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Structural analysis of the lipooligosaccharide from the commensal *Haemophilus somnus* genome strain 129Pt

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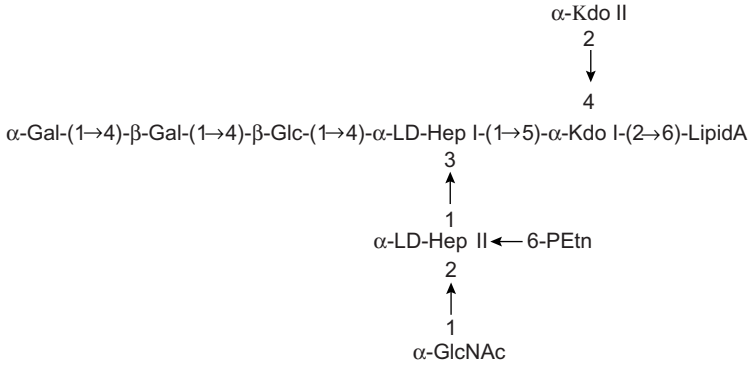
Abstract—The structure for the carbohydrate moiety of the lipooligosaccharide (LOS) from the commensal *Haemophilus somnus* strain 129Pt was elucidated. The structure of the core oligosaccharide and O-deacylated LOS was established by monosaccharide and methylation analyses, NMR spectroscopy and mass spectrometry. The following structure for the major fully extended carbohydrate glycoform of the LOS was determined on the basis of the combined data from these experiments.



In the structure Kdo is 3-deoxy-D-*manno*-octulosonic acid, Hep is L-*glycero*-D-*manno*-heptose and PEtn is phosphoethanolamine. Minor amounts of glycoforms containing nonstoichiometric substituents glycine and phosphate at the distal heptose residue were also identified.

Keywords: *Haemophilus somnus*; Lipooligosaccharide; Commensal; NMR; Mass spectrometry

1. Introduction

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. When this bacterium disseminates via septicaemia, it may cause a variety of bovine diseases, including thrombotic meningoencephalitis (TME), pneumonia, abortion, arthritis and myocarditis.[2–4] Potential virulence factors that are known for this bacterium include the presence of immunoglobulin

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binding proteins on the cell surface,[5] the capability to survive and resist phagocytosis[6] and phase variation and sialylation in the carbohydrate component of lipooligosaccharide (LOS) epitopes.[3,7–9] LOS phase variation appears to enable the bacteria to evade or delay recognition by the host immune response.[7] Pathogenic isolates also seem to be distinct from commensal isolates by their ability to cause disease in the natural host or a laboratory animal model, whereas commensals cannot.[7,10] Commensal strains have been shown not to undergo phase variation to a substantial degree and tend to produce more truncated LOS molecules.[7]

Detailed structural analysis of *Hs* LOS has so far been restricted to only one pathogenic strain termed 738[11] and one commensal strain termed 1P.[12] These studies identified several structural similarities to the LOS of the human pathogen *Neisseria meningitidis* (*Nm*), with LOS from strain 738 elaborating a phase-variable outer core oligosaccharide structure, which when fully extended presents a lacto-*N*-tetraose unit, whereas fully extended LPS from some strains of *Nm* often present a lacto-*N*-neotetraose unit. These structures differ in the type of linkage between the two terminal sugars of the tetraose unit, with a type II, β-(1 → 4)-linkage being found in *Nm* and a type I, β-(1 → 3)-linkage in *Hs*. LOS from strain 1P resembled immunotype L8 of *Nm* with only a disaccharide extension from the proximal heptose residue of the inner core LOS. Recent work from our laboratory has confirmed that *Hs* LOS can be efficiently sialylated.[9] Strain 738 and a closely related isolate 2336 were both shown to have approximately 20% of their LOS molecules capped with sialic acid.[9] These studies also illustrated the inability of several commensal strains to sialylate their LOS[9] that was borne out in the structural analysis of strain 1P.[12] This study was therefore undertaken to determine the chemical structure of another commensal strain, 129Pt, in order to examine if there was a correlation between the LOS structure and the lack of virulence for this strain. With genomic information becoming available for this strain, it was also of interest to attempt to link the structure of the LOS molecule to the putative LOS biosynthetic genes identified by genome sequence information.

2. Results and discussion

LOS from *Hs* strain 129Pt was purified by the aqueous phenol method from cells grown on blood agar plates or from broth culture. GLC–MS analysis of the derived alditol acetates from the untreated LOS showed that it was composed of glucose (Glc), galactose (Gal), 2-amino-2-deoxy-glucose (GlcN) and/or its *N*-acetyl derivative, and L-*glycero*-D-*manno*-heptose (Hep) in an approximate molar ratio of 2:1:1:2.

O-Deacylated LOS was prepared and analysed by ESIMS (Fig. 1; Table 1). A simple mass spectrum was observed with two major peaks observed at 2265.2 and 2589.4 amu, and a minor peak at 2427.5 amu. These data are consistent with a composition of Hex, 2Hep, HexNAc, 2Kdo, PEtn, Lipid A-OH for the smallest glycoform, and one additional hexose residue each for the larger two molecules (where Lipid A-OH refers to the O-deacylated lipid A molecule). This composition is consistent with the presence of a phosphoethanolamine (PEtn) residue, and also suggests that *N*-acetylhexosamine is present in the inner core oligosaccharide. CE–

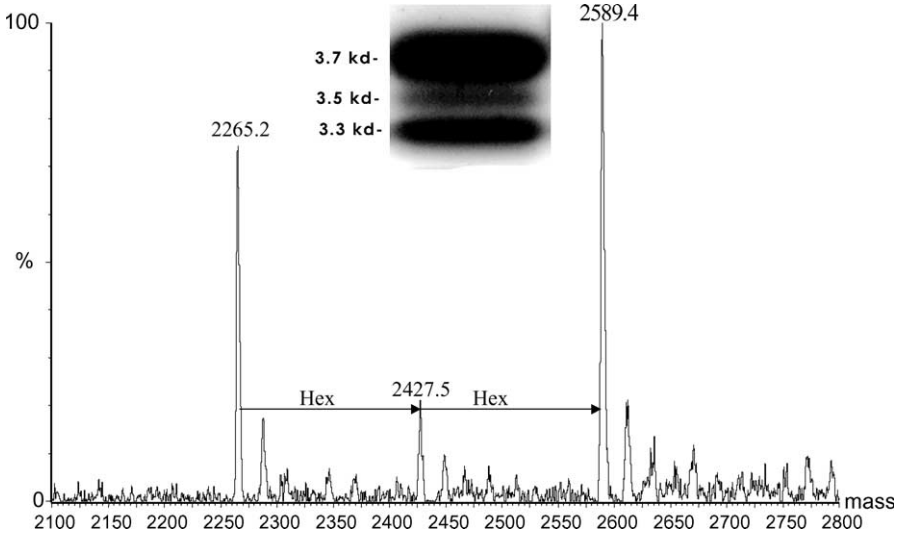

Figure 1. Negative-ion electrospray-ionisation mass spectrum of the O-deacylated LOS from *H. somnus* strain 129Pt. The transformed spectrum displayed shows the molecular ions that are derived from the actual spectrum of doubly charged ions with m/z as indicated in Table 1. Inset SDS-PAGE of LOS from *H. somnus* strain 129Pt.

Table 1. Negative-ion ESIMS and CE–ESIMS data and proposed compositions of O-deacylated LPS and core oligosaccharides from *H. somnus* strain 129Pt[a]

Strain	Observed ions (m/z)		Molecular mass (Da)		Proposed composition
	$(M-2H)^{2-}$	$(M-H)^{-}$	Observed	Calculated	
129Pt O-deac.	1131.6	—	2265.0	2265.1	Hex, HexNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH
	1212.8	—	2427.0	2427.2	2Hex, HexNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH
	1293.7	—	2589.0	2589.4	3Hex, HexNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH
Core OS					
Fr. 15–16	707.4	—	1416.8	1417.2	3Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	716.8	—	1434.6	1435.2	3Hex, HexNAc, 2Hep, PEtn, Kdo
Fr. 17–18	707.4	—	1416.8	1417.2	3Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	716.5	—	1434.0	1435.2	3Hex, HexNAc, 2Hep, PEtn, Kdo
Fr. 19–20	545.8	1091.7	1093.1	1093.0	Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	554.8	1109.8	1111.0	1111.0	Hex, HexNAc, 2Hep, PEtn, Kdo
	574.2	1148.9	1150.1	1150.0	Gly, Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	583.3	1166.9	1168.0	1168.0	Gly, Hex, HexNAc, 2Hep, PEtn, Kdo
	626.6	1254.0	1255.1	1255.1	2Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	635.7	1272.0	1273.1	1273.1	2Hex, HexNAc, 2Hep, PEtn, Kdo
	655.3	1311.0	1312.0	1312.1	Gly, 2Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	707.7	1416.1	1417.1	1417.2	3Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	716.6	1434.8	1435.8	1435.2	3Hex, HexNAc, 2Hep, PEtn, Kdo
	736.1	1473.2	1474.3	1474.2	Gly, 3Hex, HexNAc, 2Hep, PEtn, Kdo[b]
	745.6	1491.3	1492.2	1492.2	Gly, 3Hex, HexNAc, 2Hep, PEtn, Kdo
	747.7	—	1497.4	1497.2	3Hex, HexNAc, 2Hep, PEtn, Kdo,[b] P
	756.9	—	1515.8	1515.2	3Hex, HexNAc, 2Hep, PEtn, Kdo, P

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; P, 79.98, PEtn, 123.05; Gly, 57.00. O-Deacylated lipid A (Lipid A-OH) is 952.00.
[b] The major ion corresponded to [M – 18] (loss of H_2O).

MS/MS analysis (data not shown) confirmed the size of the O-deacylated lipid A as 952 amu. The O-deacylated lipid A basal species (952 amu) consists of a β-(1 → 6)-linked disaccharide of N-acylated (3-OH C 14:0) glucosamine residues, each residue being substituted with a phosphate group. Consistent with the MS analysis, SDS-PAGE of the LOS from *Hs* strain 129Pt revealed three bands corresponding in intensity to the three glycoforms observed in ESIMS (Fig. 1 inset). Presumably the most intense slowest migrating band corresponds to the highest molecular weight glycoform observed, with the faster migrating bands corresponding to the smaller glycoforms observed in ESIMS.

Core oligosaccharide was prepared and was fractionated by gel-filtration chromatography. Early eluting fractions were found to contain a 4-linked glucan as determined by methylation analysis and NMR spectroscopy (Table 2). Fractions were analysed by CE–MS. Consistent with the O-deacylated LOS MS data simple mass spectra were observed indicative of the presence of 1Hex-, 2Hex- and 3Hex-containing glycoforms (Table 1). Interestingly a series of ions were present 57 amu higher than the basal glycoforms from the latest eluting fraction to contain carbohydrate (Table 1), which is consistent with the presence of the amino acid glycine as has been observed previously[13,14] for both *Nm* and *Hi*. CE–MS/MS experiments (data not shown) localised this glycine residue to the distal heptose residue (Hep II) as we had observed previously for meningococcal LOS.[13] Also for the 3Hex glycoforms of the late eluting fraction, ions that are consistent with the presence of an additional phosphate residue were observed (Table 1). CE–MS/MS experiments (data not shown) localised this phosphate residue to the distal heptose residue (Hep II) also.

Methylation analysis of a core fraction predominantly free from glucan suggested the presence of a terminal Gal, a 4-linked Gal, a 4-linked Glc, a 3,4-linked Hep and a terminal GlcNAc residue.

In order to completely characterise the LOS structure, NMR spectroscopy was performed on the O-deacylated LOS following several lyophilisations with D_2O. The initial ^1H NMR spectrum was poor, but following the addition of deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) to the solution in D_2O, a sharp well-resolved spectrum was obtained (Fig. 2). The assignment of ^1H resonances of the sugars of the *H. somnus* strain 129Pt O-deacylated LOS was achieved by COSY and TOCSY (Fig. 3) experiments and by comparison with reported data for *Hs* strain 1P and meningococcal O-deacylated LOS[12,15,16] (Table 2). The ring sizes and relative stereochemistries of the component monosaccharides were established from the ^1H chemical shifts and the magnitude of the coupling constants.[17] Two pairs of

Table 2. [1]H NMR chemical shifts for the O-deacylated LPS from strain *H. somnus* 129Pt[a]

	H-1	H-2	H-3	H-4	H-5	H-6	H-7	Inter NOE's
Lipid A								
α-GlcN	5.50	3.85	nd[b]	nd	nd	4.08	—	—
β-GlcN	4.61	3.87	3.85	nd	nd	nd	—	4.08 α-GlcN H-6a
Inner core								
Kdo I	—	—	2.16 1.98	4.12	4.30	nd	3.84	3.68 Kdo II H-6
Kdo II	—	—	2.13 1.97	4.05	nd	3.68	nd	
Hep I	5.30	4.12	4.09	4.25	nd	4.08	nd	4.30 Kdo I H-5, 3.84 Kdo I H-7
Hep II	5.64	4.18	4.02	nd	nd	4.58	3.82 3.74	4.09 Hep I H-3
α-GlcNAc	5.08	3.87	3.87	3.52	4.09	nd	—	4.18 Hep II H-2, 5.64 Hep II H-1
Outer core								
β-Glc I	4.57	3.45	3.63	3.64	3.64	nd	—	4.25 Hep I H-4, 4.08 Hep I H-6
β-Gal I	4.53	3.58	3.77	4.05	nd	nd	—	3.64 Glc I H-4
α-Gal II	4.96	3.83	3.91	4.03	4.36	3.72 3.72	—	4.05 Gal I H-4
Glucan								
α-Glc	5.41	3.61	3.97	3.66	3.85	3.84 3.84	—	3.66 α-Glc H-4

[a] Recorded at 25 °C, in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) added. Chemical shifts referenced to internal acetone at 2.225 ppm.
[b] nd, not determined.

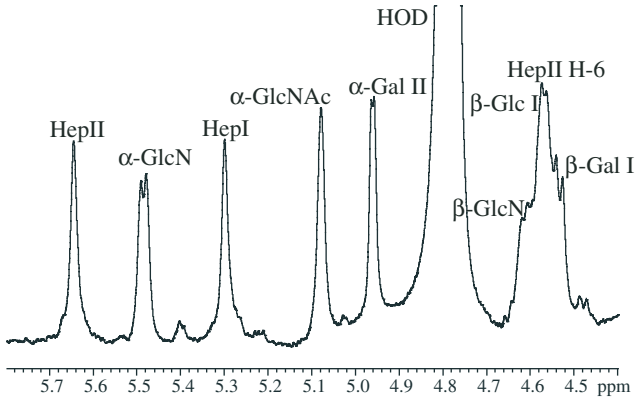

Figure 2. Anomeric region of the [1]H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 129Pt. The spectrum was recorded in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C.

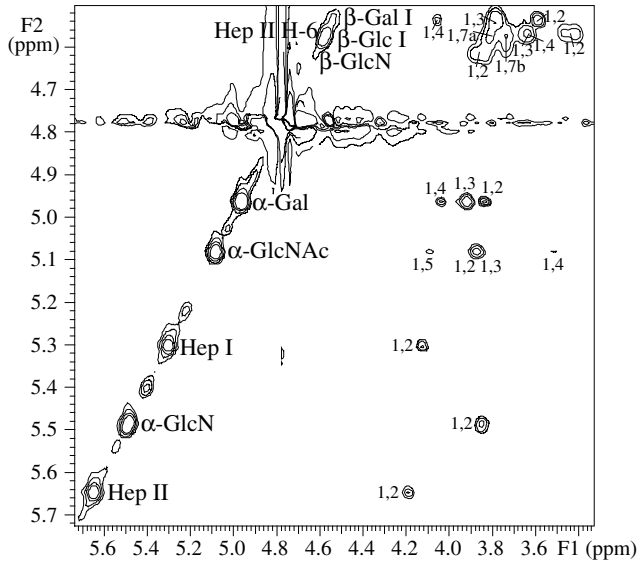

Figure 3. Region of the 2D-TOCSY [1]H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 129Pt. The spectrum was recorded in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C.

resonances in the high-field region (1.000–2.500 ppm) of the 1D [1]H NMR spectrum of the O-deacylated LOS could be attributed to the characteristic equatorial and axial methylene protons of the Kdo residues. Also in the high-field region of the [1]H NMR spectrum was a signal at 2.03 ppm, which was assigned to the methyl protons of the acetyl group of a *N*-acetylglucosamine (GlcNAc) residue.

The low-field region (4.40–5.60 ppm) of the [1]H NMR spectrum of the O-deacylated LOS revealed six major signals, one of which was unresolved (Fig. 2). Two signals at 5.30 and 5.64 ppm 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 *manno*-pyranosyl ring systems. The α configurations were evident for both of these residues from the occurrence of a single residue NOE between the H-1 and H-2 resonances.[18] The signal at 5.08 ppm was identified as the α-GlcNAc residue by virtue of a small $J_{1,2}$ vicinal proton coupling constant and large $J_{2,3}$,

$J_{3,4}$ and $J_{4,5}$ (8–10 Hz) constants. This residue was identified as an amino sugar on the basis of its C-2 chemical shift. The H-2 resonance of 3.87 ppm correlated in the ^{1}H–^{13}C HMQC experiment to a ^{13}C resonance at 54.6 ppm, the chemical shift being diagnostic of amino-substituted carbons. The expected signal from the α-GlcN residue from lipid A-OH was observed in the 1D ^{1}H NMR spectrum at 5.50 ppm, the H-2 resonance at 3.85 ppm correlating with a C-2 resonance of 55.0 ppm in the ^{1}H–^{13}C HMQC experiment. The remaining resolved resonance in the low-field region at 4.96 (Gal I) ppm was assigned to a *galacto*-pyranosyl residue from the appearance of the characteristic spin system to the H-4 resonance in a TOCSY experiment. The remaining three unresolved signals in the low-field region were all attributable to β-linked residues by virtue of their high $J_{1,2}$ (~8 Hz) coupling constants. Large vicinal proton coupling constants for $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ (8–10 Hz) indicated the presence of two hexopyranosyl residues (GlcN and Glc) having the *gluco* configuration at 4.61 and 4.57 ppm. The residue at 4.61 ppm was identified as an amino sugar in an identical manner to the GlcNAc residue detailed above. The final residue in the low-field region at 4.53 ppm was identified as a *galacto*-pyranosyl residue (Gal) from the appearance of the characteristic spin system to the H-4 resonance in a TOCSY experiment.

The sequence of glycosyl residues of the O-deacylated LOS was determined from inter-residue ^{1}H–^{1}H NOE measurements between anomeric and aglyconic protons on adjacent glycosyl residues (Fig. 4). The occurrence of

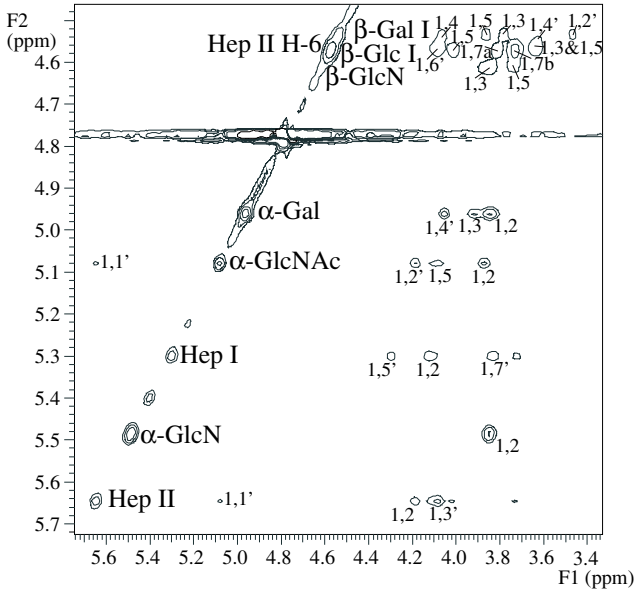

Figure 4. Region of the 2D-NOESY ^{1}H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 129Pt. The spectrum was recorded in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C. For clarity spectrum is shown at a lower intensity.

an inter-residue NOE between H-1 of the Gal II residue at 4.96 ppm and H-4 of the Gal I residue at 4.05 ppm confirmed the partial sequence of Gal II-α-(1 → 4)-Gal I. The occurrence of an inter-residue NOE between H-1 of the Gal I residue at 4.53 ppm and H-4 of the Glc I residue at 3.64 ppm confirmed the partial sequence of Gal II-β-(1 → 4)-Glc I. The linkage pattern of the core residues were confirmed by comparison to previous data, indicating a glycose arrangement of the core structure as has been observed previously for meningococcal immunotype L1 LOS, with the terminal GlcNAc residue substituting the distal heptose (Hep II) residue at the 2-position as evidenced by the characteristic H-1 to H-1 NOEs, and the Hep II residue substituting the proximal heptose residue (Hep I) at the 3-position.[15,16] Hep I is also substituted at the 4-position by Glc I as indicated by characteristic NOEs from Glc to the H-4 and H-6 resonances of Hep I. Hep I in turn substitutes Kdo at the 5-position as evidenced by characteristic NOEs to the H-5 and H-7 resonances of Kdo.

The Kdo–Lipid A region was difficult to characterise by NMR methods due to the poor resolution of resonances from residues in this region. Presumably this was due to some aggregation of this region of the molecule remaining even after the addition of deuterated SDS and EDTA. However several diagnostic NOE connectivities were identified including Kdo I H-3 to Kdo II H-6, a connectivity that is evidence for the α-(2 → 4)-Kdo II to Kdo I linkage[11] and β-GlcN H-1 to α-GlcN H-6a.

Evidence for the presence of a phosphoethanolamine (PEtn) group in the inner core was from the characteristic peak for the phosphorylation of the O-6 position of the Hep II residue at 4.58 ppm. Cross-peaks were observed in COSY and TOCSY experiments from the H-6 resonance at 4.58 ppm to the H-7 protons at 3.82 and 3.74 ppm. Additionally a cross-peak between resonances at 3.33 and 4.14 ppm was observed consistent with the CH_2–CH_2 protons of the ethanolamine group of phosphoethanolamine. Finally the proton resonance of PEtn at 3.33 ppm correlated with a ^{13}C resonance of 40 ppm in a ^{1}H–^{13}C HMQC experiment characteristic of the PEtn proton and carbon resonances adjacent to the amino group of PEtn. The NMR data for the O-deacylated LOS was consistent with the methylation analysis data for the core oligosaccharide, and therefore a simple truncated structure for the LOS from the commensal *Hs* strain 129Pt has been identified (Fig. 5).

This analysis has revealed similarities and differences between this structure and the previously published structure for the LOS from the commensal strain 1P.[12] The major differences are the presence of phosphoethanolamine (PEtn) in the inner core of this commensal strain and the presence of an additional alpha-configured galactose residue attached to the 4-position of the lactose extension from Hep I. This arrangement has been observed previously in the LOS from immunotype

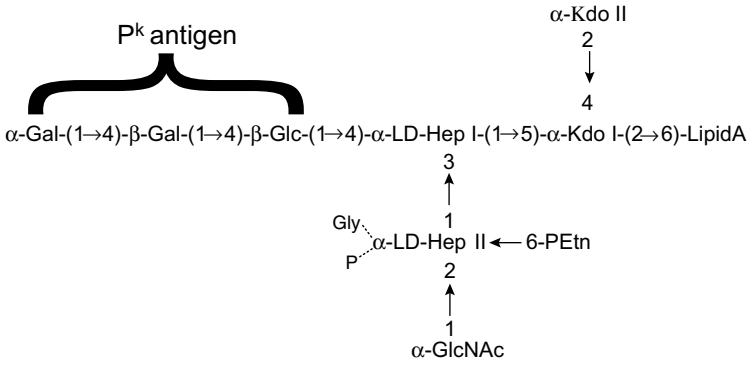

Figure 5. Structural representation of the carbohydrate component of the lipooligosaccharide from *H. somnus* strain 129Pt. The nonstoichiometric substituents glycine (Gly) and phosphate (P) are indicated at the distal heptose residue (Hep II). The location of the P^k antigen is also indicated.

L1 strain of *Nm*.[15,16] Commensal strain 129Pt also lacks the extended lacto-*N*-tetraose structure and the phosphocholine (PCho) residue of the pathogenic strain 738.[11] Strain 129Pt does, however, elaborate a GlcNAc residue attached to the Hep II residue of the inner core. The impact on the pathogenic potential of these structural variations is intriguing. PCho is recognised as in important factor for bacterial adhesion,[19] and as such is related to virulence; however, this commensal strain has successfully colonised a physiological niche, so perhaps the absence of PCho is not a significant factor in the ability of *Hs* strains to colonise. It is known that preputial isolates only live in the urogenital tract, and there is no evidence that they can disseminate and cause disease. Although PCho may not be necessary to colonise the urogenital tract, some preputial isolates do have PCho, as determined by colony blotting with a PCho-specific MAb.[20] It will be interesting to see if the evolving genome sequence reveals that the genes for incorporation of PCho into the LOS are absent or subject to phase variation in this strain.

Recently, studies in our laboratories identified the ability of *Hs* strains to sialylate their LOS as an advantage in terms of serum resistance and avoidance of antibody binding.[9] Although the P^k antigen identified here as extending from Hep I was sialylated in *Nm* strain L1,[16] no sialylation was observed for this commensal strain. This could be due to a lack of functional sialylation genes in *Hs* 129Pt. Analysis of the unfinished genome sequence of *Hs* 129Pt (http://genome.jgi-psf.org/draft_microbes/haeso/haeso.home.html) has revealed truncated, interrupted, or missing sialylation genes when 129Pt is compared to the pathogenic strain 2336 genome sequence (http://micro-gen.ouhsc.edu/h_somnus/h_somnus_home.htm).[21] Consistent with the LOS structure determined here for strain 129Pt, all of the genes anticipated to be present in the 129Pt genome sequence have been found, with homologies to characterised LOS glycosyltransferases from other species (Duncan, personal communication). This study has advanced our knowledge of the LOS structure of *Hs* and has suggested certain structural alterations that could be involved in the virulence potential of this organism.

3. Experimental

3.1. Bacterial strain, growth of organism and isolation of LOS and O-deacylated LOS

H. somnus strain 129Pt was originally isolated from the prepuce of a bull.[22] Initially, the organism was grown on Columbia blood agar (CBA) plates as described previously.[23] Cells were harvested by washing from CBA plates with phosphate-buffered saline, centrifugation and the pellet freeze dried. LOS was extracted from the freeze-dried cells (275 mg) by phenol extraction yielding 11 mg. To enable complete structural analysis, *H. somnus* strain 129Pt was grown in 5×2-L flasks in Todd Hewitt (15 g/L)/Columbia broth (17.5 g/L) 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 26 g wet wt. Freeze-dried cells were washed with organic solvents (ethanol, acetone, ether) to remove lipids and other lipophilic components to enhance the efficiency of the LOS extraction. LOS was extracted from the washed cells by the hot phenol–water method giving 123 mg of LOS. O-Deacylated LOS and core oligosaccharide were prepared by standard methods.[24,25] Briefly, purified LOS was treated with anhydrous hydrazine with stirring at 37 °C for 1 h to prepare O-deacylated LOS. The reaction was cooled in an ice bath and gradually cold acetone (−70 °C, 5 vols) was added to destroy excess hydrazine, and the precipitated O-deacylated LOS was isolated by centrifugation. The core oligosaccharide (OS) was isolated by treating

the purified LOS with 1% acetic acid (10 mg/mL, 100 °C, 1.5 h) with subsequent removal of the insoluble lipid A by centrifugation (5000 *g*). The lyophilised OS was further purified down a Bio-Gel P-2 column with individual fractions lyophilised.

3.2. Structural analysis

Alditol acetates were prepared and analysed as described previously.[19] All ESIMS (electrospray-ionisation MS) and CE–MS (capillary electrophoresis–MS) analyses were carried out as described previously.[25] Nuclear magnetic resonance experiments were performed on Varian INOVA 500, 400 and 200 NMR spectrometers as described previously.[13]

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