Characterization of Two Transposon Mutants from *Haemophilus influenzae* Type b with Altered Lipooligosaccharide Biosynthesis[†]

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ABSTRACT: Two isogenic mutants of *Haemophilus influenzae* type b (Hib) strain A2 were prepared by random m-Tn*3*(Cm) insertions into the 7.4-kb *lsg* (lipooligosaccharide synthesis genes) region of Hib DNA, which consists of seven complete and one partial open reading frames (orfs). Compared to the parent A2 strain which produces a complex mixture of lipooligosaccharides (LOS), the mutant strains 281.25 and 276.4 produced only a few LOS species. The precise locations of transposon insertions into the *lsg* loci of these mutants were determined (base 3546 in orf 4 for strain 281.25 and base 4402 in orf 5 for strain 276.4), and the effects of these mutations on LOS biosynthesis and epitope expression were evaluated. When the *O*-deacylated LOS were analyzed by mass spectrometry, both strains contained major LOS species of M_r 2601, 2439, and 2277, which consisted of a common heptose trisaccharide core structure [Hep_3(PEA)Kdo(P)-lipid A, where Hep is L-*glycero*-D-*manno*-heptose, Kdo is 3-deoxy-D-*manno*-octulosonic acid, and PEA is phosphoethanolamine] and four, three, or two hexoses, respectively. These species represent the smallest components of the wild-type LOS mixture. The major LOS oligosaccharide obtained from strain 281.25 by mild acid hydrolysis was dephosphorylated and shown by composition analysis, methylation analysis, mass spectrometry, and 2D NMR studies to be a triantennary structure consisting of a heptose trisaccharide core with two glucose disaccharide branches: Hepα1→(Glcβ1→4Glcα1→3)2Hepα1→(Glcβ1→4Glcβ1→4)3Hepα1→*anhydro*Kdo. Unlike the parent A2 strain, mutant strain 281.25 cannot add galactoses to the branches of this octasaccharide. Strain 276.4 is similarly deficient, except that it can still utilize a minor biosynthetic pathway leading to the addition of sialyl-*N*-acetyllactosamine.

Wild-type strains of *Haemophilus influenzae* type b (Hib)[1] express a complex mixture of glycolipid surface antigens, termed lipooligosaccharides (LOS). LOS consist of a conserved lipid A moiety linked to a 3-deoxy-D-*manno*-octulosonic acid (Kdo) residue bearing a core oligosaccharide with variable sugar branches. Various studies have shown that alteration of LOS oligosaccharide structures can affect the virulence of *H. influenzae* (Zwahlen et al., 1985; Cope et al., 1990; Maskell et al., 1992). To better understand the role of LOS in virulence mechanisms, we have been investigating the genetics of LOS biosynthesis in an effort to correlate precise LOS structures with specific biological function(s).

The LOS of Hib contain both stable and phase-varying epitopes (Weiser, 1992). This ability to spontaneously gain and lose epitopes has been found to enhance the invasive capacity of Hib (Weiser et al., 1990). Additionally, some of the phase-varying epitopes have been found to mimic human blood group antigens, such as the P^k antigen (Galα1→4Galβ1→4Glc) and paragloboside (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) (Mandrell et al., 1992). As also occurs in host glycolipids and in neisserial LOS, some Hib LOS are found to be sialylated, most likely on terminal *N*-acetyllactosamine structures (Mandrell et al., 1992; Gibson et al., 1993; Yamasaki et al., 1993).

Recently, we identified 11 LOS components in wild-type Hib strain A2 (Phillips et al., 1993). These structures all contained a common core region consisting of a monophosphorylated Kdo and an L-*glycero*-D-*manno*-heptose (Hep) trisaccharide moiety, and differed primarily in the number and type of branch sugars present. While the majority of the LOS oligosaccharides contained only glucose and

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[1] Abbreviations: Hib, *Haemophilus influenzae* type b; 1D, one dimensional; 2D, two dimensional; COSY, 2D *J*-correlated spectroscopy; DQF-COSY, double-quantum-filtered COSY; ESI-MS, electrospray ionization-mass spectrometry; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, L-*glycero*-D-*manno*-heptose; Hex, hexose; HexNAc, *N*-acetylhexosamine; HOHAHA, 2D homonuclear Hartmann−Hahn spectroscopy; HPLC, high-performance liquid chromatography; Kdo, 3-deoxy-D-*manno*-octulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; *lsg*, lipooligosaccharide synthesis genes; LSIMS, liquid secondary ion mass spectrometry; $(M-nH)^{n-}$, deprotonated molecular ion; NeuAc, *N*-acetylneuraminic acid or sialic acid; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; orf, open reading frame; PEA, phosphoethanolamine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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galactose as branch sugars, two minor components contained sialylated structures. Variable phosphorylation of the core with one or two phosphoethanolamines (PEA) accounted for some additional heterogeneity. Consistent with this structural heterogeneity, Hib A2 LOS exhibited significant antigenic variability (Mandrell et al., 1992; McLaughlin et al., 1992). Essentially, the LOS mixture was far too complex to evaluate the role(s) of defined carbohydrate epitopes in bacterial virulence and pathogenic processes such a cell adhesion, invasion, and dissemination.

To facilitate the elucidation of LOS biology, our laboratory has cloned an Hib gene cluster containing LOS synthesis genes (*lsg*) (Spinola et al., 1990). The *lsg* loci are contained within a 7.4-kb DNA fragment, which consists of seven complete and one partial open reading frames (orfs) (McLaughlin et al., 1994). When inserted into *Escherichia coli*, this gene cluster was shown to assemble an oligosaccharide which contained or exposed a Kdo-related epitope recognized by monoclonal antibody (MAb) 6E4 and characteristic of *H. influenzae*. Subsequently, a series of isogenic mutants of Hib A2 was generated by transposon mutagenesis using minitransposon m-Tn*3*(Cm) (McLaughlin et al., 1992). When analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), several of the transposon mutants had much simpler LOS banding patterns than the wild-type Hib A2 LOS. Preliminary immunochemical characterization of the mutant strains revealed that they no longer reacted with one or more of the MAbs recognizing phase-varying (4C4, 5G8, 3F11) or stable (6E4) Hib epitopes (McLaughlin et al., 1992). Of particular interest was mutant strain 281.25, which produced only low molecular weight LOS structures and did not express the Kdo-related 6E4 epitope or the 3F11 epitope, which is a terminal *N*-acetyllactosamine epitope common in *Haemophilus* and neisserial LOS and implicated in host mimicry (Mandrell et al., 1992). Mutant strain 276.4 produced similar low molecular weight LOS components which failed to react with MAbs 6E4 and 3F11, in addition to a minor high molecular weight species which bound MAb 6E4. However, mutant strain 281.25 was the only strain that appeared to have a phase-locked nonreactive phenotype for MAb 3F11 binding (McLaughlin et al., 1992).

In the present report, we focus on the genetic and chemical characterization of these two transposon mutants. We have established the precise locations of m-Tn*3*(Cm) insertions into orfs 4 and 5 for mutant strains 281.25 and 276.4, respectively, and compared the LOS structures observed with species previously found in the wild-type parent strain. This approach has offered insights into the functions of products of these orfs, and provided a unique opportunity to study defined subsets of the wild-type LOS population. Additionally, the reduced LOS heterogeneity characteristic of these transposon mutants has permitted us to purify sufficient quantities of the major octasaccharide to allow for a full structure assignment by application of 2D NMR methods.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Hib A2 was grown on chocolate agar supplemented with IsoVitaleX or brain heart infusion agar supplemented with 4% Fildes reagent (Difco Laboratories) at 35 °C in 5% CO_2 atmosphere. Chloramphenicol (2 μg/mL) was added to brain heart infusion medium for selection and maintenance of Hib strains containing the m-Tn*3*(Cm) transposon.

DNA Isolation and Manipulation. Plasmid DNA was isolated from *E. coli* by alkaline lysis (Birnboim & Doly, 1979). Chromosomal DNA was prepared from *H. influenzae* strains as described (McLaughlin & Hughes, 1989). T4 DNA ligase and restriction endonucleases were purchased from Promega Biotec (Madison, WI) or Bethesda Research Laboratories (Gaithersburg, MD) and used according to manufacturer's specifications.

Shuttle Mutagenesis. The *Eco*RI fragment of the *lsg* loci was mutagenized using the minitransposon m-Tn*3*(Cm) (Seifert et al., 1986) as previously described (Abu Kwaik et al., 1991).

Transformation. Hib cells were made competent by the M-IV medium procedure (Herriott et al., 1970). Cells were transformed with plasmid DNA containing m-Tn*3*(Cm) insertions and selected on plates with chloramphenicol. Chromosomal DNA from selected Hib mutants was transformed back into the parental strain and selected in the same manner.

DNA Hybridization. DNA for Southern hybridization was digested with various restriction enzymes and prepared and transferred to a nylon membrane (0.45 μm Nytran, Schleicher & Schuell) as described (Sambrook et al., 1989). Blots were blocked for 1 h with hybridization solution [5× SSPE, 2× Denhardt's, 0.1% SDS, and 100 μg/mL salmon sperm DNA (Sambrook et al., 1989)] at 68 °C. DNA fragments for use as probes were purified from vector sequences by electroelution (Sambrook et al., 1989) and labeled with [^{32}P]dCTP using random-primed oligolabeling according to the manufacturer's instructions (Pharmacia/LKB, Piscataway, NJ). Labeled probe was added to the hybridization solution (final concentration ~10^6 cpm/mL) and allowed to bind overnight at 68 °C. The blots were washed 2 times for 45 min with 1× SSPE, 0.1% SDS, and 5 mM $NaPP_i$ and 1 time for 20 min with 0.5× SSPE, 0.1% SDS, and 5 mM $NaPP_i$, air-dried, and exposed overnight at −70 °C to Kodak X-OMAT AR-5 film. Colony hybridization assays were performed as described (Sambrook et al., 1989) and probed under the same conditions as those of Southern blots.

Polymerase Chain Reaction. PCR reaction mixture was prepared according to the instruction in the GeneAmp PCR reagent kit (Perkin Elmer Cetus). PCR was utilized to amplify the DNA fragments at each end of the m-Tn*3*(Cm) insertions in strains 281.25 and 276.4. Primers were derived from the pGemLOS4 7.4kb *lsg* fragment upstream and downstream (McLaughlin et al., 1994) of the m-Tn*3*(Cm) insertions. These primers are listed in Table 1. Primers were also derived sequences from the ends of m-Tn*3*(Cm). These were provided by Dr. Hank Siefert, Northwestern University.

DNA Sequencing and Sequence Analysis. The PCR fragments were ligated into TA cloning vectors and sequenced using the common primer sites in the plasmid. The double-stranded DNA was sequenced by the dideoxynucleotide termination method (Sanger et al., 1977) and labeled with [^{35}S]ATP using the Sequenase II kit (United States Biochemicals).

SDS−PAGE Analysis of LOS. LOS from parent strain A2 and the transposon mutants was prepared by the microphenol method (Inzana, 1983) or by SDS−proteinase K treatment of whole cells (Hitchcock & Brown, 1983). The LOS were separated by SDS−PAGE in gels containing 18% acryla-

Table 1: Bacterial Strains, Vectors, and Primers

strain/plasmid	revelant characteristic	reference
E. coli		
RDP146	Δ(lac-pro)Spc^R	Seifert et al. (1986)
NS2114SM	(λ cre$^+$) Str^r	
H. influenzae type b		
A2	parental strain	Spinola et al. (1990)
281.25	Cm^R derivative of A2 expressing altered LOS	this work
276.4	Cm^R derivative of A2 expressing altered LOS	this work
plasmid		
pHSS8	Km^R	Seifert et al. (1986)
pOX38:mTnCm	Cm^R, tra$^+$	Seifert et al. (1986)
pTCA	Tc^R, *tnpA*$^+$	Seifert et al. (1986)

primers	description of primer
5′ AAG GTG CAG CGG AAA GTC GCA ATA 3′	upper primer 276.4
5′ GAG TCA CAT ATA AGT AAA CCC 3′	lower primer 276.4
5′ GAT TGG GAT GAA TTA GCC TC 3′	upper primer 281.25
5′ CCA TGA GAG GCC GAA CCA C 3′	lower primer 281.25

mide, 0.75% bis(acrylamide), and 2.5 M urea and visualized by silver staining (Tsai & Frasch, 1982).

ELISA Analysis. ELISA studies were performed by a modification of the published whole cell ELISA method (Abdillahi & Poolman, 1987). Briefly, microtiter plate wells were coated with 100 μL of a suspension of organisms in distilled water. The organisms were dried onto the microtiter well surface in a dry 37 °C incubator for 24 h. Just prior to the ELISA, the organisms on microtiter well surfaces were treated with 100 μL of 0.1 unit/mL neuraminidase in 5 mM NaOAc, 150 mM NaCl, and 4 mM $CaCl_2$, pH 5.6, for 2 h at 37 °C. Control wells contained organisms coated to the microtiter well surface and enzyme buffer alone. The wells were washed, and the ELISA was performed. Two monoclonal antibodies were used in the ELISA studies. Monoclonal antibody 3F11 was reacted with each well at a dilution of 1:80, and monoclonal antibody 6E4 was studied at a dilution of 1:40. Results were read in a Biotek Model EL311 ELISA reader at an absorbency of 560 nm.

Isolation and O-Deacylation of LOS. For large-scale isolations, LOS from the parent and mutant strains was prepared by the extraction procedure of Darveau and Hancock (1983). Approximately 1 mg of LOS was *O*-deacylated by treatment with anhydrous hydrazine (Sigma) at 37 °C for 20 min (Helander et al., 1988) as previously described (Phillips et al., 1992).

Neuraminidase Treatment of Hib 276.4 O-Deacylated LOS. A portion of the *O*-deacylated 276.4 LOS sample (≈50 μg) was treated with 50 milliunits of neuraminidase (Sigma type VI) in 50 μL of PBS, pH 6.0, for 2 h at 37 °C. The enzyme digest was desalted by microdialysis against H_2O (Pierce, microdialyzer system 500) using a 1000 MWCO membrane (Spectra/Por). The retentate was lyophilized and later redissolved in H_2O for mass spectral studies.

Isolation and Purification of Oligosaccharides. The LOS from Hib strains 276.4 and 281.25 (10 mg and 27.7 mg, respectively) was hydrolyzed in 1% acetic acid (2 mg of LOS/mL) for 2 h at 100 °C. The hydrolysates were centrifuged at 5000*g* for 20 min at 4 °C and the supernatants removed. The pellets were washed with 2 mL of H_2O and centrifuged again (5000*g*, 20 min, 4 °C). The supernatants and washings were pooled and lyophilized to give the oligosaccharide fractions. As a standard, 10 mg of LPS from *Salmonella typhimurium* TV119 Ra mutant (Sigma) was treated in the same fashion.

The 276.4 (4.0 mg) and 281.25 (14.9 mg) oligosaccharide fractions were dissolved in 0.3 mL of 0.05 M pyridinium acetate buffer (pH 5.2), centrifuge-filtered through a 0.45 μm Nylon-66 membrane (Microfilterfuge tube, Rainin), and applied to two Bio-Gel P-4 columns connected in series (1.6 × 79 cm and 1.6 × 76.5 cm, <400 mesh; BioRad). The columns were equipped with water jackets maintained at 30 °C. Upward elution at a flow rate of ≈10 mL/h was achieved with a P-1 peristaltic pump (Pharmacia), and fractions were collected at 10 min intervals and evaporated to dryness in a Speed-Vac concentrator. A refractive index detector (Knauer) was used to monitor column effluent, and chromatograms were recorded and stored with a Shimadzu C-R3A Chromatopac integrator.

Dephosphorylation of Oligosaccharides. Oligosaccharides were placed in 1.5 mL polypropylene tubes and treated with cold 48% aqueous HF (Mallinckrodt) to make 5−10 μg/μL solutions. Samples were kept for 16−24 h at 4 °C, and then aqueous HF was evaporated as previously described (Phillips et al., 1992).

Monosaccharide Composition Analysis. Dephosphorylated oligosaccharide fractions were dissolved in 400 μL of 2 M trifluoroacetic acid and heated for 4.5 h at 100 °C. The hydrolysates were evaporated to dryness in a Speed-Vac concentrator, redissolved in 20 μL of H_2O, and dried again. Hydrolysates were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (Hardy et al., 1988) using a Dionex BioLC system (Dionex, Sunnyvale, CA) with a CarboPac PA1 column as previously described (Phillips et al., 1992).

Methylation Analysis. Linkage analysis was performed using a microscale method (Levery & Hakomori, 1987) modified for use with powdered NaOH (Larson et al., 1987). Details of this procedure have been reported elsewhere (Phillips et al., 1992). Partially methylated alditol acetates were analyzed by GC/MS in the EI and CI modes on a VG70SE mass spectrometer as described previously (Phillips et al., 1990).

Liquid Secondary Ion Mass Spectrometry (LSIMS). LSIMS was performed using a Kratos MS50S mass spectrometer with a cesium ion source (Falick et al., 1986). Oligosaccharide samples (in 1 μL of H_2O) were added to 1 μL of glycerol/thioglycerol (1:1) on a stainless-steel probe tip. A Cs^+ ion primary beam energy of 10 keV was used, and the secondary sample ions were accelerated to 8 keV. Scans were taken in the negative-ion mode at 300 s/decade and recorded with a Gould ES-1000 electrostatic recorder. The spectra were mass-calibrated manually with Ultramark 1621 (PCR Research Chemicals, Inc., Gainesville, FL) to an accuracy of better than ±0.2 Da.

Electrospray Ionization Mass Spectrometry (ESI-MS). Samples were analyzed on a Bio-Q mass spectrometer (VG Instruments, Manchester, England) with an electrospray ion source operating in the negative-ion mode. The electrospray tip voltage was typically 4.0 kV. *O*-Deacylated LOS samples were dissolved in H_2O, and 1−3 μL was injected via a Rheodyne injector into a stream of H_2O/acetonitrile (1:1, v/v) containing 1% acetic acid. A flow rate of 2−4 μL/min was maintained with a μLC-500 syringe pump (Isco, Lincoln, NE). Mass calibration was carried out with an external horse heart myoglobin reference using the VG Bio-Q software.

Nuclear Magnetic Resonance Spectroscopy. For NMR analysis, the Bio-Gel P-4 column fractions containing the major octasaccharide from strain 281.25 were pooled, dephosphorylated, and rechromatographed on Bio-Gel P-4. The dephosphorylated octasaccharide (0.9 mg) was lyophilized 3−4 times from 99.96% D_2O (Aldrich) and then dissolved in 0.3 mL of 99.996% D_2O (Merck, Sharp & Dohme). A trace of acetone was added to the sample as an internal reference (δ 2.225). All 1D and 2D ^{1}H NMR spectra were recorded on a GE GN-500 spectrometer at 25 °C. The phase-sensitive double-quantum-filtered COSY (DQF-COSY) spectrum (Rance et al., 1983) was acquired with time-proportional phase incrementation of the first pulse (Redfield & Kuntz, 1975; Marion & Wüthrich, 1983). The data matrix consisted of 1024 × 1024 data points, acquired with 16 scans per t_1 value and a spectral width of ±2000 Hz. The matrix was apodized with a 45°-shifted sine-bell window function and zero-filled to 2K × 2K. The 2D homonuclear Hartmann−Hahn (HOHAHA) spectrum was obtained using the MLEV-17 sequence (Bax & Davis, 1985) and States method of quadrature detection (States et al., 1982). A spectral width of ±2000 Hz was used, with a 120-ms relay mixing time. A data matrix of 512 × 1024 data points was acquired with 16 scans per t_1 increment. The matrix was zero-filled and apodized with a Gaussian window function to 2K × 2K real points. The phase-sensitive 2D nuclear Overhauser effect (NOESY) spectrum (States et al., 1982) was acquired using a 350-ms mixing time and 3.0-s interscan delay. The spectral width was ±2000 Hz, and the data matrix contained 512 × 1024 data points, with 16 scans per t_1 value. Zero-filling and a Gaussian apodization function were used to give a final 2K × 2K matrix. All data processing was done on a VAX computer with a UNIX operating system using programs developed and modified at the UCSF NMR facility (Basus et al., 1988), including some recent improvements (M. Day and D. Kneller, unpublished data).

RESULTS

Locations of Insertions of m-Tn3(Cm) into the lsg Locus of Hib A2. *H. influenzae* A2 mutant strains 281.25 and 276.4 were generated using the shuttle mutagenesis system described by Siefert et al. (1986). The m-Tn*3*(Cm) insertions in mutant strains 281.25 and 276.4 were localized by PCR amplification of the regions in the chromosome adjacent to each end of the transposable element. The m-Tn*3*(Cm) insertions are located at base 3546 in orf 4 for strain 281.25 and at base 4402 in orf 5 for strain 276.4. Both of these orfs are in the *lsg* locus. The results of these studies are shown in Figure 1A,B.

The wild-type strain A2 reacts with both MAbs 3F11 and 6E4 (Table 2). Monoclonal antibody 3F11 has been shown to recognize the terminal *N*-acetyllactosamine of *Neisseria gonorrhoeae* LOS (Yamasaki et al., 1991), and in *Neisseria*, sialylation of the 3F11 epitope blocks binding of the antibody. Previous studies have shown that neuraminidase treatment of *H. influenzae* A2 LOS increases the binding of MAb 3F11 (Mandrell et al., 1992). Monoclonal antibody 6E4 binds to a Kdo-like epitope present on *H. influenzae* A2 LOS (Spinola et al., 1990), and sialylation of the LOS has been shown not to affect binding of this antibody. As indicated in Table 2, mutant strain 276.4 reacted with MAb 6E4 and was 3F11-negative, while mutant strain 281.25 failed to react with either MAb. Following treatment with

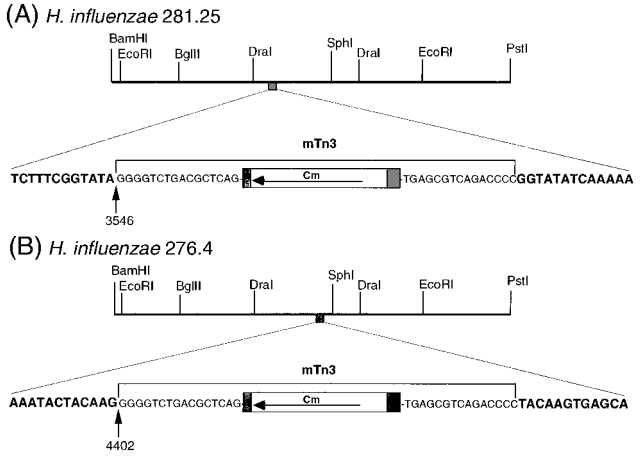


FIGURE 1: Insertion sites of the m-Tn*3*(Cm) in *H. influenzae* mutant strains (A) 281.25 and (B) 276.4.

Table 2: ELISA of MAb 3F11 and MAb 6E4 LOS Epitopes on *H. influenzae* Type b Strain A2 and Mutants 276.4 and 281.25 with and without Neuraminidase Treatment

	monoclonal antibody			
strain[c]	MAb 3F11[a]	MAb 3F11 + neuraminidase[d]	MAb 6E4[b]	MAb 6E4 + neuraminidase
Hib A2	0.694[f] (±0.095)[e]	0.972 (±0.051)	0.611 (±0.046)	0.583 (±0.107)
276.4	0.000	0.891 (±0.086)	0.632 (±0.051)	0.681 (±0.080)
281.25	0.05 (±0.015)	ND[g]	0.031 (±0.017)	ND

[a] Monoclonal antibody 3F11 was used at a dilution of 1:80. [b] Monoclonal antibody 6E4 was used at a dilution of 1:40. [c] Microtiter wells were coated with 100 μL organisms diluted to 50 Klett units in distilled water. [d] Organisms adherent to the microtiter well surface were treated with 100 μL of 0.1 unit of neuraminidase in 5 mM NaOAc, 150 mM NaCl, and 4 mM $CaCl_2$, pH 5.6, for 2 h at 37 °C. [e] ±1 standard deviation. [f] Values are read on a Biotek Model EL311 ELISA reader at an absorbance of 560 nm. [g] ND = not done.

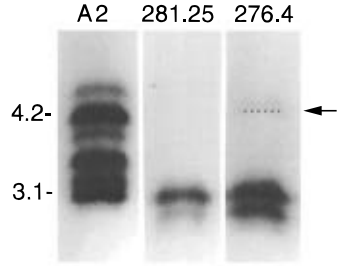


FIGURE 2: Composite SDS−PAGE gel of the LOS from wild-type *H. influenzae* type b strain A2, and mutant strains 281.25 and 276.4. The 276.4 LOS is loaded more heavily than the others to reveal the minor high molecular weight band, whose position is marked by the arrow.

neuraminidase, strain 276.4 was found to react strongly with MAb 3F11 (Table 2). To assure that the LOS phenotype was associated with these mutations, DNA was isolated from these mutants and used to re-transform *H. influenzae* strain A2. Selection was performed with chloramphenicol. Multiplate colony blots containing up to 3000 colonies were performed with each MAb to compare the LOS phenotypes of the retransformed mutants with the original mutation. The retransformed mutants had the same LOS MAb phenotype as the parent mutant.

Analysis of O-Deacylated LOS by ESI-MS. The wild-type A2 LOS mixture shows seven to eight glycolipid bands when analyzed by SDS−PAGE (Figure 2). In contrast, mutant strains 276.4 and 281.25 showed greatly simplified LOS banding patterns. Both strains showed two similar low

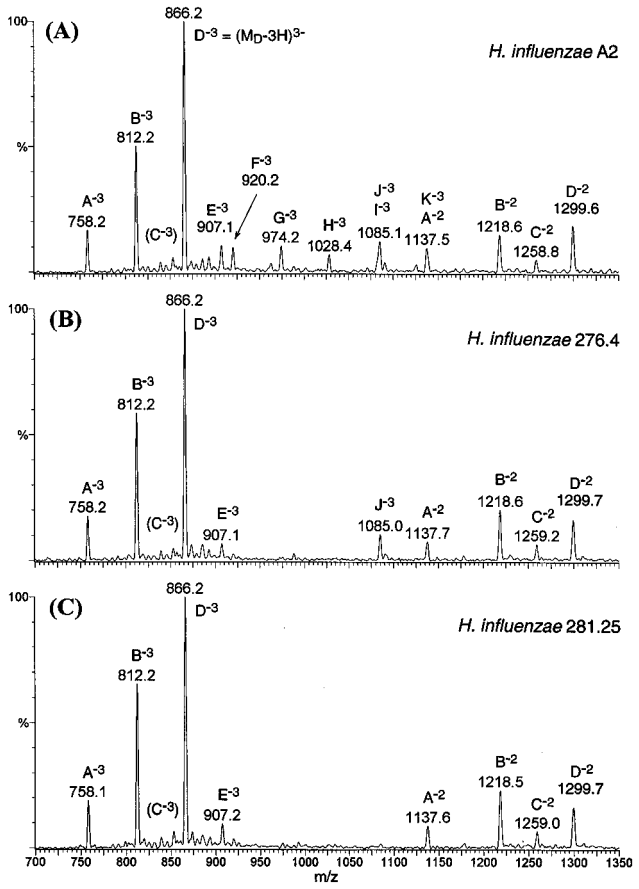


FIGURE 3: Negative-ion ESI-MS of the *O*-deacylated LOS from *H. influenzae* strains (A) A2, (B) 276.4, and (C) 281.25. When treated with neuraminidase, the J^{-3} ion in spectrum B shifted to *m/z* 987.9, consistent with the loss of sialic acid. Ions J^{-3} (*m/z* 1085.1) and K^{-3} (*m/z* $\approx$ 1139) in spectrum A were previously shown to shift upon neuraminidase treatment (Phillips et al., 1993), revealing that they overlap with the I^{-3} (*m/z* $\approx$ 1082) and A^{-2} (*m/z* 1137.5) ions.

molecular weight bands, while strain 276.4 also had a faint high molecular weight band (Figure 2). To evaluate the LOS glycoforms associated with these mutants and compare them to the parent strain, the LOS mixtures were *O*-deacylated by treatment with anhydrous hydrazine and analyzed by ESI-MS. This method has recently been shown to have excellent dynamic range for the analysis of complex LOS mixtures, where minor components at low levels ($\geq$1%) are easily observed (Gibson et al., 1993).

Previously, we identified 11 major glycoforms in the *O*-deacylated LOS from strain A2, 2 of which were shown to be sialylated, presumably on terminal *N*-acetyllactosamine moieties (Phillips et al., 1993). The negative-ion electrospray mass spectra of the *O*-deacylated LOS from strains A2, 276.4, and 281.25 are shown in Figure 3. The spectra of the two mutant strains show common doubly, $(M-2H)^{2-}$, and triply, $(M-3H)^{3-}$, charged ions corresponding to three major *O*-deacylated LOS structures of calculated M_r 2277.0,[2] 2439.1, and 2601.3 (Table 3).[3] Clearly, these components correspond to the three lowest molecular weight glycoforms present in the wild-type LOS mixture, which contain only Hex, Hep, and Kdo sugars. In addition to these common structures, which likely comprise the two major bands seen in both mutant strains by SDS−PAGE, the *O*-deacylated LOS from strain 276.4 also shows a triply charged ion at *m/z* 1085.0. This species (calculated M_r 3258.0) corresponds to a proposed composition containing HexNAc and sialic acid (NeuAc), in addition to Hex, Hep, and Kdo sugars (Table 3). To confirm the presence of sialic acid, the *O*-deacylated LOS sample was treated with neuraminidase and then reanalyzed by ESI-MS. As observed with the parent A2 strain (Phillips et al., 1993), neuraminidase treatment shifted the triply charged ion at *m/z* 1085.0 to *m/z* 987.9, consistent with the loss of a sialic acid moiety (−291.3 Da). Although strain 276.4 can produce this sialylated species, both mutants are apparently unable to produce the other higher molecular weight structures found in the parent A2 strain.

Analysis of Oligosaccharides from Strain 281.25. The LOS from strain 281.25 was hydrolyzed in 1% acetic acid for 2 h at 100 °C to cleave lipid A from the oligosaccharide. The water-soluble oligosaccharide fraction was then separated by size-exclusion chromatography, and column fractions were analyzed by LSIMS. The sample was found to contain two major species, M_r 1567.5 and 1405.5, and one minor component, M_r 1243.4. The highest molecular weight component, M_r 1567.5, corresponded to an oligosaccharide with a proposed composition of four Hex, three Hep, one PEA, and an *anhydro*Kdo. The other species represented truncated versions of this structure, lacking one (M_r 1405.5) or two (M_r 1243.4) hexoses. These three oligosaccharides clearly correspond to the major glycoforms observed in the *O*-deacylated LOS mixture from strain 281.25 (see Table 3). The generation of reducing terminal *anhydro*Kdo in *H. influenzae* oligosaccharides has been observed before (Phillips et al., 1992, 1993) and results from β-elimination of a phosphate group from the 4-position of intact Kdo during the acetic acid hydrolysis procedure (Auzanneau et al., 1991). In fact, our previous studies of the wild-type LOS mixture have indicated that all Hib A2 LOS glycoforms are phosphorylated on Kdo and produce exclusively oligosaccharides with reducing terminal *anhydro*Kdo when hydrolyzed (Phillips et al., 1993).

Oligosaccharide fractions containing primarily the highest molecular weight component, M_r 1567, were pooled and treated with aqueous HF to remove phosphoester groups, and analyzed by LSIMS. As predicted, the major component lost one PEA group (−123 Da) to give an oligosaccharide of M_r 1444. A portion of this material was hydrolyzed in 2 N TFA and analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). The dephosphorylated oligosaccharide from *S. typhimurium* Ra mutant was hydrolyzed under the same conditions to provide a known standard containing glucose, galactose, *N*-acetylglucosamine, and L-*glycero*-D-*manno*-heptose. Relative to the standard, the 281.25 hydrolysate contained 3.8 mol of glucose to 3.0 mol of L-*glycero*-D-*manno*-heptose. No other monosaccharides were detected, including *anhydro*Kdo, which is not recovered under these conditions.

[2] Ions measured by LSIMS are monoisotopic masses of the ^{12}C-containing component, and ions observed by ESI-MS are average masses.

[3] Residue mass values (monoisotopic mass, average mass) for LOS structural moieties are as follows: Hex (162.053, 162.142), Hep (192.063, 192.169), HexNAc (203.079, 203.195), Kdo (220.058, 220.179), NeuAc (291.095, 291.258), phosphate (79.966, 79.980), PEA (123.008, 123.048), and diphosphorylated *O*-deacylated lipid A (934.457, 934.994).

Table 3: Observed and Calculated Molecular Weights of *O*-Deacylated LOS

component	276.4 obsd M_r	281.25 obsd M_r	A2 obsd M_r	calcd M_r	proposed compositions
A	2277.5	2277.2	2277.3	2277.0	2 Hex, 3 Hep, PEA, Kdo(P), lipid A
B	2439.4	2439.3	2439.4	2439.1	3 Hex, 3 Hep, PEA, Kdo(P), lipid A
C	2520.4	2520.0	2519.6	2519.1	3 Hex, 3 Hep, PEA, P, Kdo(P), lipid A
D	2601.5	2601.5	2601.4	2601.3	4 Hex, 3 Hep, PEA, Kdo(P), lipid A
E	2724.3	2724.6	2724.3	2724.3	4 Hex, 3 Hep, 2 PEA, Kdo(P), lipid A
F			2763.6	2763.4	5 Hex, 3 Hep, PEA, Kdo(P), lipid A
G			2925.6	2925.6	6 Hex, 3 Hep, PEA, Kdo(P), lipid A
H			3088.2	3087.7	7 Hex, 3 Hep, PEA, Kdo(P), lipid A
I			≈3249	3249.8	8 Hex, 3 Hep, PEA, Kdo(P), lipid A
J	3258.0[a]		3258.3[a]	3257.9	NeuAc, HexNAc, 5 Hex, 3 Hep, PEA, Kdo(P), lipid A
K			≈3420[a]	3420.0	NeuAc, HexNAc, 6 Hex, 3 Hep, PEA, Kdo(P), lipid A

[a] Upon neuraminidase treatment, these peaks shifted by the loss of sialic acid.

To establish the glycosidic linkages present in the octasaccharide, a second aliquot of the dephosphorylated sample was used for linkage analysis. The partially methylated alditol acetates observed by GC/MS were derived from two terminal glucoses, two 1,4-linked glucoses, one terminal heptose, one 1,2,3-linked heptose, and one 1,3,4-linked heptose. These components were previously identified in the corresponding octasaccharide from the wild-type A2 LOS (Phillips et al., 1993).

Anomeric Assignments of the Major Octasaccharide from Strain 281.25. The 1D ^{1}H NMR spectrum of the dephosphorylated octasaccharide (Figure 4) showed seven anomeric protons, three of which were characteristic of the heptose trisaccharide core of *H. influenzae* (Phillips et al., 1992). As previously observed, some of the anomeric protons gave multiple signals, indicative of reducing terminal microheterogeneity. This phenomenon has now been observed in both *H. influenzae* (Phillips et al., 1992) and *Haemophilus ducreyi* (Melaugh et al., 1994) oligosaccharides and is caused by diastereomeric forms of *anhydro*Kdo on the reducing terminus (Auzanneau et al., 1991).

Three anomeric protons at δ 4.451, 4.551, and ≈4.56 had large coupling constants ($J_{1,2}$ = 8 Hz) indicative of β anomeric linkages, which in this case must arise from sugars in the *gluco* configuration. The remaining four anomeric protons were downfield-shifted and had small coupling constants ($J_{1,2}$ < 3 Hz), suggesting α anomeric linkages. To establish which of the four glucose residues was α-linked and confirm the oligosaccharide core structure and branching pattern, the sample was studied by 2D NMR methods. Initially, the seven monosaccharide spin systems were assigned as completely as possible using DQF-COSY and HOHAHA experiments. Where possible, apparent vicinal coupling constants were measured from the DQF-COSY cross-peaks. Starting with the β-linked sugars, the spin systems were analyzed as outlined below and summarized in Table 4.

Spin systems IV, V, and VII could all be mapped from H-1 to H-3 in the DQF-COSY spectrum (Figure 4). For those three sugars, magnetization was transferred throughout the entire spin system in the HOHAHA experiment (Figure 5A), consistent with the *gluco* configuration. In all cases, the HOHAHA cross-peaks to H-1 revealed the locations of H-5, H-6, and H-6′, whose mutual couplings were evident in the DQF-COSY spectrum. The H-4 resonance of spin system VII gave a well-resolved H-1/H-4 HOHAHA cross-peak at δ 3.430, whereas the H-4 resonances of spin systems IV and V could only be tentatively assigned at δ ≈3.50 and 3.459, respectively, based on HOHAHA cross-peak intensities.

Spin systems I, II, and III exhibited coupling networks similar to those previously associated with the *manno*-heptose core sugars of *H. influenzae* (Phillips et al., 1992; Schweda et al., 1993) and *H. ducreyi* (Melaugh et al., 1994; Schweda et al., 1994). Due to reducing terminal microheterogeneity, the anomeric proton of spin system I consisted of three resonances (δ 5.110, 5.089, and 5.039). In the DQF-COSY spectrum, these three forms of spin system I could only be mapped from H-1 to H-2. However, with the aid of the HOHAHA data, the H-3 protons could be assigned as strongly coupled to the H-2 protons, permitting the H-4, H-5, and H-6 protons to then be identified from DQF-COSY cross-peaks. The anomeric proton of spin system II also consisted of three forms (δ 5.734, 5.714, and 5.700). This spin system could be mapped from H-1 to H-3 in the DQF-COSY spectrum, and extended to H-4 using the HOHAHA data. Spin system III could be followed from H-1 (δ 5.119) to H-2 in the DQF-COSY spectrum, and additional cross-peaks to H-1 in the HOHAHA spectrum were tentatively assigned to H-3 and H-4.

The remaining spin system, VI, was mapped from H-1 to H-6/6′ using the HOHAHA cross-peaks to follow coupling connectivities in the DQF-COSY spectrum. The α-configuration of this glucose residue was confirmed by the small $J_{1,2}$ coupling constant measured from DQF-COSY cross-peaks.

The sequence of the oligosaccharide was established by analysis of cross-peaks to the anomeric protons in the 2D NOESY spectrum (Figure 5B). As previously observed, all of the *manno*-heptose residues showed H-1/H-2 intraresidue cross-peaks. The anomeric proton of heptose III showed interresidue NOE cross-peaks to H-1 and H-2 of Hep II, indicating that Hep III is the terminal heptose linked to the 2-position of Hep II. Interresidue cross-peaks between two anomeric protons are characteristic of 1,2-linkages in the α-configuration (Romanowska et al., 1988). In addition to the interresidue NOE cross-peak to H-1 of Hep III, the anomeric proton of Hep II had an interresidue cross-peak to the H-3 proton of Hep I (see Figure 5B), indicating that Hep II was linked to the 3-position of Hep I. Consistent with the linkages seen in other *Haemophilus* LOS cores, the anomeric proton of β-Glc IV showed interresidue NOE cross-peaks to H-4 and H-6 of Hep I. Considering the methylation analysis data which identified a 1,3,4-linked heptose in the sample, it was concluded that β-Glc IV was linked to the 4-position of Hep I. Consistent with its β-configuration, Glc

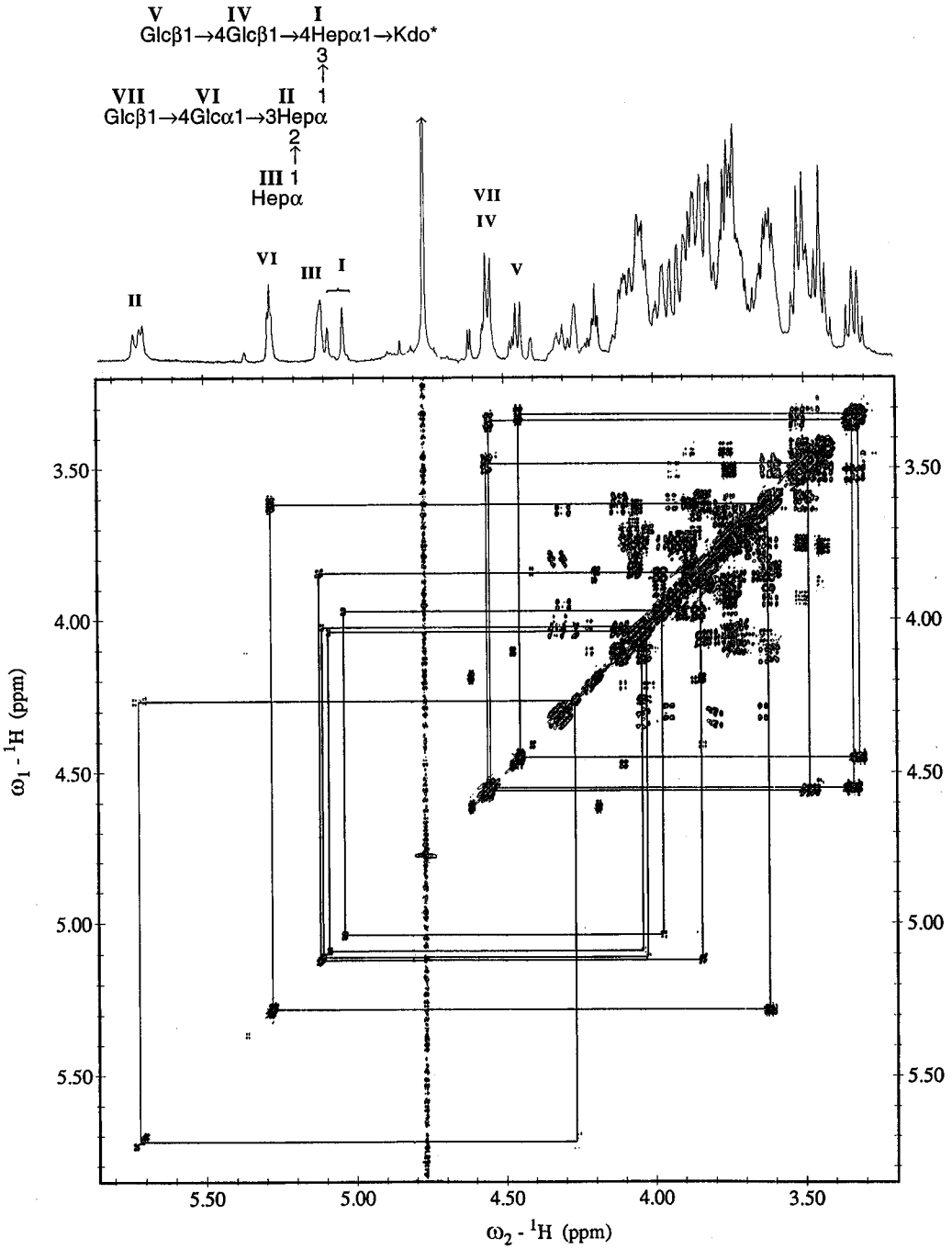


FIGURE 4: Sections of the 500 MHz 1D spectrum and phase-sensitive DQF-COSY spectrum of the major oligosaccharide from *H. influenzae* strain 281.25. Anomeric protons are labeled as shown in Table 4. H-1/H-2 cross-peaks are connected with lines on the 2D spectrum. The peak at δ 4.78 is HOD, and the peaks at δ 4.617, 4.475, and 4.409 are from the *anhydro*Kdo moiety.

IV also showed an intense cross-peak which represented intraresidue H-1/H-3 and H-1/H-5 couplings. The anomeric proton of β-Glc V showed an interresidue NOE cross-peak to the H-4 proton of β-Glc IV, which overlapped with its intraresidue H-1/H-3 cross-peak. This indicated that β-Glc V was the terminal glucose linked to the 4-position of β-Glc IV. Thus, these two glucoses comprised a Glcβ1→4Glcβ1 → branch linked to the 4-position of Hep I.

The anomeric proton of α-Glc VI showed an intraresidue cross-peak to its H-2 proton, consistent with the α-configuration, and an interresidue NOE cross-peak to H-3 of Hep II. This interresidue cross-peak indicated that α-Glc VI was the glucose residue linked to the 3-position of Hep II. The anomeric proton of the final residue, β-Glc VII, showed intraresidue couplings to H-3 and H-5, in addition to an interresidue coupling to the H-4 of α-Glc VI. This NOE cross-peak confirmed that β-Glc VII was linked to the 4-position of α-Glc VI, which established the final Glcβ1 → 4Glcα1 → branch on the 3-position of Hep II. Thus, the NMR data establish the following complete structure for the major octasaccharide from strain 281.25:

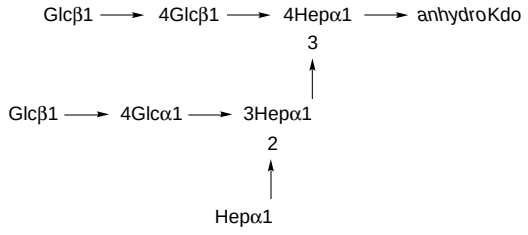
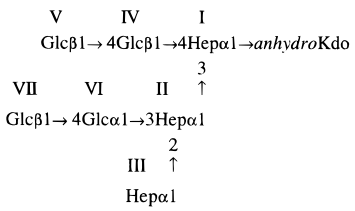


Table 4: Partial Proton NMR Assignment of the Major Dephosphorylated Oligosaccharide (M_r 1444) from *H. influenzae* Strain 281.25 (D_2O, 25 °C)[a]



proton	VII	VI	V	IV	III	II	I
H-1	4.551	5.282	4.451	4.566	5.119	5.734	5.110
				4.562		5.714	5.089
				4.557		5.700	5.039
H-2	3.341	3.617	3.321	3.475	3.844	4.265	4.023
				3.492		4.263	4.039
				3.488		4.257	3.972
H-3	3.521	3.880	3.518	≈3.60[b]	3.818[d,e]	4.040	4.030
						4.025	4.044
						4.034	3.952
H-4	3.430[d]	3.738[d]	3.459[d]	≈3.50[c,d]	3.887[d,e]	3.640[d]	4.319
						3.612[d]	4.327
						3.604[d]	4.301
H-5	3.507	3.906	3.437	3.598			3.807
							3.788
							3.638
H-6	3.747	3.852	3.756	3.834			≈4.09[c]
H-6′	3.931	3.978	3.879	4.063			
$J_{1,2}$	8	4	8	8	<3	<3	<3
$J_{2,3}$	10	10	10	10		4	sm
$J_{3,4}$	9	9	9			9−10	9−10
$J_{4,5}$							9−10

[a] Chemical shifts are reported in ppm, and *J* values are apparent coupling constants (Hz) obtained from DQF-COSY cross-peaks. [b] Average of three overlapping signals arising from reducing-terminal microheterogeneity. [c] Tentative assignments made from NOESY data. [d] Tentative assignments made from HOHAHA data. [e] Assignments could be interchanged.

The linkage position of Hep I to *anhydro*Kdo was not determined in these studies. However, it is likely that in the intact LOS, Hep I is α1,5-linked to Kdo-4-phosphate, as was found in *H. influenzae* strain 2019 (Phillips et al., 1992).

Partial Structure of the Intact LOS from Strain 281.25. In combination with the complete carbohydrate structure, the molecular weight of the *O*-deacylated LOS obtained by ESI-MS (Table 3) can be used to construct a partial structure of the intact LOS (Figure 6). This species represents the major LOS structure produced by the wild-type A2 strain and the largest structure produced by mutant strain 281.25. The two other main species produced by strain 281.25 correspond to this structure minus one and two glucoses. In the A2 parent strain, isobaric structures were found for the heptasaccharide with three glucoses, whereas the hexasaccharide was identified as a single structure with one glucose disaccharide branch on the 1,3,4-linked heptose (Phillips et al., 1993).

Based on the ESI-MS data, these same three LOS glycoforms are also the major constituents of mutant strain 276.4 LOS. However, strain 276.4 also contained an *O*-deacylated LOS species corresponding to the major structure shown in Figure 6 plus the addition of the monosaccharides Hex, HexNAc, and NeuAc. A decasaccharide corresponding to this LOS species (minus NeuAc) was observed in the oligosaccharide mixture obtained by mild acid hydrolysis of parent strain A2. Composition and methylation analyses of fractions containing this decasaccharide indicated that it likely contains terminal galactose and 1,4-GlcNAc (Phillips et al., 1993). Thus, this LOS glycoform most probably terminates in sialyl-*N*-acetyllactosamine.

DISCUSSION

Previously, we reported the sequence of the *lsg* locus from *H. influenzae* type b (McLaughlin et al., 1994) and established that the mutations producing strains 281.25 and 276.4 were in orfs 4 and 5, respectively (McLaughlin et al., 1992). In this report, we have identified the exact sites of transposon insertions into the *lsg* region of Hib A2 DNA in mutants 281.25 and 276.4 and conducted structural studies of the LOS from these strains.

ESI-MS analysis of the *O*-deacylated LOS indicated that both strains 276.4 and 281.25 produce predominantly the three lowest molecular weight species present in the wild-type strain. Strain 281.25, whose mutation is in orf 4, does not synthesize anything larger than the Hib A2 octasaccharide containing only glucose, heptose, and Kdo sugars. No LOS structures containing galactose, *N*-acetylhexosamine, or sialic acid were detected in this strain. Strain 276.4, whose mutation is in orf 5, produces quite similar glycoforms, with one important difference. Strain 276.4 can still produce one of the quantitatively sialylated structures found in the wild-type LOS population (M_r 3258.0). When strain 276.4 was treated with neuraminidase, sialic acid was lost from this LOS species, and the strain was recognized by MAb 3F11. Thus, this LOS likely contains terminal sialyl-*N*-acetyllactosamine, suggesting that the biosynthetic pathway leading to this terminal epitope is not disrupted by mutation in orf 5. However, like strain 281.25, strain 276.4 does not contain

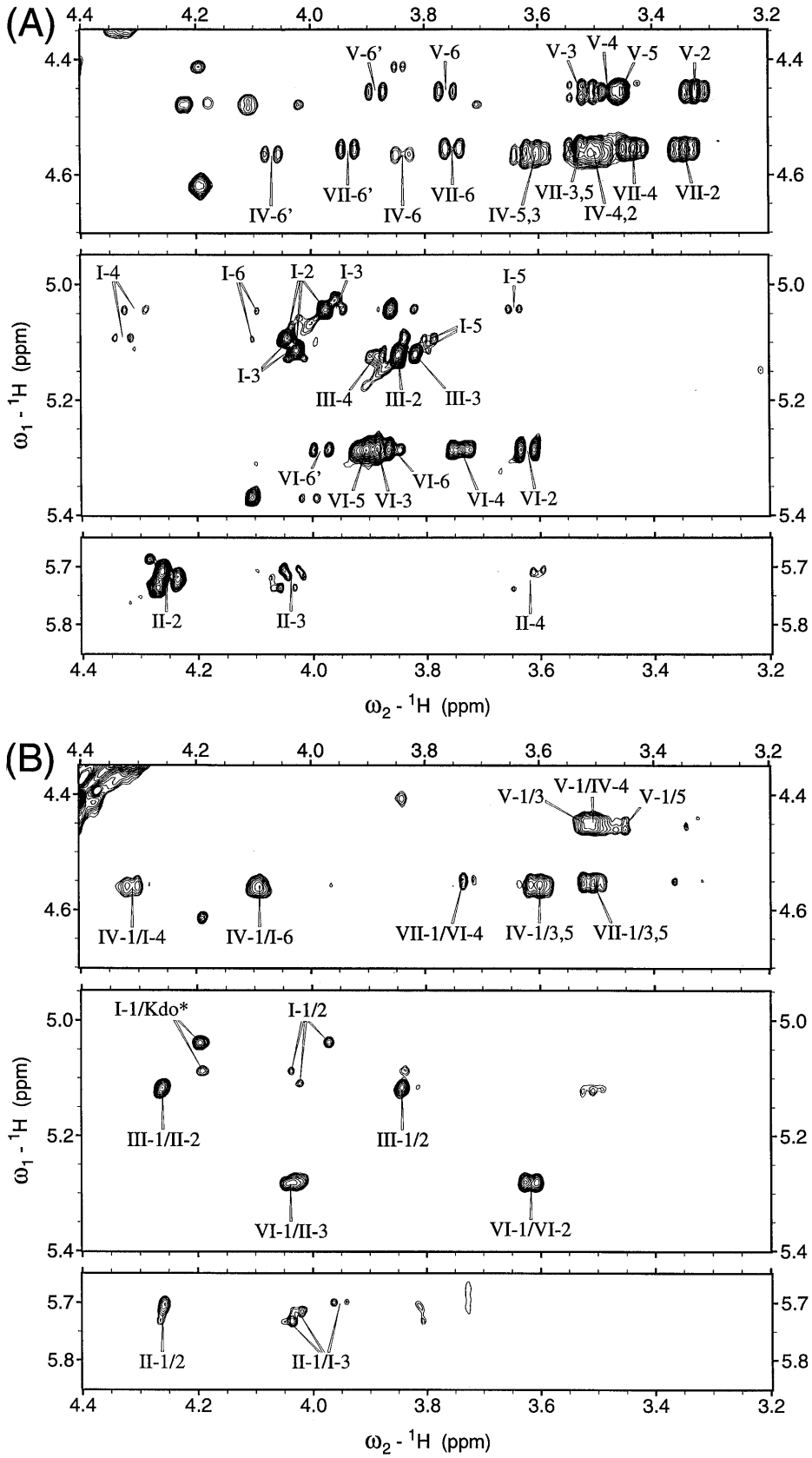


FIGURE 5: (A) Regions of the 2D HOHAHA spectrum of the major oligosaccharide from *H. influenzae* 281.25 acquired with a 120-ms mixing time. The cross-peaks at $\omega 1/\omega 2$ 5.042/3.819 and $\omega 1/\omega 2$ 5.043/3.861 arise from a minor impurity present in the sample. (B) Corresponding regions of the 2D NOESY spectrum acquired with a 350-ms mixing time. An NOE cross-peak between III-1 and II-1 is present in the full spectrum. Additional cross-peaks to the anomeric protons of III (δ 3.508) and II (δ 3.810) were not assigned.

any of the large molecular weight wild-type structures formed by the addition of 1−4 hexoses to the octasaccharide. In the wild-type Hib A2 LOS population, these structures were found to contain both di-linked and terminal galactoses, and to express the P^k epitope (Galα1→4Galβ1→4Glc) associated with wild-type *H. influenzae* LOS (Mandrell et al., 1992).

In an effort to more fully identify the LOS biosynthetic pathways affected by transposon mutagenesis of orfs 4 and

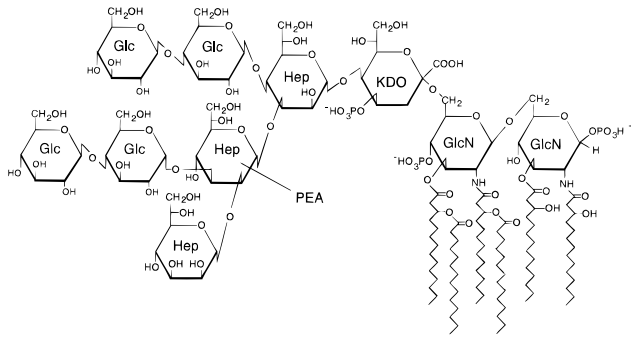


FIGURE 6: Proposed structure of the major LOS glycoform from strain 281.25. The lipid A structure shown is based in part on the LOS reported for the deep rough mutant of *H. influenzae* I-69 Rd^-/b^+ (Helander et al., 1988). All three strains showed the presence of a hexaacyl lipid A (M_r 1824). However, some differences were seen in the relative amounts of pentaacyl and tetraacyl lipid A forms (C. John and B. Gibson, unpublished results).

5, we isolated the major oligosaccharide produced by strain 281.25 and determined its complete structure by composition analysis, methylation analysis, LSIMS, and 2D NMR spectroscopy. The octasaccharide (M_r 1566) was shown to be a triantennary structure containing a heptose trisaccharide core and two glucose disaccharide branches. The 1,4-glucose residue bound to the penultimate heptose was found to be α-linked, whereas all of the other glucoses were β-linked. This structure (without anomeric assignments) was previously found to be the major component in the oligosaccharides derived from the wild-type Hib A2 strain (Phillips et al., 1993). Given that this is the largest structure made by strain 281.25, it is not surprising that MAb 3F11, which recognizes the terminal region of paragloboside, does not bind 281.25 LOS. Of all the transposon mutants initially studied, strain 281.25 had the only phase-locked nonreactive phenotype for MAb 3F11 (McLaughlin et al., 1992). The fact that LOS from strain 281.25 is also not recognized by MAb 6E4, which recognizes a Kdo-related epitope on Hib LOS (Spinola et al., 1990; Abu Kwaik et al., 1991), suggests that the 6E4 epitope may be a conformational epitope either absent or occluded in this structure.

Previous studies have indicated that the functions of the products of the individual orfs in the *lsg* locus are sequentially linked. Our studies of the 7.4-kb *lsg* locus cloned into *E. coli* have shown that successive reduction in the fragment size of the *lsg* locus results in a progressive decrease in the size of the oligosaccharide added to the *E. coli* acceptor (Abu Kwaik et al., 1991). Sequencing reveals that the entire *lsg* locus contains seven complete and one partial open reading frames (McLaughlin et al., 1994). These are organized in two sets of three orfs each which are followed by two orfs transcribed in opposite directions (McLaughlin et al., 1994). Homology search using BLASTA analysis indicates that orf 3 has homology (46.875% similarity and 22.26% identity) with asmE, a glycosyltransferase necessary for production of *Erwinia amylovora* exopolysaccharide which consists of a polymer of four galactose residues and a glucuronic acid molecule (Metzer et al., 1994; Bugert & Geider, 1995). Homology searches of orfs 4, 5, and 6 have failed to yield sequences which showed significant similarity although orfs 5 and 6 share considerable homology with each other. While we cannot assign specific functions to the orfs, based on the structural analyses described in this paper, it appears that this region is most probably involved in the transfer of galactose to the LOS structure. The *lsg* locus appears to be essential for the biosynthesis of the LOS species containing the 3F11 (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) and P^k (Galα1→4Galβ1→4Glc) epitopes. However, it also appears that both of these LOS species are synthesized separately. As previously noted (Abu Kwaik et al., 1992), the *lsg* locus is separate from the *lic* loci, and both are involved in Hib LOS biosynthesis (Maskell et al., 1992).

Through the combination of transposon mutagenesis and LOS structural analysis, we now have two mutant strains of *H. influenzae* which produce defined subsets of the wild-type LOS population. The specific structures produced by mutants 281.25 and 276.4 do not express the P^k or paragloboside epitopes implicated in host mimicry (Mandrell et al., 1992). Yet strain 276.4 does produce a minor sialylated species which likely terminates in sialyl-*N*-acetyllactosamine. It has been observed that gonococci with sialylated LOS are more resistant to the bactericidal effect of normal human serum (Parsons et al., 1988). Additionally, opaque Hib strains expressing small LOS were found to be more effective at causing bacteremia in the infant rat model (Weiser, 1992). Thus, these Hib mutants with small LOS should prove to be useful tools for evaluating the role(s) of specific carbohydrate epitopes in *H. influenzae* pathogenesis.

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