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Characterization 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*, was shown to produce a lipopolysaccharide in which the antigenic O-polysaccharide component was found by chemical, ^{1}H and ^{13}C NMR and MS analyses to be an unbranched neutral linear polymer of a repeating tetrasaccharide unit composed of 2-acetamido-2-deoxy-D-galacturonamide (D-GalNAcAN) and 2,4-diacetamido-2,4,6-trideoxy-D-glucose (D-Qui2NAc4NAc, di-*N*-acetylbacillosamine) residues (3:1) and had the structure:

$$\rightarrow 4)\text{-}\alpha\text{-D-GalNAcAN-}(1 \rightarrow 4)\text{-}\alpha\text{-D-GalNAcAN-}(1 \rightarrow 4)\text{-}\alpha\text{-D-GalNAcAN-}(1 \rightarrow 3)\text{-}\alpha\text{-D-QuiNAc4NAc-}(1 \rightarrow$$

With polyclonal murine antibody, the *F. novicida* O-antigen did not show serological cross-reactivity with the O-antigen of *F. tularensis* despite the occurrence of a common $\rightarrow$4)-D-Gal*p*NAcAN-(1$\rightarrow$4)-α-D-Gal*p*NAcAN-(1$\rightarrow$ disaccharide unit in their respective O-antigens. Thus, O-PS serology offers a practical way to distinguish between the two *Francisella* species.

1. Introduction

Francisella 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, and also considered to be a potential bioterrorism agent.[5]

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] Interestingly however, whereas the normally expressed lipopolysaccharides (LPS) of types A and B *F. tularensis* appear to be identical,[7,8] and biologically inert, the LPS of *F. novicida* is immunochemically distinct[9] and biologically more active.[9,10] Indeed, it is claimed that the enhanced biological activity of the latter triggers the host innate immune system to kill *F. novicida*, and this could account for its general avirulence for humans.[9] In support of this hypothesis, *F. tularensis* that undergoes a phase shift to express the *F. novicida* form of LPS is less virulent than the wild type.[9]

In an attempt to explain the distinct properties of the *F. novicida* LPS, we have begun a comprehensive chemical characterization of its O-antigen, core and lipid A components. Herein we present the results of the structural analysis of the O-chain of *F. novicida* lipopolysaccharide.

2. Experimental

Confluent lawns of *F. novicida* (U112) were harvested from cysteine heart agar plates containing 1% hemoglobin, following 24 h incubation at 37 °C and resuspended in 5% aq phenol for 4 h with stirring to kill it. The cells were then collected by centrifugation (5000g; 60 g wet

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weight paste) and extracted by stirring with 50% aq phenol (500 mL, 70 °C, 15 min). The separated phenol and aq layers from the cooled (4 °C) extract were dialyzed against tap water until phenol-free and were lyophilized. The respective residues were resuspended in 50 mL 0.02 M NaOAc, pH 7.0, and treated sequentially with RNase, DNase, and proteinase K (37 °C, 2 h each). Enzyme treated samples were subjected to ultracentrifugation (10,500g, 12 h, 4 °C) and the precipitated gels were dissolved in water and lyophilized to yield 580 mg (aq phase) and 120 mg (phenol phase) LPS.

LPSs from *F. novicida* and *F. tularensis* LVS were used as antigens in ELISA, dot-blot, and Western blot assays. Antisera from mice that had recovered from experimental infection with *F. tularensis* or *F. novicida* only reacted with the homologous LPS (data not shown).

Aqueous phase LPS (65 mg) was delipidated by treatment 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 volume fraction of O-polysaccharide (O-PS, 48 mg, K_{av} 0.03), a core oligosaccharide (3.1 mg, K_{av} 0.57) and a Kdo containing fraction (15 mg, K_{av} 0.97). Similar treatment of the phenol phase LPS (44 mg) yielded chemically identical O-PS (13 mg), and core (2.8 mg) to that obtained from the aq phase LPS.

^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 600 spectrometer in Me_2SO-d_6 solutions at 40 or 50 °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 200 ms), HSQC, and gHMBC (optimized for 5 Hz coupling constant) were used.

CE-ESIMS was performed as previously described.[11]

GC was performed using a HP1 column (30 m × 0.25 mm) in an Agilent 6850 chromatograph fitted with a flame ionization detector or, using a Varian Saturn 2000 ion-trap GC–MS instrument.

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 made at 220 nm.

HF treatment: Polysaccharide sample (10 mg) was dissolved in 100% HF (1 mL) in a polyethylene vial, kept at 20 °C for 30 min or 2.5 h, poured onto a polycarbonate Petri dish, to effect rapid HF evaporation, and the residue dissolved in water was neutralized with aq 25% ammonia and the polysaccharide isolated by Sephadex G-15 column chromatography.

3. Results and discussion

Lipopolysaccharide was prepared from cells of *F. novicida* (U112) grown on solid media by a modified hot aqueous phenol extraction method.[12] The LPS recovered from both the separated water and phenol layers, in a combined yield of 12% (based on dry weight cells), proved to be chemically identical. However, the initial aqueous phase product was contaminated by a mixture of amylopectin and (1 → 6)-β-D-glucan (ca. 8%), similar to the products previously found in preparations of *F. tularensis* LPS.[13] LPS was treated with 2% AcOH (100 °C, 2 h), lipid precipitate material removed by centrifugation, soluble products were separated on a Sephadex G-50 column to give a polysaccharide (O-PS) and several core fractions, differing by the presence of none, one, and two or more repeating units of the O-chain (structural analysis of the core oligosaccharide will be reported separately). Monosaccharide analysis of the polysaccharide revealed glucose (~2%) originating from starch like material produced by *F. novicida*, and a minor amount of mannose (~0.4%). Separation of the O-PS fraction on an anion-exchange column afforded several fractions which in order of elution were: starch, O-polysaccharide mixed with starch, pure O-PS (tail), and at the start of the salt gradient, a second fraction of the O-PS containing apparently short polysaccharide chains and larger proportions of the core oligosaccharide. This behavior of the O-PS on anion exchange chromatography indicated that it did not have a negative charge in the O-chain moiety, but was charged (probably due to Kdo) in the core moiety, and was thus to some extent retained on the anion-exchange column.

The NMR spectra of the O-PS (Fig. 1) showed that it is composed of a tetrasaccharide repeating unit composed of three *N*-acetyl-α-galactopyranosylaminouronic acid residues and one of 2,4-diacetamido-2,4,6-trideoxy-α-glucopyranose (bacillosamine) residue. The monosaccharides were identified on the basis of their vicinal proton coupling constants and ^{13}C NMR chemical shifts. The appearance of the C-2 signals of all residues at 50–54 ppm indicated that all the O-PS monosaccharides constituents have aminogroups at C-2. In addition, the 6-deoxy glycose (**D**) had a C-4 resonating at 58.1 ppm. Thus, residue **D** was a 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine) derivative. The residues **A**, **B**, and **C** in the HMBC spectrum showed correlations between H-5 and the signals around 175 ppm, thus these residues were derivatives of uronic acids. The anomeric configurations of the glycoses were deduced from their $J_{1,2}$ coupling constants and chemical shifts of H-1, C-1, and their C-5 signals. The glycosidic connections between monosaccharides were determined on the basis of interresidual NOE and HMBC correlations (Table 1).

In an attempt to prepare oligosaccharide fragments, the O-PS was treated with anhydrous HF (20 °C, 30 min or 2.5 h). However, this treatment resulted in the destruction of the minor starch contaminant and core components, but did not cleave the O-chain. The residual product after HF treatment was a pure polymer of the

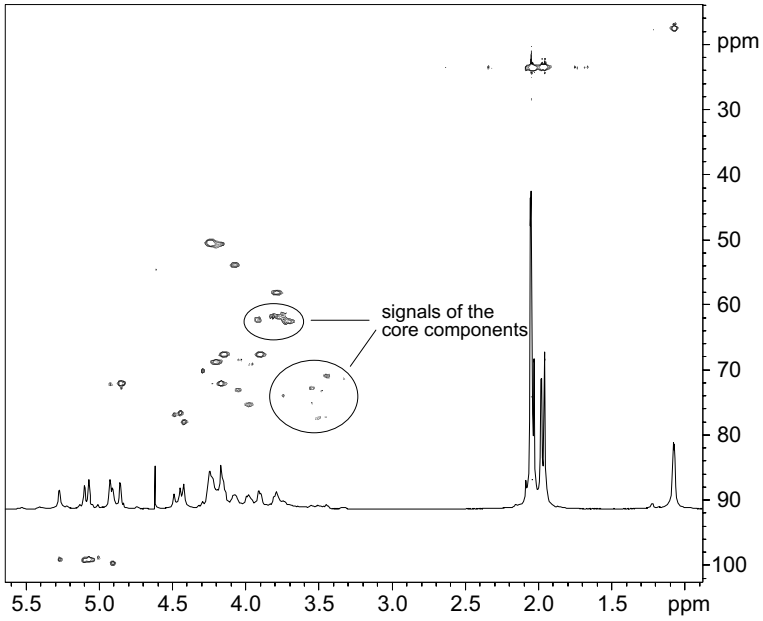

Figure 1. ^{1}H–^{13}C HSQC NMR correlation spectrum of *F. novicida* O-specific polysaccharide (D_2O, 40 °C).

Table 1. NMR data for the *F. novicida* O-specific polysaccharide (D_2O, 40 °C)

Unit, residue	Nucl.	1	2	3	4	5	6	Interresidual NOE from H-1	Interresidual HMBC from H-1
A, GalNAcAN	^{1}H	5.27	4.24	3.90	4.45	4.17		D3	D3
	^{13}C	99.1	50.6	67.7	76.6	72.1	174.4		
B, GalNAcAN	^{1}H	5.10	4.25	4.16	4.42	4.92		C3, C4, C5	C4
	^{13}C	99.1	50.6	67.6	78.0	72.1	174.5		
C, GalNAcAN	^{1}H	5.07	4.23	4.14	4.49	4.85		A3, A4, B3	A4
	^{13}C	99.1	50.6	67.6	76.9	72.0	175.2		
D, QuiNAc4NAc	^{1}H	4.90	4.08	3.98	3.79	4.20	1.08	B4	B4
	^{13}C	99.6	54.0	75.4	58.1	68.8	17.6		

repeating tetrasaccharide unit and the procedure became the method of choice for O-PS production. NMR analysis of the HF treated product was made in Me_2SO-d_6 solution in order to determine whether aminouronic acids were present in the amide form, as found in the case of *F. tularensis* O-PS.[13] Indeed, it was found that the spectra contained signals of amide protons for the NH_2 groups of uronamides present in all three of the aminouronic acids, as well as amide protons for the five acetamido groups and three hydroxyl groups (OH-3 of uronic acids) (Fig. 2). The NH_2-6 protons were identified on the basis of intramolecular NOE and TOCSY correlations (Table 2).

The O-PS had a specific optical rotation $[\alpha]_D$ +245° (*c* 0.8, water). Since the component monosaccharides could not be isolated, the direct determination of their absolute configurations was not possible. The value for the optical rotation of the glycosides of 2-acetamido-2-deoxy-α-galactopyranosyluronamide were not found in the literature, the closest related compounds described were benzyl *N*-carbobenzoxy-2-amino-2-deoxygalacturonopyranoside with $[\alpha]_D$ +140.2° (*c* 0.5, pyridine)[14] and benzyl 2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranoside with $[\alpha]_D$ +163° (*c* 0.6, MeOH).[15] From these data one can expect the optical rotation for *F. novicida* O-chain to be about +400° if all the monosaccharides had the D-configuration, or about +200° in case of three D- and one L-monosaccharide residues.

Attempts to deduce absolute configurations of the monosaccharides from the calculations of ^{13}C NMR chemical shifts[16] gave only limited success. For the fragment α-GalNAcAN-(1 → 3)-α-QuiNAc4Nac, calculations show that observed chemical shifts of the C-2 and C-4 of bacillosamine are possible only in a DD pair. The calculations for the remaining disaccharide fragments gave inconclusive results.

A number of NOE correlations from amide protons were used to determine the absolute configuration of the monosaccharide components. The structure of the polysaccharide chain was modeled using Insight II program using cvff force field. Energy minimization was

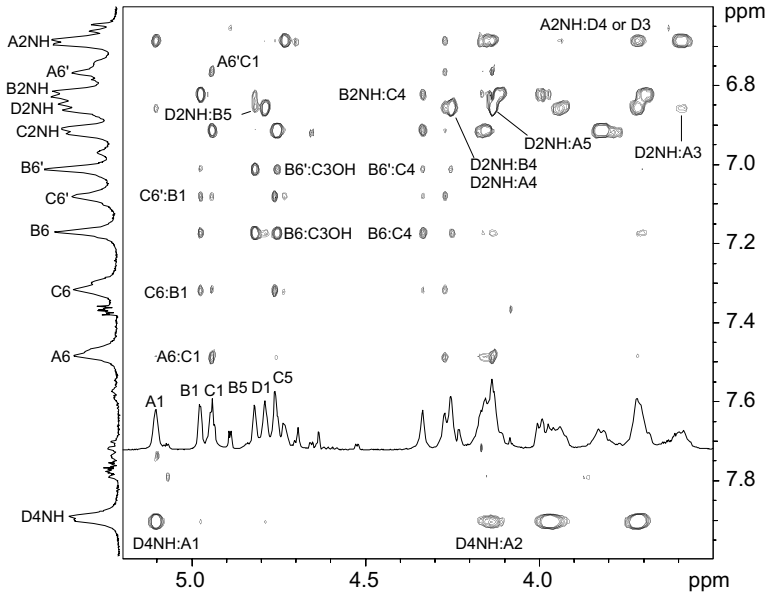

Figure 2. Fragment of the NOE spectrum of *F. novicida* O-specific polysaccharide (Me_2SO-d_6 50 °C), showing correlations from amide protons. Only interglycoside correlations are labeled.

Table 2. NMR data for the *F. novicida* O-specific polysaccharide (Me_2SO-d_6, 50 °C)

Unit, residue	Nucl.	1	2	2NAc	3	4	4NAc	5	6	6′
A, GalNAcAN	^{1}H	5.11	4.15	1.85	3.59	4.26		4.14	7.49	6.77
	^{13}C	99.5	50.6	24.6	68.3	76.3		72.4		
	^{1}H		6.69							
B, GalNAcAN	^{1}H	4.98	4.13	1.90	3.70	4.27		4.82	7.18	7.02
	^{13}C	99.8	51.3	24.6	69.8	80.4		72.7		
	^{1}H		6.82							
C, GalNAcAN	^{1}H	4.94	4.15	1.87	3.82	4.34		4.76	7.32	7.08
	^{13}C	99.4	50.7	24.4	68.5	77.6		72.7		
	^{1}H		6.92							
D, QuiNAc4NAc	^{1}H	4.79	3.94	1.86	3.72	3.72	1.76	3.97	1.05	
	^{13}C	101.0	54.5	24.6	75.4	57.9	24.3	69.9	19.0	
	^{1}H		6.86			7.90				

started from manually set glycoside linkage dihedral angles Φ(O-5–C-1–O–C′x) and Ψ[C-1–O–C′x–C-(x+1)] of ~60°, which is close to that normally observed for α-linkage. Any possible combination of the absolute configurations of the monosaccharides leads to extended stick-like minimum energy conformation of the repeating unit with no remote contacts between monosaccharide residues. For this reason, disaccharide models (DD and DL combinations of monosaccharides) were used for the modeling of interatomic distances. All observed NOEs from glycoside H-1 protons were much stronger than interglycoside correlations from amide protons, because distances between protons separated by many bonds experience much greater influence of rotation about these bonds. The observed interglycoside correlations and calculated values for minimal energy conformations are presented in Table 3. Although most of the distances differ insignificantly between DD and DL pairs, some differences are large and could not be compensated by rotation. For every pair of the monosaccharides, some observed contacts can be explained only for DD combination of the monosaccharides (marked with $ sign in Table 3). Thus we can conclude tentatively that all monosaccharides have identical absolute configurations, that is, D.

The structure of the O-PS was further confirmed by mass spectrometry. The O-PS was analyzed by ESI MS with a high orifice voltage, leading to the polymer fragmentation at the inlet. The resulting spectrum (Fig. 3) contained peaks of the mono- to tetrasaccharide fragments in complete agreement with the proposed structure:

Mass 216.2 216.2 216.2 228.2

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

[B] [C] [A] [D]

Table 3. Observed interglycoside NOEs in the *F. novicida* O-specific polysaccharide (Me_2SO-d_6, 50 °C), and calculated values for interatomic distances in minimal energy conformations, obtained by minimization of disaccharide models using Insight II—Discover 3 program with cvff force field

	1	DD	DL	2NH	DD	DL	2Ac	DD	DL	4NH	DD	DL	4Ac	DD	DL	6	DD	DL
D	B3s	4.1	4	A1w	4.8	4.8	A3w	5.6	3.9	A1ss	2.5	2.6	A1m	3.2	3.1	B1m$^{\$}$	3.5	8.2
	B4s	2.3	2.8	A5ss$^{\$}$	3.7	5.9	A4m	5	6.1	A2s	4.6	5.1				B2s$^{\$}$	3.5	7
	B6s	5.1	5.5	B4ss	4.4	5.4	B6m$^{\$}$	2.9	6.3							B4m	5	3.8
																A1s	5.8	5.7
B	C3m	4	3.9	C4m	4	5.5	C6m$^{\$}$	3.7	6.6							C4m	6.2	4.4
	C4s	2.3	2.6													C3OHs	2.3	2.1
	C5m	4.6	4.8													A6m*		
C	A3m	4	3.9	A6	3.1	5.7	A6s$^{\$}$	3.7	6.6							A4w	6.2	4.4
	A4s	2.3	2.6													B1w	5.1	5.6
A	D3s	2.4	2.3	D4s$^{\$}$	3	6										C1s	5.1	5.6
																B6m*$^{\$}$		

ss, very strong, s, strong, m, medium, w, weak; intensities cannot be compared between correlations from H-1 and from other protons.
$^{\$}$signs mark contacts where large differences in distance between DD and LD pairs are expected.
*A6B6 distance: DDD 6.8, DLD 8.8, DDL 11.1.

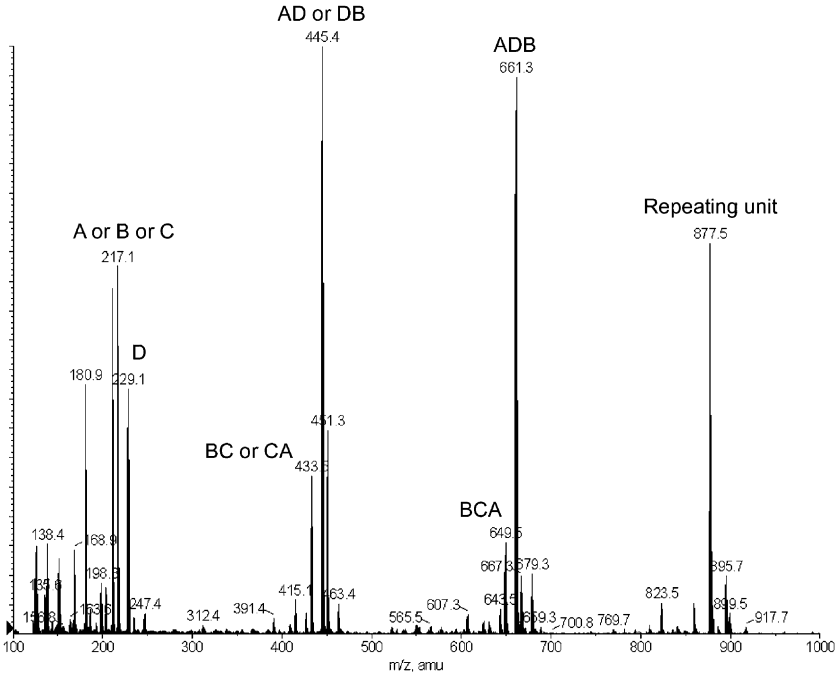

Figure 3. ESI MS spectrum of the O-specific polysaccharide from *F. novicida*. Labels refer to monosaccharide units in the polymer. Observed signals correspond to $[M\text{-}H_2O+1]^+$.

The O-PS produced by *F. novicida* was clearly structurally distinct from the previously identified *F. tularensis* O-PS.[13,17] In a variety of immunochemical assays using polyclonal murine antisera from mice that survived infection with *F. novicida* or *F. tularensis*, the respective LPSs failed to show any cross-serological binding activity despite the fact that both O-PSs share a common →4)-D-Gal*p*NAcAN-(1→4)-α-D-Gal*p*NAcAN-(1→ disaccharide component within their respective tetrasaccharide repeating units. This failure of cross-serological activity was also noted with LPS derived from *Pseudomonas aeruginosa* Fisher

F. tularensis O-PS

→4)-α-D-GalNAcAN-(1→4)-α-D-GalNAcAN-(1→3)-β-D-QuiNAc-(1→2)-β-D-Qui4NFo-(1→

F. novicida O-PS

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

P. aeruginosa Fisher immunotype 1

OAc
|3
→4)-α-D-GalNAcAN-(1→4)-α-D-GalNFoAN-(1→3)-α-D-QuiNAc-(1→2)-α-L-Rha-(1→

S. dysenteriae O7 O-PS

OAc AcGly
|3 |4
→4)-α-D-GalNAcAN-(1→4)-α-D-GalNAcA-(1→3)-α-D-GlcNAc-(1→3)-β-D-Qui4N-(1→

Figure 4. Comparative structures for *F. novicida* and related bacterial O-PS's.

immunotype 1[17], and *Shigella dysenteriae* O7 serotype,[18] which also possess the aforementioned common disaccharide (Fig. 4).

The observed lack of serological cross-reactivity remains to be explained. While the present work provides an understanding of the structural difference between the O-antigens in the LPS of *F. novicida* and *F. tularensis*, the reported differences in biological properties of these two molecules is more likely to reside in the nature of their lipid A components. The structures of the respective lipid A and cores of the two *Francisella* species and their relation to biological activity is being studied.

Acknowledgements

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