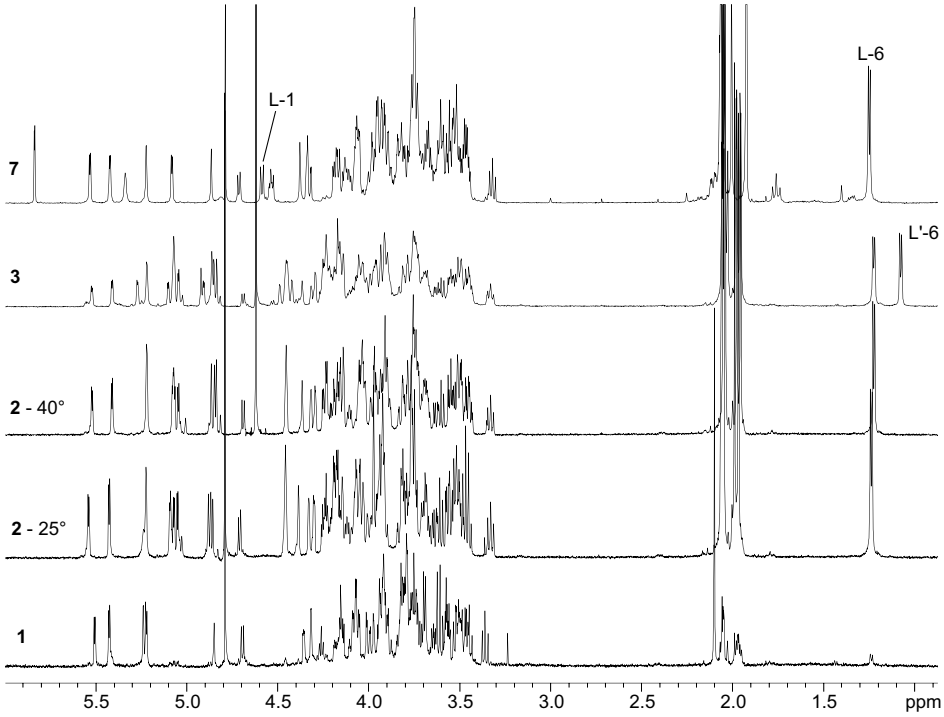


Figure 1. ^{1}H NMR spectra of compounds **1**–**3** and **7**. Spectra of **2** are shown at two different temperatures in order to illustrate that no anomeric signal is hidden under the HDO signal.

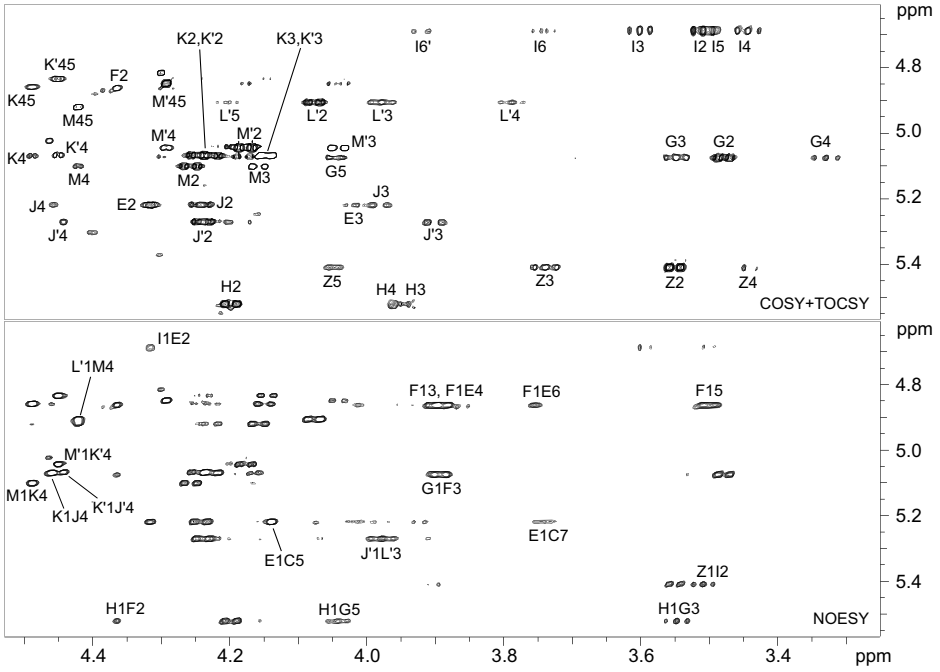


Figure 2. COSY and TOCSY (upper panel) and NOESY (lower panel) spectra of compound **3**. Intraresidual COSY and TOCSY correlations from H-1 are labelled with the second proton number only. On the NOESY panel, intraresidual correlations are not labelled except for Man F.

The signals of H-3 to H-5 of the bacillosamine residue L appeared very low and broad in the spectra of compounds **2** and **3**; the signals of H-1 and H-2 of this residue were not found at all, even in the one-dimensional ^{1}H NMR spectra (Fig. 1). Other signals of this monosaccharide were detected by TOCSY starting from the H-6 signal. The chemical shifts of C-4 and C-5 were determined from HMBC correlation between H-6 and

Table 1. NMR spectroscopy data for the isolated oligosaccharides (δ, ppm)

Residue, compound	Nucleus	1	2 (3ax)	3 (3eq)	4	5	6 (6a)	7 (6b)	8a	8b
Kdo C, **1**–**3**	^{1}H		1.92	2.00	4.16	4.14	3.92	3.74	3.63	3.80
	^{13}C		97.2	35.5	66.9	77.5	72.7	70.6	64.4	
Kdo C, **4**–**7**	^{1}H		1.76	2.11	4.18	4.13	3.74	3.84	3.68	3.94
	^{13}C		101.0	36.2	66.8	77.4	72.7	70.7	64.6	
Man E, **1**	^{1}H	5.24	4.36	4.08	3.94	4.01	3.79	3.79		
	^{13}C	99.5	77.0	69.4	77.9	72.9	60.9			
Man E, **2**,**3**	^{1}H	5.22	4.32	4.03	3.90	4.03	3.76	3.76		
	^{13}C	99.8	77.7	69.8	77.6	73.2	61.2			
Man E, **4**,**5**	^{1}H	5.22	4.34	4.06	3.89	3.99	3.76	3.76		
	^{13}C	99.7	77.3	69.6	77.3	73.0	60.9			
Man F, **1**,**4**	^{1}H	4.85	4.32	3.92	3.89	3.50	3.82	3.98		
	^{13}C	101.2	74.5	81.8	68.1	77.9	61.9			
Man F, **2**,**3**,**7**	^{1}H	4.86	4.37	3.90	4.16	3.50	3.69	3.95		
	^{13}C	100.8	73.1	83.3	72.3	77.2	61.1			
Glc G, **1**	^{1}H	5.22	3.56	3.63	3.36	3.79	3.74	3.91		
	^{13}C	101.7	72.8	74.1	71.0	74.2	62.1			
Glc G, **2**,**3**,**5**–**7**	^{1}H	5.08	3.48	3.55	3.33	4.04	3.70	3.91		
	^{13}C	103.4	73.3	75.1	71.5	74.0	62.6			
GalN H, **1**	^{1}H	5.51	4.15	4.06	4.01	4.26	3.70	3.70		
	^{13}C	97.2	51.3	68.2	69.5	71.7	61.9			
GalN H, **2**,**3**,**7**	^{1}H	5.52	4.20	3.95	3.97	4.11	3.69	3.74		
	^{13}C	95.8	51.3	69.2	69.9	72.7	62.7			
Glc I, **1**–**7**	^{1}H	4.69	3.52	3.61	3.45	3.51	3.75	3.93		
	^{13}C	101.8	77.7	75.7	71.0	77.0	62.0			
Glc Z, **1**–**7**	^{1}H	5.41	3.55	3.74	3.45	4.05	3.78	3.82		
	^{13}C	98.8	72.8	73.9	70.8	73.1	61.9			
BacN L, **2**	^{1}H			4.07	3.71	3.53	1.22			
	^{13}C			76.2	58.6	72.5	17.8			
BacN L, **3**	^{1}H			3.98	3.72	3.54	1.23			
	^{13}C				58.5	72.4	17.9			
BacN L, **5**	^{1}H	4.87	3.42	4.27	3.95	3.76	1.26			
	^{13}C	100.0	56.0	78.4	57.0	71.9	17.4			
BacN L, **6**,**7**	^{1}H	4.59	3.77	3.89	3.82	3.55	1.25			
	^{13}C	101.3	55.5	75.7	57.7	72.4	17.6			
BacN L′, **3**	^{1}H	4.91	4.08	3.98	3.79	4.20	1.07			
	^{13}C	99.6	53.9	75.4	58.2	68.8	17.6			
GalNA J, **2**	^{1}H	5.22	4.24	3.98	4.45	4.23				
	^{13}C	99.2	50.6	67.5	76.5	72.0				
Δ-GalNA J, **7**	^{1}H	5.34	3.92	4.33	5.84					
	^{13}C	98.1	53.0	64.2	110.4	144.9	170.0			
GalNA K, **2**	^{1}H	5.06	4.24	4.18	4.45	4.84				
	^{13}C	98.9	50.6	67.7	76.7	72.0	174.8			
GalNA M, **2**	^{1}H	5.04	4.18	4.04	4.30	4.85				
	^{13}C	99.1	50.7	68.4	70.1	72.7	175.7			

C-4, C-5 (the HSQC signals were of very low intensity and could not be reliably identified). The β-configuration of this monosaccharide was deduced from the low field shift of its C-5 signal ~72 ppm (compare 68.8 ppm for the α-anomer in **3**), and from the NMR data for the oligosaccharides **5**–**7**, where signals of H-1, H-2/C-1, C-2 were of normal intensity, although H-3, H-4/C-3, C-4 had low intensities. The reason for the low intensity of the signals can be a slow conformational exchange between distorted pyranose ring conformers or a fast local relaxation due to the limited mobility of the structural fragment. Distortion of the ring conformation seems to be a more probable explanation because no signal broadening is observed in the spectra of adjacent residues. The bacillosamine residue L is attached to O-4 of the 2,3,4-trisubstituted Man residue F and experiences sterical hindrances, which can lead to deformation of the $^{4}C_{1}$ chair conformation. A similar extreme broadening of the NMR signals due to conformational mobility was studied in detail for 2,4-diamino-2,4,6-trideoxygalactose.[13] Removal of the substituent from C-4 of GalNA residue J in compounds **5**–**7** led to an increased intensity of the BacN signals, probably due to decreased sterical crowding.

Deacylation of the LPS with 4 M KOH in the presence of $NaBH_4$ led to products **4**–**6**, isolated by HPAEC (last two as a mixture), that were analysed by NMR spectroscopy. Compound **4** was found to contain an N-deacylated core oligosaccharide, linked to the reduced lipid A backbone disaccharide. The LPS of *F. novicida*,

like that of *F. tularensis*,[12] contained no substituent at the reducing end of lipid A, and GlcN was therefore reduced to an alditol (some degradation of this monosaccharide, apparently epimerisation into 2-amino-2-deoxy-mannitol, was observed). The oligosaccharide contained only one unsubstituted at O-4 Kdo residue. Compounds **5** and **6** contained additionally a modified fragment of the O-chain polysaccharide. The rest of the O-chain polymer was lost due to β-elimination of the substituent from O-4 of the 4-substituted galactosaminuronamide J moiety, which was converted into β-L-*threo*-2,4-dideoxy-2-amino-hex-4-enuronic acid (Δ-GalNA). The *N*-acetyl group on the N-4 of BacN L was exceptionally stable and was not removed under the deacylation conditions; the acetyl group from N-2 of this monosaccharide was removed partially (~50%). In order to obtain a more uniform sample, the mixture of **5** and **6** was N-acetylated to give compound **7**.

NMR spectroscopy analysis of products **4**–**7** (Table 1) led to the detection of the lipid A backbone disaccharide, one Kdo, and all core monosaccharides found in products **1**–**3**. As mentioned above, compounds **5** and **6** differed by the presence or absence of the *N*-acetyl group at N-2 of the bacillosamine residue L. In these compounds, NOE and HMBC correlations from H-1 of the BacN L were clearly visible and confirmed its attachment to O-4 of the Man F residue.

ESI mass spectral data showed the mass for compound **1**: 1251.8 Da (expected 1252.1 for $C_{46}H_{77}O_{38}N_1$); **2**: 2129.6 Da (expected 2128.9 for $C_{80}H_{129}O_{57}N_9$); **3**: 3006.9 Da (expected 3005.7 for $C_{114}H_{181}O_{76}N_{17}$); **7**: 2087.6 Da (expected 2087.9 for $C_{80}H_{130}O_{57}N_6$) in agreement with the proposed structures.

Potassium hydroxide treatment of the LPS led additionally to compound **8**, which represents a disaccharide backbone of the lipid A with an α-glycosyl phosphate substituent at the reducing end. The same compound was obtained from *F. tularensis* LPS.[12]

LPS preparations of *F. novicida* also, like that of *F. tularensis*, contained various amounts of amylopectin and β-glucan:

β-D-Glc-(1 → [-6)-β-D-Glc-(1 →]$_n$-3)-Gro-(1-*P*-1)-Gro, where $n = 9$–15.

The latter compound contaminated most of the products derived from the LPS and its removal presented significant difficulties. On KOH treatment, it was partially converted into β-D-Glc-(1-[-6)-β-D-Glc-(1 →]$_n$-3)-Gro-(1-*P*.

3. Discussion

The LPS of *F. novicida* has many structural similarities to the LPS of *F. tularensis*.[12] Its lipid A carbohydrate backbone and Kdo region are identical; the core part differs by the presence of one additional glucose residue Z in *F. novicida* LPS. The polysaccharide chain is attached at the same position, with the anomeric configuration of the first monosaccharide linking PS to the core inverted compared to that present inside repeating units. Both LPSs possess an unusual lipid A structure lacking phosphate substituents in the major fraction and apparently have a reducing glucosamine (residue A) endgroup. In both LPS, a lipid A backbone disaccharide, unsubstituted by a core, with phosphate at the reducing end, was found after KOH degradation. Preparations of both LPSs contain the same additional glucan contaminants. Thus, it can be concluded at this stage that the distinctly different biological properties exhibited by each of these LPSs do not reside in the carbohydrate structures, but are possibly related to different acylation patterns present in the lipid A portion of the LPS, which is now under investigation.

4. Experimental

4.1. General methods

Bacteria were grown and LPS extracted as described previously.[11]

^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 600 spectrometer for D_2O solutions at 25 or 40 °C and referenced to the acetone standard (^{1}H, 2.225 ppm, ^{13}C, 31.5 ppm). Varian standard pulse sequences for COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 400 ms), HSQC and gHMBC (optimised for 5 Hz coupling constant) were used.

GC, GC–MS, ESIMS, were performed as previously described.[12]

Anion exchange chromatography was performed using a Hitrap Q column (5 mL, Amersham) in a gradient of water to 1 M NaCl over 1 h, with UV detection at 220 nm. Compounds were desalted by gel-chromatography on a Sephadex a G-15 column (1.6 × 80 cm).

4.2. Preparation of compounds 1–3

LPS (150 mg) was treated with 2% (v/v) AcOH (6 mL) at 100 °C for 2 h and, following removal of precipitated lipid A by low speed centrifugation, the concentrated water soluble products were fractionated by Sephadex G-50 chromatography to yield a void vol fraction of O-polysaccharide (74 mg), and several incompletely resolved oligosaccharide fractions. The oligosaccharides were purified by anion-exchange chromatography on a Hitrap Q column to give after desalting compounds **1**–**3** (6, 3 and 3 mg, respectively).

4.3. Preparation of compounds 3–8

The LPS (80 mg) was dissolved in 4 M KOH (4 mL) containing $NaBH_4$ (100 mg), kept overnight at 100 °C and neutralised with 2 M HCl. The precipitated material was removed by centrifugation and the soln was applied to a Sephadex G-50 column. The oligosaccharide fraction was further separated by HPAEC in a gradient of 0.1 M NaOH (A) to 1 M NaOAc in 0.1 M NaOH (B), 3–50% of B. Products were desalted on a Sephadex G-15 column to give oligosaccharide **4**, and a mixture of compounds **5** and **6**. The latter two compounds were N-acetylated (2 mL water, 0.1 mL Ac_2O, excess $NaHCO_3$, 1 h) to give after desalting oligosaccharide **7**. Compound **8** was eluted after the main oligosaccharide fraction from the Sephadex G-50 column and was additionally desalted on a Sephadex G-15 column and purified by HPAEC in the same conditions.

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