Structure of the Variable and Conserved Lipopolysaccharide Oligosaccharide Epitopes Expressed by *Haemophilus influenzae* Serotype b Strain Eagan[†,‡]

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Received August 8, 1996; Revised Manuscript Received November 14, 1996[⊗]

ABSTRACT: Lipopolysaccharide (LPS) is a major virulence determinant of *Haemophilus influenzae*. The organism is capable of expressing a heterogeneous population of LPS which exhibits extensive antigenic diversity among multiple oligosaccharide (OS) epitopes. Structural elucidation of variable and conserved OS epitopes of *H. influenzae* serotype b strain Eagan was determined by the application of high-field NMR techniques and MS-based methods on oligosaccharides obtained from LPS samples by a deacylation strategy. LPS extracted by the hot aqueous phenol method gave complex electrophoretic patterns consisting of at least six low-molecular mass bands. Electrospray ionization−mass spectrometry of *O*-deacylated LPS revealed a series of related structures differing in the number of hexose residues as well as subpopulations of glycoforms containing additional phosphoethanolamine (PEA) groups. It was demonstrated that the LPS contains a conserved PEA-substituted, heptose-containing trisaccharide inner core moiety attached *via* a KDO 4-phosphate unit to a lipid A component. Tandem MS experiments unambiguously established the presence of a KDO 4-pyrophosphoethanolamine unit in the subpopulation of LPS containing additional PEA groups. The occurrence of LPS containing this structural feature was found to be dependant on the isolation procedure used. Each heptose of the common inner core element L-α-D-Hep*p*(1→2)-L-α-D-Hep*p*(1→3)-L-α-D-Hep*p*(1→5)-α-KDO is substituted by a hexose residue with further chain elongation from the central unit. The structures of the major glycoforms containing four (three Glcs and one Gal), five (three Glcs and two Gals), and six (three Glcs and three Gals) hexoses were determined in detail. The Hex6 glycoform contains the terminal structure, α-D-Gal*p*(1→4)-*β*-D-Gal*p*(1→4)-*β*-D-Glc, providing, for the first time, definitive structural evidence for the expression of the P^k-blood group antigen in *H. influenzae* LPS. Moreover, an analogue of the Hex4 glycoform was identified in which the third heptose residue carries phosphate at *O*-4.

Haemophilus influenzae is a major cause of disease worldwide. In developed countries, serotype b capsular strains are associated with invasive diseases, including meningitis and pneumonia (Turk, 1981), while nontypable (i.e. acapsular) *H. influenzae* strains are primary pathogens in otitis media and respiratory tract infections (Murphy & Apicella, 1987). Lipopolysaccharide (LPS)[1] is an essential and characteristic surface component of these pathogens (Moxon & Maskell, 1992). Both capsular and nontypable strains of *H. influenzae* express heterogeneous populations of rough-type LPS which exhibit extensive antigenic diversity among multiple oligosaccharide (OS) epitopes (Flesher & Insel, 1978; Inzana, 1983). These carbohydrate regions of the LPS molecules provide targets for recognition by host immune systems, and expression of certain OS epitopes is implicated in virulence potential (Kimura & Hansen, 1986; Cope *et al.*, 1990; Maskell *et al.*, 1992). A structural model has been advanced for *Haemophilus* LPS in which a conserved heptose-containing inner core trisaccharide moiety is attached *via 3*-deoxy-D-*manno*-octulosonic acid (KDO) 4-phosphate to a lipid A component [see Gibson *et al.* (1996)]. Each heptose within this triad can provide a point for further OS chain elongation, leading to a vast array of possible structures (Phillips *et al.*, 1992, 1993; Schewda *et al.*, 1993, 1995). Recent immunochemical studies (Borelli *et al.*, 1995) confirm the presence of this common inner core element in LPS of a number of *Haemophilus* strains.

H. influenzae LPS can undergo rapid switching or phase variation between defined structures which leads to an extensive repertoire of OS epitopes within a single strain (Kimura & Hansen, 1986; Maskell *et al.*, 1991; Weiser, 1992). It is believed that phase variation provides a mechanism whereby the pathogen can adapt to variations in environmental conditions within and between individual hosts (Weiser *et al.*, 1990; Weiser, 1993). In serotype b capsular

[†] This work was supported in part by Programme Grants from the Medical Research Council, U.K., and the Wellcome Trust.

[‡] Presented in part at the XVIIIth International Carbohydrate Symposium in Milan, Italy, on July 21−26, 1996. This is National Research Council of Canada Publication Number 39540.

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[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: DOC−PAGE, deoxycholate−polyacrylamide gel electrophoresis; GLC−MS, gas liquid chromatography−mass spectrometry; ESI−MS, electrospray ionization−mass spectrometry; COSY, correlated spectroscopy; TOCSY, total correlated spectroscopy; HMQC, heteronuclear multiple-quantum coherence; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; KDO, 3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharide; Mab, monoclonal antibody; LPS-OH, *O*-deacylated LPS; lipid A-OH, *O*-deacylated lipid A; OS, oligosaccharide; PEA, phosphoethanolamine; PPEA, pyrophosphoethanolamine.

S0006-2960(96)01989-7 CCC: $14.00

strains, monoclonal antibody binding studies have revealed phase-variable expression of the galactose-containing P^k epitope, α-D-Gal*p*(1→4)-*β*-D-Gal*p* (Virji *et al.*, 1990; Mandrell *et al.*, 1992), an antigen also found on the surface of human epithelial cells (Lund *et al*., 1987). The molecular structure of this LPS OS epitope has not been established by chemical means. Here we employ a combination of NMR- and MS-based techniques for the elucidation of variable and conserved structural features exhibited by LPS of *H. influenzae* serotype b strain Eagan, providing, for the first time, definitive evidence for expression of the terminal α-D-Gal*p*(1→4)-*β*-D-Gal*p*(1→4)-*β*-D-Glc*p* unit. Our studies confirm the presence of L-α-D-Hep*p*(1→2)-L-α-D-Hep*p*(1→3)-L-α-D-Hep*p*(1→5)-KDO as a common LPS inner core element of this *H. influenzae* serotype b strain. It is established that triantennary structures constitute the major LPS population group in which each heptose is substituted by a hexose residue with further chain elongation from the central unit leading to full expression of the P^k epitope.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

H. influenzae strain Eagan is an encapsulated serotype b clinical isolate (Anderson *et al*., 1972). For this study, strains from two sources were employed: one from the Oxford culture collection (Oxford; NRCC 4247) and the other kindly supplied by Dr. P. Anderson (Rochester; NRCC 4246). Eagan strains were characterized by their binding to monoclonal antibodies (Mabs) 4C4, 6A2, and 12D9 but not to Mab 5G8 in colony blot assays (Gulig *et al*., 1987). Mabs were kindly supplied by Dr. E. H. Hansen.

Bacterial strains were cultivated in liquid media by using the following conditions. (1) Small-scale incubations were done with inocula of Oxford log phase cells at 37 °C for 20 h in brain heart infusion broth (BHI) (Oxoid) containing 2 mg/L NAD (Sigma) and 10 mg/L Hemin (Sigma) in 10 L batches as previously described (High *et al*., 1993). Cells were harvested by low-speed centrifugation (5000*g*), the pellet was resuspended in phosphate buffer saline containing 0.5% (w/v) phenol and then collected by centrifugation. (2) For large-scale preparations of LPS, Oxford or Rochester strains were first resuscitated from frozen stocks on chocolate agar plates (Quelab) and then cultivated at 37 °C in a medium containing BHI (3.7% w/v, Difco) supplemented with 2 mg/L Hemin (Sigma) in 28 and 75 L fermenters (New Brunswick Scientific). Stationary phase cells were killed by addition of phenol (1% final concentration) and harvested with a Sharples continuous-flow centrifuge.

Preparation of Lipopolysaccharide

The wet cell mass obtained by centrifugation of the bacterial growth was washed successively, once with ethanol, twice with acetone, and twice with light petroleum ether, and LPS was extracted from the air-dried cellular material by the hot phenol−water extraction procedure of Westphal *et al*. (1952). LPS was obtained from the aqueous phase either after extensive dialysis and lyophilization (method A) or by precipitation with 4 volumes of ethanol (method B), followed by purification by repeated ultracentrifugation (105000*g* at 4 °C for 2 × 5 h).

Deoxycholate−Polyacrylamide Gel Electrophoresis (DOC−PAGE)

PAGE was performed using the buffer system of Laemmli and Favre (1973) as modified by Komuro and Galanos (1988) with DOC as the detergent. LPS bands were visualized by silver staining as described by Tsai and Frasch (1982).

Preparation of Oligosaccharides

(1) Core Oligosaccharides. LPS (25−100 mg) was hydrolyzed in 1−2% aqueous acetic acid (5−20 mL) for 2.5 h at 100 °C; the solution was then cooled (4 °C), and the precipitated lipid A was removed by low-speed centrifugation (Masoud *et al*., 1994a). The supernatant solution was lyophilized, and the water soluble components were fractionated by gel filtration. A Bio-Gel P2 column (2.6 × 140 cm, 200−400 mesh, Bio-Rad) was used for this purpose, and elution was performed with pyridinium acetate (0.05 M, pH 4.5). Column eluants were monitored for neutral glycoses (Dubois *et al*., 1956), and the core oligosaccharide fractions were collected by lyophilization.

(2) O-Deacylated LPS. LPS was *O*-deacylated with anhydrous hydrazine under mild conditions (Holst *et al*., 1991) as previously described (Masoud *et al*., 1994a). For ESI−MS analysis, a sample (1−14 mg) was treated with anhydrous hydrazine (0.2−1.0 mL) and stirred at 37 °C. After 1 h, the reaction mixture was cooled (0 °C) and hydrazine destroyed by addition of cold acetone (5 mL), and the product was obtained by centrifugation. The precipitated product was washed with acetone (4 × 2 mL) and acetone−water (4:1, 5 mL) and then lyophilized from water.

(3) LPS Backbone Oligosaccharide. Backbone OS was prepared from LPS (400 mg) by *O*-deacylated with anhydrous hydrazine under mild conditions, followed by dephosphorylation with aqueous HF, reduction of the reducing terminus with $NaBH_4$, and *N*-deacylated with anhydrous hydrazine under more vigorous conditions as described previously (Holst *et al.*, 1991; Masoud *et al*., 1994b). The oligosaccharide products were fractionated on the Bio-Gel P2 gel filtration system as described above. Oxford LPS afforded high- and low-molecular mass OS fractions (labeled HMW and LMW, respectively) as the major components, while Rochester LPS gave a HMW fraction as the major product. The Rochester HMW fraction was resolved into Hex5 and Hex6 backbone OS glycoforms by further fractionation on the Bio-Gel P2 system.

Analytical Methods and Methylation Analysis

Glycoses were determined by GLC as their alditol acetate derivatives. Samples (0.2−0.5 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 90 min at 125 °C and evaporated to dryness under a stream of nitrogen. The liberated glycoses were reduced ($NaBH_4$) and acetylated (Ac_2O) as previously described (York *et al*., 1985b). The configuration of peracetylated heptitol derivatives was determined to be L-*glycero*-D-*manno* (or D-*glycero*-L-*manno*) by comparison of their GLC retention times with that of an authentic standard. On the basis of biosynthetic grounds (Coleman, 1983), the L-*glycero*-D-*manno* absolute stereochemistry is assumed. Hexoses were determined to have the D-configuration by GLC analysis of their acetylated (*R*)-

2-octylglycoside derivatives (Gerwig *et al*., 1979). The absolute configuration of KDO was previously reported to be D-*manno* (Helander *et al*., 1988).

GLC analysis was performed with a Hewlett-Packard model 5890 series II gas chromatograph fitted with a hydrogen-flame ionization detector, using a fused-silica capillary column (0.3 mm × 25m) containing 3% OV 17; an initial column temperature of 180 °C was held for 2 min, followed by an increase to 240 °C at 2 °C/min.

Methylation analysis was performed on oligosaccharide samples (2−5 mg) with iodomethane in dimethyl sulfoxide containing an excess of potassium (methylsulfinyl) methanide (Hakomori, 1964). Excess iodomethane was evaporated under a stream of nitrogen and water (3 mL) added, and the methylated products were extracted into chloroform (2 × 5 mL). The chloroform extracts were evaporated under N_2, and the methylated OS was purified on a Sep-Pak C-18 cartridge as previously described (Mort *et al*., 1983). For locating phosphate substituents, methylated OS was treated with aqueous 48% HF (0 °C for 48 h), dried under N_2, and remethylated with perdeuteiro iodomethane (CDN Isotopes Inc.) as above.

Purified methylated oligosaccharides were hydrolyzed with 0.25 M H_2SO_4 in 95% acetic acid at 85 °C overnight, reduced ($NaBD_4$), and acetylated according to the acetolysis procedure of Stellner *et al.* (1973). Partially methylated alditol acetates were separated by GLC and identified by electron impact on a Hewlett-Packard 5958 B or Varian Saturn II Iontrap GLC−MS system fitted with a DB-17 fused-silica capillary column (0.25 mm × 25 m), utilizing the temperature program of 180 °C for 2 min followed by an increase to 320 °C at 5 °C/min.

Electrospray Mass Spectrometry

Samples were analyzed on a VG Quattro Mass Spectrometer (Micromass, Manchester, U. K.) fitted with an electrospray ion source. Backbone oligosaccharide and *O*-deacylated LPS samples were dissolved in water, which was then mixed in a 1:1 ratio with 50% aqueous acetonitrile containing 1% acetic acid for mass spectral analysis in either the negative- or positive-ion mode. Samples were injected by direct infusion at 4 μL/min with a Harvard 22 syringe pump. The electrospray tip voltage was 2.7 kV, and the mass spectrometer was scanned from *m/z* 50 to 2500 with a scan time of 10 s. Data were collected in multichannel analysis mode, and data processing was handled by the VG data system (Masslynx). *O*- and *N*-deacylated LPS samples were analyzed as described above except samples were dissolved in acetonitrile−H_2O−methanol−10% aqueous ammonia (4:4:1:1), and a scan time of 15 s was used. For negative-ion MS−MS experiments, precursor ions were selected using the first quadrupole mass analyzer and fragment ions, formed by calycinal activation with argon in the RF-only quadrupole cell, were mass analyzed by scanning the third quadrupole. Collision energies were typically 60 eV (laboratory frame of reference).

Nuclear Magnetic Resonance Spectroscopy

NMR spectra were obtained with a Bruker AMX 500 spectrometer using standard Bruker software. All measurements were made on D_2O solutions at 37 °C after several lyophilizations with D_2O. Proton spectra were recorded at 500 MHz by using a spectral width of either 3 or 6 kHz and a 90° pulse. Broad-band proton-decoupled ^{13}C-NMR spectra were obtained at 125 MHz using a spectral width of 33.3 kHz, a 90° pulse, and WALTZ decoupling (Shaka *et al*., 1983). Acetone was used as the internal standard, and chemical shifts were referenced to the methyl resonance (δ_H, 2.225 ppm; δ_C, 31.07 ppm). Two-dimensional homonuclear proton chemical shift correlation experiments (COSY) (Bax *et al*., 1981) were measured over a spectral width of 2.2 or 1.5 kHz, using data sets ($t_1 \times t_2$) of 256 × 2048 or 512 × 2048 points; 32 or 64 scans were acquired, respectively. Spectra were processed in the magnitude mode with symmetrization about the diagonal. Two-dimensional total correlated (TOCSY) (Subramanian & Bax, 1987) and nuclear Overhauser effect (NOESY) (Kumar *et al*., 1980) experiments were performed in the phase-sensitive mode over a spectral width of 2.2 kHz, by using a data set of 256 × 2048 points. Mixing times of 66 and 400 ms were employed, and 64 and 128 scans were acquired for each t_1 value for TOCSY and NOESY, respectively. Heteronuclear two-dimensional ^{1}H−^{13}C chemical shift correlations were measured in the ^{1}H-detected mode *via* multiple-quantum coherence (HMQC) (Bax *et al*., 1983) with proton decoupling in the ^{13}C domain, by using data sets of 2048 × 256 points and spectral widths of 4.5 and 13.9 kHz for ^{1}H and ^{13}C domains, respectively. Sixty-four scans were acquired for each t_1 value. ^{31}P spectra were measured at 202 MHz with a spectral width of 13 kHz, and phosphoric acid (85%) was used as the external standard (δ_P, 0.0 ppm). ^{1}H−^{31}P correlations (HMQC) were made in the ^{1}H-detected mode by using a data matrix of 16 × 1024 points, sweep widths of 10 kHz for ^{31}P and 1.3 kHz for ^{1}H, and a mixing time of 60 ms.

RESULTS

Characterization of LPS from H. influenzae Eagan. H. influenzae Eagan from two sources (Oxford and Rochester) was grown in liquid culture, and cell wall LPS was isolated by the hot phenol−water extraction method (Westphal, 1952). LPS was obtained from the aqueous phase following extensive dialysis against tap water or by ethanol precipitation in yields of *ca.* 2−3% from dried bacterial cells. DOC−PAGE analysis of the LPS revealed a heterogeneous mixture of at least six low-molecular mass components having electrophoretic mobilities between those of the Ra (core) and SR (core + one repeat unit) bands of S-type *Salmonella* LPS (Figure 1). The R-type nature of *H. influenzae* LPS is consistent with results of earlier investigations (Flesher & Insel, 1978; Inzana, 1983). Apart from the relative intensity of the individual bands, similar LPS banding patterns were observed for the different strains of *H. influenzae* Eagan. Bacterial growth conditions[2] and the methods employed for LPS isolation were also found to influence the relative band intensities. A comparison of the DOC−PAGE banding patterns from Oxford and Rochester LPS revealed a preponderance of the intermediates to high-M_r LPS components in the latter (Figure 1). In agreement with previous reports (Zamze & Moxon, 1987; Inzana *et al*., 1985), compositional

[2] LPS obtained from *H. influenzae* Eagan grown in liquid culture, on solid media, and in the interstitial space of chicken eggs in our laboratories gave similar patterns of PAGE banding, differing only in the relative intensity of the individual bands (A. Martin, H. Masoud, M. E. Deadman, E. R. Moxon, and J. C. Richards, unpublished results).

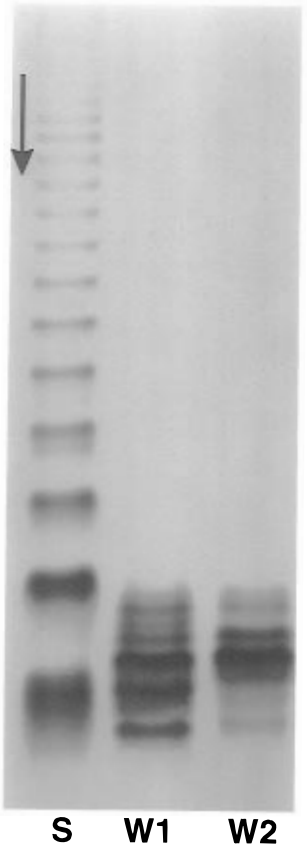


FIGURE 1: DOC−PAGE patterns of LPS from *Salmonella milwaukee* and *H. influenzae* serotype b Eagan: lane S, *S. milwaukee* (S-type LPS, 10 μg); lane W1, *H. influenzae* strain Oxford (4 μg); and lane W2, *H. influenzae* strain Rochester (4 μg).

analysis of the LPS samples indicated D-glucose (Glc), D-galactose (Gal), L-*glycero*-D-*manno*-heptose (Hep), and 2-amino-2-deoxy-D-glucose (GlcN) as the constituent glycoses which were identified by GLC−MS of the corresponding alditol acetate and (*R*)-2-octylglycoside derivatives. Significantly more galactose was detected in Rochester LPS (Glc:Gal ratio of 3:2) than in Oxford LPS (Glc:Gal ratio of 3:1.3).

Partial acid hydrolysis of LPS with dilute acetic acid afforded an insoluble lipid A and core OS fraction which was purified by gel filtration chromatography on the Bio-Gel P2 system. The core OS was composed of Glc, Gal, and Hep; GlcN was not detectable in the total acid hydrolysates of core OS. The presence of KDO has been previously reported (Zamze *et al.*, 1987).

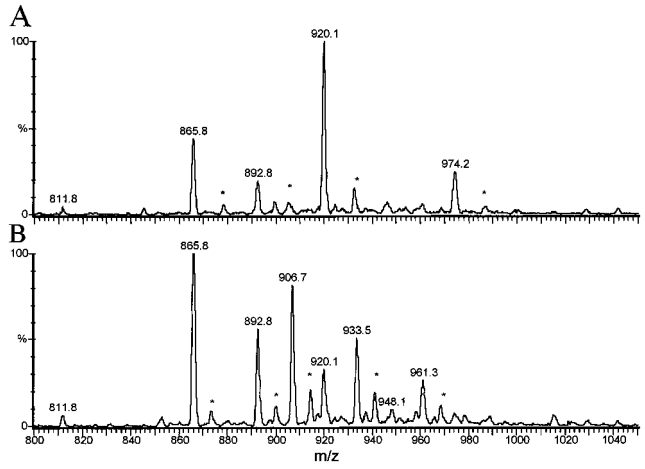


FIGURE 2: Negative-ion ESI−MS of the triply charged molecular ion region of *O*-deacylated LPS from *H. influenzae* Eagan strain Oxford obtained by phenol−water extraction following (A) extensive dialysis and (B) ethanol precipitation. The calculated molecular masses and proposed structures are given in Table 1. Ions indicated by an asterisk are due to potassium (spectrum A) or sodium (spectrum B) aducts of the major LPS-OH species.

Treatment of the LPS samples with anhydrous hydrazine under mild conditions (37 °C, 1 h) afforded water soluble *O*-deacylated material (LPS-OH). Electrospray ionization−mass spectrometry (ESI−MS) in the negative-ion mode of LPS-OH revealed a series of related structures differing in the number of hexose residues (Table 1). The ESI mass spectra were dominated by molecular peaks corresponding to doubly and triply deprotonated ions. The triply charged region of the ESI−MS of Oxford LPS-OH is shown in Figure 2. The MS data are consistent with each molecular species containing a conserved PEA-substituted, heptose-containing trisaccharide inner core moiety attached *via* a phosphorylated KDO linker to the putative *O*-deacylated lipid A (lipid A-OH) (Phillips *et al.*, 1993; Gibson *et al.*, 1993). *H. influenzae* lipid A-OH is known (Helander *et al.*, 1988) to be composed of a bis-phosphorylated β-1,6-linked glucosamine disaccharide substituted by 3-hydroxytetradecanoamide groups at C-2 and C-2′. Thus, the triply charged ions at *m/z* 865.8, 920.1, and 974.2 (Figure 2A), together with the corresponding doubly charged counterparts (Table 1), indicated that the major LPS glycoforms contain four (Hex4), five (Hex5), and six (Hex6) hexose residues, respectively.

Table 1: Negative-Ion ESI−MS Data and Proposed Compositions for *O*-Deacylated LPS of *H. influenzae* Strain Eagan[a]

LPS glycoform	observed ion (*m/z*)		molecular mass (Da)		proposed composition
	$[M - 3H]^{3-}$	$[M - 2H]^{2-}$	observed	calculated[b]	
Hex2[e]	757.8[c]	1137.1[c]	2276.2	2277.1	Hex_2·Hep_3·PEA_1·P_1·KDO_1·lipid A-OH
Hex3[e]	811.8	1218.3	2438.4	2439.2	Hex_3·Hep_3·PEA_1·P_1·KDO_1·lipid A-OH
Hex4	865.8	1299.3	2601.3	2601.3	Hex_4·Hep_3·PEA_1·P_1·KDO_1·lipid A-OH
	892.8	1339.7	2681.4	2681.3	Hex_4·Hep_3·PEA_1·P_2·KDO_1·lipid A-OH
	906.7	1360.9	2723.5	2724.4	Hex_4·Hep_3·PEA_2·P_1·KDO_1·lipid A-OH
	933.5	−	2803.5	2804.4	Hex_4·Hep_3·PEA_2·P_2·KDO_1·lipid A-OH
Hex5	920.1	1380.8	2763.6	2763.5	Hex_5·Hep_3·PEA_1·P_1·KDO_1·lipid A-OH
	961.3	−	2886.9	2886.5	Hex_5·Hep_3·PEA_2·P_1·KDO_1·lipid A-OH
Hex6	974.2[c,d]	1461.2[d]	2925.4	2925.6	Hex_6·Hep_3·PEA_1·P_1·KDO_1·lipid A-OH
	1015.1	−	3048.1	3048.6	Hex_6·Hep_3·PEA_2·P_1·KDO_1·lipid A-OH

[a] Unless indicated, data obtained on LPS-OH sample from Oxford strain using extraction condition B. [b] Average mass units were used for calculation (Gibson *et al.*, 1993) of molecular mass values based on proposed compositions as follows: Hex, 162.15; Hep, 192.17; KDO, 220.18; phosphate, 79.98; PEA, 123.05; and lipid A-OH, 953.03. [c] Data obtained on LPS-OH sample from Oxford strain using extraction condition A. [d] Data obtained on LPS-OH sample from Rochester strain using extraction condition A. [e] Minor components.

Table 2: Methylation Analysis of the LPS Backbone OS Fractions Derived from *H. influenzae* Strain Eagan

		relative detector response[c]		
		Oxford strain		Rochester strain
methylated sugar[a] (as alditol acetate)	T_m[b]	LMW fraction	HMW fraction	HMW fraction
2,3,4,6-Me_4-D-Glc	1.00	1.2	1.0	1.0
2,3,4,6-Me_4-D-Gal	1.06	0.9	1.9	1.8
2,3,6-Me_3-D-Gal	1.30	−	nd[d]	0.1
2,3,6-Me_3-D-Glc	1.31	1.2	2.3	2.1
2,3,4,6,7-Me_5-L,D-Hep	1.51	−	nd[d]	0.1
3,4,6,7-Me_4-L,D-Hep	1.71	0.7	1.0	0.9
2,6,7-Me_3-L,D-Hep	1.94	0.8	1.0	0.7
4,6,7-Me_3-L,D-Hep	2.03	1.0	1.4	0.9

[a] 2,3,4,6-Me_4-D-Glc represents 1,5-di-*O*-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol-d_1, etc. Data for neutral sugars are reported only. Partially methylated glycose derivatives from the GlcN(1→6)GlcN moiety were not detected, probably due to the relative resistance of the GlcN glycosidic linkage to the conditions used for acid hydrolysis (see Experimental Procedures). [b] Retention times (T_m) are quoted relative to that of 2,3,4,6-Me_4-D-Glc. [c] Values are not corrected for differences in detector response factors. [d] Value not determined.

LPS-OH glycoforms having similar compositions have been previously reported (Phillips *et al*., 1993, 1996) for *H. influenzae* strain A2. In strain Eagan, an abundant ion at *m/z* 892.8 indicated the presence of an additional Hex4 glycoform that contains an extra phosphate group (Figure 2A). Moreover, a parallel series of ions displaced by *ca.* 41 *m/z* units to higher mass in the triply charged region could be attributed to a subpopulation of glycoforms containing an additional PEA group (Table 1). This subpopulation of glycoforms was particularly evident in the MS of LPS-OH samples obtained following ethanol precipitation (Figure 2B). The ESI−MS of Rochester LPS-OH was similar to that of LPS-OH derived from the Oxford strain except that a preponderance of the Hex5 glycoform is observed (data not shown).

Characterization of Backbone OS Fractions. Backbone OS samples were obtained from LPS by a procedure involving deacylation, dephosphorylation, and reduction of the lipid A terminal GlcN residue (Masoud *et al*., 1994a). Oxford LPS afforded high- and low-molecular mass (HMW and LMW, respectively) backbone OS fractions following size exclusion chromatography on the Bio-Gel P2 system, while Rochester gave mainly a HMW fraction. The backbone OS fractions were found to contain Glc, Gal, Hep, GlcN, and KDO. Methylation analysis data for the HMW and LMW fractions are presented in Table 2. The three fractions gave qualitatively similar results, indicating that the major backbone OS components contain 3,4-di-*O*-substituted Hep*p*, 2,3-di-*O*-substituted Hep*p*, 2-*O*-substituted Hep*p*, 4-*O*-substituted Glc*p*, and terminal nonreducing Glc and Gal residues. The hydrolysis products from the HMW fractions contained significantly more 2,3,4,6-tetra-*O*-methyl-D-Gal and 2,3,6-tri-*O*-methyl-D-Glc, together with a lesser amount of 2,3,4,6-tetra-*O*-methyl-D-Glc than the LMW fraction. This is consistent with the major OS component in the HMW fraction (Hex5) containing an additional D-Gal residue. Results from methylation analysis of the Rochester HMW fraction revealed small amounts of 2,3,6-tri-*O*-methyl-D-Gal and 2,3,4,6,7-penta-*O*-methyl-L,D-Hep due to minor OS components.

The HMW samples from the two strains gave similar ESI−MS and ^{1}H-NMR spectra. A major doubly charged

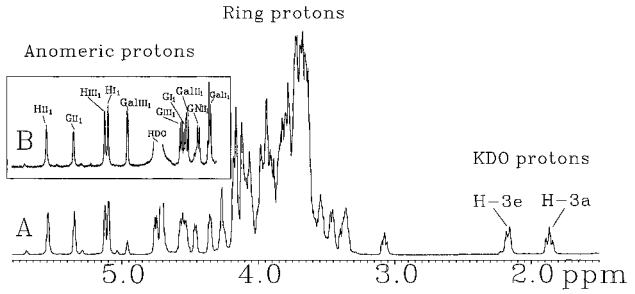


FIGURE 3: ^{1}H-NMR spectra of the backbone oligosaccharides from (A) the HMW fraction consisting predominantly of the Hex5 glycoform and (B) the derived Hex6 LPS glycoform produced from *H. influenzae* Eagan strain Rochester. Anomeric resonances are indicated using the following abbreviations: GlcI-GlcIII, GI-GIII; GlcN, GNII; and HepI-HepIII, HI-HIII.

$[M + 2]^{2+}$ ion at *m/z* 975.6 from the Hex5 glycoform (M_r of 1949.2) was observed in the positive-ion ESI−MS of the HMW fractions together with minor peaks at *m/z* 1056.5 (M_r of 2111.0) and 894.4 (M_r of 1786.8) from the Hex6 and Hex4 glycoforms, respectively. Nine major anomeric ^{1}H resonances were observed in the low-field region (5.6−4.3 ppm) of the ^{1}H-NMR spectrum of the HMW fraction (Figure 3A). This is particularly evident in the two-dimensional ^{1}H−^{13}C chemical shift correlation (HMQC) spectrum (Figure 4A). A minor anomeric resonance (relative signal area of *ca.* 0.2H) observed at 4.95 ppm could be attributed to an additional hexose residue from the Hex6 oligosaccharide (Figure 3A). The Hex6 backbone OS was obtained in a pure form by further fractionation of Rochester HMW material on the Bio-Gel P2 system. It showed 10 resonances in the anomeric region of the ^{1}H-NMR spectrum (Figure 3B) and a single doubly protonated molecular ion in its ESI−MS at *m/z* 1056.8. In the positive-ion ESI−MS of the LMW backbone OS fraction, an abundant doubly charged $[M + 2H]^{2+}$ ion was observed at *m/z* 894.4, consistent with the Hex4 LPS glycoform. As expected, the ^{1}H-detected HMQC spectrum of the LMW fraction revealed eight anomeric resonances from the Hex4 backbone OS in the low-field ^{1}H (5.6−4.3 ppm) and ^{13}C (105−90 ppm) regions. The ^{1}H- and ^{13}C-NMR resonances from the Hex4, Hex5, and Hex6 oligosaccharides were fully assigned (see below) by two-dimensional homo- and heteronuclear chemical shift correlation techniques (Masoud *et al*., 1994a,b), and the data are recorded in Tables 3 and 4.

Structure of the Hex5 Backbone OS. The HMW backbone OS sample from Rochester LPS was subjected to detailed NMR analysis which led to determination of the sequence of glycoses within the Hex5 glycoform. The ^{1}H-NMR resonances of this OS were fully assigned by COSY (Figure 5A) and TOCSY (data not shown) experiments. Subspectra corresponding to all the glycosyl residues were identified on the basis of connectivity pathways delineated in the ^{1}H chemical shift correlation maps (Masoud *et al*., 1994a,b), the chemical shift values (Bock & Thøgerssen, 1982), and the vicinal proton coupling constants (Altona & Haasnoot, 1980). The chemical shift data (Table 3) are consistent with each D-sugar residue being present in the pyranosyl ring form. Further evidence for this conclusion was obtained from NOE data (Figure 5B) which also served to confirm the anomeric configurations of the linkages.

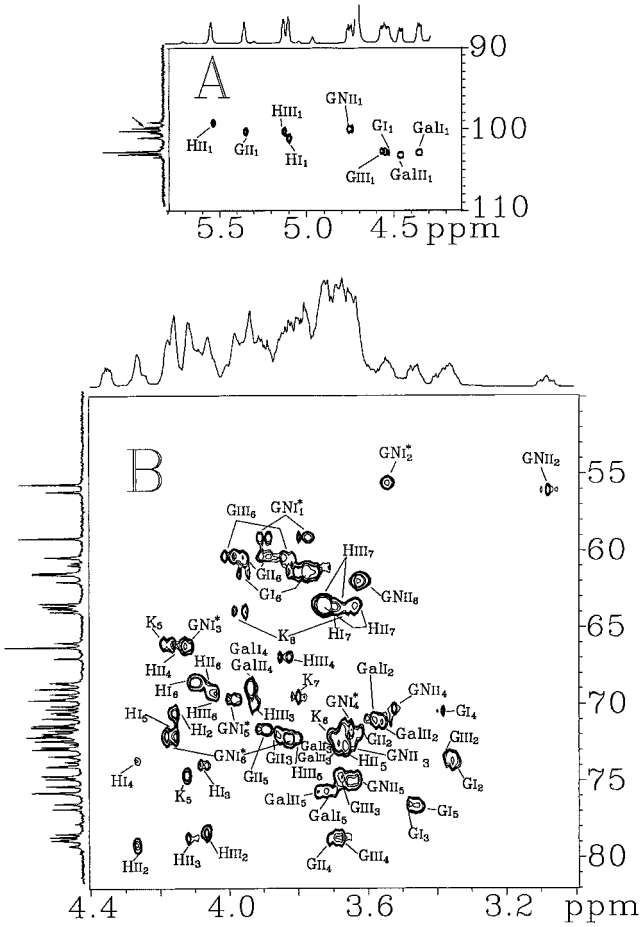


FIGURE 4: Heteronuclear 2D $^1H-^{13}C$ chemical shift correlation map of (A) the anomeric and (B) the ring regions of the HMW backbone OS (predominantly Hex5). Resonance assignments are indicated. Abbreviations are as follows: GlcNol, GNI*; and KDO, K; the abbreviations are also as indicated in Figure 3.

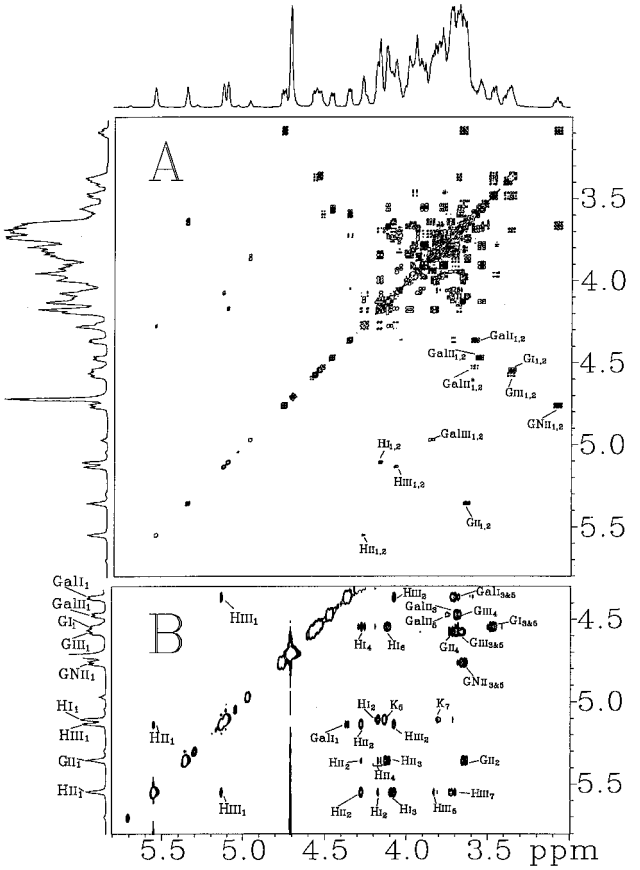


FIGURE 5: Partial 2D homonuclear (A) COSY and (B) NOESY spectra of the HMW backbone OS (predominantly Hex5). H-1−H-2 cross-peaks and NOE connectivities are labeled in spectra A and B, respectively. Cross-peaks labeled by GalIII* and GalII* are from the α-D-Gal(1→4)-β-D-Gal(1→ end group arising from minor amounts of the Hex6 glycoform in the sample. Abbreviations are given in Figures 3 and 4.

Assignments of the ^{13}C resonances were made by one-bond correlation with the ^1H resonances of the directly attached protons in the HMQC experiment (Figure 4). In the one-dimensional ^{13}C-NMR spectrum, resonances were observed in the low-field region (90−105 ppm) corresponding to the anomeric carbons from 10 sugar residues. Nine of these residues were directly correlated to the corresponding anomeric ^1H resonances in the HMQC experiment (Figure 4A). A ^{13}C resonance showing an absence of a $^1H-^{13}C$ connectivity in the HMQC experiment (100.44 ppm) was assigned to the C-2 (quaternary) resonance from the KDO residue. In addition, a diagnostic signal from the methylene carbon (C-3) of the KDO residue was observed at 35.56 ppm. The corresponding H-3 methylene protons showed characteristic resonances (Masoud *et al.*, 1994a,b; Carlson *et al.*, 1988; York *et al.*, 1985a), at 1.86 (t, 1H, H-3_{ax}) and 2.17 ppm (dd, 1H, H-3_{eq}), indicative of an α-linked KDO residue.

The ^1H subspectra corresponding to the three heptose residues (HepI−HepIII) were identified on the basis of the observed small $J_{1,2}$ (<1 Hz) and $J_{2,3}$ (4.4 Hz) values, indicative of the *manno*-pyranose ring systems, and by the fact that eight proton resonances were associated within each subspectra. The anomeric ^1H and ^{13}C chemical shift values indicated (Masoud *et al.*, 1994a, 1995) that each of the Hep ring systems has the α-D-configuration, and this was confirmed by the occurrence of single intraring NOE between the respective H-1 and H-2 Resonances (Richards & Perry, 1988). Two subspectra typical of β-D-galactopyranose ring systems (labeled GalI and GalII) were identified from the magnitude of their respective $J_{1,2}$ (7.5 Hz), $J_{2,3}$ (8.8 Hz), $J_{3,4}$ (3.2 Hz), and $J_{4,5}$ (∼1 Hz) values. On the basis of the observed large $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$, ring couplings (8−10 Hz), three ^1H subspectra were attributed to hexopyranosyl residues having the *gluco* configuration (GlcI, GlcII, and GlcN). From the anomeric proton couplings, GlcII was assigned the α-D configuration ($J_{1,2}$ = 3.8 Hz), while GlcI and GlcN were identified as having the β-D-configuration ($J_{1,2}$ = 7.5 Hz). The later residue could be attributed to the lipid A-derived β-D-GlcN residue from the ^{13}C chemical shift value (56.63 ppm) of its C-2 (Bock & Pedersen, 1974). Assignment of the ^1H spin system from the reduced terminal glucosamine residue (GlcNol) of the β-1,6-linked D-glucosamine disaccharide moiety of the deacylated lipid A was facilitated by correlation between its C-2 (56.15 ppm) and H-2 (3.53 ppm) in the HMQC experiment (Figure 4B). The relative stereochemistry of the subspectrum for GlcIII was not readily discernible from the ^1H spin system since second-order effects rendered the proton couplings difficult to determine (Table 3). Since methylation analysis indicated three Glc residues in the HMW fraction, this subspectrum could be attributed to a glucopyranose residue. In accord with this assignment, the occurrence of a $^1H-^1H$ NOE between H-1, H-3, and H-5 (Figure 5B) pointed to the β-D-pyranosyl ring

Table 3: Proton Chemical Shifts (Parts per Million) and Coupling Constants (Hertz) for the LPS Backbone Oligosaccharide Derived from *H. influenzae* Eagan[a]

LPS glycoform[b]	residue	glycose unit	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$, $J_{6,6'}$)	H-7 ($J_{6,7}$)	H-7′ ($J_{6,7'}$, $J_{7,7'}$)	H-8 ($J_{7,8}$)	H-8′ ($J_{7,8'}$, $J_{8,8'}$)
Hex4	GlcNol	→6)-D-GlcNol	3.89[c]	3.53	4.11	3.65	3.98	4.16	3.82				
			(4.4)	(4.4)	(≈1.0)	(8.8)		(3.2)	(7.3, 11.1)				
	GlcN	→6)-β-D-Glc*p*N(1→	4.74	3.07	3.64	3.51	3.63	3.62	–[f]				
			(7.5)	(–)[e]	(–)	(–)		(–)					
	KDO	→5)-α-KDO*p*(2→			1.86[d]	4.17	4.11	3.68		3.79		3.96	3.64
					(12.4)	(5.8)		(≈1.0)		(8.8)		(4.4)	(5.8, 11.7)
	HepI	→3)-L-α-D-Hep*p*(1→	5.09	4.15	4.06	4.25	4.16	4.10		3.71	3.71		
		4	(≈1.0)	(4.4)	(9.1)	(10.2)		(≈1.0)		(≈1.0)	(≈1.0, 11.7)		
		↑											
	HepII	→2)-L-α-D-Hep*p*(1→	5.53	4.26	4.10	4.15	3.68	4.08		3.62	3.72		
		3	(≈1.0)	(4.4)	(9.3)	(9.3)		(≈1.0)		(5.8)	(8.8, 10.2)		
		↑											
	HepIII	→2)-L-α-D-Hep*p*(1→	5.11	4.05	3.92	3.83	3.80	4.04		3.72	3.68		
			(≈1.0)	(4.4)	(8.8)	(9.3)		(≈1.0)		(4.4)	(9.1, 11.1)		
	GalI	β-D-Gal*p*(1→	4.35	3.57	3.67	3.92	3.69	3.75	3.81				
			(7.5)	(8.8)	(3.2)	(≈1.0)		(2.9)	(7.3, 11.7)				
	GlcI	β-D-Glc*p*(1→	4.53	3.34	3.46	3.37	3.45	3.95	3.77				
			(7.5)	(8.8)	(8.8)	(8.8)		(≈1.0)	(5.8, 11.7)				
	GlcII	→4)-α-D-Glc*p*(1→	5.34	3.62	3.85	3.70	3.89	3.96	3.88				
			(3.8)	(9.3)	(9.3)	(9.3)		(≈1.0)	(–,–)				
	GlcIII*	β-D-Glc*p*(1→	4.53	3.31	3.53	3.42	3.51	3.94	3.74				
			(7.5)	(9.2)	(9.2)	(9.2)		(3.0)	(5.7, 11.9)				
Hex5	GlcIII	→4)-β-D-Glc*p*(1→	4.56	3.35	≈3.68	≈3.62	≈3.62	3.99	3.84				
			(7.5)	(8.8)	(–)	(–)		(–)	(–, –)				
	GalII	β-D-Gal*p*(1→	4.45	3.54	3.66	3.93	3.72	3.78	–				
			(7.5)	(8.8)	(3.2)	(≈1.0)		(–)					
Hex6	GalII*	→4)-β-D-Gal*p*(1→	4.51	3.58	3.74	4.04	3.79	3.85	3.93				
			(8.1)	(9.6)	(4.2)	(≈1.0)		(4.2)	(7.5, 12.5)				
	GalIII*	α-D-Gal*p*(1→	4.95	3.83	3.91	4.03	4.35	3.72	3.69				
			(3.6)	(10.2)	(4.2)	(≈1.0)		(–)	(6.9, ≈11.1)				

[a] Measured at 32 °C (Hex5) or 27 °C (Hex4 and Hex6) in D_2O (pD ~6) from COSY spectra. [b] Chemical shift values for conserved residues (i.e., residues GlcNol to GlcII) in Hex4, Hex5, and Hex6 glycoforms are the same (±0.01 ppm). In Hex4, the central oligosaccharide chain carries GlcIII* as a terminal residue. Hex5 contains the disaccharide GalII-GlcIII as the terminal end group; Hex6 contains the disaccharide GalIII*-GalII* as the terminal end group. [c] Values for H-1 and $J_{1,2}$: H-1′ = 3.78 ppm; $J_{1',2}$ = 6.4 Hz, $J_{1,1'}$ = 11.7 Hz. [d] Values for H-3a and $J_{3a,4}$: H-3_e = 2.17 ppm; $J_{3e,4}$ = 4.1 Hz; $J_{3a,3e}$ = 12.4 Hz (a and e correspond to the axial and equatorial protons of KDO, respectively). [e] (–), coupling constant unresolved. [f] –, chemical shift unresolved.

Table 4: ^{13}C Chemical Shifts (Parts per Million) for the LPS Backbone Oligosaccharides Derived from *H. influenzae* Eagan[a]

LPS glycoform	residue	glycose unit	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Hex4[b]	GlcNol	→6)-D-GlcNol	59.67	56.15	66.75	71.79	70.18	72.57		
	GlcN	→6)-β-D-Glc*p*N(1→	100.57	56.63	73.24	70.78	75.3	62.51		
	KDO	→5)-α-KDO*p*(2→	175.17	100.44	35.56	66.47	75.09	72.79	70.07	64.44
	HepI	→3)-L-α-D-Hep*p*(1→	101.69	71.08	74.48	74.10	72.33	69.07	64.20[c]	
		4								
		↑								
	HepII	→2)-L-α-D-Hep*p*(1→	99.83	79.70	79.10	66.75	≈72.79	69.07	64.08[c]	
		3								
		↑								
	HepIII	→2)-L-α-D-Hep*p*(1→	100.89	78.90	70.54	67.47	72.79	69.78	63.92[c]	
	GalI	β-D-Gal*p*(1→	103.45	71.35	73.38[d]	69.40	76.15	61.95[e]		
	GlcI	β-D-Glc*p*(1→	103.40	74.10	76.92	70.99	77.25	62.04[e]		
	GlcII	→4)-α-D-Glc*p*(1→	100.89	72.43	≈72.33	79.36	72.13	60.90		
	GlcIII*	β-D-Glc*p*(1→	102.7	73.2	75.6	69.8	75.9	60.3		
Hex5	GlcIII	→4)-β-D-Glc*p*(1→	103.35	73.81	75.09	79.2	–[f]	60.90		
	GalII	β-D-Gal*p*(1→	103.77	71.35	73.33[d]	69.40	76.19	61.86[e]		
Hex6	GalII*	→4)-β-D-Gal*p*(1→	104.2	≈71.8	73.1	78.2	76.2	61.2		
	GalIII*	α-D-Gal*p*(1→	101.2	69.4	70.0	69.9	71.8	61.5		

[a] Measured at 32 °C (Hex5) or 27 °C (Hex4 and Hex6) in D_2O (pD ~6). Chemical shifts were measured from a one-dimensional spectrum for the Hex5 glycoform and from two-dimensional HMQC experiments for the Hex4 and Hex6 glycoforms. Chemical shift values for conserved residues (i.e. residues GlcNol to GlcII) in Hex4, Hex5, and Hex6 glycoforms are the same (±0.10 ppm). Values for the variable OS residues in Hex4 and Hex6 are given to one decimal place only. [b] See Table 3 for definitions. [c] Assignments may be reversed. [d] Assignments may be reversed. [e] Assignments may be reversed. [f] Value not determined.

system. Moreover, the $J_{1,2}$ value (7.5 Hz) was consistent with the β-D-configuration.

The sequence of the glycoses within this backbone OS was determined from transglycosidic proton NOEs between anomeric and aglyconic protons on contiguous residues. NOE measurements were made in the two-dimensional mode; part of the NOESY contour plot is shown in Figure 5B. The occurrence of intense transglycosidic NOE connectivities between the proton pairs HepIII H-1/HepII H-2, HepII H-1/ HepI H-3, and HepI H-1/ KDO H-5 established the sequence

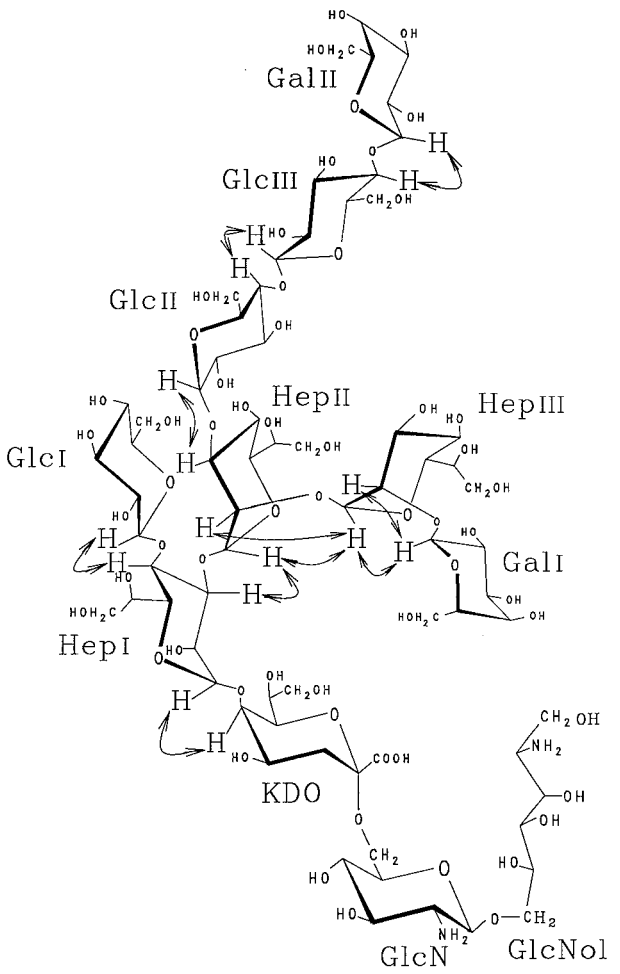


FIGURE 6: Structure of the backbone OS of the Hex5 glycoform illustrating the network of observed transglycosidic NOE connectivities.

of the heptose-containing trisaccharide unit in the inner core region and the point of attachment to KDO as follows:

L-α-D-Hep*p*(1→2)-L-α-D-Hep*p*(1→3)-L-α-D-Hep*p*(1→5)-α-KDO*p*

Intraresidue NOEs were observed between H-1 and H-2 of each of the heptose units, confirming the assigned α-D configurations.

HepIII is substituted at O-2 by one of the β-D-Gal*p* end groups, and this was determined from a transglycosidic NOE between GalI H-1 and HepIII H-2. Interresidue NOE between the anomeric proton resonances (H-1) of these two residues as well as between those of HepIII and HepII (Figure 6) provided confirmation of the 1,2-linkages (Romanowska *et al*., 1988). Moreover, strong NOEs were also observed between the H-1 resonance of HepII and the proton resonances at C-5 and C-7 of HepIII (Figure 5B). A similar pattern of interresidue NOE connectivities (i.e. H-1/H-5) is well documented for α-1,2-linked D-mannoses (DiFabio *et al.*, 1988; Helander *et al.*, 1992; Masoud & Perry, 1996). The NOE data indicate that both HepI and HepII form branch points for further substitution, and this is consistent with the methylation analysis results (Table 2). Thus, the occurrence of an interresidue NOE between H-1 of GlcI and the HepI H-4/H-6 proton pair pointed to substitution of this heptose at *O*-4 by the β-D-Glc*p* end group. The trisaccharide unit, β-D-Gal*p*(1→4)-β-D-Glc*p*(1→4)-α-D-Glc*p*, was identified from the observed interresidue NOE between GalII H-1/GlcIII H-4 and GlcIII H-1/GlcII H-4 (Figure 5B). An intense transglycosidic NOE between GlcII H-1 and HepII H-3 indicated substitution at *O*-3 of the central heptose by the α-D-Glc*p* moiety of the trisaccharide unit. Thus, it is HepII that provides the point for further chain extension in the Hex5 glycoform. The connectivities and the linkage positions of the glycosyl residues of the Hex5 backbone OS are illustrated in Figure 6.

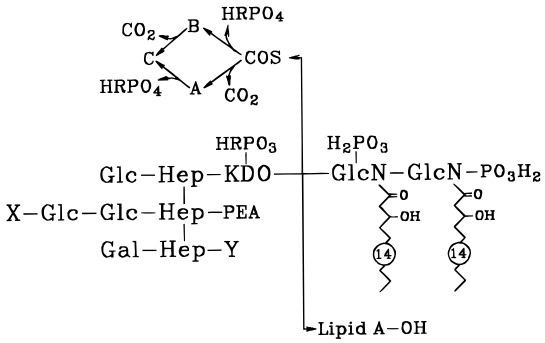
The observed rapid liberation of lipid A from the LPS on treatment with dilute acid concurs with the KDO unit of the backbone OS providing the link to the nonreducing glucosamine (Helander *et al*., 1988). In accord with the glycosylation patterns determined by analysis of the NOE and methylation data, the ^{13}C chemical shifts of the indicated linkage carbons showed significant downfield shifts compared to those of unsubstituted reference compounds (Bock & Pedersen, 1983). As previously noted (Masoud *et al*., 1994a), a similar deshielding effect is not observed for the GlcN C-6 resonance in the →5)-α-KDO(2→6)-β-D-GlcN-(1→linkage.

Structure of Hex4 and Hex6 Backbone Oligosaccharides. The LMW fraction comprised primarily of Hex4 backbone OS was found to be structurally similar to the Hex5 OS but lacking the terminal β-D-Gal*p* residue (GalII) of the trisaccharide chain. Methylation analysis indicated only one *O*-4-substituted D-Glc*p* residue together with higher proportions of D-Glc*p* end groups in this fraction. The ^{1}H spin system corresponding to the exposed terminal β-D-Glc*p* residue (GlcIII*) in the Hex4 OS was identified from a COSY experiment (Table 3). ^{13}C-NMR chemical shift values determined from HMQC experiments (Table 4) for this residue were typical of an unsubstituted β-D-glucopyranose (*cf.* residue GlcI, Table 4) (Bock & Pedersen, 1983). Apart from this residue and the absence of GalII, the chemical shift assignments were identical to those of the Hex5 OS (Tables 3 and 4). The structure of the Hex4 backbone OS was confirmed by NOE measurements (data not shown).

Detailed NMR analysis of the Hex6 backbone OS revealed the presence of an additional α-D-Gal*p* residue that is 1,4-linked to the terminal β-D-Gal (GalII) present in the Hex5 trisaccharide chain. This was established from the occurrence of an intense interresidue NOE between H-1 of this residue (GalIII*, 4.95 ppm) and H-4 of the β-D-Gal*p* (GalII*, 4.04 ppm). Assignments of the ^{1}H resonances from these residues were made from the patterns of connectivities in the COSY spectrum (Table 3). In the ^{13}C spectrum (Table 4), the downfield-shifted value of the GalII* C-4 resonance (78.2 ppm) compared to that of the corresponding resonance (GalII, 69.40 ppm) in the Hex5 OS is consistent with substitution at *O*-4. The ^{1}H and ^{13}C chemical shift values are closely similar (±0.02 ppm) to values reported previously (Masoud *et al*., 1994b) for the α-D-Gal*p*(1→4)-β-D-Gal*p*(1→ terminal structure present in *Moraxella catarrhalis* LPS.

Structures of the Major LPS Glycoforms. Tandem ESI−MS experiments on *O*-deacylated LPS samples provided information leading to the location of phosphate and PEA groups. The MS−MS data for the major LPS-OH glycoforms produced by low-energy collisional activation of the corresponding triply deprotonated molecules $[M - 3H]^{3-}$ are given in Table 5. In each spectrum, a major fragment ion is observed at *m/z* 951 (lipid A-OH) arising from cleavage of the KDO−β-D-GlcN bond in which the ketosidic oxygen

Table 5: MS−MS Data for Selected Precursor Ions from *H. influenzae* Strain Eagan LPS-OH



LPS glycoform	X	Y	R	parent ion (*m/z*) [M − 3]$^{3-}$	product ion (*m/z*)[a] lipid A-OH [P]$^{-}$	COS [P]$^{2-}$	A [P]$^{2-}$	B [P]$^{2-}$	C [P]$^{-}$	$HRPO_4$ [P]$^{-}$
Hex4	H	H	H	866[b,c]	951[d]	822	801	774	1504	97
	H	H_2PO_3	H	893[b]	951	862	−	−	1585[e]	97
	H	H	PEA[f]	907[c]	951	884	863	−	1505	220
Hex5	Gal	H	H	920[b]	951[d]	904	883	855	1665	97
Hex6	Gal-Gal	H	H	974[b]	951[d]	985	−	936	1830	97

[a] Proposed assignments of product ions are indicated in the structure: COS (core oligosaccharide bearing terminal anhydro-KDO moiety), M-(lipid A-OH); A, M-(lipid A-OH)-CO_2; B, M-(lipid A-OH)-$HRPO_4$; C, M-(lipid A-OH)-CO_2-$HRPO_4$. The data are presented as integer values (±1 amu). [b] Data obtained on LPS-OH sample from Oxford strain using extraction condition A (see Figure 2A). [c] Data obtained on LPS-OH sample from Oxford strain using extraction condition B (see Figure 2B). [d] Doubly charged [P]$^{2-}$ ion observed at *m/z* 475. [e] Intense doubly charged [P]$^{2-}$ ion observed at *m/z* 792. [f] PEA; $HPO_3(CH_2)_2NH_2$.

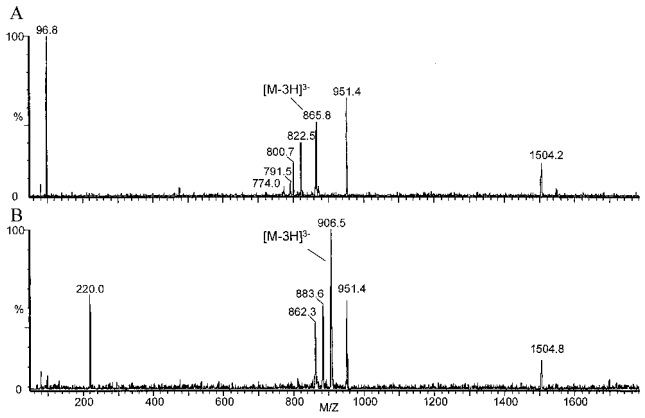


FIGURE 7: Tandem mass spectra (MS−MS) of triply deprotonated precursor ions from (A) the Hex4 glycoform at *m/z* 865.8 and (B) the Hex4 analogue containing an additional PEA group at *m/z* 906.5. Fragment ion assignments are given in Table 5.

is retained by the *O*-deacylated lipid A fragment. Cleavage of this linkage in LPS-OH to yield the lipid A-OH singly charged ion appears to be the dominant fragmentation pathway in the negative-ion mode (Auriola *et al.*, 1996; Gibson *et al.*, 1996; Kelly *et al.*, 1996). The mass of this fragment ion is consistent with that expected (Helander *et al.*, 1988) for *H. influenzae* lipid A-OH (Table 5, structure insert). In addition, the core OS fragment terminating in anhydro-KDO (COS) is observed as a doubly charged ion. This is seen at *m/z* 822.5 for the Hex4 LPS-OH glycoform (Figure 7A). This fragment can subsequently lose carbon dioxide or phosphate (Gibson *et al.*, 1996) from the anhydro-KDO moiety to give the respective doubly charged fragments (Table 5, fragments A and B), while expulsion of both groups affords a singly charged fragment (Table 5, fragment C). In accord with this fragmentation pathway, the Hex4 glycoform gave peaks at *m/z* 800.7, 774.0 (minor), and 1504.2 corresponding to the respective product ions (Figure 7A). Moreover, the fragment at *m/z* 791.5 corresponds to a loss of a neutral PEA moiety from the triply charged precursor ion. These data are consistent with the Hex4 glycoform containing a single PEA moiety in the core OS region. For the Hex4 (M_r of 2601.3), as well as for the higher glycoforms (M_r of 2763.6 and 2925.4) belonging to the subpopulation of LPS-OH containing a single PEA moiety, a fragment ion arising from phosphoric acid anion ($H_2PO_4^-$) was observed at *ca. m/z* 97 (Figure 7A). MS-MS of the Hex4 species from the subpopulation of LPS-OH that contains an additional PEA group (M_r of 2723.5) gave product peaks having the same mass for the lipid A-OH (*m/z* 951.4) and the singly charged fragment ion C (*m/z* 1504.8), indicating that the second PEA is located on the KDO moiety (Figure 7B). The occurrence of an abundant fragment ion in this spectrum at *m/z* 220.0 attributed to monoanionic pyrophosphoethanolamine (PPEA) pointed to PEA substitution of the KDO 4-phosphate group (Table 5, fragmentation scheme). Moreover, the Hex5 and Hex6 glycoforms from this subpopulation of LPS-OH (M_r of 2886.9 and 3048.1, respectively) gave the corresponding fragments in their respective MS−MS spectra (data not shown). In agreement with this conclusion, a group of signals in the region characteristic of pyrophosphate diesters (−10 to −12 ppm) was observed in the ^{31}P-NMR spectrum (Rosner *et al.*, 1979) of LPS-OH obtained from Oxford LPS which had been purified following ethanol precipitation.

MS−MS of the triply charged ion at *m/z* 893 from the phosphorylated Hex4 LPS-OH analogue (M_r of 2681.4) afforded the normal lipid A-OH ion (*m/z* 951.4) and a singly charged C fragment ion (*m/z* 1585.1) which is shifted 80 units to high mass compared to the corresponding fragment derived from the Hex4 glycoform (Table 5). This latter fragment, together with its doubly charged counterpart (*m/z* 791.8), was consistent with this LPS-OH species [Hex4(P)] containing an additional phosphate group in the core OS region. Results from a modified methylation analysis procedure provided further insight into the location of this phosphate substituent. Methylation of the core OS sample

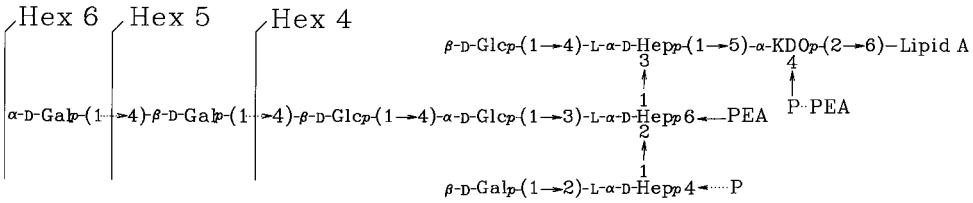


FIGURE 8: Structural model showing the major LPS glycoforms from *H. influenzae* serotype b strain Eagan. As indicated, the KDO moiety is substituted at the *O*-4 position by either phosphate or PPEA, giving rise to two subpopulations of LPS molecules. The Hex4 glycoform provides the framework for further substitutions. In the phosphate-containing analogue, Hex4(P), HepIII carries P at the *O*-4 position. An additional phosphate at this position is only detected in this glycoform. Hex5 and Hex6 glycoforms arise from sequential addition of *β*-D-Gal*p* and α-D-Gal*p*(1→4)-*β*-D-Gal*p* to the growing OS chain from the penultimate heptose.

obtained from Rochester LPS followed by dephosphorylation and remethylation with perdeuteriomethyl iodide afforded a similar mixture of the partially methylated sugars listed in Table 2. Using this procedure, small amounts of 3,4,6,7−tetra-*O*-methylheptose containing a deuterated methyl group at *O*-4 was also detected in the hydrolysis products. This pointed to the presence of a phosphate substituent at C-4 of HepIII and can be attributed to the phosphate-substituted Hex4 LPS-OH species detected by ESI−MS (M_r of 2681.4).[3] In addition, methylation analysis by this procedure afforded 4,6,7-tri-*O*-methylheptose in which both *O*-6 and *O*-7 carried deuteriomethyl groups (*ca.* 50% CD_3 at each site) which was consistent with PEA substitution at one or the other position in HepII. Migration of phosphate substituents under alkaline conditions employed in methylation analysis is well documented (Brown *et al.*, 1954; Zähringer *et al.*, 1990). Phosphorus coupling to H-6 of HepII was established in a $^1H-^{31}P$ chemical shift correlation map of a LPS-OH sample, confirming the former position as the site of attachment.[4] PEA has been reported at C-6 of the central heptose (Schweda *et al*., 1993) in LPS of *an H. influenzae* mutant.

DISCUSSION

Previous studies have indicated that *H. influenzae* serotype b strains elaborate a heterogeneous population of LPS showing considerable interstrain variability (Inzana, 1983; Zamze & Moxon, 1987; Gulig *et al.*, 1987; Weiser *et al*., 1989). On the basis of studies of colonies (Kimura & Hansen, 1986) and individual bacterial cells (Weiser *et al.*, 1989; Mertsola *et al.*, 1991), the LPS of *H. influenzae* shows spontaneous, high-frequency reversible acquisition and loss of reactivity with monoclonal antibodies which are specific for oligosaccharide structures. A number of surface-exposed LPS OS epitopes, defined by their reactivity with monoclonal antibodies (Patrick *et al*., 1989), have been correlated to phase-variable expression and to changes in *H. influenzae* virulence potential (Kimura & Hansen, 1986; Kimura *et al*., 1987). The potential repertoire of variant OS structures is extensive, providing an important mechanism whereby *Haemophilus* can adapt to varied environmental conditions, leading to an enhanced capacity to evade host immune defenses (Weiser *et al.*, 1990; Weiser, 1993). Extensive use of four Mabs, namely 4C4, 12D9, 6A2, and 5G8, has been made to study expression of LPS OS epitopes in a wide range of *H. influenzae* serotype b strains (Gulig *et al*., 1987; Patrick *et al.*, 1989; Weiser *et al*., 1989). The most prevalent *H. influenzae* disease isolates have been shown to express the Mab 4C4-reactive epitope (Gulig *et al*., 1987), and this epitope has been associated with altered virulence (Cope *et al.*, 1991). The LPS epitope recognized by Mab 4C4 was recently shown (Virji *et al*., 1990) to comprise the terminal structure α-D-Gal(1→4)-*β*-D-Gal. This was determined from binding studies with synthetic galabiose-containing protein glycoconjugates. Moreover, the B subunit of shiga toxin which is known to bind the α-D-Gal(1→4)-*β*-D-Gal structure (Karlsson *et al*., 1986) showed specific binding to *H. influenzae* Mab 4C4-reactive LPS (Virji *et al*., 1990). Expression of the galabiose-like epitope on *Haemophilus* LPS was recently confirmed by binding studies with a Mab specific for the P^k blood group antigen (Mandrell *et al*., 1992).

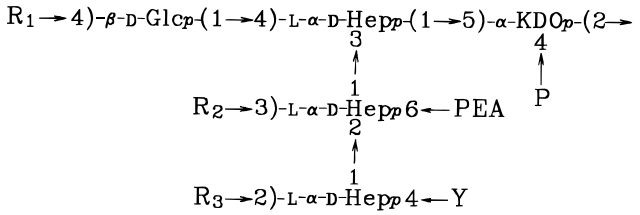
To date, only limited information is available on the structural features of variable OS epitopes of *H. influenzae* serotype b LPS (Virji *et al.*, 1990; Mandrell *et al.*, 1992; Phillips *et al*., 1993). The molecular structure of the galabiose-containing P^k epitope and the structural framework on which it is expressed have not yet been determined. Results from this study indicate that strain Eagan elaborates a heterogeneous population of LPS molecules. A series of related structures (glycoforms) differing in the number of hexose residues as well as subpopulations containing additional phosphate and/or PEA groups was identified by mass spectrometric techniques (Tables 1 and 5). Populations of LPS from strains from different sources, while qualitatively similar, showed differences in the relative amounts of the individual LPS components. As previously observed (Maskell *et al.*, 1991), strain Eagan (obtained from two different laboratories) showed variable binding to Mab 4C4 in colony blot assays. Detailed NMR studies of backbone OS samples obtained from the mixtures of LPS led to full structural elucidation of the variable OS epitopes.

A structural model representative of the major LPS populations elaborated by *H. influenzae* strain Eagan is shown in Figure 8. It contains a conserved L-*glycero*-D-*manno*-heptose-containing trisaccharide inner core attached *via* a phosphorylated KDO unit to the *β*-D-GlcN component of the hexaacylated lipid A moiety (Helander *et al.*, 1988). Each of the three heptose units is substituted by a hexose residue with further chain elongation from the central HepII unit. Hex4, Hex5, and Hex6 were identified as the major LPS glycoforms. In the Hex4 glycoform, HepII is substituted by a *β*-D-Glc*p*(1→4)-α-D-Glc*p* dissaccharide unit. The

[3] The presence of a phosphate substituent at C-4 of HepIII has been confirmed by detailed NMR analysis of LPS-derived OS from an *H. influenzae* Eagan-derived mutant strain (*lgt C*) which elaborates only Hex4 and its phosphorylated analogue Hex4(P) (H. Masoud, D. Hood, E. R. Moxon, and J. C. Richards, unpublished result).

[4] The Hep residues of the LPS-OH sample were assigned by COSY, TOCSY, and NOESY−TOCSY−NOESY experiments, and $^1H-^{31}P$ correlations were made using the HMQC pulse sequence (H. Masoud, D. Uhrin, and J. C. Richards, unpublished result).

Table 6: Reported Major Structural Features of Variable LPS Oligosaccharides Expressed by *H. influenzae* LPS



strain	R_1	R_2	R_3	Y	reference
2019[a]	β-D-Gal	H	H	PEA[b]	Phillips *et al.*, 1992
AH1−3[c]	β-D-Glc	H	β-D-Gal	H	Schweda *et al.*, 1993
GalE/GalK[c]	β-D-Glc	β-D-Glc(1→4)-α-D-Glc	β-D-Gal	H	Schweda *et al.*, 1995
	H	β-D-Glc(1→4)-α-D-Glc	H	H	
	β-D-Glc	H	H	H	
281.25[d] Eagan	β-D-Glc	β-D-Glc(1→4)-α-D-Glc	H	H	Phillips *et al.*, 1996
Hex4	H	β-D-Glc(1→4)-α-D-Glc	β-D-Gal	H	present investigation
Hex4(P)	H	β-D-Glc(1→4)-α-D-Glc	β-D-Gal	H_2PO_3	
Hex5	H	β-D-Gal(1→4)-β-D-Glc(1→4)-α-D-Glc	β-D-Gal	H	
Hex6	H	α-D-Gal(1→4)-β-D-Gal(1→4)-β-D-Glc-(1→4)-α-D-Glc	β-D-Gal	H	

[a] Nontypable *H. influenzae* strain. [b] The linkage position of the PEA substituent was not determined. [c] Mutant derived from *H. influenzae* serotype b strain RM 7004. [d] Mutant derived from *H. influenzae* serotype b strain A2. The location of PEA was not determined; it is assumed to be located at *O*-6 of HepII. This OS is also expressed by the major LPS glycoform in the parent strain (Phillips *et al.*, 1993).

terminal β-D-Glc*p* of this unit is capped by a β-D-Gal*p* residue in the Hex5 glycoform, while the P^k epitope [α-D-Gal*p*(1→4)-β-D-Gal*p*(1→4)-β-D-Glc*p*] is expressed in the Hex6 glycoform. A Hex4 LPS glycoform in which HepIII is substituted at the *O*-4 position by a phosphate group [Hex4 (P)] was also identified. It is noteworthy that the corresponding phosphate-containing Hex5 and Hex6 glycoforms were not detected. We propose that phosphorylation may provide a mechanism for attenuating expression of the galabiose epitopes. In support of this, certain mutants of *H. influenzae* Eagan generated by inactivation of genes required for galabiose expression have populations of LPS containing both Hex4 and its phosphorylated analogue, while HepIII 4-phosphate containing LPS is absent from mutants containing deeper truncations (Hood *et al.*, 1996).

Detailed characterization of LPS glycoforms containing up to four hexose units attached to the common trisaccharide heptose-containing template has been previously reported for other wild-type (Phillips *et al.*, 1992, 1993) and mutant (Schweda *et al.*, 1993, 1995; Phillips *et al.*, 1996) strains of *H. influenzae* (Table 6). In contrast to the case for strain Eagan, chain extension from HepI is observed in a nontypable as well as other serotype b strains. LPS from nontypable *H. influenzae* 2019 comprises predominantly Hex2 glycoforms in which HepI is substituted by a lactose unit, while the major LPS glycoform of the serotype b strain, A2 (Spinola *et al.*, 1990), carries β-D-Glc*p*(1→4)-D-Glc*p* units at both HepI and HepII (Phillips *et al.*, 1993, 1996). The major LPS components from mutants of the serotype b strain, RM 7004, have also been shown to be substituted at HepI by this glucose-containing disaccharide (Schweda *et al.*, 1993, 1995). *H. influenzae* strain A2 has also been reported to express minor populations of higher LPS glycoforms containing, in addition to Glc, Gal, HexNAc, and sialic acid residues, although detailed structural information is not available (Phillips *et al.*, 1993, 1996; Gibson *et al.*, 1996).[5] The present investigation provides unequivocal structural evidence for expression of the phase-variable P^k epitope by an *H. influenzae* serotype b strain. The OS structure, α-D-Gal*p*(1→4)-β-D-Gal*p*, is also present in LPS of *Neisseria meningitidis* (Di Fabio *et al.*, 1990), *Neisseria gonorrhoeae* (John *et al.*, 1991), and *M. catarrhalis* (Masoud *et al.*, 1994b), but in these organisms, it is expressed within different molecular environments.

Two subpopulations of LPS are identified in which the KDO moiety carries either a 4-phosphate substituent or its pyrophosphoethanolamine analogue. The relative abundance of the two subpopulations was found to be dependent on the method employed for LPS isolation. The subpopulation of LPS containing an additional PEA, attached *via* a pyrophosphate linkage (KDO-4-PPEA), was almost completely absent when LPS was isolated by extensive dialysis of the aqueous phase from phenol−water extractions. This would suggest that the PPEA groups are lost during this isolation procedure since pyrophosphate linkages are known to be acid labile, even under extremely mild conditions (Masoud *et al.*, 1994c). In accord with this view, the KDO-PPEA subpopulation accounted for more than 50% of the LPS fraction when it was precipitated (with ethanol) from the aqueous phase immediately following phenol−water

[5] On the basis of ESI−MS data (Phillips *et al.*, 1993; Gibson *et al.*, 1996), sialylated HexNAc·Hex6 (M_r of 3416.4) and HexNAc. Hex5 (M_r of 3256.2) were identified as minor LPS-OH components in *H. influenzae* strain A2 from triply deprotonated ions at *m/z* 1137.8 and 1084.4. We found no evidence of these species from ESI−MS analysis of Eagan-derived LPS-OH samples, although minor peaks were detectable at *m/z* 1041.9 and 987.9 (data not shown) which may correspond to the respective asialo analogues (M_r of 3128.6 and 2966.6). The presence of sialylated LPS has not been demonstrated in strain Eagan (Weiser, 1993).

[6] LPS from a deep rough *H. influenzae* Eagan mutant lacking OS extension beyond the KDO residue (strain *opsX*) isolated by using condition B afforded a mixture of KDO(4-P)/lipid A-OH and KDO-(4−PPEA)/lipid A-OH (Hood *et al.*, 1996). It is noteworthy that PEA was not detected in the LPS from an analogous mutant strain, I-69 Rd^-/b^+; it is possible that this is related to the LPS extraction procedure employed (Helander *et al.*, 1988).

extraction.[6] The structural studies reported by others (Table 6) have typically relied on the acid lability of the KDO ketosidic linkage and subsequent dissociation of the lipid A moiety for determination of major OS components. In this study, an alternative approach (Masoud *et al*., 1994a,b) involving removal of some, or all, of the fatty acyl groups from the lipid A moiety was employed to obtain oligosaccharides that are representative of the complete LPS backbone structures. This has enabled us to identify, for the first time, subpopulations containing PPEA in the KDO region of these molecules.

ACKNOWLEDGMENT

We thank Dr. P. Anderson for providing *H. influenzae* Eagan (Rochester), Dr. E. J. Hansen for Mabs, D. Griffith for large-scale production of cells, M. Deadman for small-scale preparation of LPS, and F. Cooper for GLC−MS analyses. We also thank Dr. P. Thibault for helpful discussions.

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BI961989Y