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Note

Structural analysis of the lipooligosaccharide-derived oligosaccharide of *Histophilus somni* (*Haemophilus somnus*) strain 8025

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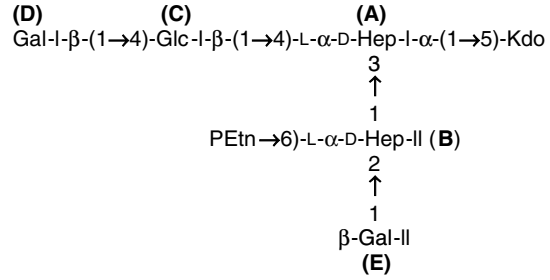
Abstract—Previous structural studies in our laboratory on lipooligosaccharide (LOS) inner core oligosaccharide (OS) had identified structures from several strains of *Histophilus* (*Haemophilus*) *somni* (738, 2336, 1P, 129Pt). Recently a type strain 8025 was proposed for this species and we therefore sought to determine the core OS structure of this *H. somni* strain. Core OS was isolated by standard methods from Westphal purified LOS. Structural information was established by a combination of monosaccharide and methylation analyses, NMR spectroscopy and mass spectrometry. The following structure for the core OS was determined on the basis of the combined data from these experiments:



The structure determined contains aspects of other *Histophilus somni* core OS structures, such as the β-Gal attached at the 2-position of Hep II (2336), PEtn only at the 6-position of Hep II (738, 129Pt) and a lactose extension from Hep I (1P). Since genetic manipulation has been achieved with this strain, the identification of the core OS structure will enable experiments designed to identify the role of glycosyltransferases involved in LOS biosynthesis.

Keywords: *Histophilus somni* (*Haemophilus somnus*); LOS; Core oligosaccharide; Mass spectrometry; NMR spectroscopy

Histophilus somni (*Haemophilus somnus*) (*Hs*) is a Gram-negative bacterium that causes significant economic loss to the beef and dairy cattle industries.[1] *Hs* may be isolated as a commensal or pathogen from the genito-urinary or respiratory tracts of cattle.

Detailed structural analysis of *Hs* LOS has so far been restricted to two related pathogenic strains termed 738 and 2336[2,3] and two commensal strains termed 1P and 129Pt.[4,5] This study was undertaken in order to determine the chemical structure of the LOS of the newly

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designated type strain 8025,[6] which was isolated in the USA from a bovine brain with lesions of thromboembolic meningoencephalitis. This strain was one of the first strains of *Hs* isolated and as such has been passed extensively in the laboratory.

LOS from *Hs* strain 8025 was extracted by the aqueous phenol method from cells grown from broth culture. GLC–MS analysis of the derived alditol acetates from LOS identified glucose (Glc), galactose (Gal), 2-amino-2-deoxy-glucose (GlcN) and/or its *N*-acetyl derivative and L-*glycero*-D-*manno*-heptose (Hep). The amount of glucitol identified was consistent with the presence of a glucan. The relative intensities of the alditol acetates would suggest that the amount of glucose present constituted approximately 75% of the LOS sample.

O-Deacylated LOS (LOS-OH) was prepared and analysed by CE–ESIMS in the negative-ion mode (Table 1). Major peaks were observed at *m*/*z* 2224.6 and 2387.6 in a ratio of 1:2 from both the LOS- and 8K-derived LOS-OH, which is consistent with a composition of 2Hex, 2Hep, PEtn, 2 Kdo, Lipid A-OH for the smallest glycoform with an additional Hex residue for the larger glycoform (where Lipid A-OH refers to the O-deacylated lipid A molecule). CE–MS/MS analysis (data not shown) confirmed the size of the O-deacylated lipid A as 952 amu for each glycoform. The O-deacylated lipid A basal species (952 amu) consists of a disaccharide of N-acylated (3-OH C 14:0) glucosamine residues, each residue being substituted with a phosphate group. The core oligosaccharide was prepared and fractionated by gel-filtration chromatography. Early eluting fractions were found to contain a 4-linked glucan as determined by methylation analysis. Fractions were analysed by CE–MS. Consistent with the LOS-OH MS data, simple mass spectra were observed that were indicative of the presence of 2Hex- and 3Hex-containing glycoforms (Table 1). CE–MS/MS analysis (data not shown) confirmed the PEtn residue to be present on the distal heptose residue using the same techniques as described previously.[2,3]

Methylation analysis of a core OS fraction free from glucan suggested the presence of a terminal Gal, a 4-linked Glc and a 3,4-linked Hep residues. The identification of only one heptose residue in the methylation analysis corroborated the CE–MS/MS data that one of the heptose residues was substituted with a PEtn residue, and the presence of the 3,4-linked Hep residue (commonly encountered in *H. somni*, *Neisseria meningitidis* and *Haemophilus influenzae* as the proximal heptose) was consistent with the distal heptose residue bearing the PEtn moiety.

In order to completely characterise the LOS structure, NMR spectroscopy was performed on a glucan-free core OS fraction (~2 mg). The assignment of ^{1}H resonances of the sugars of the *Hs* strain 8025 OS was achieved by TOCSY (Fig. 1) and COSY experiments (Table 2) and by comparison with reported data for *Hs* strains 738,[2] 2336,[3] 1P[4] and 129Pt.[5] The ring sizes and relative stereochemistries of the component monosaccharides were established from the ^{1}H chemical shifts and the magnitude of the coupling constants.[7]

The region of the ^{1}H NMR spectrum (5.00–5.60 ppm) of the OS downfield of the HOD signal revealed two

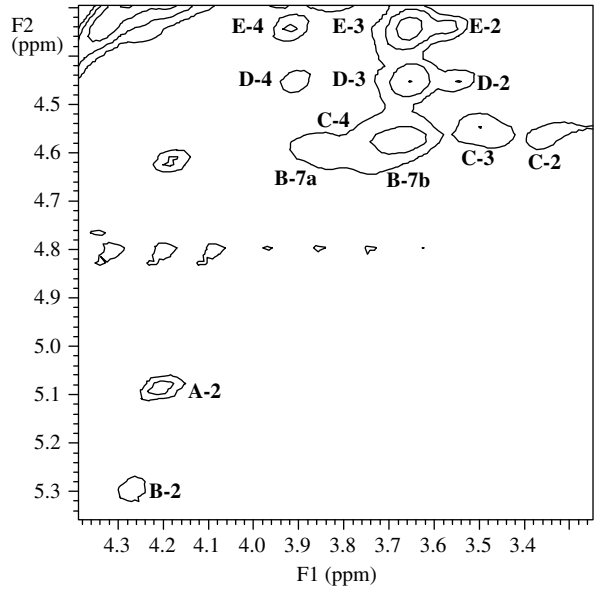

Figure 1. Region of the 2D TOCSY ^{1}H NMR spectrum of the core OS from *Hs* strain 8025. The spectrum was recorded at 25 °C in D_2O at pH 7.0. Letter designations are as in Table 2.

Table 1. Negative-ion CE–ESIMS data and proposed compositions of O-deacylated LOS and core OS from *H. somni* strain 8025

Preparation	Observed ions (*m*/*z*)		Molecular mass (Da)		Relative intensity	Proposed composition
	$(M-2H)^{2-}$	$(M-H)^{3-}$	Observed	Calculated		
8025						
O-Deacylated	1111.3	740.3	2224.6	2224.1	0.50	2Hex, 2Hep, PEtn, 2Kdo, Lipid A-OH[a]
LOS	1192.8	794.8	2387.6	2386.2	1.00	3Hex, 2Hep, PEtn, 2Kdo, Lipid A-OH
Core OS	533.8	—	1069.6	1069.9	0.75	2Hex, 2Hep, PEtn, Kdo
	615.0	—	1232.0	1232.0	1.00	3Hex, 2Hep, PEtn, Kdo

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; Kdo, 220.18; PEtn, 123.05. O-Deacylated lipid A (Lipid A-OH) is 952.00.

Table 2. ^{1}H NMR chemical shifts[a] for the core OS from *H. somni* strain 8025

	H-1	H-2	H-3	H-4	H-5	H-6	H-7	Inter-residue NOE's (from anomeric protons)
Kdo	—	—	2.08 1.92	4.10	4.21	nd	nd	
Hep I (**A**)[b]	5.09	4.21	4.09	4.12	nd	nd	nd	4.21 Kdo H-5
Hep II (**B**)	5.30	4.28	3.83	nd	nd	4.60	3.84 3.75	4.09 Hep I H-3, 4.35 Gal II H-1
β-Glc I (**C**)	4.58	3.37	3.48	3.69	nd	nd	—	4.12 Hep I H-4
β-Gal I (**D**)	4.46	3.57	3.65	3.91	nd	nd	—	3.69 Glc I H-4
β-Gal II (**E**)	4.35	3.59	3.66	3.92	nd	nd	—	4.28 Hep II H-2, 5.30 Hep II H1
PEtn	4.16	3.30						

[a] Chemical shifts are given in parts per million relative to internal acetone at 2.225 ppm.
[b] Letter designation used for residues in figures is as indicated in parentheses.

major signals at 5.30 (**B**) and 5.09 ppm (**A**) were attributed to the H-1 protons of heptose (Hep) residues due to their small $J_{1,2}$ (<2 Hz) and $J_{2,3}$ (~3 Hz) coupling constant values, which pointed to mannopyranosyl ring systems. The α-configurations for both of these residues were evident from the occurrence of a single residue NOE between the H-1 and H-2 resonances[8] coupled with the absence of H-1/H-3 and H-1/H-5 intraresidue NOE's. The remaining signals in the low-field region upfield of the HOD signal were characterised by a TOCSY experiment (Fig. 1). Characteristic spin systems for two β-galactose residues (**D** and **E**) and a β-glucose residue (**C**) were observed from the anomeric proton resonances at 4.46, 4.35 and 4.58 ppm, respectively. The intensity of residues (**D** and **E**) the two terminal galactose residues in the TOCSY spectrum (Fig. 1) would suggest that residue (**D**) is non-stoichiometrically attached. Other resonances in this region of the spectrum included the H-6 signals for heptose residue (**B**) at 4.60 ppm, identified by virtue of a spin system including the H-7a, H-7b signals at 3.84 and 3.75 ppm. The downfield chemical shift for the ^{1}H-resonance of **B-6** is consistent with phosphorylation at this location (see below).

The sequence of glycosyl residues of the core OS was determined from inter-residue ^{1}H–^{1}H NOE measurements between anomeric and aglyconic protons on adjacent glycosyl residues. The occurrence of an inter-residue NOE between H-1 of the Gal II residue (**E**) at 4.35 ppm and the distal heptose residue (**B**) at the 2-position as evidenced by the characteristic H-1 to H-1 NOEs was of interest as this arrangement has recently been observed in the core OS of *Hs* strain 2336.[3] Hep II residue (**B**) was found to be substituting the proximal heptose residue (**A**) at the 3-position as has been observed previously in *Hs*.[2–5] **A** is also substituted at the 4-position by Glc I (**C**) as indicated by characteristic NOEs from **C** to the H-4 and H-6 resonances of **A**. **A** in turn substitutes Kdo at the 5-position as evidenced by a characteristic NOE to the H-5 resonance of Kdo.

The extension from the proximal heptose residue **A** was found to consist of typical linkages for a lactose unit as in strain 1P by virtue of characteristic inter-residue NOE's (Table 2).

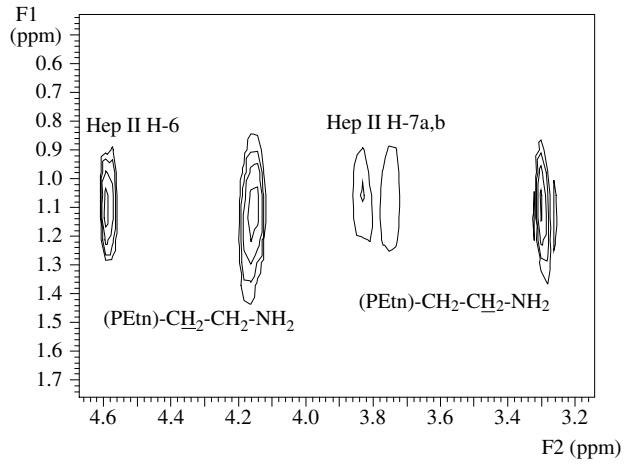

Figure 2. Region of the 2D ^{31}P–^{1}H HSQC–TOCSY NMR spectrum of the core OS from *Hs* strain 8025 showing correlations between the ^{31}P-resonance (*x*-axis) and ^{1}H-resonances (*y*-axis) for the heptose residue (**B**) and the ethanolamine resonance as indicated. The spectrum was recorded at 25 °C in D_2O at pH 7.0.

In order to complete the characterisation of the core OS region of the molecule, it was necessary to confirm the linkage position of phosphoethanolamine (PEtn) residue that was inferred to be at the 6-position of heptose residue **B** from the combined MS, NMR and methylation analysis data above, where the location of the PEtn residue was inferred from characteristic chemical shifts as detailed in Table 2. Confirmation of the linkage positions was obtained from a 2-D ^{31}P–^{1}H HSQC experiment on the core OS that showed correlations between the ^{31}P-resonance and a signal at 4.60 ppm assigned to the H-6 ^{1}H-resonances of heptose residue **B** (data not shown). This was confirmed in a 2-D ^{31}P–^{1}H HSQC–TOCSY experiment (Fig. 2) that identified the H-7a and H-7b ^{1}H-resonances from the H-6 ^{1}H-resonance of residue **B**.

The structural analysis of the core OS from *Hs* strain 8025 revealed some interesting structural features. The structure determined contains aspects of other *H. somni*

core OS structures, such as the β-Gal attached at the 2-position of Hep II (2336), PEtn only at the 6-position of Hep II (738, 129Pt) and a lactose extension from Hep I (1P). Since genetic manipulation has been achieved with this strain,[9] the identification of the core OS structure will enable experiments designed to identify the role of glycosyltransferases involved in LOS biosynthesis.

1. Experimental

1.1. Growth of bacteria, isolation and fractionation of LOS

H. somni strain 8025 (NRCC#6253) was grown and isolated as described previously.[3] Briefly, *H. somni* strain 8025 was grown in 6×2 L flasks in Todd Hewitt (15 g L^{-1})–Columbia broth (17.5 g L^{-1}) for 24 h at 37 °C. The cells were killed by addition of phenol to 2% before harvesting cells by using a Sharples continuous flow centrifuge giving 41 g wet wt. Freeze-dried cells (11.5 g) were washed with organic solvents (ethanol, acetone, ether) to remove lipids and other lipophilic components to enhance the efficiency of the LOS extraction.[10] Washed cells (10 g) were extracted by the hot phenol–water method,[11] and the aqueous phases were combined and dialysed against running water for 48 h. The retentate was freeze-dried yielding ~0.48 g, made up to a 2% solution in water and treated with DNase and RNase at 37 °C for 4 h, followed by proteinase K treatment at 37 °C for 4 h. Small peptides were removed by dialysis. After freeze-drying, the retentate (~0.32 g) was made up to a 2% solution in water, centrifuged at 8000*g* for 15 min (yielding an '8K pellet' of ~67 mg) followed by further centrifugation of the supernatant at 100,000*g* for 5 h. The pellet, containing purified LOS, was redissolved and freeze-dried (yielding ~67 mg). LOS-OH and core OS were isolated and fractionated as described previously.[12] Briefly, 8K pellet material (~5 mg) and LOS (~5 mg) were treated with anhydrous hydrazine with stirring at 37 °C for 1 h to prepare LOS-OH yielding ~2 mg from each preparation. The core OS was isolated by treating the LOS (~60 mg) with 1% acetic acid (8 mg/mL, 100 °C, 1 h) with subsequent removal of the insoluble lipid A by centrifugation (5000*g*). The lyophilised supernatant was subsequently further purified down a Bio-Gel P-2 column with separate fractions lyophilised.

1.2. Analytical methods

Sugars were determined as their alditol acetate derivatives[13] by GLC–MS as described previously.[12] Methylation analysis was carried out by the NaOH–DMSO–methyl iodide procedure[14] and analysed by GLC–MS as described previously.[12]

1.3. Mass spectrometry and NMR spectroscopy

All mass spectrometry and NMR experiments were performed as described previously.[12]

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