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Structural and Genetic Characterisation of Variant Glycoforms of *Haemophilus influenzae* Lipopolysaccharide; Implications for Virulence*

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Abstract: *Haemophilus influenzae* (Hi) type b transformants RM135 and RM133 were previously found to differ in virulence and capsule content [Zwahlen *et al*., Microb Pathog 1986; 1: 465-73]. The more virulent strain RM135 produced less capsule than RM133, but was indicated to differ in its lipopolysaccharide (LPS). We have now performed detailed analyses of the respective LPSs and correlate structure, genetics and the original virulence observation. We found that RM133 expressed identical major LPS structures as the parent strain Rd whereas strain RM135 expressed shorter glycoforms. The genetic basis for this difference was found to be the expression of the phase-variable gene *lic 2A*, which could be shown to be compromised in RM135. The glycosyltransferase Lic2A is involved in the expression of a digalactoside (α-D-Gal*p*-(1→4)-β-D-Gal*p*(1→) and plays a role in chain elongation from the inner-core region of *Hi* LPS. One consequence of altered oligosaccharide extension, possibly contributing to the heightened virulence of RM135, was the sialylation of the LPSs. RM135 expressed *N*-acetylneuraminic acid whereas strain RM133 did not. Sialylation of LPS has been shown to be important for resistance of *Hi* to the killing effect of normal human serum. We thus propose that *lic 2A* expression can modulate virulence and that the effect of changes in LPS can outweigh that of capsule copy number.

Key words: *Haemophilus influenzae*, lipopolysaccharide, *Lic2A*, virulence, sialic acid, digalactoside

Introduction

Haemophilus influenzae (*Hi*) is an important commensal and pathogen of humans. *Hi* commonly colonises the upper respiratory tract and is found in both encapsulated (types a through f) and non-encapsulated, so-called non-typeable (NT), forms. Through contiguous spread, NT*Hi* are a relatively common cause of respiratory tract infections (e.g., otitis media, pneumonia) whereas encapsulated, mostly type b strains, can be invasive and cause bacteraemic infections such as meningitis and septicaemia. Lipopolysaccharide (LPS) is a major and essential component of the cell wall of *Hi* and is also a virulence determinant for both encapsulated and non-encapsulated strains. LPS can be involved at each stage of the pathogenesis of *Hi* infections, causes cytotoxic injury to target tissues and is a target for host immune responses (Hood and Moxon, 1999b).

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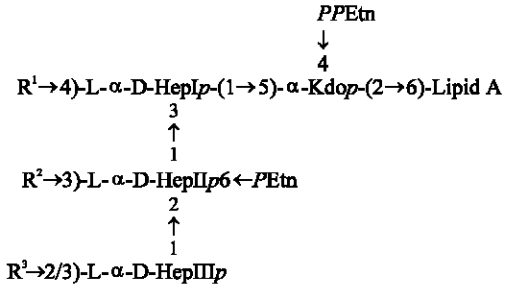
LPS from a number of different *Hi* strains has been analysed and shown to be composed of an oligosaccharide portion including a common L-*glycero*-D-*manno*-heptose-containing inner-core trisaccharide unit attached to the membrane bound lipid A moiety *via* a phosphorylated 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue (Månsson *et al*., 2001; Månsson *et al*., 2002; Månsson *et al*., 2003a; Månsson *et al*., 2003b; Masoud *et al*., 2003; Masoud *et al*., 1997; Phillips *et al*., 1992, 1993; Rahman *et al*., 1999; Risberg *et al*., 1999a, b; Risberg *et al*., 1997; Schweda *et al*., 1993, 1995, 2000). Each of the heptose (Hep) residues can provide a point for the addition of a hexose (Hex) residue, which in turn can lead to oligosaccharide chain extensions (Structure 1).



1 (R^1, R^2, R^3 = H or sugar residues)

The nature of the oligosaccharide chains and the degree to which they contain non-carbohydrate substituents shows intra- and inter-strain variation that can have profound effects upon the virulence of the organism. Several of the surface exposed epitopes of *Hi* LPS are subject to high frequency phase-variation (Hood and Moxon, 1999b; Jarosik and Hansen, 1994; Weiser *et al*., 1989), an adaptive mechanism which is advantageous for survival of bacteria confronted by the differing microenvironments and immune responses of the host. In type b strains, glycose substitution has been found to occur at each of the heptoses. The highest glycoform (Hex6) characterized for the *Hi* type b strain Eagan (Masoud *et al*., 1997) contains a β-D-Glc*p*-(1→ residue, an α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→ unit and a β-D-Gal*p*-(1→ residue which are linked to the heptoses at R^1, R^2 and R^3, respectively. In *Hi* strain Rd (RM118; Rd), a spontaneous, capsule-deficient derivative of a type d nasopharyngeal isolate, no elongation from the 2,3-di-substituted heptose is observed (i.e. R^2 = H) (Risberg *et al*., 1999b). In this strain HepI is substituted at O-4 by a *P*Cho→6)-β-D-Glc*p* unit (R^1), with chain elongation in the major glycoforms from O-2 of HepIII; a Hex5 glycoform that carries a globotetraose side-chain (R^3 = β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*) from which sequentially truncated Hex4 and Hex3 glycoforms contain globoside (R^3 = α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*) and lactose (R^3 = β-D-Gal*p*-(1→4)-β-D-Glc*p*) chains, respectively. In addition, minor components in the LPS containing sialic acid or *P*Etn-GalNAc have been identified (Cox *et al*., 2002; Hood *et al*., 2001a; Hood *et al*., 2004).

When strain Rd was transformed using whole cell genomic DNA obtained from the type b strain Eagan, two type b transformants (RM133 and RM135) were obtained which revealed significant differences in the degree of capsulation, the composition of LPS and virulence (Zwahlen *et al*., 1986). The capsule polysaccharide of type b *Hi* is composed of D-ribofuranosyl-D-ribitol phosphate repeating units (Crisel *et al*., 1975). RM133 makes about twice as much capsular polysaccharide as RM135. In addition, RM133 undergoes spontaneous, high frequency loss of capsule production. The LPS of RM135 is altered, as evidenced by loss of reactivity with monoclonal antibodies (Zwahlen *et al*., 1986). When inoculated into the infant rat, a well-characterized model of *Hi* infection, both transformants produced bacteremia and meningitis, but RM135 was consistently more virulent (Zwahlen *et al*., 1986). Since the more virulent strain RM135 produces less capsule than RM133, but differs in its LPS, we sought to characterize the LPS from each of the transformants and investigate

further the genetic basis of these differences and their association with the observed differential virulence properties.

Materials and Methods

Bacteria and Cultivation

The *Haemophilus influenzae* strains RM133 (Rd:b$^+$:01) and RM135 (Rd:b$^+$:02), obtained after transformation of strain RM118 (strain Rd) using genomic DNA obtained from the type b strain Eagan (RM153), have been described (Zwahlen *et al*., 1986). Bacteria were cultured in brain heart infusion (BHI) medium as described earlier (Hood *et al*., 1999c), with kanamycin (10 μg mL^{-1}) added for selection following transformation, when appropriate.

LPS Preparation

The LPS was extracted from bacteria by the hot phenol-water procedure (Schweda and Richards, 2003), followed by extensive dialysis. Crude LPS was purified by repeated ultracentrifugation (105 000 g at 4°C for 5 h).

Chromatography

Gel Permeation Chromatography (GPC) was performed using a Sephadex G50 column (2.5×80 cm) with pyridinium acetate (0.1 M, pH 5.3) as eluent. Column eluents were continuously monitored for changes in refractive index using a differential refractometer and collected fractions were assayed colorimetrically by the phenol-sulfuric acid method (Dubois *et al*., 1951) followed by lyophilization. GLC was performed on a Hewlett-Packard 5890 instrument using a DB-5 fused silica capillary column (25 m×0.25 mm ×0.25 μm) and a temperature gradient of 160°C (1 min) →250°C (1 min) at 3°C/min.

Preparation of Oligosaccharides

O-Deacylation of LPS with Hydrazine

O-Deacylation of LPS was performed as previously described (Schweda and Richards, 2003). Briefly, LPS was stirred in anhydrous hydrazine (200 μL) at 37°C for 1 h. The reaction mixture was cooled and 1 mL of cold acetone was added in small portions. The precipitated *O*-deacylated LPS (LPS-OH) was centrifuged (48 200 g, 20 min). The pellet was washed twice with cold acetone and dissolved in water for lyophilization.

Mild Hydrolysis of LPS

LPS from strain RM135 was hydrolysed in 1% aqueous acetic acid (pH 3.1, 100°C, 2 h) in the presence of borane-*N*-methylmorpholine complex. The reaction mixture was cooled and the precipitated lipid A was removed by centrifugation (48 200 g, 20 min). The water soluble part was purified by GPC and a major oligosaccharide fraction (OS-1) was obtained which was investigated further.

Mass Spectrometry

GLC-MS analyses were performed with a Delsi Di200 chromatograph equipped with a NERMAG R10-10H quadruple mass spectrometer. ESI-MS was performed with a VG Quattro Mass Spectrometer (Micromass, Manchester, UK.) in the negative mode. LPS-OH and the oligosaccharide (OS) sample were dissolved in a mixture of water: acetonitrile (1:1). Sample solutions were injected via the loop into a running solvent of water: acetonitrile (1:1) at a flow rate of 10 μL min^{-1}.

NMR Spectroscopy

^{1}H-NMR chemical shifts are reported in ppm and referenced to sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00). NMR spectra of OS-1 were obtained on a Varian UNITY

600 MHz spectrometer using standard pulse sequences for COSY, TOCSY and NOESY. Measurements were recorded in solutions of deuterium oxide at 30°C. Mixing times of 50 ms and 180 ms were used for 2D TOCSY experiments and a mixing time of 250 ms was used in the 2D NOESY experiment. A ^{1}H-NMR spectrum was acquired for LPS-OH of RM133 on a Bruker AMX 500 spectrometer at 25°C. The LPS-OH sample was solubilized by adding perdeutero-EDTA (2 mM) and perdeutero-SDS (10 mg mL^{-1}) to the D_2O solution.

Analytical Methods

Sugars were identified as their alditol acetates as described earlier (Sawardeker *et al*., 1965). Methylation analysis was accomplished on acetylated material which was obtained by treatment of the oligosaccharides with acetic anhydride (0.5 mL) and 4-diethylaminopyridine (0.5 mg) at room temperature (20-22°C) for 24 h. Methylation was then performed with methyl iodide in dimethylsulfoxide in the presence of lithium methylsulfinylmethanide (Blakeney and Stone, 1985). The methylated compounds were recovered using a SepPak C18 cartridge and subjected to sugar analysis. The relative proportions of the various alditol acetates and partially methylated alditol acetates obtained in sugar- and methylation analyses, discussed below, correspond to the detector response of the GLC-MS. Neu5Ac was determined by treating LPS-OH (0.2 mg) with 20 mU of neuraminidase in 0.2 mL 10 mM NaOAc, pH 5.0, at 37°C for 4 h. The reaction mixture was analyzed by high-performance anion-exchange chromatography (HPAEC) as previously described without further work-up (Bauer *et al*., 2001). The enzyme cleaves terminal Neu5Ac residues linked α-2,3, α-2,6 or α-2,8 to oligosaccharides.

DNA Methodology and Strain Construction

Restriction endonucleases and DNA polymerases were obtained from Boehringer Mannheim and used according to the manufacturers instructions. Chromosomal DNA was prepared from *Hi* strains as described previously (High *et al*., 1993).

To sequence the repeat region of the *lic2A* gene, PCR primers lic2a (5'-ACTGAACGTCGCAAAC) and lic2b (5'-GCTAATTAAACAGCCT) were designed to amplify a region of DNA from the 5' end of the gene from chromosomal DNA. Primer lic2b was labelled with biotin so that PCR amplified products could be sequenced directly using Dynabeads (Dynal, UK). PCR conditions were 1 min periods of denaturation (94°C), annealing (50°C) and polymerization (72°C) for 30 cycles.

The source of DNA used for transformation of *Hi* was the amplification products of the *lic2* locus from PCR of chromosomal DNA of strains RM118 *lic2A* (Hood *et al*., 2001b) and RM7004 *lic2A* ΔCAAT (High *et al*., 1996) using primers lic2A 121F (5'-ACTGAACGTCGCAAACAT-3') and lic2D (5'-AAACACTTAGGCCATACG-3'). These primers amplify the relevant *lic2A* gene and the adjacent gene, *ksgA*, encoding a kasugamycin resistance determinant (High *et al*., 1993). PCR conditions were as described above.

Hi strains were transformed by the MIV procedure (Herriott *et al*., 1970) using 14 μL of the relevant PCR product. Transformants were recultured on BHI kanamycin then were confirmed by PCR and or Southern analysis. For the lic2AΔCAAT transformants, replacement with a constitutive gene was verified by a smaller PCR product containing no repeats, when compared to wild type.

Analysis of LPS by Electrophoresis

The patterns of LPS isolated from wild type and mutant strains were determined after fractionation by tricine-SDS-polyacrylamide gel electrophoresis (T-SDS-PAGE) as described previously (Hood *et al*., 1999a).

Table 1: Negative ion ESI-MS data and proposed compositions for *O*-deacylated LPS (LPS-OH) of *Hi*strains RM133 and RM135. Average mass units were used for calculation of molecular weight values based on proposed compositions as follows: Hex, 162.14; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; *P*, 79.98; *P*Etn, 123.05; *P*Cho, 165.13 and lipid A-OH, 953.02. Relative abundance was estimated from the area of the ions relative to the total area (expressed as percentage). Signals representing less than 5% of the base peak are not included in the table

Observed ions (*m/z*)		Molecular mass (Da)		Relative abundance (%)		
$(M-3H)^{3-}$	$(M-2H)^{2-}$	Observed	Calculated	RM133	RM135	Proposed composition
703.7	1056.0	2113.1	2115.0	-	3	Hex_1•Hep_3•*P*Etn•*P*•Kdo•lipid A-OH
757.8	1137.2	2276.4	2277.1	8	22	Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH
812.9	1219.9	2441.8	2442.2	23	65	*P*Cho•Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH
853.9	1281.3	2564.7	2565.3	7	4	*P*Cho•Hex_2•Hep_3•$PEtn_2$•*P*•Kdo•lipidA-OH
866.3*	1300.4	2602.4	2604.3	19	3	*P*Cho•Hex_3•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH
920.5*	1381.8	2765.1	2766.3	23	1	*P*Cho•Hex_4•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH
988.6*	1483.0	2968.4	2969.5	16	2	*P*Cho•HexNAc•Hex_4• Hep_3•*P*Etn•*P*•Kdo•lipidA-OH
1029.1	-	3090.3	3092.6	4	-	*P*Cho•HexNAc•Hex_4•Hep_3 •$PEtn_2$•*P*•Kdo•lipidA-OH

* values are taken from the spectrum of RM133

Results

Structural Characterization of LPS from RM133

Compositional analysis of the LPS sample from strain RM133 indicated glucose, galactose, 2-amino-2-deoxy-glucose, 2-amino-2-deoxy-galactose and L-*glycero*-D-*manno*-heptose were present as identified by GLC-MS as their alditol acetate derivatives. Methylation analysis of LPS showed terminal glucose, terminal galactose, 4-substituted Gal, 4-substituted Glc, 3-substituted Gal, 2-substituted Hep and 3,4 substituted Hep in the relative proportions 8:17:11:18:8:20:16. Traces of terminal GlcNAc and GalNAc were also observed. The LPS of RM133 did not show any detectable amounts of *N*-acetylneuraminic acid (Neu5Ac) when subjected to HPAEC following treatment with neuraminidase (Bauer *et al*., 2001). Mild hydrazinolysis of LPS afforded water soluble *O*-deacylated LPS (LPS-OH). The ESI-MS spectrum of LPS-OH (Table 1) recorded in the negative mode showed major peaks in the triply charged region, at *m/z* 812.9, 866.3, 920.5 and 988.6 corresponding to *P*Cho•Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipid A-OH, *P*Cho•Hex_3•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH, *P*Cho•Hex_4•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH and *P*Cho•HexNAc•Hex_4•Hep_3•*P*Etn•*P*•Kdo•lipidA-OH, respectively. In addition, minor amounts of the *P*Cho-deficient Hex2 glycoform at *m/z* 757.9 corresponding to Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipid A-OH were observed. The ^{1}H-NMR spectrum of LPS-OH revealed, *inter alia*, signals at δ 5.09, 5.18, 5.58 and 5.48 corresponding to H-1 of the three heptose residues (HepI-HepIII) in the inner-core region of *Hi* LPS and the *N*-acylated, glycosidically phosphorylated α-D-GlcN residue in the lipid A region, respectively. An intense singlet corresponding to the *P*Cho methyl protons was observed at 3.24 ppm. It was concluded that the glycoforms of RM133 were similar, probably identical, to those of the parent strain (ie., RM118) (Risberg *et al*., 1999b) showing *P*Cho-containing Hex3, Hex4 and Hex5 glycoforms as major species. Thus, in the Hex3 glycoform a lactose unit, β-D-Gal*p*-(1→4)-β-D-Glc*p*, is attached at the O-2 position of the terminal heptose of the inner-core element. The Hex4 glycoform contains the P^K epitope, α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*, while in the Hex5 glycoform, this OS is elongated by the addition of a terminal β-D-Gal*p*NAc residue, giving the P antigen, β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p*.

Structural Characterization of LPS from RM135

Compositional analysis of the LPS sample from strain RM135 indicated glucose, 2-amino-2-deoxy-glucose and L-*glycero*-D-*manno*-heptose as identified by GLC-MS as their alditol acetate

derivatives. Methylation analysis of LPS showed terminal glucose, 2-substituted Hep and 3,4 substituted Hep in the relative proportions 1.4:1.0:1:0. Neu5Ac (c = 0.5 pmol μg^{-1}) was detected as a substituent of RM135 LPS by HPAEC following treatment with neuraminidase (Bauer *et al*., 2001). The negative ion ESI-MS spectrum of LPS-OH obtained by mild hydrazinolysis (Table 1) showed two major peaks in the triply charged region, at *m/z* 757.8 and at *m/z* 812.9 corresponding to Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipid A-OH and *P*Cho•Hex_2•Hep_3•*P*Etn•*P*•Kdo•lipid A-OH, respectively. This data clearly indicated that *Hi* strain RM135 produced more truncated glycoforms than strain RM133.

Mild acid hydrolysis of LPS from RM135 in the presence of borane-*N*-methylmorpholine complex gave an insoluble lipid A and Kdo-reduced core OS components which were purified by GPC on a Sephadex G-50 column to give one water soluble major fraction OS-1. Methylation analysis of OS-1 showed terminal glucose, 2-substituted Hep and 3,4 substituted Hep in the relative proportions 40:20:40. The data are consistent with biantennary structures, containing the common conserved inner core element L-α-D-Hep*p*-(1→2)-L-α-D-Hep*p*-(1→3)-[β-D-Glc*p*-(1→4)-]-L-α-D-Hep*p*-(1→5)-α-Kdo of *H. influenzae* LPS. In the ESI-MS spectrum of OS-1, a major, doubly charged [$(M-2H)^{2-}$] ion at *m/z* 704.3 was consistent with *P*Cho•Hex_2•Hep_3•*P*Etn•*An*Kdo-ol. A minor ion at *m/z* 621.7 corresponded to Hex_2•Hep_3•*P*Etn•*An*Kdo-ol. Trace amounts of an ion at *m/z* 785.2 corresponded to *P*Cho•Hex_3•Hep_3•*P*Etn•*An*Kdo-ol.

The ^{1}H-NMR resonances were assigned by ^{1}H-^{1}H chemical shift correlation experiments (COSY and TOCSY). Subspectra corresponding to all the glycosyl residues were identified on the basis of *J* connectivity pathways delineated in the ^{1}H chemical shift correlation maps, the chemical shift values and the vicinal coupling constants. The chemical shift data of OS-1 are summarized in Table 2. The chemical shift data are consistent with each D-sugar residue being present in the pyranosyl form. Further evidence for this conclusion was obtained from NOE data which also served to confirm the anomeric configurations of the linkages and monosaccharide sequence (Table 3). The ^{1}H-NMR spectra of OS-1 gave familiar patterns in the anomeric region which are characteristic of the triheptose-containing inner core element (Schweda and Richards, 2003). Spin systems for ethylene protons from *P*Etn were observed in the COSY/TOCSY spectra and were similar to those observed earlier (Risberg *et al*., 1999b). Anomeric resonances were observed at δ 5.66-5.61 (1 H, not resolved), δ 5.10 (1 H, not resolved) and δ 5.08-5.03 (1 H, not resolved) which corresponded to the three heptose residues (HepI-HepIII). The identities of the Hep ring systems and their α-configurations were confirmed from the observed small $J_{1,2}$ values and by the occurrence of NOE connectivities between the respective H-1 and H-2 resonances (Table 3) (Schweda and Richards, 2003). Signals for methylene protons of *An*Kdo-ol were observed in the COSY spectrum in the region 2.86-1.78 ppm. As observed earlier (Schweda *et al*., 1993), anhydro-forms of Kdo are formed during mild acid hydrolysis by elimination of the phosphate group from C-4 of Kdo which gives rise to microheterogeneity in the sample. As a consequence, several anomeric signals of HepI and HepII are observed (Table 2). Two anomeric resonances were observed at δ 4.63 and 4.50 and their relatively large $J_{1,2}$ (7.6 Hz and 7.4 Hz, respectively) indicated each to have the β-anomeric configuration. These residues could be attributed to terminal glucoses, designated GlcI and GlcII and are in agreement with methylation analysis. The arrangement of the glycoses within the inner core region was firmly established from transglycosidic proton NOEs between anomeric and aglyconic protons on contiguous residues. NOE measurements were made in the two-dimensional mode and are summarized in Table 3. The occurrence of intense transglycosidic NOE connectivities between the proton pairs HepIII H-1/HepII H-2, HepII H1/HepI H-3 confirmed the sequence of the heptose-containing trisaccharide unit L-α-D-Hep*p*-(1→2)-L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*-(1→ in OS-1. GlcI was linked to HepI as indicated by the NOE connectivities between H-1 of the terminal Glc and H-4/ H-6 of Hep I (Table 2). Inter residue NOEs were observed between H-1 of GlcII and H-1 and H-2 of HepIII, confirming the presence of the β-D-

Table 2: ^{1}H NMR chemical shifts for oligosaccharide preparation OS-1 derived from *H. influenzae* strain RM135. Data was recorded in D_2O at 25°C. $^{3}J_{H,H}$ values for anomeric ^{1}H resonances (H-1) are given in parentheses; n.r., not resolved (small coupling). The signal corresponding to *P*Cho methyl protons was observed at 3.24 ppm. Pairs of deoxyprotons of reduced, *An*Kdo were identified in the DQF-COSY at 2.16-1.85 ppm

Residue	Glycose unit	H-1	H-2	H-3	H-4	H-5	H-6_A	H-6_B	H-7_A	H-7_B
HepI	→3,4)-L-α-D-Hep*p*-(1→	5.03-5.08[a] (n.r.)	3.98-4.04[a]	4.00	4.22[b]	3.63	4.12[b]		-[c]	-
HepII	→2)-L-α-D-Hep*p*-(1→ 6 ↑ *P*etn	5.62-5.66 (n.r.)	4.31-4.32	3.96	-	-	4.54		3.73	3.87
HepIII	→3)-L-α-D-Hep*p*(1→	5.10 (n.r.)	4.22	3.96	3.77	-	-		-	-
GlcI	β-D-Glc*p*-(1→ 6 ↑ *P*Cho	4.50 (7.4)	3.38	3.46	3.59	3.53	4.15	4.25		
GlcII	β-D-Glc*p*-(1→	4.63 (7.6)	3.32	3.53	3.41	3.53	3.73	3.91		
*P*Etn		4.13	3.28							
*P*Cho		4.39	3.68							

[a] Several signals were observed for HepI and HepII due to heterogeneity in the *An*Kdo moiety. [b]H-4/H-6 of HepI are observed by NOE from H-1 of GlcI. [c] -, not obtained

Table 3: Proton NOE data for the oligosaccharide preparation OS-1 derived from *His*train RM135. Measurments were made from NOESY experiments

	Observed proton	
Anomeric proton	Intraresidue NOE	Interresidue NOE
Hep I	H-2	n.r. [a]
Hep II	H-2	H-3 of HepI; H-1of HepIII
Hep III	H-2	H-1, H-2 of Hep II; H-1 of Glc II
Glc I	H-3, H-5	H-4, H-6 of Hep I
Glc II	H-3, H-5	H-1, H-2 of Hep III

[a]n. r.; not rationalized

Glc*p*-(1→2)-L-α-D-Hep*p*(1→unit. The spin system of GlcI showed significant downfield shift displacements of H-6A (δ 4.15), H-6B (δ 4.25), H-5 (δ 3.59) compared to H-6A (δ 3.73), H-6B (δ 4.91), H-5 (δ 3.41) of GlcII. Thus, it was concluded, that GlcI was substituted with *P*Cho at the O-6 position. On the basis of the summarized data, OS-1 is concluded to have the structure shown for RM135 in Fig. 1.

The Differences in LPS Phenotype Between RM133 and RM135 Reflect Differences in lic2A

A parsimonious genetic explanation for the difference in the LPS structure between strains RM133 and RM135 would be differential expression of *lic*2A, the phase variable transferase responsible for adding the β-D-Gal*p* as the second hexose sugar in the extension from HepIII, as observed in the LPS structures of RM118 and RM133. *Lic2A* is phase variable as a consequence of a hypermutable tetranucleotide repeat tract within the reading frame. Thus, the number of repeats within this gene was determined in the same inocula of the transformants (RM133 and RM135) used to culture bacteria for LPS structural analysis and strains RM118 (the recipient for the donor DNA) and Eagan (the source of the DNA for the transformation of RM118). RM118 and RM133 had 22 copies of the 5'-CAAT-3' tetranucleotide and Eagan had 16 copies of the repeat, numbers that would place the different initiation codons, designated ATGx and ATGy, in frame (High *et al*., 1993). Strain RM135 had 23 copies of the repeat, a number of repeats that would place ATGz, a translational start

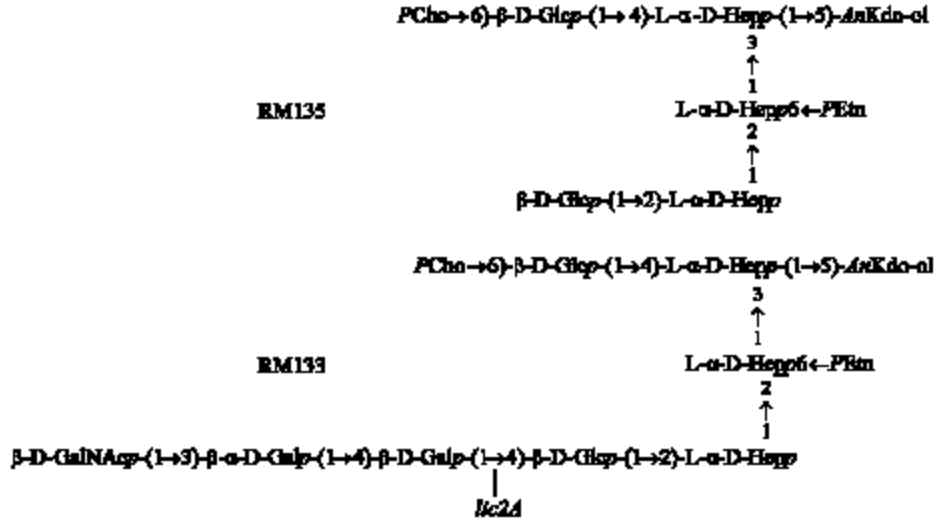


Fig. 1: Oligosaccharide structures identified for LPS isolated from *H. influenzae* strains RM135 (upper) and RM133 (lower). The proposed site of action of the gene *lic2A* is indicated. Abbreviations: *An*Kdo-ol, reduced anhydro Kdo; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; Hep, heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; *P*Cho, phosphocholine; *P*Etn, phosphoethanolamine

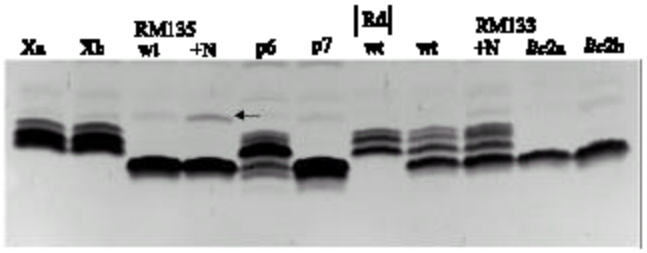


Fig. 2: Profiles of LPS isolated from strains RM118 (Rd), RM133 and RM135 and derived strains following transformation. wt indicates the parental strain in each case. Xa and Xb are independent transformants of strain RM135 obtained using PCR amplified products from chromosomal DNA and p6 and p7 are transformants using plasmid DNA, each input DNA has the *lic2A* gene fixed ON. *lic*2a and *lic*2b are independent transformants of strain RM133 with the *lic2A* gene mutated. +N indicates that the bacteria were grown in the presence of sialic acid. The arrow indicates the position of the sialylated lmsg

codon in a different frame from ATGx and ATGy, in frame. Thus, assuming that ATG_x/ATG_y and ATG_x reading frames both allow efficient translation of *lic*2A, each strain had a number of tetranucleotide repeats that should be permissive for translation of *lic2A* (High *et al*., 1993). DNA sequences 120 bp upstream and 350 bp downstream of the repeats were also obtained and showed no deviation from the *lic2A* gene in the published Rd genome sequence (http://www.tigr.org). Thus, an explanation for the differences in LPS structure was not obviously attributable to phase variable expression of *lic2A*.

However, expression of *lic2A* in strain RM135 might have been compromised by some other change in the sequence of the *lic2A* gene. To test this possibility, we transformed strain RM135 with DNA containing a *lic2A* gene with the tetranucleotide repeats removed that would constitutively express the gene product and some flanking DNA. When LPS from RM135 *lic*2AΔCAAT transformants was analysed by T-SDS-PAGE, a number of banding patterns were observed (Fig. 2). For several transformants there was no change when compared to RM135, but for others there was a restoration of a banding pattern similar to that seen in the parent strain, RM118 and strain RM133. These variant patterns remained stable following repeated subculture of the relevant strains. Thus, it is likely that this variation of phenotype is dependent upon the precise site of recombination between the transforming DNA and the target genes within strain RM135. Be that as it may, expression of the *lic2A* gene present in strain RM135 is evidently compromised and, whatever the mechanism(s), provides an explanation for the truncated LPS seen in RM135. In a parallel experiment, the *lic2A* gene was inactivated in strain RM133. As expected, LPS from strain RM133*lic2A* showed a banding pattern on T-SDS-PAGE that is typical of significant truncation and identical to that of strain RM135 (Fig. 2). Again, the findings are consistent with the hypothesis that the different glycoforms of RM133 and RM135 are the result of differences in the expression of *lic2A*.

The only other discernible difference between the LPS of RM133 and RM135 was the more prominent sialylated high molecular weight glycoforms (hmsg) in strain RM135 (Fig. 2). This hmsg is a four sugar lacto-N-neotetraose capped with sialic acid, added *en bloc* as a complete unit, as recently described for the LPS from strain RM118 (Cox *et al*., 2002).

Discussion

The LPS structures of *Hi* strains RM133 and RM135 have been investigated. LPS is a virulence determinant of *Hi* and its structure in the two strains might explain, in whole or in part, the observed differences in virulence between them in previous animal model experiments (Zwahlen *et al*., 1986). RM133 expresses the major higher glycoforms (Hex3, Hex4 and Hex5: Fig. 1) that are characteristic of the parent strain RM118 (Risberg *et al*., 1999b) whereas Hex3 and Hex4 glycoforms are at the limits of detection in RM135. Instead, RM135 expresses predominantly the Hex2 glycoform (Fig. 1), a structure that is not observed in the parent strain, RM118.

Strain RM118 produces LPS containing a number of LPS glycoforms, predominantly comprising Hex3 to Hex5 structures. The majority of this heterogeneity within the LPS population is recognised as being due to the number of phase variable genes involved in LPS synthesis in this organism (Hood *et al*., 2001). A bias in the normal pattern of random switching of expression, particularly for the *lic2A* gene, would provide an explanation for the results obtained from analysis of the LPS from strain RM135. *Lic*2A adds a β-D-Gal*p* as the second sugar in the globotetraose extension from HepIII of RM118 LPS. Non-translation of this gene through either an inappropriate number of repeats within the reading frame or some other mechanism would explain the truncated LPS observed. However, sequence analysis of the repeat tract from *lic2A* indicated that the number of repeats in RM135 was compatible with its translation, although we cannot exclude the possibility that differences in LPS expression might result from the use of the alternative start codon (ATGz) in RM135, as compared to the ATGx and ATGy start codons of strains RM133 and RM118. Both of these reading frames have been shown to permit Lic2A expression in the type b strain Eagan but ATG_z has not been associated with Lic2A expression in strain RM7004 (High *et al*., 1993). Alternatively, some other change in the unsequenced 3' portion of the *lic2A* gene or its regulatory region in strain RM135 was responsible for the difference in LPS phenotype. Irrespective of the basis of the defect in *lic2A* expression, compelling evidence for its involvement was provided by transformation of RM135 with a cloned *lic2A* gene in which the repeats had been deleted. Transformants of RM135 were obtained

that could no longer phase vary *lic2A* expression and that displayed a restored wild type LPS pattern. Depending on the precise positions of recombination, correction of the defect in *lic2A* would be expected to occur in only some of the transformants, thus explaining the different LPS phenotypes obtained. Although we did not pursue the precise genetic basis for the truncated LPS phenotype, the expression of the *lic2A* gene of RM135 is evidently compromised. Further confirmation of the crucial role of *lic2A* in synthesising different LPS in strains RM133 and RM135 was obtained by inactivating the *lic2A* gene in strain RM133 and obtaining a strain (RM133*lic2A*) in which the LPS was equivalent to that from strain RM135 and a *lic2A* mutant of strain RM118. Thus, changes in *lic2A* are sufficient to explain the altered LPS structure and potentially differences in the virulence phenotypes of RM133 and RM135.

It is somewhat counter-intuitive that truncation of the globotetraose LPS structure enhanced the virulence of strain RM135. In general, truncation of LPS results in attenuation rather than heightening of virulence in gram-negative bacteria, including *Hi* (Hood *et al*., 1996). However, it is possible that epitopes associated with the globotetraose structure are specifically targeted by the rat immune system and could enhance clearance. Although the prevalence of α1-4 linked digalactoside (e.g., globoseries glycolipids) on human cells has been suggested as an explanation, through mimicry, for the evolution of this self-antigen on the cell surface of *Hi* and *Neisseria meningitidis*, rats possess an abundance of α1-3, not α1-4, cell surface digalactosides and would not be constrained by mimicry from making an antibody response to these epitopes (Weiser and Pan, 1998). Another possible factor contributing to the heightened virulence of RM135 can be seen in Fig. 2. Sialylation of LPS has been shown to be important for resistance of *Hi* to the killing effect of normal human serum (Hood *et al*., 1999a). Strain RM118 can produce several sialylated LPS glycoforms (Hood *et al*., 2001a) and one of these, the hmsg, is evidently produced in greater quantities in strain RM135 when compared to RM133. This would be consistent with our previous finding that progressive truncation of the oligosaccharide extension from HepIII in strain RM118 results in an increase in the relative proportion of sialylated LPS molecules containing the hmsg (Cox *et al*., 2002). Significant levels of free sialic acid are available in serum and can be incorporated into LPS resulting in enhanced virulence (Hood *et al*., 2004).

A mutant strain of *Hi* (strain RM118-28), previously analysed in our laboratory, constructed by transforming strain RM118 with subclones of the *lic1* locus isolated from a type b strain (strain RM7004), also predominantly expressed a Hex2 glycoform in its LPS. In this strain, the location of the *P*Cho had been switched to O-6 of the β-D-Glc*p* residue attached to the terminal Hep residue in the inner-core region of LPS (Structure 1, R^3 = PCho→6)-β-D-Glc*p*; R^1 = β-D-Glc*p*, R^2= H) (Risberg *et al*., 1997). This location of *P*Cho is presumed to abrogate further chain extension from this heptose. In the current study, the transformation of RM118 to give strains RM135 and RM133 does not alter the *lic1* locus in the transformants and *P*Cho is expressed as in the parent strain.

In summary, we report the structural differences in LPS phenotype that were observed, but not detailed, in previous studies of RM133 and RM135 and provide evidence that the gene *lic2A* is responsible for these differences. These findings therefore offer a clear demonstration of how a single phase variable gene, *lic2A*, can modulate virulence and that the effect of *lic2A* can outweigh that of capsule copy number, since RM133 is less virulent than RM135, but makes twice as much type b capsule.

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