A *Haemophilus influenzae* Strain Associated with Fisher Syndrome Expresses a Novel Disialylated Ganglioside Mimic†

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ABSTRACT: The non-typeable *Haemophilus influenzae* strain DH1 was isolated from a 25 year old male patient with Fisher syndrome, a postinfectious autoimmune condition characterized by the presence of anti-GQ1b IgG antibodies that target and initiate damage to peripheral nerves. DH1 was found to display an αNeuAc(2−8)αNeuAc(2−3)βGal branch bound to the tetraheptosyl backbone core of its lipooligosaccharide (LOS). The novel sialylation pattern was found to be dependent on the activity of a bifunctional sialyltransferase, Lic3B, which catalyzes the addition of both the terminal and subterminal sialic acid residues. Patient serum IgGs bind to DH1 LOS, and the reactivity is significantly influenced by the presence of sialylated glycoforms. The display by DH1, of a surface glycan that mimics the terminal trisaccharide portion of disialosyl-containing gangliosides, provides strong evidence for its involvement in the development of Fisher syndrome.

Fisher syndrome (FS[1]) is a postinfectious immune disorder characterized by the presence of autoantibodies targeted against gangliosides of the oculomotor and primary sensory neurons (*1*). Most individuals that develop FS have circulating IgG antibodies which react principally to GQ1b (*2*), as well as other gangliosides possessing disialosyl moieties, such as GT1a, GD2, and GD3 (*3*). There is strong evidence that autoantibodies found in patients with FS, and its clinical variant Guillain−Barré syndrome (GBS), are elicited following exposure to a pathogen bearing surface glycans that mimic host gangliosides (*1*).

Bacterial lipooligosaccharide (LOS) has been identified as an agent that triggers the production of autoreactive IgGs in humans. Support for this assertion has come principally through investigations focused on *Campylobacter jejuni*, the organism that most frequently infects patients prior to the onset of GBS and FS (*4*, *5*). Animals injected with *C. jejuni* LOS produce anti-ganglioside antibodies and develop GBS-like symptoms (*6*, *7*). The carbohydrate component of the LOS in several strains shows a close resemblance to specific gangliosides (*5*, *8*, *9*), and genetic studies have demonstrated that the vast majority of strains associated with GBS and FS possess sialyltransferases necessary to synthesize ganglioside mimics (*10*, *11*).

In a small number of cases (<10%), the bacterium *Haemophilus influenzae* has been isolated from individuals with FS (*5*). The oligosaccharide component of its LOS is known to resemble mammalian glycolipids (*12*) and often contains *N*-acetylneuraminic acid (NeuAc) (*13*). Recent epidemiological studies have established a causal relationship between *H. influenzae* infection and the onset of FS (*5*); however, its association with GBS and FS remains an area of controversy. Because this organism naturally inhabits the respiratory tract of the majority of humans, strains cultured from patients with FS cannot be easily linked to the disease without rigorous serological characterization to exclude the potential involvement of other pathogens (*14*). Furthermore, while there have been several investigations that have established the similarity between the structure of gangliosides and the LOS of *C. jejuni* strains isolated from individuals with GBS and FS (*5*, *8*, *9*), no analogous structural studies have confirmed the presence of a surface-bound ganglioside mimic in a *H. influenzae* strain isolated from a FS patient.

In this report, we describe the isolation and characterization of a non-typeable *H. influenzae* (NTHi) strain from an individual suffering from FS, who had elevated titers for the ganglioside GQ1b. The LOS structure of DH1 was found to incorporate a novel αNeuAc(2→8)αNeuAc(2→3)βGal extension from the tetraheptosyl backbone of its core oligosaccharide. The similarity of this branch to the nonreducing end of GQ1b, and other disialosyl-containing gangliosides, suggests that anti-GQ1b IgGs in this patient were elicited as a result of exposure to an LOS-bound GQ1b mimic presented by DH1.

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[1] Abbreviations: COS, core oligosaccharide; GBS, Guillain−Barré syndrome; FS, Fisher syndrome; LOS, lipooligosaccharide; NeuAc, *N*-acetylneuraminic acid; NTHi, non-typeable *Haemophilus influenzae*; *P*Cho, phosphocholine; *P*EA, phosphoethanolamine.

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Table 1: Summary of the DH1 Glycosyltransferase Genes That Were Probed by PCR and Sequenced When Amplified

glycosyltransferase	detected (+) or not detected (−)	GenBank accession No.	proposed activity[a]	comment
LsgB	+	EF428564	sialyltransferase	
Lic3A	+	EF428562	(α-2,3-sialyltransferase)	inactive because of insertion of 10-YK repeats
Lic3B	+	EF444927	α-2,3/8-sialyltransferase	
SiaA/OrfY	−	n/a	(sialyltransferase)	
LgtF	+	EF428561	*β*-1,4-glucosyltransferase	
Lic2C	−	n/a	(α-1,3-galactosyltransferase)	
LpsA	+	EF428563	*β*-1,2-glucosyltransferase	
LosA	+	EF444928	*β*-1,4-galactosyltransferase	
LosB	+	EF444928	(heptosyltransferase)	inactive because of a frame-shift mutation
RfbB	+	EF444927	dTDP-glucose 4,6-dehydratase	

[a] Parentheses are used to indicate glycosyltransferases that are either inactive or absent in DH1.

MATERIALS AND METHODS

Patient Case Report. Two days after contracting upper respiratory infectious symptoms, a 25 year old man noticed blurred vision. He could not open his left eye and could not walk straight. Five days after neurological onset, left blepharoptosis was present and bilateral ocular movements were severely limited. He had no weakness in the four limbs, but he showed limb and truncal ataxia. Deep tendon reflexes were diminished in the arms and absent in the legs. Hypesthesia was present at the distal limbs. Serum IgG antibody titers against GQ1b and GT1a were significantly increased in the acute phase of the condition, which gradually decreased during the recovery phase. IgG antibodies against GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, and GT1b were not detected. The NTHi strain DH1 was isolated from the patient from a swab culture.

Bacterial Growth and LOS Extraction. Suspension growth of DH1 cells was in brain−heart infusion broth supplemented with haemin (10 μg/mL), NAD (2 μg/mL), and NeuAc (10 μg/mL). LOS was extracted from cell biomass using the hot-phenol water procedure (*15*). Plated *H. influenzae* cultures were grown on chocolate agar plates, supplemented in some instances with NeuAc (5 mg/plate), and microscale extraction of LOS for analytical characterization was performed as described previously (*16*). Anhydrous hydrazine was used for *O*-deacylation of the LOS (*16*, *17*). Hydrolyzed core oligosaccharide (COS) was obtained by incubating the LOS in 1% acetic acid for 90 min at 100 °C, and recovered in the aqueous phase following centrifugation (8000*g*, 20 min).

MS Analysis of the LOS from DH1 and Its Associated Mutants. *O*-Deacylated LOS was characterized by capillary electrophoresis (CE)−MS and multiple-step MS (MS^n) fragmentation experiments performed using previously described methods (*18*). Tandem CE−MS/precursor ion scan experiments were used to screen for the presence of sialylated species among the complex mixture of LOS glycoforms present. Specifically, analysis for the diagnostic ion at $m/z = 290.1$ was used to detect all species that fragmented to a common ion corresponding to NeuAc. GLC−MS was carried out with a Hewlett-Packard 6890 chromatograph connected to a Micromass quadrupole mass spectrometer using a DB-5 fused silica capillary column and a temperature gradient from 130 to 250 °C, varying by 3 °C/min. Tandem LC−ESI-MS^n experiments on permethylated COS were performed in the positive ion mode on a Waters 2690 HPLC system (Waters, Milford, MA) coupled to a Finnigan LCQ iontrap mass spectrometer (Finnigan-MAT, San Jose, CA). Separation was achieved by loading the material onto a microbore C18-column (Phenomenex LUNA, Torrance, CA) and eluting with a methanol gradient against an H_2O/acetate solution (1% acetic acid, 0.1 mM sodium acetate).

NMR Analysis of DH1 LOS. Both *O*-deacylated LOS and COS were characterized by NMR spectroscopy. Highly purified material used for NMR analysis was obtained by anion exchange chromatography using HiTrap Q HP columns run on an AKTA explorer system (Amersham Biosciences), followed by the removal of NaCl with HiTrap desalting columns. *O*-Deacylated LOS was dispersed at a concentration of ∼1 mM in phosphate buffered d_{25}-SDS micelles (50 mM, pD 7.0) containing d_4-EDTA (1 mM), while the COS was dissolved in D_2O at a concentration of ∼1 mM. Standard 2D ^1H homonuclear and $^1H-^{13}C$ and $^1H-^{31}P$ heteronuclear spectra were acquired as described previously (*19*), at various temperatures on Varian Inova spectrometers operating at either 500 or 600 MHz. The data were processed using the software TOPSPIN (Bruker Biospin, Billerica, MA).

Chemical Analysis of DH1 LOS. Sugars were identified by GLC−MS as their alditol acetates as previously described (*20*). Methylation was performed with methyl iodide in dimethyl sulfoxide in the presence of lithium methylsulfinylmethanide (*20*). The methylated compounds were recovered using a SepPak C18 cartridge and subjected to sugar analysis or LC−ESI-MS^n. The relative proportions of the various alditol acetates and partially methylated alditol acetates obtained in sugar and methylation analyses correspond to the detector response in GLC−MS. Dephosphorylation was performed with 48% hydrogen fluoride as described previously (*20*).

DH1 Genetic Characterization and Generation of Mutant Strains. We designed primers (see Supporting Information Table S6) to amplify the genes encoding glycosyltransferases that were putatively associated with the synthesis of the DH1 LOS core glycan. PCR products were generated using an Advantage 2 PCR kit (Clontech Laboratories), and sequencing was performed using an Applied Biosystems (Montreal, Canada) model 3100 genetic analyzer. To help elucidate the sialylation pattern, three isogenic DH1 mutants were constructed by inserting a kanamycin resistance cassette (from pUC4K, Amershan Biosciences, Montreal, Canada) into the *lic3A* (DH1*lic3A*), *lic3B* (DH1*lic3B*), and *lpsA* (DH1*lpsA*) genes. The genetic loci corresponding to these genes were amplified from DH1 genomic DNA and cloned into pBluescript II SK (Stratagene, La Jolla, CA). For *lpsA* it was necessary to engineer into the sequence a *Pst*I restriction site.

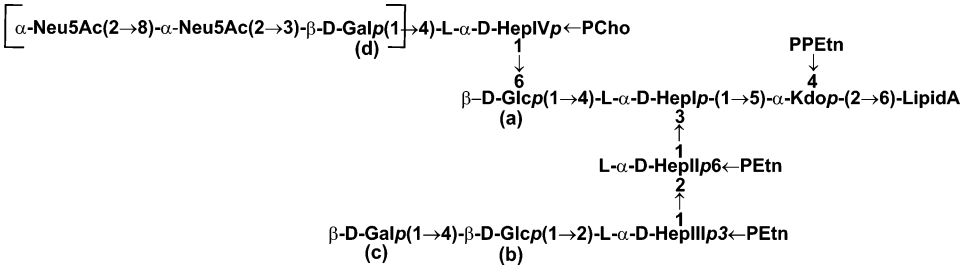

FIGURE 1: The core LOS structure adopted by DH1. The trisaccharide unit common to all disialosyl-containing gangliosides is delineated by square brackets. Sialylated species account for <10% of the total isolated LOS glycoforms, a level of sialylation that is consistent with previously characterized *H. influenzae* strains grown under similar conditions (*24*). The hexose units are labeled alphabetically by lower case letters (a−d) for the purpose of peak labels in NMR spectra and chemical shift assignments (Figure 2). Heptose units are labeled with roman numerals, according to standard nomenclature for published *H. influenzae* LOS structures (*23*).

The pBluescript constructs were digested with either *Bgl*II (for *lic3A* and *lic3B* constructs) or *Pst*I and fused with the kanamycin resistance cassette. PCR analysis was used to select the clones with the resistance gene in the same orientation as the disrupted gene. The resulting plasmids containing the disrupted genes were linearized and used to transform DH1 cells using the MIV method (*21*). Following growth on BHI plates supplemented with kanamycin (10 μg/mL), haemin (10 μg/mL), and NAD (2 μg/mL), individual colonies were restreaked onto chocolate agar plates. PCR analysis and DNA sequencing was used to confirm site-specific recombination with the kanamycin-inserted gene.

Serum Reactivity to DH1 LOS. ELISA was used to measure the level of IgG reactivity for crude LOS extracted from DH1 cells and its associated mutants based on previously described methods (*5*). Following growth on chocolate agar plates, the bacterial cells were suspended in a PBS solution at an optical density of 0.4 (at 650 nm). 1.5 mL aliquots were pelleted (14000*g*, 1.5 min), and the cells were resuspended in H_2O, boiled for 10 min, and then treated with proteinase K for 60 min at 60 °C. Serum samples at various dilutions were combined with the crude LOS in microplate wells, and peroxidase-conjugated rabbit anti-human IgG was used as the secondary antibody.

RESULTS

DH1 LOS Is Sialylated and Contains a Lactosyl and βGal-HepIV branch. The glycan component of the LOS in NTHi strains adopts a conserved glucose-substituted triheptosyl inner-core bound to the lipidA anchor via 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) 4-phosphate (Figure 1) (*20*, *22*). Structural heterogeneity arises from variable branching patterns off of the three heptose residues that form the backbone of the glycan core. In addition to the triheptosyl backbone, the major DH1 core oligosaccharide consists of a βGal(1→4)βGlc (lactose) extension from C2 of HepIII, and a βGal(1→4)αHepIV branch from C6 of the βGlc residue bound to HepI (Figure 1). The tetraheptose (Hep4) arrangement has been observed previously in *H. influenzae* (*23*). DH1 LOS also contains two phosphoethanolamine (*P*EA) residues, bound to C3 and C6 of HepII and HepIII respectively, and a phosphocholine substituent bound to HepIV (*P*Cho) (Figure 1). *P*EA and *P*Cho are common substituents found in *H. influenzae* LOS.

The structure of the DH1 LOS glycan was elucidated using homo- and heteronuclear NMR analysis of purified core oligosaccharide and was confirmed through analytical and MS techniques. A 1D ^{1}H spectrum of COS is presented in Figure 2 with the anomeric protons of each glycose unit labeled. The precise arrangement of the sugar moieties was established through the observation of NOEs between protons of adjoining residues, and similarly confirmed using HMBC experiments. ^{1}H and ^{13}C chemical shift assignments of the COS are provided as Supporting Information, as well as the results from compositional and methylation analysis, and branching information obtained using LC−ESI-MS^n. The results are fully consistent with the structure presented in Figure 1, which represents the predominant glycoform displayed by DH1. Minor species with truncated extensions from the backbone were detected, as well as trace amounts of higher molecular weight glycoforms corresponding to elongated extensions with hexose residues from HepIV.

When DH1 cells were grown in the presence of exogenous NeuAc, the LOS was found to be sialylated. This 9-carbon sugar is easily identified when *O*-deacylated DH1 LOS is dispersed in micelles, from the well-resolved H3 methylene protons that appear in the upfield region of ^{1}H-detected spectra (Figure 2, inset). MS analysis of the LOS indicated the presence of both mono- and disialylated LOS species, and there was clear evidence from fragmentation patterns for the presence of disialate residues. Sialylated species represent only a minor fraction (<10%) of the total DH1 LOS glycoforms isolated from cells grown in liquid culture, which is consistent with previous observations for *H. influenzae* strains that incorporate LOS-bound NeuAc. The level of sialylation is dependent on growth conditions (*24*, *25*). It is unknown whether a significantly higher proportion of *H. influenzae* LOS is decorated with this residue in a host environment.

Several glycosyltransferases have been identified in *H. influenzae* that are involved in the synthesis of the core oligosaccharide. These include the enzymes responsible for branch initiation from one of the three backbone heptose residues: LgtF (HepI), Lic2C (HepII), and LpsA (HepIII). To help rationalize the deduced LOS structure, we used PCR to confirm the presence or absence of specific *H. influenzae* glycosyltransferases in DH1 (Table 1, Figure 3). LgtF, which catalyzes the addition of βGlc to HepI and was found in 100% of *H. influenzae* strains in a survey performed by Hood et al. (*26*), was sequenced and shares 98% identity with its homologue in the well-characterized strain Rd (RM118). LpsA, which adds either glucose or galactose to HepIII depending on the allelic variant (*27*), is also carried by DH1, and its sequence is identical to that found in strain Rd; the

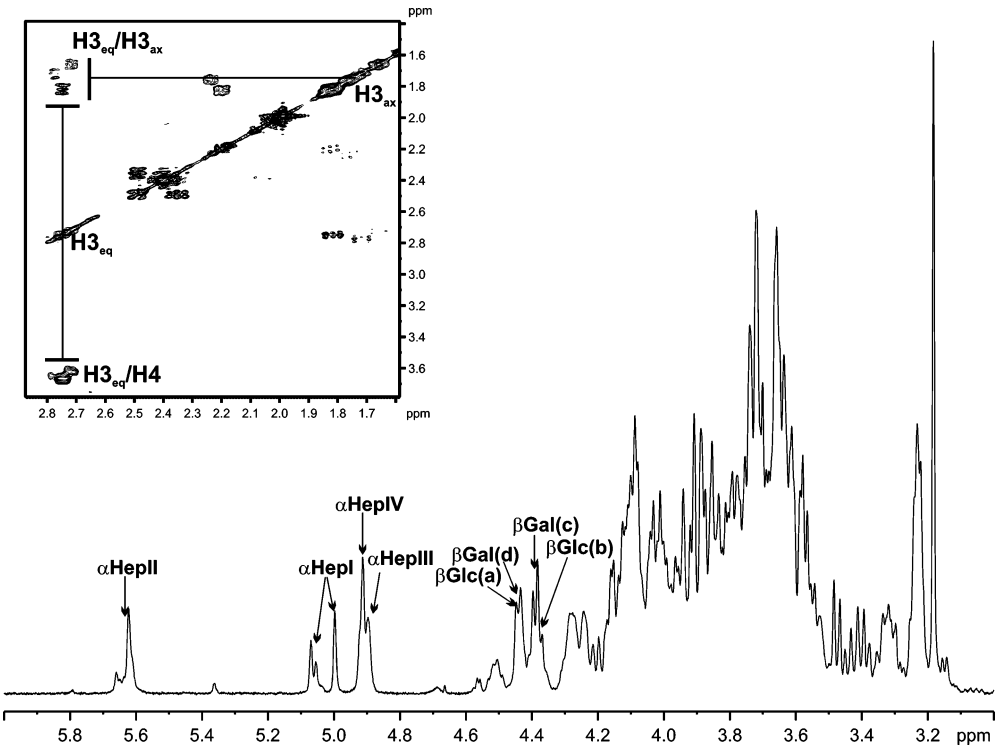

FIGURE 2: NMR spectra of purified LOS from DH1. Displayed is a 1D ^{1}H spectrum of purified COS material from DH1, with anomeric protons of the heptose and hexose residues labeled based on the structure presented in Figure 1. Inset shows the upfield region of a 2D COSY spectrum of *O*-deacylated DH1 LOS dispersed in SDS micelles. Labeled is the connectivity pattern formed by the $H3_{ax}$, $H3_{eq}$, and H4 spin network for at least three distinct NeuAc residues, consistent with the presence of both mono- and disialylated species within the ensemble of LOS glycoforms.

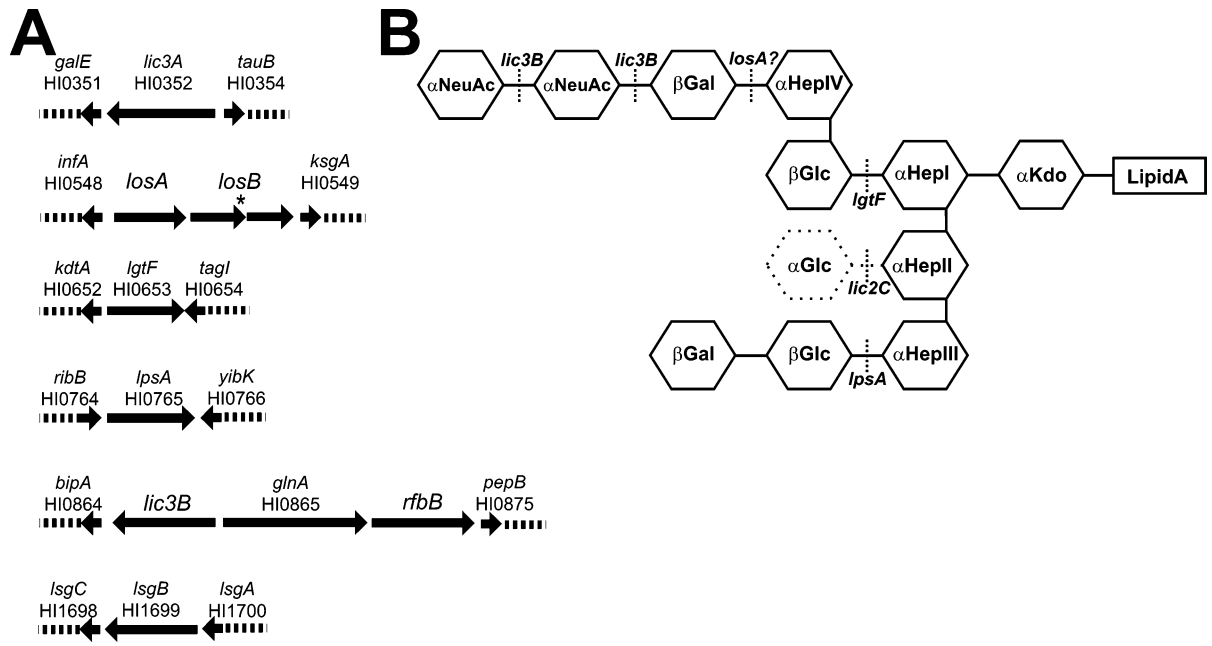

FIGURE 3: Organization and role of LOS-associated genes in DH1. The LOS biosynthesis genes are not confined to a single locus in *H. influenzae*, but are distributed among different locations in the genome. (A) The solid arrows represent genes that were amplified and sequenced, with the arrowhead oriented with respect to the direction of translation, and the length proportional to the gene size. The genes flanking those sequenced, represented by dashed lines, are numbered according to assignments made for those of *H. influenzae* strain Rd (GenBank accession L42023). An asterisk (*) indicates a premature translational stop in *losB*. (B) Function of the glycosyltransferases involved in the synthesis of the LOS core glycan, outlined in a structural cartoon representation based on Figure 1. DH1 does not carry the *lic2C* gene, which is required for branch initiation form HepII (dashed αGlc residue), but is replaced by the genes *losA* and *losB*. *LosA* is putatively assigned the function of a galactosyltransferase based on homology with a similar enzyme identified in *H. ducreyi (30)*.

LOS in both DH1 and Rd possesses a glucose residue bound to HepIII in the β1,2 configuration. The *lic2C* gene, which is required for branching from HepII, is not found in the DH1 genome, consistent with its elucidated LOS structure. This gene, when present, is found in a locus flanked by *infA* and *ksgA*. *Lic2C* is replaced by the genes *losA* and *losB* in DH1, an arrangement found in roughly 20% of NTHi strains (*28*, *29*) (Figure 3). LosA was previously proposed to be a

galactosyltransferase based on homology with a similar enzyme from *Haemophilus ducreyi* and may be involved in the transfer of galactose to HepIV. LosB in *H. ducreyi* functions as a heptosyltransferase (*30*); however, in DH1, this enzyme appears to be inactive as a result of a premature translational stop codon (Figure 3, Table 1).

DH1 Exhibits a Novel Lic3B-Dependent Sialylation Pattern. To date, investigations focused on the LOS structure of NTHi strains have identified two known NeuAc-containing structural motifs: sialyllactose and sialyllacto-*N*-neotetraose (*13*, *31*, *32*). These motifs have been identified as branches from either HepIII or HepI, respectively. The sialyltransferase Lic3A, which has been cloned and characterized *in vitro*, catalyzes the transfer of αNeuAc to lactose moieties (*24*), whereas the sialyltransferases LsgB and SiaA are the most probable candidates for the synthesis of sialyllacto-*N*-neotetraose-like units (*32*−*35*). Disialosyl moieties (i.e., αNeuAc(2−8)αNeuAc) bound to lactose have also been observed; in strains with these units the addition of the terminal NeuAc is catalyzed by the bifunctional sialyltransferase Lic3B (*36*).

Because of the small fraction of sialylated glycoforms, we were unable to elucidate the linkage position(s) of the NeuAc residues on the LOS of DH1 using NMR spectroscopy. For this determination, we constructed isogenic DH1 mutant strains where specific LOS-associated glycosyltransferases were inactivated, and analyzed their LOS using CE−MS. The sialylation pattern was confirmed through the use of MS^n fragmentation analysis. DH1 exhibits a completely novel mode of sialylation, with the addition of NeuAc residues directed to the *β*Gal-αHepIV branch of the LOS (Figure 1 and Figure 4).

PCR analysis established that both the *lic3A* and *lic3B* genes are found in the DH1 genome (Figure 3, Table 1). DH1 mutant strains were constructed in which these sialyltransferases were individually knocked out. The LOS of DH1*lic3A* exhibited the same sialylation pattern as wild-type DH1, indicating that Lic3A is inactive in this strain (data not shown). This is likely due to the presence of multiple TATAAA repeats in the genomic sequence of Lic3A, which encode for consecutive −YK− residues located between positions 104 and 123 within the 346 residue protein. These repeats are absent in Lic3A homologues of previously characterized strains and are likely responsible for its inactivation. In contrast, a knock-out mutation of *lic3B* (DH1*lic3B*) completely eliminated LOS sialylation (Figure 4B), demonstrating that this bifunctional sialyltransferase was responsible for addition of the terminal and subterminal NeuAc residues.

The sialyltransferases OrfY and SiaA, which are found in the *hmg* locus when present in other NTHi strains (*34*), are not carried by DH1 (Figure 3, Table 1). We amplified this region and found that it contained a single gene, *rfbB* (a homologue of dTDP-glucose 4,6-dehydratase). This organization of the *hmg* region is similar to that observed in strain 86-028NP (GenBank No. CP000057). The sialyltransferase LsgB, which putatively targets *N*-acetyllactosamine moieties (*32*), is present in the DH1 genome. We did not observe the presence of *N*-acetyllactosamine in DH1 and propose that LsgB has no role in LOS biosynthesis in this strain, since inactivation of Lic3B resulted in complete loss of sialylated glycoforms.

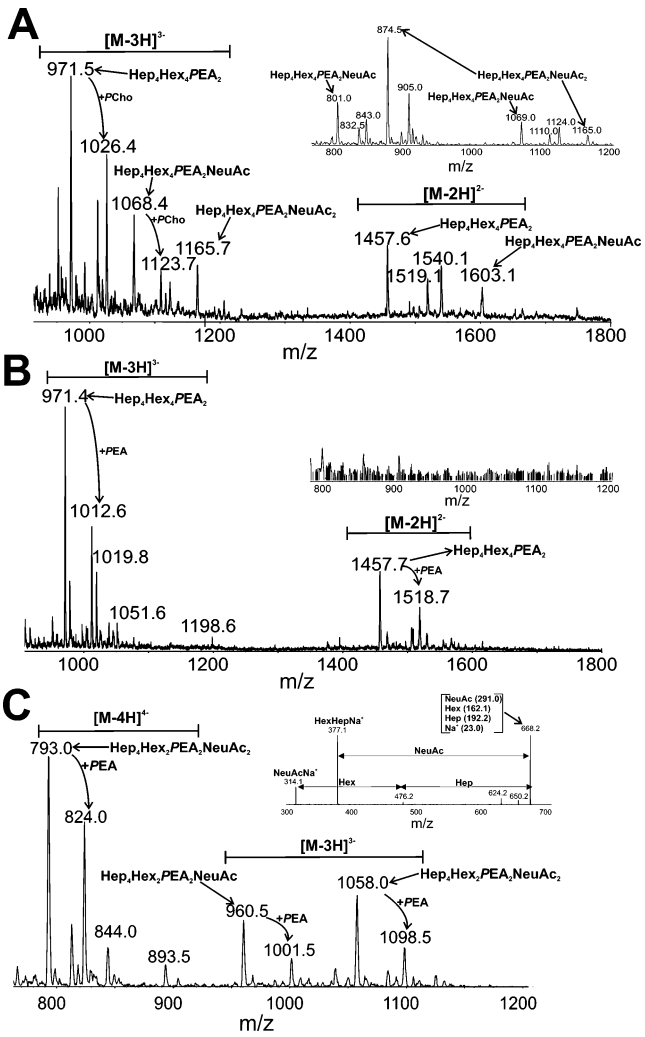

FIGURE 4: DH1 expresses a disialosyl moiety on the *β*Gal-αHepIV branch of its LOS. (A) A CE−MS spectrum of *O*-deacylated LOS from DH1 shows the presence of both mono- and disialylated species, confirmed by (inset) the presence of analogous peaks using a precursor ion scan for sialylated species. (B) Knock-out of the bifunctional sialyltransferase, Lic3B, eliminates the presence of all sialylated species in CE−MS spectra of the LOS, and a corresponding loss of peaks in the precursor ion mode (inset). (C) An *lpsA* mutation eliminates the lactose moiety (-2Hex) bound to HepIII. The presence of sialylated species in DH1*lpsA* LOS confirms that sialylation is directed to the *β*Gal-αHepIV branch. This is further demonstrated by the detection and fragmentation of an ion at *m*/*z* 668.2 which corresponds to a NeuAc-Hex-Hep species. In addition to the Hep (192.17), Hex (162.15), *P*EA (123.05), *P*Cho (165.05), and NeuAc (291.05) units which combine to account for the mass of ions in the CE−MS spectra, each labeled species also contains a Kdo-P (300.2) and lipidA (953.0) moiety.

In our initial assessment of the DH1 LOS structure, we viewed the lactose branch linked to HepIII as the most likely acceptor position for the NeuAc residues, since lactose is frequently sialylated in NTHi strains. However, MS fragmentation analysis of the LOS from DH1 clearly showed the presence of ions corresponding to NeuAc-Hex-Hep, and not NeuAc-Hex-Hex (Figure 4C). This indicated that sialylation was likely directed to the *β*Gal-αHepIV unit rather than to lactose (see structure in Figure 1). To confirm these fragmentation results, we constructed an *lpsA* mutant. LpsA catalyzes the transfer of glucose (or galactose in some instances) to the distal heptose of the glycan backbone, and must be present for chain elongation from HepIII (*27*). LC−

ESI-MS^n analysis of LOS isolated from DH1*lpsA* cells demonstrated that the lactose unit bound to HepIII was lost; nevertheless, the LOS from this isogenic mutant contains mono- and disialylated species, confirming that βGal-αHepIV serves as the acceptor (Figure 4C). DH1 LOS glycoforms therefore possesses an unusually positioned αNeuAc(2−8)αNeuAc(2−3)βGal trisaccharide unit. This unit corresponds to the terminal glycan residues of several disialosyl-containing gangliosides.

Patient Serum IgG Reactivity toward DH1 LOS Is Influenced by Sialylation. LOS extracts from DH1 cells react with patient serum antibodies. This was demonstrated based on ELISA measurements, in which the optical response was based on IgG-specific binding (Figure 5). To establish that this response was the result of DH1-specific epitopes, extracts from a number of NTHi strains were probed; however, only the LOS from DH1 gave rise to a strong response (Figure 5A). The serum reactivity is markedly influenced by the presence or absence of sialylated species. Side-by-side measurements were made for DH1 cells grown in media with and without supplemented NeuAc. *H. influenzae* requires an exogenous source of sialic acid in order for this residue to be incorporated into its surface glycans (*37*). There was a significant decrease in IgG serum reactivity for DH1 cells that were grown in media lacking NeuAc (Figure 5B). Similarly, in comparison to DH1, patient serum was also much less reactive toward DH1*lic3B* LOS, which lacks sialylated glycoforms (Figure 5C). However, the reactivity did not differ noticeably for the LOS isolated from DH1*lic3A* (Figure 5C) or DH1*lpsA* cells (Figure 5C), which do possess sialylated species. These results demonstrate that for a large fraction of patient antibodies that bind to the LOS, sialic acid residues comprise part of their recognition epitope.

DISCUSSION

The most frequently isolated infectious organism from patients with FS is *C. jejuni*, which in one study accounted for close to 1/5 of the identified antecedent agents (*5*). There is substantial evidence to support the premise that molecular mimicry between its LOS and host gangliosides is the triggering mechanism for the onset of this syndrome (*1*, *38*). There have been several other identified organisms putatively associated with the development of FS (*39*), including *H. influenzae*; however, there are few mechanistic clues as to how they may be involved in initiating the production of autoreactive antibodies.

We recently used immunological binding techniques to demonstrate that FS-associated *H. influenzae* isolates are more likely to react with anti-GQ1b antibodies than those cultured from patients with uncomplicated respiratory infections (*5*). We now provide clear evidence that the strain DH1 possesses a structural mimic of the terminal trisaccharide unit of GQ1b. This strain was isolated from a patient who suffered from FS and possessed elevated titers against GQ1b. ELISA measurements indicate not only that serum IgGs react with DH1 LOS, but also that the reactivity is influenced by the level of sialylation. Serum reactivity for LOS isolated from DH1 cells that lack sialylated species, such as from DH1*lic3B* cells, is significantly diminished in comparison to those that are sialylated (Figure 5). This provides clear evidence that the NeuAc residues form part of the epitope recognized by many of the anti-LOS immunoglobulins. Anti-ganglioside antibodies can be elicited in mice and rabbits upon injection with LOS from *C. jejuni* strains associated with Guillain−Barré and Fisher syndromes (*5*−*7*); however, to date, we have not been able to similarly stimulate anti-GQ1b antibody production in animals injected with DH1 LOS. This may be because of the very low percentage of LOS-associated sialylated glycoforms synthesized by DH1, and other NTHi strains, when grown in laboratory conditions.

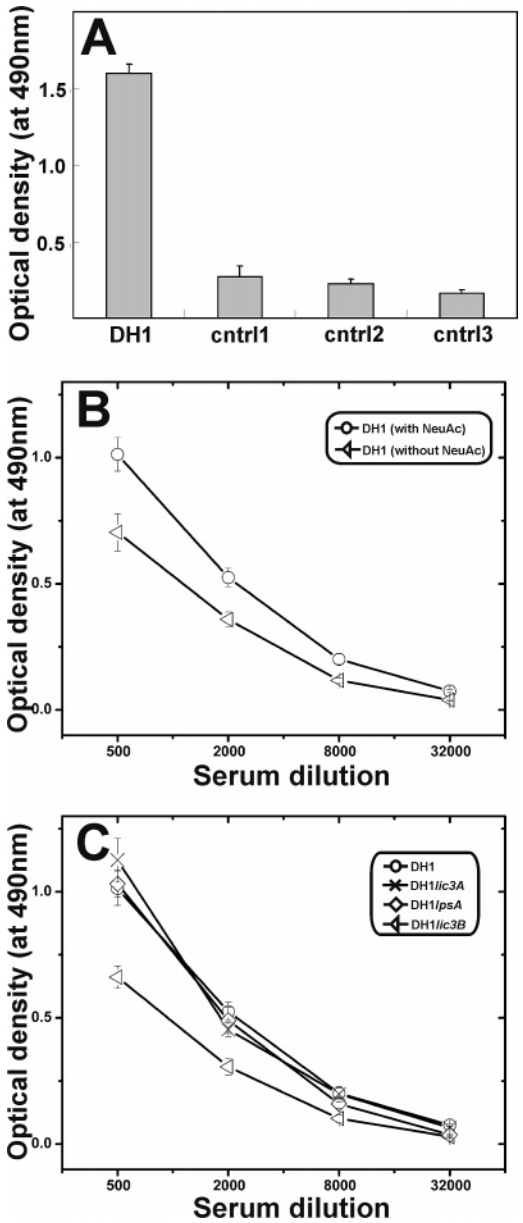

FIGURE 5: Patient serum IgGs react with DH1 LOS. Serum reactivity was measured using ELISA. (A) Patient immunoglobulins exhibit significantly higher reactivity toward the LOS of DH1 in comparison with NTHi control strains isolated from individuals with uncomplicated respiratory infections. (B) Serum IgGs bind more strongly to the LOS of DH1 cells grown in the presence of exogenous NeuAc versus those that were not. (C) The binding profiles for the LOS of DH1*lic3A* and DH1*lpsA* reveal no significant differences in the level of reactivity in comparison to DH1. These two isogenic mutants possess sialylated glycoforms. For DH1*lic3B* LOS, which does not incorporate NeuAc, IgG binding is significantly diminished. All comparisons of serum antibody binding to the LOS of DH1 and its associated mutants were from cells grown in the presence of NeuAc. Error bars represent ± one standard deviation unit from mean values.

Bifunctional sialyltransferases, possessing α2−3 and α2−8 transferase activity, have been identified in *H. influenzae* (*36*) and *C. jejuni* (*40*), and with dual α2−3 and α2−6 activity, from *Neisseria meningitidis* (*41*). Bifunctional isozymes are generally close homologues with monofunctional sialyltransferases carried by the same organism; the functionality can in some instances hinge on a single amino acid difference between the two forms (*41*). All known NTHi strains carry the *lic3A* gene, which encodes a monofunctional sialyltransferase (*24*), whereas roughly 60% carry the related bifunctional *lic3B* gene (*36*). In DH1, Lic3A is inactive, and sialylation of the LOS is catalyzed exclusively by Lic3B (Figure 4). A study of *C. jejuni* isolates from GBS/FS patients found that 100% of strains that synthesized a GQ1b-like LOS structure carried a bifunctional sialyltransferase gene (*11*, *42*). This is not surprising, since the synthesis of LOS-bound mimics of terminal GQ1b epitopes is dependent on the ability to add terminal disialosyl moieties.

There is considerable inter- and intrastrain variation with respect to the glycan component of the LOS in *H. influenzae*, and this is also true for other mucosal pathogens. Much of the variability arises from accumulated mutations in the nucleotide sequence of LOS-associated glycosyltransferases that modulate their expression and corresponding activity (*43*, *44*). As a result, there is an ever increasing ensemble of LOS structures known to be synthesized by this organism. In DH1, we have uncovered a novel sialylation pattern. To date, characterized sialylated structures have been limited to sialyllactose and sialyllacto-*N*-neotetraose branches off of the triheptosyl backbone. In DH1, the βGal-αHepIV branch is the acceptor for NeuAc residues, even though there is a lactose unit bound to HepIII (Figure 1). *In vitro* studies have indicated that both lactose and sialyllactose should serve as acceptors for Lic3B (*36*). We have no verifiable explanation to account for why NeuAc is added to βGal-αHepIV as opposed to the lactose branch, and it is difficult to draw comparisons from other known structures because there are few examples of the tetraheptosyl arrangement. It is possible that the βGal-αHepIV branch may be in a more conformationally accessible position for Lic3B during LOS synthesis.

The serum from FS patients often reacts with a number of gangliosides that possess terminal disialosyl units, such as GQ1b, GT1a, GD3, GD2, GD1b, and GT1b (*3*). This binding promiscuity may be due to the presence of individual antibodies that recognize the shared terminal epitopes in these molecules. We have identified an FS-associated mAb that recognizes several disialosyl-containing gangliosides as a result of a binding site that is confined to the terminal αNeuAc residue (*45*). Recent work has indicated that the ligand recognition domain for the majority of antiganglioside antibodies in individuals suffering from FS and related neuropathies is contained within the terminal αNeuAc(2−8)αNeuAc(2−3)βGal trisaccharide unit (*46*). The production of pathogenic anti-GQ1b antibodies appears to have been triggered by this critical epitope found in LOS glycoforms presented by DH1.

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SUPPORTING INFORMATION AVAILABLE

Tables are provided with analytical, MS, and NMR data in support of the elucidated structure of the DH1 LOS glycan, as well as the primer sequences used to amplify LOS-associated DH1 glycosyltransferases. This material is available free of charge via the Internet at http://pubs.acs.org.

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