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# Structural analysis of the lipooligosaccharide from the commensal *Haemophilus somnus* strain 1P

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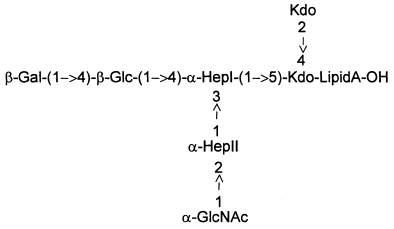
**Abstract**

The structure of the lipooligosaccharide (LOS) from the commensal *Haemophilus somnus* strain 1P was elucidated. The structure of the O-deacylated LOS was established by monosaccharide analysis, NMR spectroscopy and mass spectrometry. The following structure for the O-deacylated 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 lipid A-OH refers to O-deacylated Lipid A. The elucidation of this structure has increased our understanding of the relationship between the variability in LOS structure and the pathogenic potential of this organism. Specifically, the inability of this commensal strain to sialylate its LOS suggests that LOS sialylation could be a crucial virulence factor for *H. somnus*. 

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

*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 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's 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] This study identified several structural similarities to the LOS of the human pathogen *Neisseria meningitidis* (*Nm*),[12] as both pathogens elaborate phase-variable outer core oligosaccharide structures, which when fully extended, present a lacto-*N*-neotetra-

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ose unit (*Nm*) or lacto-*N*-tetraose unit (*Hs*). 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*. However, unlike *Nm* LOS, LOS from the *Hs* pathogenic strain 738 did not contain an *N*-acetylglucosamine (GlcNAc) residue linked to the distal heptose residue (Hep II) in the inner core OS. Interestingly, recent work from our laboratory has confirmed that *Hs* LOS can be efficiently sialylated.[9] Strain 738 and a closely related strain 2336 were both shown to have approx 20% of their LOS molecules

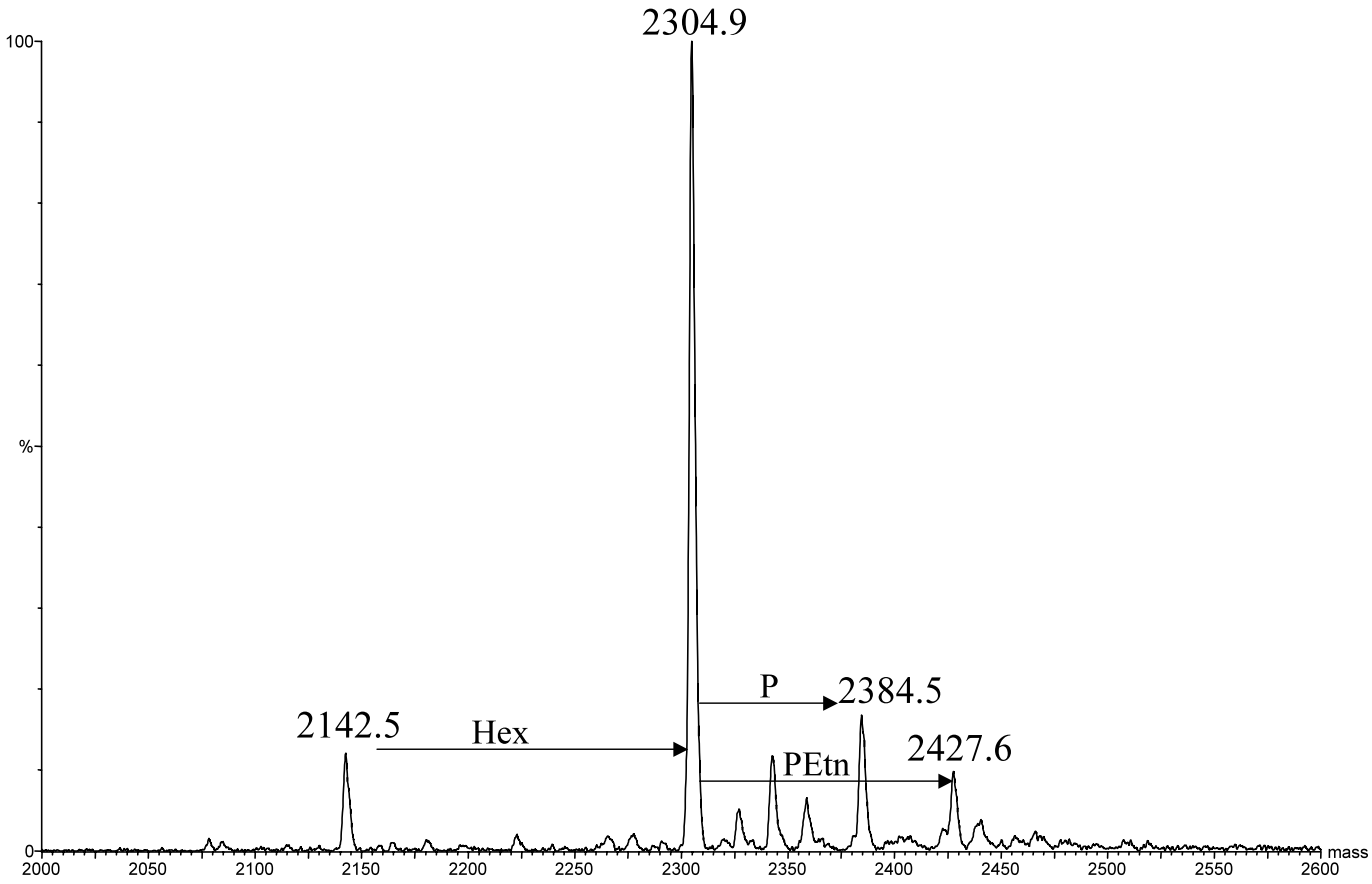


Fig. 1. Negative-ion electrospray mass spectrum of the O-deacylated LOS from *H. somnus* strain 1P. The transformed spectrum displayed shows the molecular ions that are derived from the actual spectrum of doubly charged ions with *m*/*z* as indicated in Table 1.

Table 1
Negative-ion ESIMS data and proposed compositions of O-deacylated LOS from *H. somnus* strain 1P [a]

| Strain | Observed ions (*m*/*z*) $(M-2H)^{2-}$ | Molecular mass (Da) | | Relative intensity | Proposed composition |
|---|---|---|---|---|---|
| | | Observed | Calculated | | |
| 1P | 1070.3 | 2142.6 | 2142.0 | 0.12 | Hex, HexNAc, 2Hep, 2Kdo, lipid A-OH (952.0) |
| | 1151.3 | 2304.6 | 2304.2 | 1.00 | 2Hex, HexNAc, 2Hep, 2Kdo, lipid A-OH (952.0) |
| | 1191.2 | 2384.4 | 2384.2 | 0.15 | 2Hex, HexNAc, 2Hep, 2Kdo, P, lipid A-OH (1032.0) |
| | 1212.8 | 2427.6 | 2427.2 | 0.08 | 2Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH (952.0) |

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNAc, 203.19; Kdo, 220.18; P, 79.98. The average molecular weight of the O-deacylated lipid A (lipid A-OH) is as indicated.

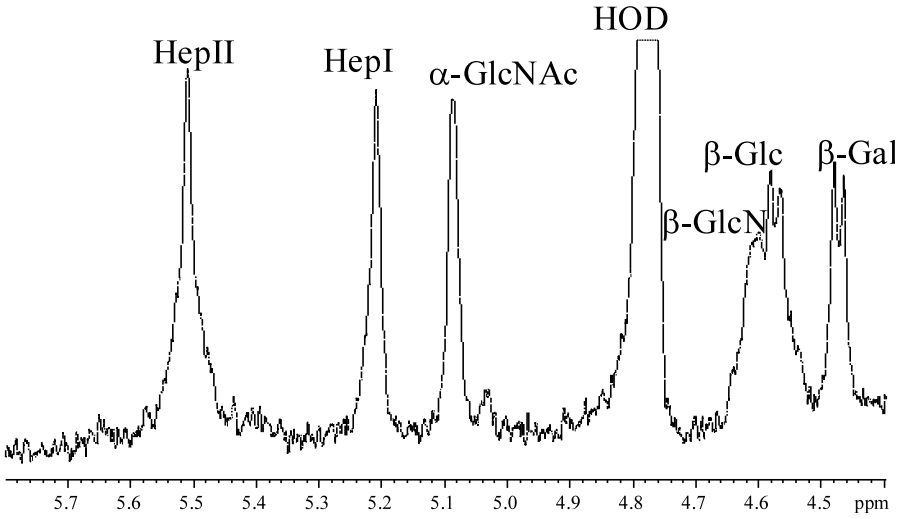


Fig. 2. Anomeric region of the $^1$H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 1P. The spectrum was recorded in $D_2O$ with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C.

capped with sialic acid.[9] These studies also illustrated the inability of several commensal strains to sialylate their LOS.[9] This study was therefore undertaken to determine the chemical structure of one such commensal strain, 1P, in order to examine if there was a correlation between the LOS structure and the lack of virulence from this strain.

LOS from *Hs* strain 1P was purified by the aqueous phenol method from cells grown on blood agar plates, (approx 5% yield). 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-D-3 glucose (GlcN) and/or its *N*-acetyl derivative and L-*glycero*-D-*manno*-heptose (Hep) in an approximate molar ratio of 1:1:1:2. O-deacylated LOS was prepared and analysed by electrospray-ionisation–mass spectrometry (ESIMS) (Fig. 1; Table 1). A simple mass spectrum was observed with a single major peak at 2304.9 amu consistent with a composition of 2 Hex, 2 Hep, HexNAc, 2 Kdo, lipid A-OH. This composition is consistent with the absence of a phosphoethanolamine (PEtn) residue, and also suggests that *N*-acetylhexosamine is present in the inner core oligosaccharide. Minor peaks were observed at 2142.5 amu, presumably due to the absence of a hexose residue, and at 2384.5 due to the presence of an additional phosphate residue, and at 2427.6 due to the presence of an additional PEtn residue. Capillary electrophoresis–tandem mass spectrometric (CE–MS/MS) analysis (data not shown) confirmed the size of the O-deacylated lipid A as 952 amu for the two smaller molecules and 1032 amu, which is consistent with an additional phosphate group in the lipid A region for the larger O-deacylated LOS species. 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. CE–MS/MS analysis (data not shown) confirmed the size of the O-deacylated lipid A as 952 amu for the ion at $m/z$ 2427 amu, consistent with the additional PEtn group localised in the core OS of this glycoform. However, the very small amount of this glycoform precluded any subsequent attempts to localise this residue to a specific residue in the core OS.

In order to completely characterise the O-deacylated LOS structure, NMR spectroscopy was performed following several lyophilisations with $D_2O$. The initial $^1$H NMR spectrum was poor, but following the addition of

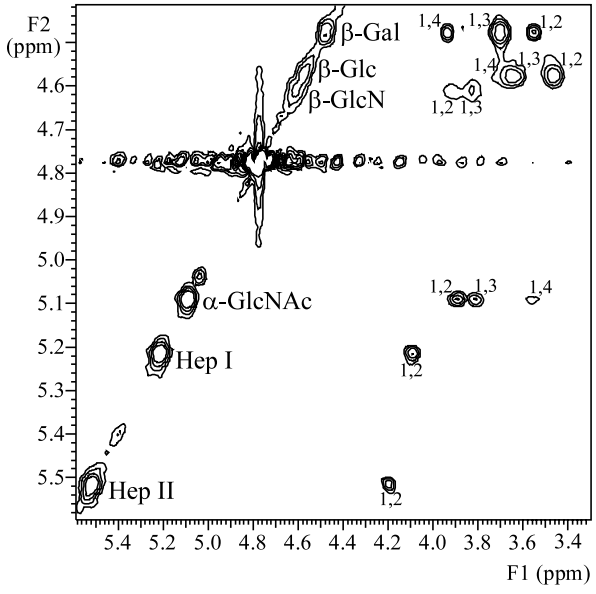


Fig. 3. Region of the 2D-TOCSY $^1$H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 1P. The spectrum was recorded in $D_2O$ with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C.

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

| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-7 | NOEs | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Inter | Intra | Long Range |
| *Lipid A* | | | | | | | | | | |
| α-GlcN | 5.40 | nd | nd | nd | nd | nd | – | – | nd | |
| β-GlcN | 4.61 | 3.92 | 3.82 | nd | nd | nd | – | 4.01 α-GlcN H-6 | 3.82 β-GlcN H-3 | |
| *Inner core* | | | | | | | | | | |
| Kdo I | – | – | 2.14<br>1.95 | 4.13 | 4.32 | nd | nd | 3.66 Kdo II H-6 | nd | |
| Kdo II | – | – | 2.01<br>1.94 | 4.04 | nd | 3.66 | nd | nd | nd | |
| Hep I | 5.21 | 4.09 | 4.10 | 4.26 | nd | 4.08 | nd | 4.32 Kdo I H-5<br>3.86 Kdo I H-7 | 4.09 Hep I H-2 | |
| Hep II | 5.51 | 4.20 | 3.99 | nd | nd | nd | nd | 4.10 Hep I H-3 | 4.20 Hep II H-2 | 3.87 nd |
| α-GlcNAc | 5.09 | 3.88 | 3.82 | 3.55 | nd | nd | – | 4.20 Hep II H-2<br>5.51 Hep II H-1 | 3.88 GlcNAc H-2 | 4.47 Gal H-1<br>3.47 Glc H-2 |
| *Outer core* | | | | | | | | | | |
| β-Glc | 4.57 | 3.47 | 3.64 | 3.72 | 3.64 | 4.06<br>3.84 | – | 4.26 Hep I H-4<br>4.08 Hep I H-6 | 3.64 Glc H-3, H-5<br>3.47 Glc H-2 | |
| β-Gal | 4.47 | 3.55 | 3.70 | 3.94 | 3.79 | nd | – | 3.72 Glc H-4 | 3.70 Gal H-3<br>3.79 Gal H-5<br>3.94 Gal H-4 | 5.09 GlcNAc H-1<br>4.02 Glc H-6a<br>3.47 Glc H-2 |

[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. nd, not determined.

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 $^1$H NMR resonances of the sugars of the *H. somnus* strain 1P O-deacylated LOS was achieved by COSY and TOCSY (Fig. 3) experiments and by comparison with reported data for *Hs* strain 738 and meningococcal O-deacylated LOS[11,13,14] (Table 2). The ring sizes and relative stereochemistries of the component monosaccharides were established from the $^1$H NMR chemical shifts and the magnitude of the coupling constants.[15] Two of the 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 residue. 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 an *N*-acetylglucosamine 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 (Fig. 2). Two signals at 5.21 and 5.51 ppm were attributed to the H-1 protons of heptose (Hep) residues due to their small $J_{1,2}$ ($<2$ Hz) and $J_{2,3}$ ($\sim 3$ Hz) coupling constant values, which pointed to mannopyranosyl 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.[16] The signal at 5.09 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 3.88 ppm correlated in the $^1$H, $^{13}$C HMQC experiment to a $^{13}$C resonance at 55.7 ppm, the chemical shift being diagnostic of amino-substituted carbons. The expected signal from the α-GlcN residue from lipid A-OH was not observed in the 1D $^1$H NMR spectrum and was only weakly observed in the 2D experiments, presumably due to aggregation of this region of the molecule to suppress the signal. The remaining three signals in the low-field region were all attributable to β-linked residues by virtue of their high $J_{1,2}$ ($\sim 8$ 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 4.47 ppm was identified as a galactopyranosyl residue (Gal) from the vicinal ring proton coupling constant values of $J_{2,3}$ ($\sim 10$ Hz), $J_{3,4}$ ($\sim 4$ Hz) and $J_{4,5}$ (1 Hz). The sequence of glycosyl residues of the O-deacylated LOS was deter-

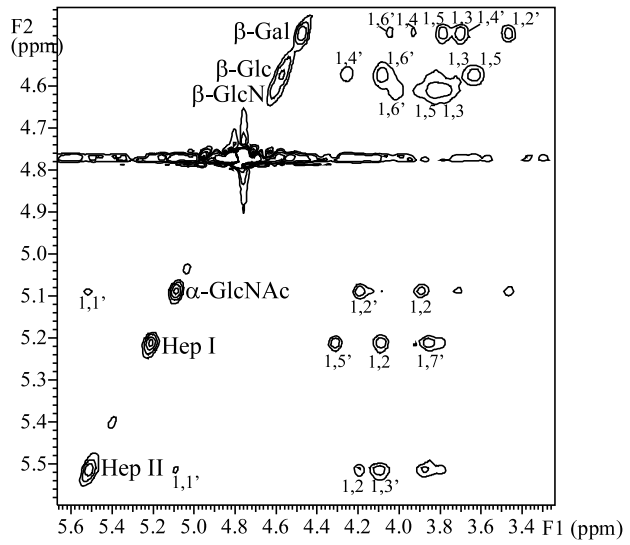


Fig. 4. Region of the 2D-NOESY $^1$H NMR spectrum of the O-deacylated LOS from *H. somnus* strain 1P. The spectrum was recorded in $D_2O$ with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0 and 25 °C.

mined from inter-residue $^1$H–$^1$H NOE measurements between anomeric and aglyconic protons on adjacent glycosyl residues (Fig. 4). Several long-range NOE connectivities were observed at higher intensity levels necessary to visualise some of the expected linkages, and those that could be attributed to specific resonances have been detailed in Table 2. The occurrence of an inter-residue NOE between H-1 of the Gal residue at 4.47 ppm and H-4 of the Glc residue at 3.72 ppm confirmed the partial sequence of β-Gal-(1→4)-β-Glc. Interestingly NOEs were also observed to the H-2 and H-6 protons of the Glc residue from the H-1 of the Gal residue as has been observed previously for a meningococcal strain with a similar linkage.[13]

The linkage pattern of the inner core residues were confirmed by comparison to previous data, indicating a common inner core structure as has been observed previously for meningococcal 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.[13,14] Hep I is also substituted at the 4-position by Glc 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 phosphate groups in the inner core was limited, consistent with the ESIMS data that revealed only a very small population of glycoforms with PEtn in the core OS, as characteristic peaks for the phosphorylation of the O-3 or O-6 positions of the Hep II residue were not observed. However, a cross peak between resonances at 3.33 and 4.14 ppm was observed, which is consistent with the $–CH_2–CH_2–$ protons of the ethanolamine group of PEtn.

This study has identified a simple truncated structure for the LOS from the commensal *Hs* strain 1P. This analysis has revealed similarities and differences between this structure and the previously published structure for the LOS from the pathogenic *Hs* strain 738.[11] The major differences are the complete absence of phosphocholine (PCho) and partial absence of PEtn from the inner core of this commensal strain. This strain also lacks the extended lacto-*N*-tetraose structure of the pathogenic strain. The commensal strain 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,[17] and as such is related to virulence; however, these commensal strains have successfully colonised a physiological niche, so perhaps the absence of PCho is not a significant factor in the ability of *Hs* strains to be considered pathogenic. It is known that preputial isolates only live in the urogenital tract, and there is no evidence that they can disseminate and cause disease, and although PCho may not be necessary to colonize the urogenital tract, some preputial isolates do have PCho, as determined by colony blotting with a PCho-specific Mab.[18] Recently, studies in our laboratory 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 lactose-like structure identified here as extending from Hep I is very often a good acceptor for sialylation, no sialylation was observed for this commensal strain. It is interesting to note that the LOS from the immunotype L8 strain of *Nm* has a similar structure to the LOS from strain 1P, apart from the presence of a PEtn residue at the 3-position of Hep II, and this LOS structure has also been found not to be sialylated. The absence of PEtn from the vast majority of glycoforms in the inner core of the commensal strain LOS when compared to the pathogenic strain is of interest. In the meningococcal inner core PEtn at the 3-position of Hep II has been found to be immunodominant.[19] It is likely that the presence or absence of PEtn in the inner core

LOS will have an impact on the conformation and charge characteristics of the LOS molecule, but the relationship to the pathogenic potential of the organism is again unclear. The presence of the inner core GlcNAc residue only in the commensal strain is of interest. The role of this residue in pathogenesis is not clear, but all meningococcal clinical strains that have been examined do contain this residue, suggesting its importance. It is apparent that more *Hs* LOS structures need to be determined before any firm conclusions can be drawn as to the importance of certain LOS structural features in relation to the pathogenic potential of a strain. Nonetheless, this study has advanced our knowledge of the LOS structure of *Hs* and suggested certain structural alterations that could be involved in the virulence potential of this organism.

## 1. Experimental

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

*H. somnus* strain 1P was originally isolated from the prepuce of a bull.[20] The organism was grown on Columbia blood agar (CBA) plates as described previously.[21] Cells were harvested by washing from CBA plates with phosphate-buffered saline, followed by centrifugation. The pellet was then freeze-dried. LOS was extracted from the freeze-dried cells (275 mg) by phenol extraction yielding 11 mg. O-Deacylated LOS was prepared by standard methods.[19]

### 1.2. Structural analysis

Alditol acetates were prepared and analysed as described previously.[17] All electrospray-ionisation–mass spectrometric (ESIMS) and capillary electrophoresis–mass spectrometric (CE–MS) analyses were carried out as described previously.[13] Nuclear magnetic resonance experiments were performed on Varian INOVA 500, 400 and 200 NMR spectrometers as described previously.[14]

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