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Structural analysis of the oligosaccharide of *Histophilus somni* (*Haemophilus somnus*) strain 2336 and identification of several lipooligosaccharide biosynthesis gene homologues

Frank St. Michael,[a] Jianjun Li,[a] Michael D. Howard,[b] A. Jane Duncan,[b] Thomas J. Inzana[b] and Andrew D. Cox[a,*]

[a]*Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, ON, Canada K1A 0R6*
[b]*Centre for Molecular Medicine and Infectious Diseases, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0342, USA*

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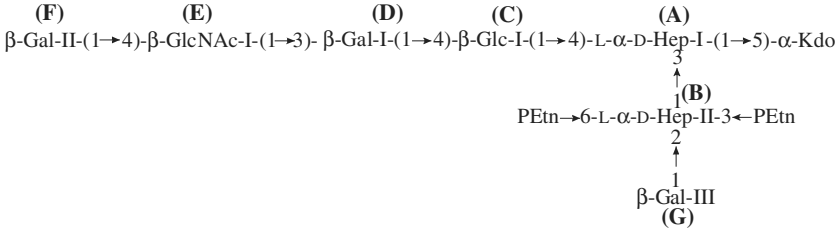
Abstract—The structure of the core oligosaccharide from a pneumonic *Histophilus somni* (*Haemophilus somnus*) strain 2336 was elucidated. The lipooligosaccharide (LOS) was subjected to a variety of degradative procedures. The structures of the purified products were established by monosaccharide and methylation analyses, NMR spectroscopy and mass spectrometry. The following structure for the core oligosaccharide was determined on the basis of the combined data from these experiments:



The structural elucidation was intriguing as it suggested several differences in the LOS structures between strain 2336 and the related strain 738. Strain 738 originated following passaging of strain 2336 through a calf. The differences between the two structures are a different linkage between Gal II and GlcNAc (1→4 here; 1→3 in 738), the absence of phosphocholine (PCho) from 2336 and the presence of two phosphoethanolamine (PEtn) residues and Gal III (at the 2-position) of Hep II in 2336. Although pulse-field gel electrophoresis data following digest with only one restriction enzyme showed identical profiles suggesting that strains 738 and 2336 are the same strain, the structural data does suggest that, if strain 738 is indeed a phase variant of strain 2336, considerable variation occurred on calf passaging and could therefore be an intriguing example of how broadly this bacterium can adapt itself in the host.

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

1. Introduction

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. When this bacterium disseminates via septicaemia, it may cause a variety of bovine diseases, including thrombotic meningo-encephalitis (TME), pneumonia, abortion, arthritis and myocarditis.[2–4] Potential virulence factors that are

* Corresponding author. Tel.: +1 613 991 6172; fax: +1 613 952 9092; e-mail: andrew.cox@nrc-cnrc.gc.ca

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known for this bacterium include the presence of immunoglobulin binding proteins on the cell surface,[5] the capability to survive and modulate 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] Commensal strains have been shown not to undergo phase variation to a substantial degree and tend to produce more truncated LOS molecules.[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]

Detailed structural analysis of *Hs* LOS has so far been restricted to only one pathogenic strain termed 738[11] and two commensal strains termed 1P and 129Pt.[12,13] These studies identified several structural similarities to the LOS of the human pathogen, *Neisseria meningitidis* (*Nm*). LOS from strain 738 elaborates a phase-variable outer core oligosaccharide structure, which, when fully extended, presents a lacto-*N*-tetraose unit, whereas fully extended LOS from some strains of *Nm* often present a lacto-*N*-neotetraose unit.[14] 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,[12] and LOS from strain 129Pt resembled immunotype L1 of *Nm*,[15] with a trisaccharide extension from the proximal heptose residue.[13] Recent work from our laboratory has confirmed that *Hs* LOS can be efficiently sialylated.[9] Strain 738 and a closely related isolate, 2336, the subject of this study, 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] which was borne out in the structural analysis of strains 1P and 129Pt.[12,13] Strain 738 was derived following calf passaging of strain 2336.[18] This study was therefore undertaken to determine the chemical structure of the LOS of the pathogenic strain 2336. In addition, the genome of strain 2336 is being sequenced to completion. Therefore, 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

LOS from *Hs* strain 2336 was extracted 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 LOS derived from different growths showed similar compositions 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 (LOS-OH) were prepared from the different growths and analysed by CE–ESMS in the negative-ion mode (Table 1). Similar mass spectra were observed with major peaks observed at 2347.6, 2509.5, 2713.5, 2874.6 and 3166.4 amu, consistent with a composition of 2Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH for the smallest glycoform with an additional Hex, HexNAc, Hex and sialic acid residues, respectively, for each of the larger molecules (where Lipid A-OH refers to the O-deacylated lipid A molecule). These compositions are consistent with the presence of two

Table 1. Negative-ion CE–ESIMS data and proposed compositions of O-deacylated LOS from *Histophilus somni* (*Haemophilus somnus*) strain 2336

Strain	Observed ions (*m/z*)		Molecular mass (Da)		Relative intensity	Proposed composition
	$(M-2H)^{2-}$	$(M-3H)^{3-}$	Observed	Calculated		
2336	1172.8	—	2347.6	2347.1	0.60	2Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Blood agar	1253.6	—	2509.5	2509.3	0.55	3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Grown	1355.8	903.5	2713.5	2712.5	0.20	HexNAc, 3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
O-deAc	1436.4	957.2	2874.7	2874.6	1.00	HexNAc, 4Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
	1582.0	1054.6	3166.4	3166.8	0.50	Sial, HexNAc, 4Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
2336	1172.8	—	2347.6	2347.1	1.00	2Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Broth	1253.6	—	2509.5	2509.3	0.65	3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Grown	1355.8	903.5	2713.5	2712.5	0.20	HexNAc, 3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
O-deAc	1436.4	957.2	2874.7	2874.6	0.80	HexNAc, 4Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
2336	1173.0	781.5	2347.6	2347.1	1.00	2Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Fermenter	1254.0	836.0	2509.5	2509.3	0.65	3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
Grown	1355.5	903.5	2713.5	2712.5	0.30	HexNAc, 3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
O-deAc	1436.5	957.5	2874.7	2874.6	0.65	HexNAc, 4Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH
	—	1054.5	3166.4	3166.8	0.30	Sial, HexNAc, 4Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH

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; PEtn, 123.05; Sial, 292.18. O-Deacylated lipid A (Lipid A-OH) is 952.00.

phosphoethanolamine (PEtn) residues. 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 identification of the basal lipid A-OH species therefore demonstrated that the two PEtn residues were both located in the core OS. In order to confirm the location of the PEtn residues and other structural features of the core OS, several CE–MS/MS experiments were performed in the positive-ion mode. MS/MS on the doubly charged ion at *m/z* 1256 that corresponds to the 3Hex, 2Hep, 2PEtn, 2Kdo, Lipid A-OH glycoform revealed a singly charged peak at *m/z* 1338 that corresponded to loss of a Kdo residue and the Lipid A-OH species due to the lability of the ketosidic bonds following fragmentation (Fig. 1a). Tentative conclusions could also be drawn from the fragmentation pattern observed in this experiment, which identified an ion at *m/z* 601, which could correspond to a [PEtn-Hep-PEtn]-Hex moiety. MS/MS/MS on the singly charged ion at *m/z* 1338 revealed an informative fragmentation pattern (Fig. 1b). Of most significance was the identification of a singly charged ion at *m/z* 439 diagnostic for a PEtn-Hep-PEtn species. The tentative identification of the [PEtn-Hep-PEtn]-Hex moiety was confirmed following MS/MS/MS of the singly charged ion *m/z* 601 whose fragmentation pattern showed that the two PEtn species were attached to the Hep residue, and that this species was substituted by a Hex residue (Fig. 1c).

Methylation analysis of LOS-OH isolated from blood agar plate grown cells suggested the presence of a terminal Gal, a 3-linked Gal, a 4-linked Glc, a 3,4-linked Hep and a 4-linked HexNAc residue.

In order to completely characterise the LOS structure, NMR spectroscopy was performed on the LOS-OH isolated from blood agar plate grown cells 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 well-resolved spectrum was obtained (Fig. 2). The assignment of ^1H resonances of the sugars of the *Hs* strain 2336 LOS-OH was achieved by TOCSY (Fig. 3) and COSY experiments (Table 2) and by comparison with reported data for *Hs* strains 738,[11] 1P[12] and 129Pt.[13] 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]

The region of the ^1H NMR spectrum (5.10–5.60 ppm) of the LOS-OH downfield of the HOD signal revealed three major signals (Fig. 2). Two signals at 5.27 (**B**) and 5.22 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,

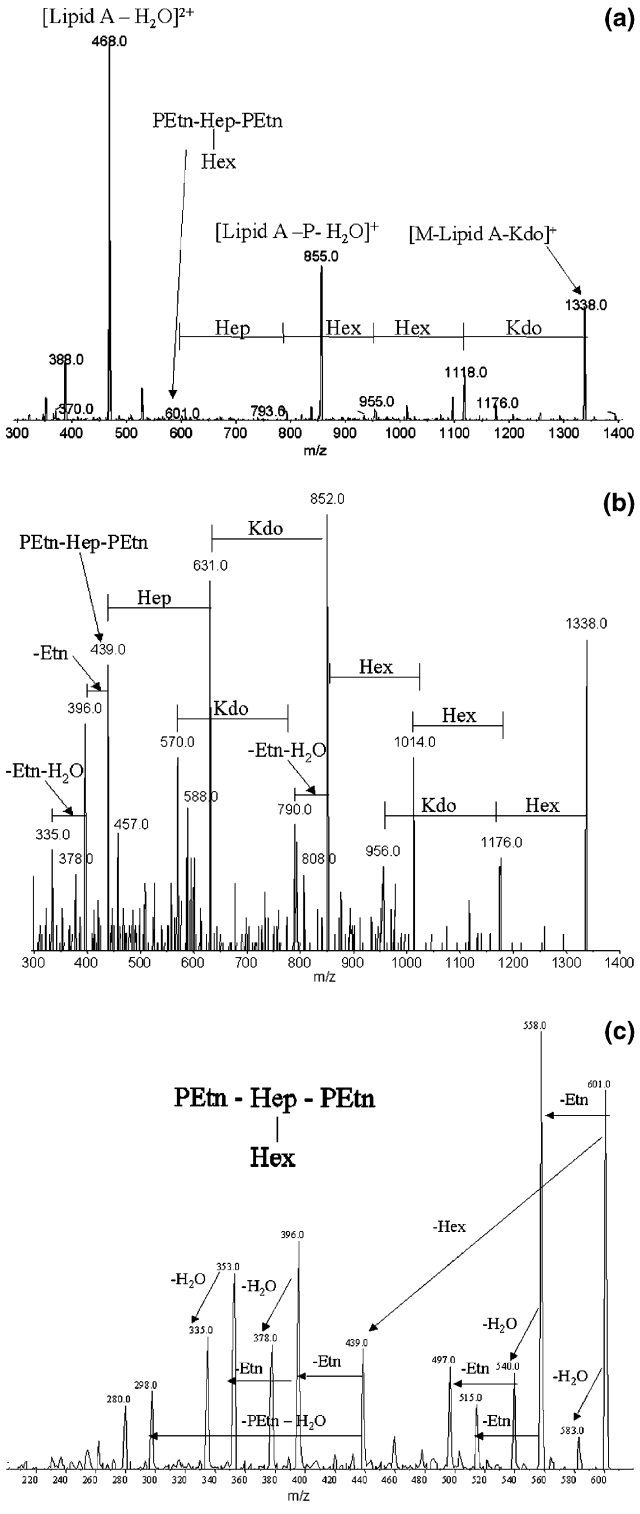

Figure 1. Positive-ion capillary electrophoresis–electrospray-ionisation mass spectra (CE–ESIMS) of the LOS-OH from *Hs* strain 2336. (a) Product ion spectrum from *m/z* 1256^{2+}; (b) Product ion spectrum from *m/z* 1338^{+}; (c) Product ion spectrum from *m/z* 601^{+}.

which pointed to *manno*-pyranosyl ring systems. The α-configurations for both of these residues were evident from the occurrence of a single-residue NOE between

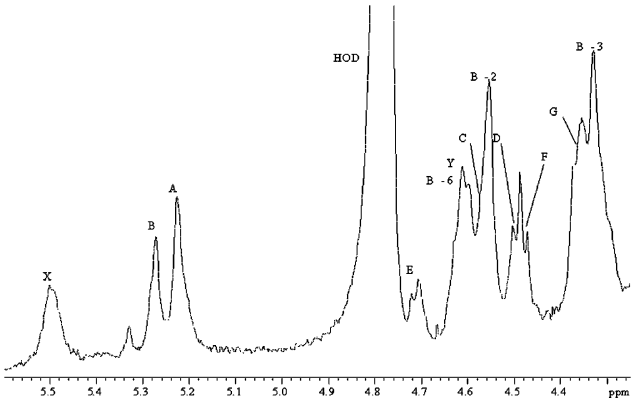

Figure 2. Anomeric region of the ^{1}H NMR spectrum of the O-deacylated LOS from *Hs* strain 12336. The spectrum was recorded at 25 °C in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0.

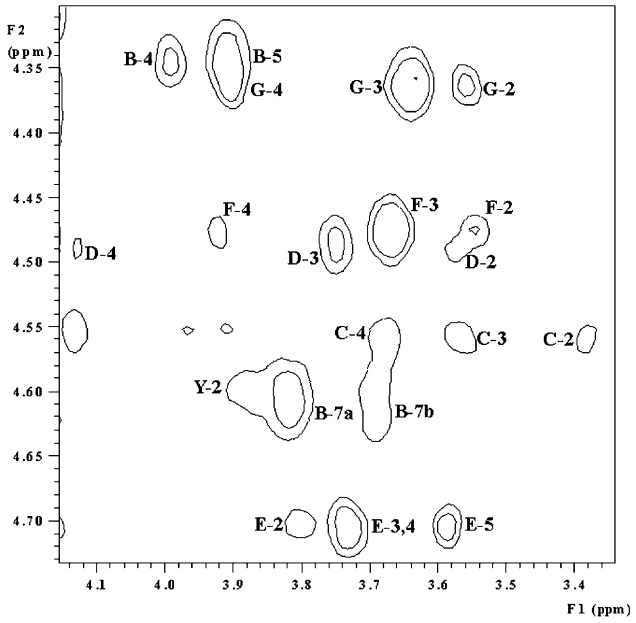

Figure 3. Region of the 2D-TOCSY ^{1}H NMR spectrum of the O-deacylated LOS from *Hs* strain 2336. The spectrum was recorded at 25 °C in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0. Letter designations are as in Table 2.

the H-1 and H-2 resonances.[18] The signal at 5.50 ppm was shown to be from a *gluco*-configured residue (**X**) 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 3.86 ppm correlated in the ^{1}H–^{13}C HMQC experiment to a ^{13}C resonance at 54.6 ppm, this chemical shift being diagnostic of amino-substituted carbons. The remaining signals in the low-field region up-field of the HOD signal were characterised by a TOCSY experiment (Fig. 3). Characteristic signals for a lacto-*N*-neo-tetraose unit were observed with two β-galactose residues (**D** & **F**), a β-glucose residue (**C**) and a β-*N*-acetylglucosamine residue (**E**). An additional β-galactose residue (**G**) was also observed with a ^{1}H-resonance of 4.36 ppm. Other resonances in this region of the spectrum included the H-3 and H-6 signals for heptose residue **B** at 4.34 and 4.62 ppm, identified by virtue of spin-systems including the H-4, H-5 and the H-7a, H-7b signals, respectively. The downfield chemical shifts for the ^{1}H-resonances of

Table 2. ^{1}H and ^{13}C NMR chemical shifts for the O-deacylated LOS from *Histophilus somni* (*Haemophilus somnus*) strain 2336

	H-1	H-2	H-3	H-4	H-5	H-6	H-7	NOEs
α-GlcN (**X**)	5.50 (92.5)	3.86 (55.0)	nd	nd	nd	nd	—	—
β-GlcN (**Y**)	4.61 (100.6)	3.89 (54.6)	nd	nd	nd	nd	—	nd
Hep I (**A**)	5.22 (100.4)	4.18 (70.4)	4.18 (73.2)	4.18 (74.6)	nd	4.09 (70.5)	nd	4.33 Kdo H-5
Hep II (**B**)	5.27 (100.3)	4.56 (77.8)	4.34 (74.5)	4.01 (70.5)	nd	4.62 (74.2)	3.90 3.75 (61.6)	4.18 **B**-3, 4.36 **G**-1
β-Glc I (**C**)	4.57 (102.7)	3.39 (73.4)	3.58 (74.9)	3.68 (76.8)	3.55 (75.4)	nd	—	4.18 **A**-4, 4.09 **A**-6
β-Gal III (**G**)	4.36 (104.2)	3.56 (71.1)	3.64 (73.1)	3.90 (68.9)	3.58 (78.1)	nd	—	4.56 **B**-2, 5.27 **B**-1
β-Gal I (**D**)	4.50 (102.7)	3.57 (71.8)	3.75 (81.8)	4.13 (68.6)	nd	nd	—	3.68 **C**-4
β-GlcNAc (**E**)	4.71 (102.9)	3.81 (55.2)	3.74 (75.4)	3.73 (78.2)	3.59 (75.4)	nd	—	3.75 **D**-3
β-Gal II (**F**)	4.48 (102.8)	3.55 (70.8)	3.68 (72.8)	3.93 (68.8)	nd	nd	—	3.73 **E**-4
PEtn (**3**)	4.16 (62.0)	3.30 (40.0)						
PEtn (**6**)	4.19 (62.1)	3.29 (40.1)						

Recorded at 25 °C, in D_2O. Chemical shifts referenced to internal acetone at 2.225 ppm.

B-3 and **B-6** being consistent with phosphorylation at these locations (see below). An anomeric ^{1}H-resonance for the β-configured GlcN residue of lipid A (**Y**) was assigned at 4.61 ppm by virtue of a cross peak to the H-2 resonance at 3.89 ppm, which correlated to a ^{13}C-resonance of 54.2 ppm in a ^{13}C–^{1}H-HSQC experiment, consistent with an amino sugar.

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. The occurrence of an inter-residue NOE between H-1 of the Gal III residue (**G**) at 4.36 ppm and the distal heptose residue (**B**) at the 2-position as evidenced by the characteristic H-1 to H-1 NOEs (Fig. 4), was of interest as this arrangement has not been observed before in the LOS of *Hs*. 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*.[11–13] **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 characteristic NOEs to the H-5 and H-7 resonances of Kdo (Fig. 4).

The extension from the proximal heptose residue **A** was found to consist of typical linkages for the lacto-*N*-neo-tetraose unit by virtue of characteristic inter-residue NOEs (Table 2). Interestingly, when compared to the related strain 738,[11] strain 2336 exhibits a type II β-(1→4)-linkage between the terminal galactose residue (**F**) and the *N*-acetylglucosamine residue (**E**) as illustrated when one compares the 1-D TOCSY spectra for the core OS of the 3-substituted GlcNAc residue (**F**) from strain 738, with the 2-D TOCSY spin system of 4-substituted GlcNAc residue (**E**) from strain 2336 (Fig. 5).

In order to complete the characterisation of the core OS region of the molecule, it was necessary to confirm the linkage positions of the two phosphoethanolamine (PEtn) residues that were inferred to be at the 3- and 6-positions of heptose residue B from a combination of MS and TOCSY data above. The location of the two PEtn residues 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 ^{31}P-resonances and signals at 4.34 and 4.62 ppm assigned to the H-3 and H-6 ^{1}H-resonances of heptose residue **B** (Fig. 6). This was confirmed in a 2-D ^{31}P–^{1}H-HSQC-TOCSY experiment (data not shown) that identified the H-1, H-2 and H-4 ^{1}H-resonances and the H-7a and H-7b ^{1}H-resonances from the H-3 and H-6 ^{1}H-resonances of residue **B**, respectively. Finally, to unequivocally prove the location of PEtn substitution at Hep II (**B**), methylation analysis was performed on the core OS, but subsequent to the initial methylation step the PEtn residues were removed by HF treatment, and the resulting product was re-methylated with iodomethane-d_3. Following derivatisation to give the partially methylated alditol acetates, the points of attachment of the two PEtn residues were confirmed at the 3- and 6-positions of a heptose residue.

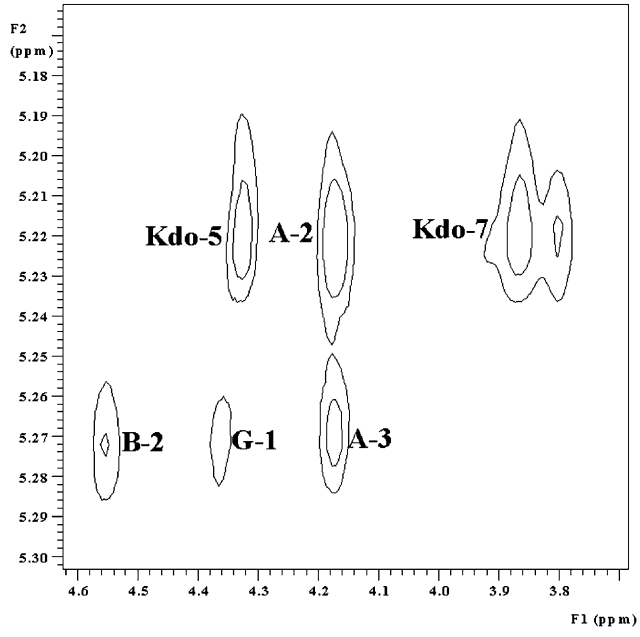

Figure 4. Region of the 2D-NOESY ^{1}H NMR spectrum of the O-deacylated LOS from *Hs* strain 2336. The spectrum was recorded at 25 °C in D_2O with deuterated SDS (5 mg) and deuterated EDTA (0.5 mg) at pH 7.0. Letter designations are as in Table 2.

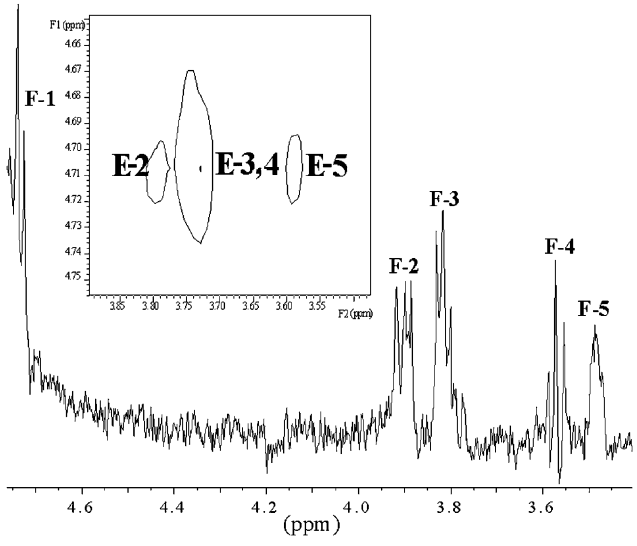

Figure 5. 1D-TOCSY spectrum of 3-substituted GlcNAc residue (F) from *Hs* strain 738 core OS and inset a region of 2D-TOCSY spectrum of 4-substituted GlcNAc residue (E) from *Hs* strain 2336 core OS. The spectra were recorded at 25 °C in D_2O at pH 7.0.

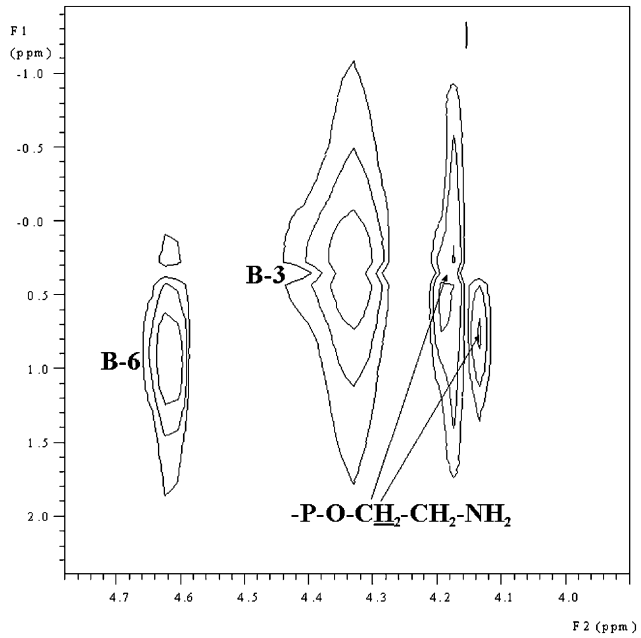

Figure 6. Region of the 2D-^{31}P–^{1}H-HSQC NMR spectrum of the core OS from *Hs* strain 2336 showing correlations between the ^{31}P-resonances (*x*-axis) and ^{1}H-resonances (*y*-axis) for the heptose residue (**B**) and the ethanolamine resonance as indicated. The spectrum was recorded at 25 °C in D_2O at pH 7.0.

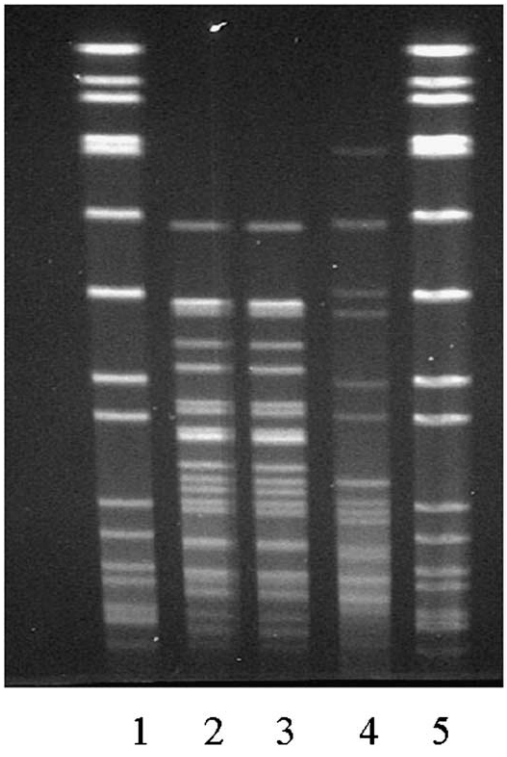

Figure 7. Pulsed-field gel electrophoresis profile of *Hs* strains as described in the Experimental. Lanes: 1, *Staphylococcus aureus*; 2, 2336; 3, 738; 4, 129Pt; 5, *Staphylococcus aureus*.

Due to the identification of several structural differences between the core OSs of strain 2336 and 738, a pulsed-field gel electrophoresis profile was obtained (Fig. 7), which illustrated that the two strains shared identical profiles following restriction digest with restriction enzyme *SmaI*.

3. Discussion

The structural analysis of the core OS from *Hs* strain 2336 revealed some interesting structural features and some significant structural differences from strain 738,[11] which is reported to be a phase variant of strain 2336 following passaging of strain 2336 through a calf.[16] The presence of a galactose residue (Gal III, **G**) at the second position of heptose residue **B** is unusual. In several species this heptose residue is indeed 2-substituted, but by another heptose residue in *Haemophilus influenzae*[19] and *Actinobacillus pleuropneumoniae*,[20] for example, or by an *N*-acetylglucosamine residue in *N. meningitidis*[14] and some strains of *Hs*.[12,13] The presence of two PEtn molecules at the same heptose residue has not been observed previously in *Hs* but has been observed before in *N. meningitidis*.[21] The genes responsible for the transfer of PEtn to the 3- and 6-positions of this heptose residue, *lpt-3* and *lpt-6* have recently been identified in *N. meningitidis* for *lpt-3*[22] and in both *H. influenzae* and *N. meningitidis* for *lpt-6*.[23] Examination of the 2336 genome revealed homologues for both *lpt-3* and *lpt-6* encoding for proteins with ~50% identity and 70% similarity to the neisserial proteins (Table 3). The absence of phosphocholine (PCho) from the core OS of strain 2336 was also a difference from strain 738. Four genes *lic1A–D* have been shown in *H. influenzae* to be involved in the transfer of PCho to the LOS.[24]

Examination of the 2336 genome revealed homologues to *lic1A–D*. However, there was a repeat tract in the *lic1A* gene, and in 2336 this would result in the gene being phase-varied off, whereas in *Hs* 738 the number of repeats is permissible for gene function (Elswafi, S., personal communication). The difference in the linkage of the terminal Gal-GlcNAc disaccharide between the two strains was also of interest, with strain 2336 elaborating a β-(1→4)-linked Gal-GlcNAc terminal disaccharide and strain 738 elaborating the β-(1→3)-linked disaccharide. Consistent with the structural analyses of these strains *Hs* strain 738 has been found to have galactosyl β-(1→3)- and β-(1→4)-transferase activity, whereas strain 2336 has no detectable β-(1→3)-activity (Wakarchuk, W. W., personal communication). The availability of the genome sequence for the *Hs* strain 2336, along with a thorough knowledge of the OS structure, enabled several candidate glycosyltransferases for the biosynthesis of strain 2336 inner core OS to be identified (Table 3). Outer core biosynthesis is likely controlled by six genes, *lob1* and *lob2a–e*, that have been identified as a locus in the evolving genome sequence

Table 3. Putative glycosyltransferases for the LOS inner core biosynthesis in *Hs* strain 2336

Hs gene designation	Putative function	Best homologues	Aa identity (%) Aa similarity (%)
kdtA	Kdo to lipid A α-(2→6)-Kdo transferase	KdtA *Pm* (PM 1305)	70 81
opsX	Hep I to Kdo α-(1→5)-heptosyltransferase	OpsX *Pm* (PM 1843)	66 79
rfaF	Hep II to Hep I α-(1→3)-heptosyltransferase	RfaF *Pm* (PM 1844) RfaF *Hi* (HI 1105)	73 84
lpt3	PEtn to 3-position of Hep II	Lpt3 *Nm* (NMB 2010)	50 70
lpt6	PEtn to 6-position of Hep II	Lpt6 *Nm* (NMA 0408)	53 70

and show similarity to several glycosyltransferases. All of these genes except *lob2c* contain repeat tracts indicative of the potential for phase variation. Previous work had suggested that *lob2a* was the α-(1→3)-*N*-acetylglucosaminyltransferase due to the absence of the terminal disaccharide in a *lob2a* mutant of strain 738.[25] Studies are underway to mutate the remaining *lob* genes and elucidate the structural consequences on the LOS structure in order to determine the specific function of each gene in LOS biosynthesis. This study has therefore identified the structure of strain 2336 OS, and coupled with genomic information, suggested putative glycosyltransferases responsible for the biosynthesis of this structure. The fact that there are several significant structural differences between the OS of these two variant strains 2336 and 738 illustrates the degree to which *Hs* can utilise phase variation in order to adapt itself to its host environment.

4. Experimental

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

Hs strain 2336 was originally isolated from the lung of a calf with pneumonia.[26] Initially, the organism was grown on Columbia blood agar (CBA) plates supplemented with 0.1% thiamine monophosphate (TMP) as described previously.[13] *Hs* strain 2336 was also grown in flasks of Columbia broth with 0.1% TMP yielding 1 g of freeze-dried cells, the LOS from which was isolated by a small-scale phenol extraction[27] (20 mL) giving ~50 mg of LOS. To enable complete structural analysis, *Hs* strain 2336 was grown in a 28-L fermenter in Todd Hewitt (15 g L^{-1})/Columbia broth (17.5 g L^{-1}) supplemented with 0.1% TMP for 24 h at 37 °C. The cells were killed by addition of phenol to 2% and harvested by using a Sharples continuous flow centrifuge giving 38.5 g wet wt. LOS was extracted from the cells by the hot phenol–water method (yield 120 mg). O-Deacylated LOS and core oligosaccharide were prepared by standard methods.[13]

4.2. Structural analysis

Alditol acetates were prepared and analysed as described previously.[20] Methylation analysis was carried out by the NaOH–DMSO–iodomethane procedure and analysed by GLC–MS as described previously.[20] To determine the location of phosphate residues, the methylation analysis procedure was modified whereby, following the initial methylation step, the methylated carbohydrate was treated with aqueous hydrofluoric acid (HF) (4 °C, 24 h) to remove the phosphate substituents and then re-methylated with iodomethane-d_3 (Aldrich) before continuing with the remaining methylation analysis procedure. All ESIMS (electrospray-ionisation MS) and CE–MS (capillary electrophoresis MS) analyses were carried out as described previously.[20] Nuclear magnetic resonance experiments were performed on Varian INOVA 500, 400 and 200 NMR spectrometers as described previously.[20]

4.3. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was performed on *H. somnus* strains 2336, 738 and 129Pt. DNA was extracted and digested using the restriction enzyme *SmaI* (New England Biolabs, Beverly, MA). A molecular weight ladder, *Staphylococcus aureus* (NCTC 8325), also digested with *SmaI*, was used as a control. Restriction fragments were separated by PFGE on a 1% agarose gel in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]) at 14 °C with the Bio-Rad GenePath system (Bio-Rad, Hercules, CA). The gel parameters were 6 V/cm for 24 h, using 5–50 s pulse times. Gels were then stained with ethidium bromide and analysed by Molecular Analyst Fingerprinting Plus (Bio-Rad, Hercules, CA) software.

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