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Structure of the LPS O-chain from *Fusobacterium nucleatum* strain 10953, containing sialic acid

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Abstract

Fusobacterium nucleatum is an anaerobic bacterium found in the human mouth where it causes periodontitis. Recently, it has been gaining attention as a potential causative agent for colorectal cancer and is strongly linked with pregnancy complications including pre-term and still births. Little is known about virulence factors of this organism and thus we have initiated studies to examine the bacterial surface glycochemistry. Consistent with a recent paper suggesting that *F. nucleatum* strain 10593 can synthesize sialic acid, a staining technique identified sialic acid on the bacterial surface. We isolated lipopolysaccharide from this *F. nucleatum* strain and performed structural analysis on the O-antigen. Our studies identified a trisaccharide repeating unit of the O-antigen with the following structure:

where Ac indicates 4-N-acetylation of ~30% FucNAc4N residues.

The presence of sialic acid as a constituent of the O-antigen is consistent with recent data identifying *de novo* sialic acid synthesis in this strain.

Keywords

Fusobacterium nucleatum; Lipopolysaccharide; Sialic acid

1. Introduction

Fusobacterium nucleatum is a Gram-negative anaerobic oral bacterium associated with periodontitis, a common inflammatory disease of the gums and tooth supporting apparatus that also affects systemic health in humans. *F. nucleatum* acts as a bridge bacterium for the early and late colonizing bacteria of the dental plaque. *In vivo*, *F. nucleatum* together with the oral pathogen *Tannerella forsythia* dominate the intermediate layers of the mature subgingival biofilms [1], and synergistically enhances alveolar bone loss when co-infected

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with periodontal pathogens in mice [2, 3]. While *F. nucleatum* is normally recognized as a dental pathogen, it is now being increasingly found associated with a wide spectrum of human diseases including colorectal cancer (CRC) and adverse pregnancy outcomes [4], and thus is considered an opportunistic pathogen. *F. nucleatum* has been identified as a driver of colorectal tumorigenesis, initiating signaling events that stimulate cancer growth [5, 6]. In human pregnancies, *F. nucleatum* is a predominant species detected in amniotic fluid and fetal membrane associated with preterm birth [4].

F. nucleatum has been classified into 5 subspecies: *animalis*, *nucleatum*, *polymorphum*, *vincentii* and *fusiforme*, though recent analysis has suggested that the *fusiforme* and *vincentii* subspecies be classified into a single subspecies [7]. Little is known about virulence factors of *F. nucleatum*, however the adhesins Fap2 [8] and FadA [6] may play a pro-inflammatory role in early tumorigenesis. *F. nucleatum*, being a Gram-negative bacterium, elaborates a lipopolysaccharide (LPS), which includes an O-antigen polysaccharide, a core oligosaccharide and lipid A. It was shown that serum antibody levels to *F. nucleatum* LPS did correlate to poor oral health and the degree of periodontitis [9]. There are thought to be many serovars of oral *F. nucleatum* strains, with up to 19 serovars suggested in an earlier immunochemical study [10], and 6 chemotypes identified based on sugar compositions of LPS isolated from 20 strains [11]. Limited structural information is available on the LPS of *F. nucleatum* from several preliminary studies restricted to determination of chemical composition of lipid A [12] and carbohydrate components of the LPS [13–15]. We have been undertaking a study to establish O-antigen structures of the *F. nucleatum* LPS in order to develop a better understanding of the surface glycochemistry of this emerging pathogen and recently established the O-antigen repeating unit structure for *F. nucleatum* subspecies *nucleatum* strain 25586 [16]. In this study, we performed structural characterization of the O-antigen of *F. nucleatum* subspecies *polymorphum* strain 10953 and identified sialic acid as a component of the O-antigen repeat, consistent with the recent identification of de novo sialic acid biosynthesis in this strain [17].

2. Results and discussion

By genome mining we previously suggested that sialic acid metabolic pathways might exist in many oral bacteria, including *F. nucleatum* [18]. Specifically, we were interested in determining if this opportunistic oral pathogen decorates its cell surface with sialic acid, which may provide the bacterium a unique advantage to interact with oral microbiota as well as the host immune system. We detected sialic acid on the surface of *F. nucleatum* strain 10953 by a specific staining technique that uses mild periodate oxidation of sialic acid followed by aniline-catalyzed oxime ligation and detection protocol described in the Experimental section. This method detected a significant proportion of cells positive for sialic acid in a *F. nucleatum* 10953 culture (Fig. 1).

The finding that some but not all cells expressed sialic acid was intriguing, but nevertheless, these initial experiments suggested that *F. nucleatum* 10953 could indeed sialylate its cell surface. Staining shows that probably not all cells produce LPS O-chain. The current study was therefore undertaken to determine if LPS, a molecule that undergoes sialylation modification in other pathogens, could be sialylated in strain 10953. For this purpose, *F.*

nucleatum strain 10953 cells were grown and LPS was extracted with hot aqueous phenol. LPS was hydrolyzed with 1% AcOH and the polysaccharide O-antigen was isolated by GPC on Sephadex G-50. The attribution of the polysaccharide as a part of LPS followed from the facts that LPS was precipitated upon ultracentrifugation and produced typical polysaccharide, core and lipid A fractions upon mild acid hydrolysis.

Monosaccharide analysis by GC-MS of the alditol acetates showed the presence of galactose. GC-MS of the acetylated products of methanolysis indicated additionally the presence of a sialic acid. Absolute configuration of galactose was determined as D by GC-MS of the acetylated glycosides with chiral 2-butanol.

2D NMR spectra (^1H - ^1H gCOSY, ^1H - ^1H TOCSY, ^1H - ^1H NOESY, ^1H - ^{13}C HSQC (Fig. 2), ^1H - ^{13}C HMBC) of the polysaccharide contained main spin systems for three monosaccharides, which were identified by characteristic NMR chemical shifts and correlation patterns. They were α -N-acetylneuraminic acid (α -Neu5Ac, N), β -galactopyranose (B), and 2-acetamido-4-amino-2,4,6-trideoxy- α -galactopyranose (α -FucNAc4N, A) together with minor variants of A and B as well as β -Neu5Ac due to partial hydrolysis of the glycosidic linkages of α -Neu5Ac N. Another reason for the heterogeneity was partial 4-N-acetylation of α -FucNAc4N A, which caused a significant downfield shift of the H-4 signal (Table 1). The assignment of the NMR signals of both variants of A was confirmed by a comparison with published data for β -D-GlcP-(1 $\rightarrow$ 3)- α -D-FucpNAc4N [19] and β -D-GlcP-(1 $\rightarrow$ 3)- α -D-FucpNAc4NAc [20] (a large difference between H-5 chemical shifts of this monosaccharide, $\sim$ 4.70 ppm in both published papers and 4.17 ppm in FucpNAc4N or 4.06 ppm in FucNAc4NAc in this work, remains without explanation). ^{13}C NMR chemical shifts of C-2, C-3, and C-4 of α -FucNAc4N and C-1 of β -Gal that is linked to O-3 of FucNAc4N, were close to the published values [19, 20] and supported the D configuration of FucNAc4N.

A1:N3eq,4 and B1:A3 NOE correlations as well as the corresponding ^1H - ^{13}C HMBC correlations, A1(H):N4(C) and B1(H):A3(C), indicated the A1:N4 and B1:A3 linkages. The N2:B4 linkage followed from the HMBC correlation between C-2 of N to H-4 of B. Taken together these data indicate the following structure of the polysaccharide repeating unit:

where Ac indicates 4-N-acetylation of $\sim$ 30% FucNAc4N residues.

The structure was confirmed by positive ion mode high orifice voltage mass spectrum of the polysaccharide [21], which showed ions for the repeating unit at m/z 639.9 (calculated 639.25 Da for $\text{C}_{25}\text{H}_{41}\text{N}_3\text{O}_{16}$) and for a variant with FucNAc4NAc at m/z 682.2 (Fig. 3).

While the specific role of sialic acid in *F. nucleatum* LPS remains to be determined, sialylated LPS may have a significant role at the immune interface. Sialylation can block serum complement attack [22, 23], cause immuno-suppression through activation of sialic acid binding immunoglobulin-like receptors (Siglecs) [24], and help avoiding host immune

surveillance through glycomimicry [25]. Specifically, with respect to dental plaque development, LPS sialylation in fusobacteria might have a role in the ecology and development of dental biofilms as well. Sialic acid can be catabolized as a source of carbon and growth factor by cohabiting species of the dental plaque. In this regard, the periodontal pathogen *T. forsythia*, which is found in close association with *F. nucleatum* in dental plaque [1], could harvest sialic acid from *F. nucleatum* LPS. Our previous studies have indicated that utilization of sialic acid from host and cohabiting bacterial partners such as fusobacteria likely plays a role in the survival of *T. forsythia* in the oral environment [26]. The identification of FucNAc4N is of interest as this monosaccharide is often found, as it is here, as a component of zwitterionic polysaccharides that may have biological implications [27, 28].

3. Experimental

3.1. Isolation of lipopolysaccharide and O-antigen

Bacteria were grown as described [16]. Bacterial cells (71 g wet wt) were lyophilized and washed sequentially with ethanol (2×150 mL), acetone (2×150 mL) and ether (2×150 mL), pelleting the cells between each wash ($8000 \text{ rpm} \times 15 \text{ min}$) resulting in 35 g of defatted cells. LPS (460 mg) was extracted from the washed cells with aqueous 45% phenol and purified as described [16].

A LPS sample (100 mg) was hydrolyzed with 1% AcOH (8 mL) at 100°C for 1.5 h and the polysaccharide O-antigen was purified by GPC on a Sephadex G-50 column ($60 \times 2.5 \text{ cm}$) in 1% acetic acid, monitored using a Gilson refractive index detector to give early (31 mg), middle (16 mg) and late (7 mg) eluting fractions.

3.2. Sialic acid staining

F. nucleatum 10953 cells were analyzed for surface sialic expression by adopting a previously described method for living cells [29]. Bacterial cells harvested from agar plates were washed twice with phosphate buffered saline (PBS) and then resuspended ($D_{600} = 0.2$) in 0.5 mL of 1 mM sodium periodate in PBS. After incubation at 4°C for 30 min cells were pelleted, washed twice with 1 mL PBS and resuspended in 0.2 mL of 100 μM aminooxy-biotin and 10 mM aniline in PBS, followed by rocking at 4°C for 90 min. Bacteria were washed twice with PBS and stained by incubating with streptavidin conjugated Texas red solution (0.5 mL of 2 $\mu\text{g/mL}$ for 30 min at 37°C). After washing twice with PBS, bacteria were mounted on a slide under a coverslip with mounting medium that contains 4',6-diamidino-2-phenylindole (DAPI) and visualized with a fluorescent microscope. The phase contrast and fluorescence images were recorded using Zeiss Axio Observer and Axio Imager wide-field fluorescence microscope (Carl Zeiss) at the University at Buffalo Confocal and Imaging Core Facility. Texas-red signal was collected with the filter set ex560/40 and em630/75, and DAPI signal with the filter set ex365/50 and em445/50. Differential interference contrast filter was used for phase contrast. Phase contrast and fluorescence images were obtained from the same area and matched using Axio Vision software (release 4.8). All image data were taken at $600 \times$ magnification.

3.3. Chemical methods

A polysaccharide sample (0.5 mg) with inositol added as internal standard was hydrolyzed with 3 M TFA (120 °C, 3 h), dried, reduced with NaBD₄, the reagent was destroyed with 0.5 mL of AcOH, the solution dried by stream of air, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL acetanhydride in 0.4 mL pyridine for 30 min at 100 °C, dried, and analyzed by GC-MS (Varian Saturn 2000 ion-trap instrument, capillary column DB-17, 160–260 °C at 4°/min).

A polysaccharide sample (0.5 mg) in methanol (1 mL) was cooled with dry ice and 0.1 mL of acetyl chloride was added, the mixture was heated at 90 °C for 3 h, dried, acetylated, and analyzed as described above.

For determination of the absolute configuration, a polysaccharide sample (0.2 mg), (*R*)-2-butanol (0.2 mL) and acetyl chloride (0.02 mL) were mixed at 20 °C, heated at 90 °C for 3 h, dried by stream of air, acetylated, and analyzed as described above. Reference compounds were prepared from D-galactose by the same procedure using (*R*)- and (*S*)-2-butanol.

3.4. NMR spectroscopy and mass spectrometry

NMR experiments were carried out on a Varian INOVA 500 MHz (¹H) spectrometer with 3 mm gradient probe at 25 °C with acetone as internal reference (2.225 ppm for ¹H and 31.45 ppm for ¹³C), using standard pulse sequences gCOSY (gradient COrelation SpectroscopY), TOCSY (Total Correlation Spectroscopy) (mixing time 120 ms), NOESY (Nuclear Overhauser Effect Spectroscopy) (mixing time 300 ms), gHSQC (gradient Heteronuclear Single-Quantum Coherence), and gHMBC (gradient Heteronuclear Multiple-Bond Correlation) (100 ms long range transfer delay). Acquisition time was ~0.8 s for ¹H-¹H correlations and 0.25 s for HSQC. 256 increments were acquired for t₁ in all 2D spectra, except for 512 increments in gCOSY.

Positive ion mode high orifice voltage mass spectrometry was carried out as described [21].

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Fig. 1.

Analysis of surface sialylation in *F. nucleatum* 10953. Plate grown bacterial cells were stained by mild periodate oxidation followed by aniline-catalyzed aminoxy-biotin labeling and detection with streptavidin conjugated Texas-red. Bacteria were mounted on slides with DAPI (blue) mounting medium and visualized with a fluorescent microscope. Sialic acid positive cells are shown as red-pink as opposed to the sialic acid negative blue stained cells. Phase contrast and fluorescence images were obtained and matched using Axio Vision software as described in the Experimental section. All image data were taken at $600\times$ magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.
 ^1H - ^{13}C HSQC spectrum of the *F. nucleatum* 10953 polysaccharide.

Fig. 3. MS/MS spectrum of the *F. nucleatum* 10953 polysaccharide. All ions are $[M-H_2O+H]^+$. RU indicates repeating unit.

Table 1
NMR data for *F. nucleatum* 10953 polysaccharide (δ , ppm). Spectra were recorded at 25 °C.

Residue	H/C-1	H/C-2	H/C-3 (3ax, 3eq)	H/C-4	H/C-5	H/C-6 (6a, 6b)	H/C-7	H/C-8	H/C-9
α -FucpNAc4N A	5.03	4.23	4.11	3.93	4.17	1.31			
	95.4	48.8	75.8	56.3	64.1	16.8			
α -FucpNAc4NAc	5.00	4.33	3.97	4.47	4.06	1.11			
	95.4	49.6	77.5	54.4	67.3	16.8			
α -Neup5Ac N			1.77, 2.82	3.69	4.04	3.71	3.62	3.85	3.63, 3.86
	173.8	101.9	37.6	75.7	50.4	74.0	69.0	73.0	63.9
β -Galp B	4.50	3.52	3.60	4.49	3.70	3.64, 3.73			
	106.2	72.2	72.9	73.4	76.3	62.1			

Signals for Me groups of the NAc groups were at δ H/ δ C 2.02/23.3 and 2.06/23.3.