THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 52, pp. 40024–40032, DECEMBER 29, 2006

Identification of a Bifunctional Lipopolysaccharide Sialyltransferase in *Haemophilus influenzae*

*INCORPORATION OF DISIALIC ACID**[S]

Received for publication, March 13, 2006, and in revised form, October 26, 2006 Published, JBC Papers in Press, October 27, 2006, DOI 10.1074/jbc.M602314200

Kate L. Fox[‡1], Andrew D. Cox[§], Michel Gilbert[§], Warren W. Wakarchuk[§], Jianjun Li[§], Katherine Makepeace[‡], James C. Richards[§], E. Richard Moxon[‡2], and Derek W. Hood[‡2]

From the [‡]Molecular Infectious Diseases Group, University of Oxford Department of Paediatrics, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DS, United Kingdom and [§]Institute for Biological Sciences, National Research Council, Ottawa, Ontario, K1A OR6, Canada

The lipopolysaccharide (LPS) of non-typeable *Haemophilus influenzae* (NT*Hi*) can be substituted at various positions by *N*-acetylneuraminic acid (Neu5Ac). LPS sialylation plays an important role in pathogenesis. The only LPS sialyltransferase characterized biochemically to date in *H. influenzae* is Lic3A, an α-2,3-sialyltransferase responsible for the addition of Neu5Ac to a lactose acceptor (Hood, D. W., Cox, A. D., Gilbert, M., Makepeace, K., Walsh, S., Deadman, M. E., Cody, A., Martin, A., Månsson, M., Schweda, E. K., Brisson, J. R., Richards, J. C., Moxon, E. R., and Wakarchuk, W. W. (2001) *Mol. Microbiol.* 39, 341–350). Here we describe a second sialyltransferase, Lic3B, that is a close homologue of Lic3A and present in 60% of NT*Hi* isolates tested. A recombinant form of Lic3B was expressed in *Escherichia coli* and purified by affinity chromatography. We used synthetic fluorescent acceptors with a terminal lactose or sialyllactose to show that Lic3B has both α-2,3- and α-2,8-sialyltransferase activities. Structural analysis of LPS from *lic3B* mutant strains of NT*Hi* confirmed that only monosialylated species were detectable, whereas disialylated species were detected upon inactivation of *lic3A*. Furthermore, introduction of *lic3B* into a *lic3B*-deficient strain background resulted in a significant increase in sialylation in the recipient strain. Mass spectrometric analysis of LPS indicated that glycoforms containing two Neu5Ac residues were evident that were not present in the LPS of the parent strain. These findings characterize the activity of a second sialyltransferase in *H. influenzae*, responsible for the addition of di-sialic acid to the LPS. Modification of the LPS by di-sialylation conferred increased resistance of the organism to the killing effects of normal human serum, as compared with mono-sialylated or non-sialylated species, indicating that this modification has biological significance.

Sialylation of lipopolysaccharide (LPS)[3] is a widely conserved structural modification among mucosal pathogens, with a reported role in virulence in a number of organisms. In *Haemophilus influenzae* LPS sialylation has been demonstrated to be important in resistance to the killing effects of normal human serum. A significant decrease in bacterial survival in human serum was observed in sialylation-deficient mutants, in which the CMP-Neu5Ac synthetase gene (*siaB*) had been disrupted, compared with the wild-type organisms (2). Furthermore, the role of sialylation as a critical virulence factor in the pathogenesis of experimental otitis media has been demonstrated, Neu5Ac-deficient mutants of non-typeable *H. influenzae* (NT*Hi*) were profoundly attenuated in a chinchilla model of infection (3). Related to this, it has been proposed that LPS sialylation may be implicated in the formation of biofilms (4–6). These studies provide evidence that Neu5Ac-containing LPS promotes bacterial persistence *in vivo*, potentially indicating a multi-faceted role for Neu5Ac in bacterial colonization and disease.

Virtually all NT*Hi* strains tested include Neu5Ac in their LPS (2, 7), but the patterns of sialylation and the repertoire of putative sialyltransferases differ between strains. Two common sialylated species have been identified to date in *H. influenzae* LPS, sialylated lactose and sialylated lacto-*N*-neotetrose (2, 8, 9). Lic3A, the only biochemically characterized sialyltransferase in *H. influenzae*, is responsible for the addition of Neu5Ac in a α-2,3-linkage to a lactose acceptor on the LPS of *H. influenzae* (1). Just within the 5′ end of the *lic3*A open reading frame are tandem repeats of the tetranucleotide 5′-CAAT (10), and hence, the encoded sialyltransferase is subject to phase variation. In some strain backgrounds, terminal *N*-acetyllactosamine expressed as part of a lacto-*N*-neotetrose-like structure is also sialylated. Likely candidate enzymes to sialylate such structures in *H. influenzae* are encoded by the sialyltransferase gene homologues *lsgB* and *siaA* (originally termed *orf Y*) (6, 9, 11, 12). In a minority of NT*Hi* strains, disialic acid residues have been detected in a small proportion of LPS glycoforms (2), indi-

* This work was supported in part by the Medical Research Council, United Kingdom. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

[1] Recipient of a studentship. To whom correspondence should be addressed: Dept. of Microbiology and Parasitology, School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, QLD 4072, Australia. Tel.: 61-73365-1892; Fax: 61-73365-4620; E-mail: k.fox@uq.edu.au.

[2] Funded by a Program Grant.

[3] The abbreviations used are: LPS, lipopolysaccharide; NT*Hi*, non-typeable *H. influenzae*; Neu5Ac, *N*-acetylneuraminic acid; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-mannoheptose; PEtn, phosphoethanolamine; Neu5Ac, *N*-acetylneuraminic acid; PCho, phosphocholine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MS, mass spectroscopy; Mes, 4-morpholineethanesulfonic acid; Lac, lactose; FCHASE, 6-(5-fluorescein-carboxamido)-hexanoic acid succinimidyl ester.

cating a potential role for another sialyltransferase in these strains able to use Neu5Ac as an acceptor. Alternatively, a sialyltransferase enzyme capable of performing both reactions may exist in some *H. influenzae* strains as has been reported in *Campylobacter jejuni* (13), where a bifunctional sialyltransferase has been described. The findings from the current study provide the first evidence that a bifunctional sialyltransferase exists in *H. influenzae*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—Twenty-six NT*Hi* isolates used in this study, designated 162, 176, 285, 375, 432, 477, 486, 667, 723, 981, 1003, 1008, 1124, 1158, 1159, 1180, 1181, 1200, 1207, 1209, 1231, 1232, 1233, 1247, 1268, and 1292, were obtained from the Finnish Otitis Media Study Group. Isolates were obtained from the middle ears of patients with otitis media and selected as representative of the genetic diversity of *H. influenzae* after ribotyping analysis (14). Thirty-two typeable *H. influenzae* isolates were also used in this study, representing serotypes a–f (15). *H. influenzae* was grown at 37 °C in brain heart infusion broth supplemented with hemin (10 μg/ml) and NAD (2 μg/ml). Brain heart infusion plates were prepared with 1% agar and supplemented with 10% Levinthals base (16) and, when appropriate, kanamycin (10 μg/ml), tetracycline (4 μg/ml), or Neu5Ac (25 μg/ml). *Escherichia coli* strain DH5α was used to propagate plasmids and was grown at 37 °C in LB broth (17) supplemented when appropriate with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (12 μg/ml).

Mutation of lic3B and lic3A—Southern analysis identified a novel 5′-CAAT repeat tract which co-hybridized with a *lic3A* probe. DNA from a genomic restriction digest of a size corresponding to the positive hybridization signal was isolated after electrophoresis of EcoRI-digested DNA. DNA was isolated from the gel fragment using Qiaex resin (Qiagen), and purified DNA was cloned into the pUC18 vector (Amersham Biosciences). After transformation, transformants were screened by colony hybridization (17) using a radiolabeled *lic3A* gene probe. The sequence of the cloned insert was confirmed by DNA sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (ABI Prism, Applied Biosystems) and an ABI 377 sequencer (ABI Prism, PerkinElmer Life Sciences). The plasmid containing the novel repeat-associated locus, *lic3B*, was designated pAA2. The cloned *lic3B* gene from NT*Hi* isolate 375, contained within pAA2, was disrupted by digestion with restriction endonuclease BglII and insertion of a kanamycin resistance cassette, released from pUC4Kan (Amersham Biosciences) by digestion with BamHI, to give plasmid pAA2kan. To construct *lic3A* mutants, the cloned *lic3A* gene previously described (1) was inactivated after digestion with BglII by insertion of a tetracycline resistance cassette derived from Tn10 to give plasmid plic3Atet. After transformation into *E. coli*, all mutant strains were confirmed by restriction and PCR analyses.

Construction of Sialyltransferase Mutant Strains of NTHi—Mutant strains were constructed in NT*Hi* isolates 375 and 1003 after transformation by the MIV procedure (18). All transformants were confirmed by re-culturing on the appropriate selective media and by PCR analyses of both the *lic3A* and *lic3B* loci. Mutant strain 375*lic3A* had previously been constructed (1), and to make a 1003*lic3A* mutant, isolate 1003 was transformed with plasmid plic3Atet. To construct *lic3B* mutants, pAA2kan, containing the interrupted *lic3B* gene, was used to transform NT*Hi* isolates 375 and 1003. In this case, transformants were further confirmed by Southern analysis. To construct a 375*lic3Alic3B* double mutant, a 375*lic3B* mutant strain was transformed with linearized plic3Atet. To construct a 1003*lic3Alic3B* double mutant, strain 1003*lic3A* was transformed with chromosomal DNA isolated from strain 1003*lic3B*. Mutant strain 375*siaB* has been previously described (2). To construct a 1003*siaB* mutant, plasmid pDJ1, containing the *siaB* gene, inactivated by insertion of a kanamycin resistance cassette (2) was used to transform NT*Hi* isolate 1003.

Transfer of lic3B into a lic3B-deficient Strain Background—A kanamycin resistance cassette was inserted into the *bipA* gene (HI0864), the gene upstream of *lic3B*, in plasmid pAA2 to act as a selectable marker after transformation. Plasmid pAA2 was digested with NcoI, and a BamHI-digested kanamycin resistance cassette from pUC4Kan was inserted at the site. Plasmid pAA2*bipA*kan was verified by restriction and PCR analyses, then used to transform NT*Hi* isolate 1003. Chromosomal DNA isolated from 1003*bipA* was used to transform strains Rd and Rd*lgtC* (1). Transformants in each case were confirmed by PCR analysis.

Analysis of LPS by Electrophoresis—Bacterial lysates were prepared from cells grown overnight on brain heart infusion plates supplemented with Neu5Ac, then suspended in phosphate-buffered saline (pH 5.8) to a concentration with an absorbance of 1 at 260 nm. Lysates were then treated with neuraminidase and analyzed by Tricine-SDS-PAGE (19) and staining with silver (Quicksilver; Amersham Biosciences). The neuraminidase, purified from *Clostridium perfringens*, cleaves terminal sialic acids bound to oligosaccharides.

Structural Analysis of LPS—Structural characterization of LPS was achieved using electrospray ionization-MS techniques, as described previously (8). Briefly, LPS was *O*-deacylated by treatment with anhydrous hydrazine at 37 °C for 1 h. Samples were analyzed in the negative ion mode by using a Prince CE system (Prince Technologies, The Netherlands) coupled to an API 3000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a microIonSpray interface. The separations were obtained on ~90-cm-length bare fused-silica capillary using 30 mM morpholine in deionized water, pH 9.0, containing 5% methanol. A voltage of 30 kV was applied at the injection, and a sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1.0 μl/min.

Expression and Purification of Recombinant Lic3A and Lic3B—The *lic3A* and *lic3B* genes were amplified by PCR from NT*Hi* isolate 375 chromosomal DNA using *Pwo* polymerase. The primers HI-06 (5′-CTTAGGAGGT*CATATG*TCAAAGTCTGTCATTATTGCAGGTAATGG 3′; the NdeI site is in italics) and HI-07 (5′-CCTAG*GTCGAC*CTAATCCCATTTTCTTGATTTTAAGGCGTG 3′; the SalI site is in italics) were used to amplify the *lic3A* gene. The following primers were used to amplify *lic3B*; HI-15 (5′-CTTAGGAGGT*CATATG*TCAAAG-

CCTGTCATTATTGCAGGTAATGG-3′; the NdeI site is in italics) and HI-16 (5′-CCTAG*GTCGAC*TTATTTGCGTAGTCTCATTTTCTTTGC 3′; the SalI site is in italics). The PCR products were digested with NdeI and SalI and cloned in pCWori+(−*lacZ*) containing the sequence encoding the *E. coli* maltose-binding protein (MalE) (without the leader peptide) and the thrombin cleavage site. *E. coli* AD202 strains containing either construct HST-06 (*lic3B* from NT*Hi* isolate 375 in pCWori+) or HST-08 (*lic3A* from NT*Hi* isolate 375 in pCWori+) were grown in 2 × YT medium (17) containing 150 μg/ml ampicillin and 2 g/liter glucose. The cultures were incubated at 37 °C until A_{600} = 0.35, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and then incubated overnight at 25 °C. The cells were broken using an Avestin C5 Emulsiflex cell disruptor (Avestin, Ottawa, CA), and the MalE-Lic3A and MalE-Lic3B fusions were purified by affinity chromatography on amylose resin following the manufacturer's instructions (New England Biolabs, Beverly, MA).

Enzymatic Assay Conditions—The sialyltransferase assays were performed at 37 °C for 5 min. The α-2,3-sialyltransferase activity was assayed using CMP-Neu5Ac as donor and Galβ-1,4-Glc-FCHASE as acceptor in 50 mM Hepes, pH 7.5, containing also 10 mM $MnCl_2$. The α-2,8-sialyltransferase activity was assayed using CMP-Neu5Ac as donor and Neu5Acα-2–3-Galβ-1,4-Glc-FCHASE as acceptor, in 50 mM Hepes, pH 7.5, containing also 10 mM $MnCl_2$. The transialidase activity was assayed at 37 °C for 20 min using Neu5Acα-2–3-Galβ-1,4-Glc-FCHASE as donor and CMP as acceptor in 50 mM Mes, pH 6.0, containing also 10 mM EDTA. All the reactions were stopped by the addition of acetonitrile (25% final concentration). The purified enzymes were diluted to observe between 3 and 10% conversion of the substrate into product during the incubation times indicated above. The samples were analyzed by capillary electrophoresis as described previously (31). Quantitation of the reactions was performed by integration of the capillary electrophoresis trace peaks using the MDQ 32 Karat software (Beckman, CA). The sialyltransferase and transialidase activities are expressed in milliunits (nmol of product/min)/mg of purified fusion proteins.

Serum Bactericidal Assay—Bacteria cultured to mid-log phase in brain heart infusion broth supplemented with Neu5Ac (100 μg/ml) were resuspended in phosphate-buffered saline. 10^6 organisms were taken and incubated in 10% pooled normal human serum for 30 min. After incubation, surviving bacteria were enumerated by dilution and culture on plates.

RESULTS

A Novel Locus Containing a 5′-CAAT Tetranucleotide Repeat Tract Is Homologous to lic3A—A comprehensive survey of tetranucleotide repeats in a set of 25 NT*Hi* isolates by Southern analysis identified a novel *lic3A* homologue (20). Several NT*Hi* isolates exhibited two restriction fragments that hybridized correspondingly to both an internal *lic3*A probe and a $(5'\text{-CAAT})_5$ oligonucleotide probe. The novel *lic3A* hybridizing restriction fragment from NT*Hi* isolate 375, *i.e.* that which showed no equivalent banding pattern in identical hybridization analyses with the genome sequenced strain, Rd, was cloned to give plasmid pAA2. After DNA sequencing of the cloned insert, an open reading frame encoding a protein of 333 amino acids with high homology to Lic3A was identified. This Lic3A homologue was flanked upstream by HI0865, a GTP-binding protein with homology to *bipA* (21), and downstream by HI0864, a glutamine synthetase gene, *glnA*. *glnA* is immediately adjacent to the *hmg* locus (HI0866 to HI0874), which contains multiple genes responsible for the synthesis and incorporation of the lacto-*N*-neotetrose-like structure, expressed on the LPS of a range of capsular and NT*Hi* strains (12). This second copy of *lic3A* has been designated *lic3B*.

Primers HI0864 and HI0865 (supplemental data), designed against the DNA sequences flanking *lic3B* were used to amplify by PCR portions of HI0864 and HI0865 that are contiguous in the Rd genome sequence and any DNA located between them. 11 of 25 NT*Hi* isolates tested gave an amplification product of 1.6 kilobases, indicating a gene organization like that of strain Rd; however, in 14 of the isolates a product of 2.6 kilobases was amplified indicating an insertion of DNA in this region of the chromosome. Upon Southern analysis, all of the larger PCR amplification products hybridized to a *lic3*A probe, indicating that the inserted DNA is conserved between strains (data not shown). A survey of the presence of *lic3B* in 32 *H. influenzae* capsular strains, representing serotypes *a–f*, by PCR amplification indicated that *lic3B* was present in a lower proportion (31%) of capsular *H. influenzae* strains than observed with NT*Hi* (60%). The *lic3B* gene was found to be absent from the serotype c and d strains tested and was present in 86% of serotype a strains, 50% of serotype f strains, 25% of serotype e strains, and 17% of serotype b strains tested (data not shown).

The *lic3A* gene is phase variably expressed due to a 5′-CAAT tetranucleotide repeat tract located within the reading frame (22). *Lic3B* also contains a 5′-CAAT repeat tract located at the same position relative to the downstream open reading frame. The DNA sequence of the tetranucleotide repeat tracts within *lic3A* and *lic3B* in the 25 NT*Hi* isolates was obtained, and the number of repeated motifs was found to vary from 14 to 41 in *lic3A* and from 12 to 28 in *lic3B*. For both genes two of the three possible reading frames are predicted to allow for translation of full-length gene products from alternative initiation codons (ATG1 and ATG2), immediately upstream of the repeats. The amino acid sequences of Lic3A (GenBank™ accession number DQ447744) and Lic3B (GenBank™ accession number DQ444277) from NT*Hi* isolate 375 share 93% similarity and 91% identity (supplemental data).

Disruption of lic3B Eliminates Disialylation of LPS—To investigate the function of *lic3B*, the cloned gene from NT*Hi* isolate 375 was inactivated by insertion of a kanamycin resistance cassette then used to transform NT*Hi* isolates 375 and 1003. To confirm that only *lic3B* had been specifically targeted and that *lic3A* had not been altered, PCR amplification using *lic3A*- and *lic3B*-specific primers along with Southern hybridization analysis was used to confirm the integrity of the transformants (data not shown). NT*Hi* isolate 1003 was chosen as a second strain background in which to study the function of Lic3B since the detailed structure of the LPS has been determined (Fig. 1), and Neu5Ac occupies the same molecular environment as in isolate 375, which is attached to the β-galactose of lactose attached to the distal heptose (23). In addition, disia-

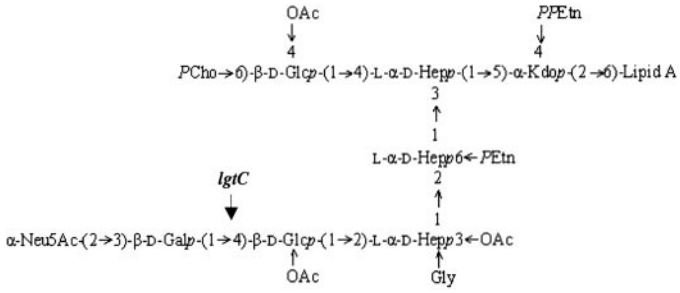


FIGURE 1. **Schematic representation of the LPS structure of NT*Hi* isolate 1003 as presented in Månsson *et al.* (23).** The site of attachment of Neu5Ac, as shown here for NT*Hi* isolate 1003, is identical in the LPS of NT*Hi* isolate 375 (2). The site of action of the glycosyltransferase, LgtC, is also indicated.

lylated species have also been detected in the LPS of this isolate (23). To analyze LPS sialylation in a comprehensive manner in these two NT*Hi* isolates, mutant strains in which *lic3A* (1) and *siaB*, the CMP-Neu5Ac synthetase gene, had been disrupted were constructed along with a double *lic3Alic3B* mutant.

Bacterial lysates prepared from wild-type isolates 375 and 1003 and mutant strains were electrophoresed to visualize LPS profiles. LPS from the wild-type and *lic3A* and *lic3B* mutant strains of NT*Hi* 375 and 1003 was then purified, and the glycan profiles were determined using electrospray ionization-MS techniques (8). The LPS profile of wild-type isolate 1003 exhibits four bands; the upper two bands (*bands 1* and *2* indicated on Fig. 2*a*) were removed upon neuraminidase treatment, results that are consistent with there being mono- and disialylated species (23). In 1003*lic3A* and 1003*lic3B* mutant strains only one sialylated band (*band 2* in Fig. 2*a*) was observed; the upper sialylated band evident in wild-type 1003 LPS is absent from the LPS profiles of the mutants. In the 1003*lic3Alic3B* mutant strain all sialylated glycoforms are absent (Fig. 2*b*). Structural analysis of the LPS of 1003*lic3B* revealed that glycoforms containing only a single Neu5Ac residue are present in this strain, whereas analysis of 1003*lic3A*-derived LPS identified disialylated species (Table 1, *top*). The absence of the upper sialylated band in the LPS profile of strain 1003*lic3A* may be due to decreased levels of disialylation in this mutant strain compared with wild type.

Wild-type isolate 375 displays a LPS electrophoretic profile comprising three distinguishable bands, the upper of which is a minor band that shifts in molecular weight after neuraminidase treatment (Fig. 2*c*). This minor upper band is believed to represent the highest molecular weight disialylated LPS species present in this bacterium. The major middle band remains, whereas a more intense lower band appears in neuraminidase-treated LPS. Isolated 375 possesses monosialylated LPS species and a minor proportion of disialylated species (2). In the 375*lic3B* mutant, the minor upper band observed with wild-type LPS was not present, indicating the likely absence of disialylated species in this mutant. However, a sialylation phenotype was evident upon neuraminidase treatment; this shift in the mobility of the LPS suggests that mono-sialylated species are present in this mutant strain. In the 375*lic3*A mutant LPS sialylation is significantly higher than that observed in wild type. Neuraminidase treatment of this hypersialylated LPS resulted in the loss of the intense upper bands. The profile of neuraminidase-treated 375*lic3A* LPS is altered from that of neuraminidase-treated 375 wild-type LPS; this result was unexpected and cannot be simply explained. No sialylation phenotype was observed on neuraminidase treatment of the LPS in the 375*lic3Alic3B* (Fig. 2*c*) or 375*siaB* mutant strains (data not shown). Similar to our findings with NT*Hi* 1003, mass spectrometric analysis of the LPS of 375*lic3B* revealed that glycoforms containing only a single Neu5Ac residue are present in this strain, whereas analysis of 375*lic3A* LPS identified disialylated species (Table 1, *bottom*). In addition, data from MS/MS analysis of 375*lic3A* LPS showed clear proof of the disialic acid glycoform consistent with Neu5Ac being directly linked to Neu5Ac in the LPS molecule (data not shown).

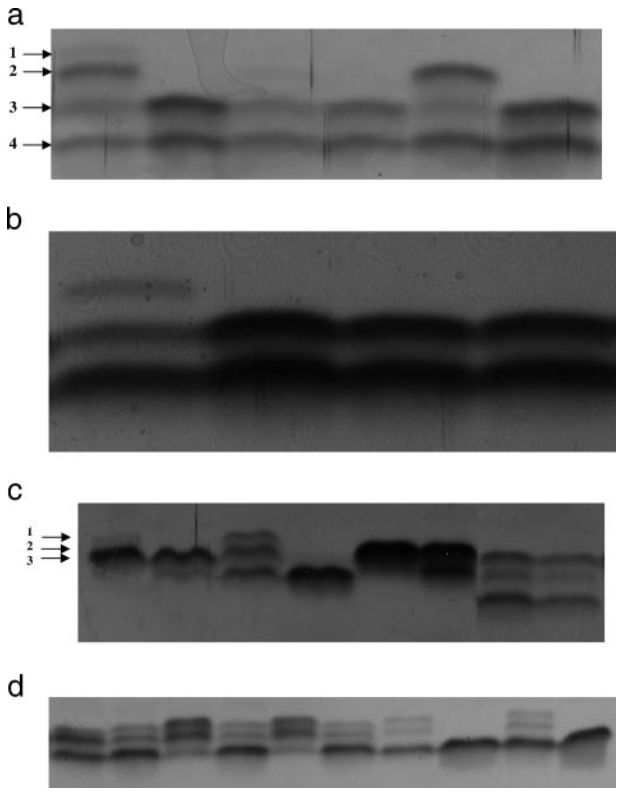


FIGURE 2. Electrophoretic profiles of LPS isolated from NTHi isolate 1003 and derived mutant strains in which putative sialyltransferase encoding genes are disrupted (*panel a, lanes 1* and *2*, 1003 wild type; lanes 3 and *4*, 1003*lic3A*; *lanes 5* and *6*, 1003*lic3B* and *panel b*, *lanes 1* and *2*, 1003*lic3A*; *lanes 3* and *4*, 1003*lic3Alic3B*). NT*Hi* isolate 375 and derived mutant strains in which putative sialyltransferase encoding genes are disrupted (*panel c*, *lanes 1* and *2*, 375 wild type; *lanes 3* and *4*, 375*lic3A*; *lanes 5* and *6*, 375*lic3B*; *lanes 7* and *8*, 375*lic3Alic3B*), and *H. influenzae* strain Rd and the derived mutant strains containing the marked *lic3B* allele, Rd*bipA*$^-$*lic3B*$^+$ and Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+$ *(panel d*, *lanes 1* and *2*, Rd wild type; *lanes 3* and *4*, Rd*bipA*$^-$*lic3B*$^+{}_1$; *lanes 5* and *6*, Rd*bipA*$^-$*lic3B*$^+{}_2$; *lanes 7* and *8*, Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+{}_1$; *lanes 9* and *10*, Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+{}_2$). A pair of profiles, without (−) and with (+) neuraminidase treatment, are shown for each strain, In *panels a* and *c*, distinct LPS glycoforms are indicated by *numbered arrows*.

Measurement of Sialyltransferase Activity of Lic3B—Lic3A and Lic3B from NT*Hi* isolate 375 were expressed in *E. coli* fused with the maltose-binding protein and purified on amylose resin. In both cases the construct was made without the 5′-CAAT repeats to minimize the effect of phase variation in the recombinant constructs. The 5′-CAAT repeats encode corresponding SINQ repeats in the amino acid sequence and their deletion resulted in the truncation of 32 and 35 residues at the

TABLE 1

Negative ion electrospray-MS data and proposed compositions of *O*-deacylated LPS from *H. influenzae* strains 1003 wild-type (wt), 1003*lic3A*, 1003*lic3B*, and 1003*lic3Alic3B* (top) and 375 wt, 375*lic3A*, 375*lic3B*, and 375*lic3Alic3B* (bottom)

Average mass units were used for calculation of molecular weight based on proposed composition as follows: Lipid A-OH, 953.00 Da; hexose (Hex), 162.15 Da; Hep, 192.17 Da; Kdo-P, 300.13 Da; PEtn, 123.05 Da; PCho, 165.05 Da; sialic acid (Sial), 291.05 Da.

Strain	Observed ions		Observed mass	Calculated mass	Relative intensity[a]	Proposed composition
	$[M-3H]^{3-}$	$[M-2H]^{2-}$	*Da*	*Da*	*%*	
1003						
wt	854.3	1281.7	2565.7	2565.1	22	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	908.3	1363.1	2728.0	2727.3	9	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1005.4	1508.6	3019.2	3019.4	43	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1102.7		3311.1	3311.6	26	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
lic3A	854.4	1282.1	2566.2	2565.1	27	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	908.5	1362.8	2728.0	2727.3	9	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1005.6	1508.8	3019.7	3019.4	46	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1102.7		3311.1	3311.6	18	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
lic3B	854.3	1282.1	2566.1	2565.1	20	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	908.4	1362.8	2727.9	2727.3	13	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1005.5	1508.7	3019.5	3019.4	67	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
lic3A lic3B	854.3	1282.1	2566.1	2565.1	47	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	908.4	1363.2	2728.3	2727.3	53	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
375						
wt	962.6	1444.2	2890.6	2889.5	45	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1003.3	1505.7	3013.1	3012.5	41	PCho, 4Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	1005.7	1508.7	3019.7	3019.4	9	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1102.7		3311.1	3311.6	5	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
lic3A	908.4	1362.7	2727.9	2727.3	18	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	949.5	1424.6	2851.3	2850.4	9	PCho, 3Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	962.4	1444.2	2890.8	2889.5	7	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1005.5	1509.0	3019.9	3019.4	23	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1046.7		3143.1	3142.5	4	Sial, PCho, 3Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	1102.7	1654.7	3311.2	3311.6	23	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1143.7		3434.1	3434.7	16	2Sial, PCho, 3Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
lic3B	962.5	1444.2	2890.5	2889.5	63	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1003.6	1505.7	3013.1	3012.5	31	PCho, 4Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	1005.6		3019.8	3019.4	6	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
lic3A lic3B	854.3	1282.1	2566.1	2565.1	31	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	895.3	1343.6	2689.1	2688.2	16	PCho, 2Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	908.3	1363.2	2728.2	2727.3	22	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	949.5	1424.7	2851.5	2850.4	9	PCho, 3Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH
	962.4	1444.1	2890.2	2889.5	13	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1003.5	1505.3	3013.0	3012.5	9	PCho, 4Hex, 3Hep, 3PEtn, Kdo-P, Lipid A-OH

[a] Relative intensities were calculated from the average of the integrals of the double-, triple-, and quadruple-charged ions as appropriate.

TABLE 2

Comparison of the activity of the Lic3A and Lic3B sialyltransferases from NT*Hi* 375

Lic3A and Lic3B were expressed in *E. coli* as fusions with the maltose-binding protein in the vector pCWori+ and purified on amylose resin.

Sialyltransferase	Specific activity		
	α-2,3-Sialyltransferase[a]	α-2,8-Sialyltransferase[b]	Transialidase[c]
	milliunits/mg		
Lic3A	240.9	Not detectable	5.6
Lic3B	100.0	5.6	0.6

[a] The α-2,3-sialyltransferase activity was assayed using CMP-Neu5Ac as donor and **Gal**β-1,4-Glc-FCHASE as acceptor. The sialic acid (Neu5Ac) residue was transferred to the Gal residue (in bold).

[b] The α-2,8-sialyltransferase activity was assayed using CMP-Neu5Ac as donor and **Neu5Ac**α-2–3-Galβ-1,4-Glc-FCHASE as acceptor. The sialic acid (Neu5Ac) residue was transferred to the Neu5Ac residue (in bold).

[c] The transialidase activity was assayed using Neu5Acα-2–3-Galβ-1,4-Glc-FCHASE as donor and CMP as acceptor.

N termini of Lic3A and Lic3B, respectively. We assayed the sialyltransferase activity of purified recombinant Lic3A and Lic3B with fluorescent model compounds (Table 2). Both Lic3A and Lic3B transfer sialic acid to lactose (Lac)-FCHASE (Galβ-1,4-Glc-FCHASE), whereas only Lic3B transfers to sialyllactose-FCHASE (NeuAcα-2,3-Lac-FCHASE). These profiles of activities suggest that Lic3A can transfer sialic acid only to terminal galactose residues, whereas Lic3B can transfer sialic acid either to a terminal galactose residue or to a terminal Neu5Ac residue. We have shown that Lic3B can form disialic acid on a terminal galactose residue by adding sequentially two Neu5Ac residues using CMP-Neu5Ac as donor. After a 120-min incubation time, we observed a distribution of 24.2% Lac-FCHASE, 65% Neu5Acα-2,3-Lac-FCHASE, and 10.8% Neu5Acα-2,8-Neu5Acα-2,3-Lac-FCHASE (supplemental data). Adding more enzyme or donor did not significantly increase the yield of sialylated products, which suggested that the reaction reached an equilibrium between the acceptor and the sialylated products. While assaying Lic3A activity with siallylactose-FCHASE as acceptor, a significant production of Lac-FCHASE rather than the transfer of a second sialic acid was observed. This "sialidase" activity is 4.6-fold higher when it is assayed in the presence of CMP. Thus, Lic3A has significant transialidase activity and performs the "backward" reaction in the presence of sialyllactose and CMP. It is likely that there is a small amount of CMP in the CMP-Neu5Ac used as donor, and this explains

TABLE 3

Negative ion electrospray-MS data and proposed compositions of *O*-deacylated LPS from *H. influenzae* strains Rd wild-type (wt), Rd*lgtC*$^-$, Rd*bipA*$^-$*lic3B*$^+$, and Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+$

Average mass units were used for calculation of molecular weight based on proposed composition as follows: Lipid A-OH, 953.00 Da; hexose (Hex), 162.15 Da; Hep, 192.17 Da; HexNAc, 203.19 Da; Kdo-P, 300.16 Da; PEtn, 123.05 Da; PCho, 165.05; Sial, 291.05 Da.

Strain	Observed ions		Observed mass	Calculated mass	Relative intensity	Proposed composition
	$[M-3H]^{3-}$	$[M-2H]^{2-}$				
			Da	*Da*	%	
Rd wt	867.7	1302.1	2606.1	2604.2	10	PCho, 3Hex, 3Hep, PEtn, Kdo-P, lipid A-OH
	908.4	1363.2	2728.2	2727.3	24	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	962.5	1444.2	2890.5	2889.5	22	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	989.0		2970.0	2969.6	12	PCho, HexNAc, 4Hex, 3Hep, PEtn, Kdo-P, Lipid A-OH
	1005.6	1508.5	3019.6	3019.4	15	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1030.2	1545.7	3093.5	3092.7	17	PCho, HexNAc, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
Rd*lgtC*	853.6		2563.8	2565.1	33	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	867.0	1301.4	2604.4	2604.2	14	PCho, 3Hex, 3Hep, PEtn, Kdo-P, Lipid A-OH
	908.4	1363.1	2728.0	2727.3	48	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
		1509.5	3021.0	3019.4	5	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
Rd*bipA* *lic3B*$^+$	908.4	1362.7	2727.9	2727.3	15	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	962.4	1443.7	2889.8	2889.5	17	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	989.9		2972.7	2969.6	12	PCho, HexNAc, 4Hex, 3Hep, PEtn, Kdo-P, Lipid A-OH
	1005.5		3019.5	3019.4	12	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1030.3	1545.3	3093.1	3092.7	15	PCho, HexNAc, 4Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1102.7		3311.1	3311.6	29	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
Rd*lgtC* *bipA lic3B*$^+$	908.4	1363.2	2728.3	2727.3	50	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1005.5	1508.6	3019.4	3019.4	20	Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH
	1102.7		3311.1	3311.6	30	2Sial, PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, Lipid A-OH

why we observed degradation of sialyllactose when we assayed Lic3A for α-2,8-sialyltransferase activity. This transialidase activity is much less significant in the case of Lic3B (Table 2).

Transfer of lic3B into a lic3B-deficient Strain Background Confers Increased Sialylation—To further analyze the function of Lic3B, the *lic3B* gene was transferred into a *lic3B*-deficient strain background, strain Rd, to determine whether a sialylation phenotype specific to *lic3B* would be acquired. An antibiotic resistance cassette was first introduced into *bipA* (HI0864), the gene upstream of *lic3B*, to act as a selectable marker after transformation. The electrophoretic profiles of 1003*bipA* mutants were examined to confirm that inactivation of *bipA* had no detectable effect on the LPS profiles when compared with wild type (data not shown). Transformants containing the *lic3B* allele in strain Rd exhibited increased LPS sialylation (Fig. 2*d*) when compared with the parent strain. Three bands are detectable in the LPS profile of wild-type strain Rd, the middle of which is sialylated, as illustrated by the fact that treatment with neuraminidase decreases the intensity of this band. The decrease in intensity of this band rather than complete removal on treatment with neuraminidase likely indicates that a non-sialylated band co-migrates with the sialylated LPS glycoform in this strain background. In the Rd*bipA*$^-$*lic3B*$^+$ mutants the upper of the three bands is also sialylated, with a decrease in intensity of these two upper bands observed upon neuraminidase treatment. To clarify this observation, strain Rd*lgtC*, containing a mutation in the gene encoding the α-1,4-galactosyltransferase, *lgtC*, was used as this precludes any competition for the lactose acceptor and the formation of non-sialylated extensions. This has been shown to increase the proportion of observed sialylated glycoforms (1). Two bands representing sialylated glycoforms are evident in the Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+$ mutants that are both removed upon neuraminidase treatment (Fig. 2*d*). These findings are consistent with our prediction that the introduction of *lic3B* into strain Rd increases the degree and complexity of LPS sialylation in that strain.

Mass spectrometric analysis of LPS isolated from strain Rd*bipA*$^-$*lgtC*$^-$*lic3B*$^+$ showed clear evidence of glycoforms containing 1 and 2 sialic acid residues (Table 3). These glycoforms were present in relatively equal abundance. Only glycoforms displaying a single Neu5Ac residue were apparent upon analysis of LPS isolated from strain Rd*lgtC* (Table 3).

Serum Resistance of Sialyltransferase-deficient Strains—Neu5Ac is known to influence the resistance of organisms to the killing effect of human sera (2). The set of sialyltransferase mutants in NT*Hi* isolate 1003 were subject to bactericidal assays to analyze the effect of inactivation of single and combinations of putative sialyltransferase genes upon biological activity of the LPS. The wild-type strain along with the *siaB* mutant was included in these assays for comparison, and assays were repeated multiple times. For NT*Hi* isolate 1003, the wild type strain was most resistant and the *siaB* mutant least resistant (>2 log drop) to the killing effect of human sera (Fig. 3). The 1003*lic3Alic3B* double mutant was sensitive to serum similar to 1003*siaB*. *Lic3* single mutants displayed intermediate resistance phenotypes. Student's *t* test showed a significant difference ($p = 0.000336$) between strains lacking sialic acid in their LPS (1003*lic3Alic3B* and 1003*siaB*) compared with strains possessing 1 or 2 sialic acid residues in their LPS (wild-type 1003, 1003*lic3A*, 1003*lic3B*). The difference in serum resistance was not statistically significant between the *lic3A* and *lic3B* mutant strains. However, a significant difference ($p = 0.05$) was shown between wild-type 1003 and the single *lic3A* and *lic3B* mutants, indicating that disruption of either one of these sialyltransferase genes reduces serum resistance significantly. The results obtained in the serum resistance assay correlated well with the LPS phenotypes described by the MS data. The most heavily sialylated strains corresponded to the most serum-resistant phenotypes, and the strains unable to sialylate LPS corresponded to the most serum-sensitive phenotypes.

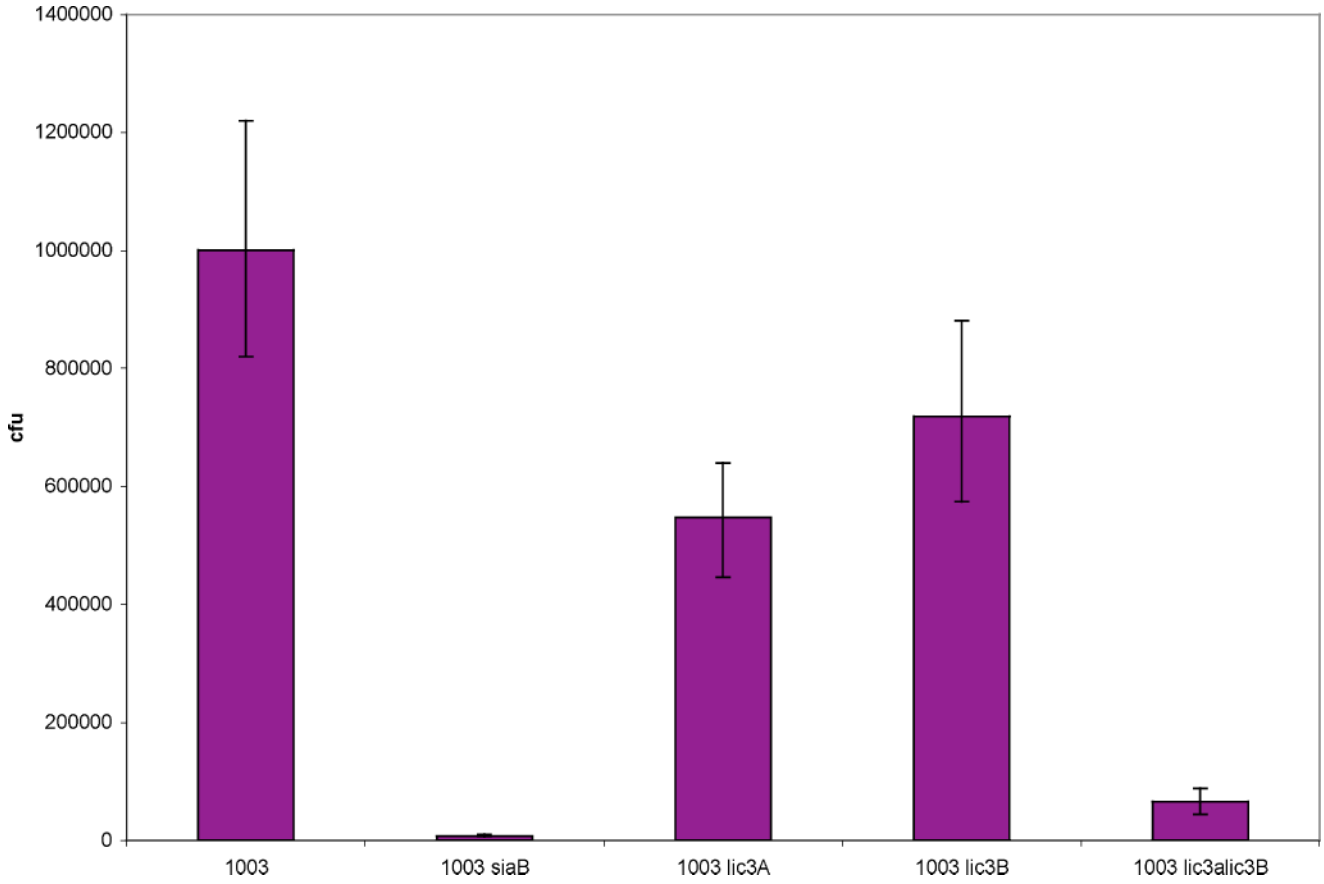


FIGURE 3. **Resistance of NT*Hi* isolate 1003 mutated in sialyltransferase genes or the CMP-Neu5Ac synthetase gene to the killing effect of pooled human serum.** Results are expressed as number of surviving bacteria after incubation with human serum. Results are expressed as average values from two experiments each done in triplicate; S.D. are shown. *cfu*, colony-forming units.

DISCUSSION

Sialic acid has been implicated as a virulence factor in several bacterial species. The mechanism by which sialylation of LPS contributes to both the commensal and pathogenic behavior of NT*Hi* might be key to understanding the pathogenesis of disease caused by this organism. Sialylation of LPS is a critical virulence factor *in vivo* required for NT*Hi* to cause otitis media in a chinchilla model of infection (3). *H. influenzae* lacks the genes required for *de novo* synthesis of Neu5Ac and must therefore access an external source of the sugar as either a substrate for growth (24) or for modification of LPS via a number of specific sialyltransferases. Sialyltransferases determine the frequency and pattern of LPS sialylation across NT*Hi* strains.

In this study we investigated the function of the sialyltransferase Lic3B, encoded by a gene identified as a close homologue of *lic3A*. *lic3A* encodes the only previous biochemically characterized α-2,3-sialyltransferase required for sialylation of lactose in *H. influenzae* LPS (2). Lic3B and Lic3A are closely related enzymes that have overlapping functions. Evidence presented here from biochemical, structural, and mutational analyses supports the role of Lic3B as a bifunctional enzyme with α-2,3- and α-2,8-sialyltransferase activity able to utilize both terminal galactose of lactose and Neu5Ac of sialyllactose as acceptor molecules. The recombinant purified Lic3A protein showed no activity on sialyllactose (1). Mass spectrometric analysis of LPS isolated from *lic3A* and *lic3B* mutant strains of NT*Hi* demonstrated that upon inactivation of *lic3B* only monosialylated species were detectable, whereas after inactivation of *lic3A*, disialylated species were detected. Furthermore, the introduction of *lic3B* into a *lic3B*-deficient strain background revealed a significant increase in LPS sialylation. The presence of disialylated species was confirmed in this modified strain.

Bifunctional sialyltransferase enzymes have previously been described in other mucosal pathogens including *C. jejuni* and *Neisseria meningitidis* (13, 25). In *C. jejuni*, the *cstI* gene encodes an α-2,3-sialyltransferase, and the *cstII* gene encodes either a monofunctional α-2,3-sialyltransferase or a bifunctional enzyme with α-2,3- and α-2,8-sialyltransferase activity, depending on the allele of Cst-II (13). Interestingly, Lic3A and Lic3B of *H. influenzae* and Cst-I and Cst-II of *C. jejuni* are classified in the same family of carbohydrate-active enzymes as defined by the CAZy classification system, indicating the relatedness of these enzymes. In *N. meningitidis*, the *lst* gene encodes an enzyme with α-2,3-sialyltransferase activity in strain MC58 (L3 immunotype) and a bifunctional enzyme with α-2,3- and α-2,6-sialyltransferase activity in strain 126E (L1 immunotype) (25). The monofunctional enzyme from MC58 can be changed into a bifunctional 126E-like enzyme by site-directed mutagenesis of a single amino acid (25). Work is ongoing to determine the specific amino acids that confer bifunctionality of the Lic3B sialyltransferase enzyme.

A strong correlation exists between the epitopes of *H. influ-*

enzae LPS that are known to have specific biological function and synthesis dependent upon phase variable gene expression. Lic3A and likely Lic3B are both expressed in a phase variable manner. Indeed, all enzymes competing to use the terminal lactose off the distal heptose as an acceptor are phase variably expressed, as is Lic2A, which adds the terminal β-galactose of the lactose itself. This makes the sialyllactose or the alternative digalactoside synthesized when LgtC is dominant, potentially one of the most highly variable LPS epitopes expressed by NT*Hi*. This likely indicates an advantage to the organism in expressing Neu5Ac or, alternatively, a digalactoside in its LPS under certain conditions. These variable conditions may correspond to changing microenvironments in different host compartments or between hosts. No information is available on the level of expression of the *lic3A* and *lic3B* genes, but an added level of complexity may be inferred by the fact that both genes have two potential initiation codons. In other phase-variable genes it has been shown that different reading frames can relate to differing levels of expression of the gene product (26, 27). There may, therefore, be modulation of LPS sialylation phenotypes depending on the relative expression levels of both of these genes.

The *lic3A* gene is present in all strains tested, whereas *lic3B* is present in only a proportion. It is not clear whether *lic3B* arose by horizontal transfer or by duplication within the genome. Because of the proximity of *lic3B* to the *hmg* locus, it is possible that these isolates belong to the same lineage where some complex ancestral insertion/deletion event has occurred. Because of the lack of redundancy generally associated with pathogenic organisms containing small genomes, it is unlikely that two alleles carrying out identical functions would be maintained within the genome. It has been demonstrated in other organisms that minor sequence changes can confer bifunctionality of sialyltransferase genes (25). It would, therefore, be reasonable to hypothesize that after gene duplication, minor sequence changes would have been sufficient to confer distinct functional specificities of the Lic3A and Lic3B proteins, favoring the maintenance of the corresponding genes within the genome.

Two other putative sialyltransferases SiaA and LsgB can also be present in NT*Hi* isolates, but neither 375 nor 1003 contain the *siaA* gene. The *lsgB* gene was mutated both singly and in combination with the *lic3A* and *lic3B* sialyltransferase genes. No effect on sialylation was observed when *lsgB* was mutated in our NT*Hi* isolate backgrounds, confirming that Lic3A and Lic3B alone are responsible for sialylation of sialyllactose. SiaA and LsgB proteins may be involved in sialylation of the high molecular weight glycoform expressed by some NT*Hi* isolates (12).

The findings from our study indicate that details of the pathway and mechanism of LPS sialylation in NT*Hi* isolate 375 is somewhat more complex than other isolates studied. The unexpected observation of a hypersialylated phenotype in the 375*lic3A* mutant could possibly be explained by the transialidase activity of Lic3A under *in vitro* conditions. In addition to this unexpected hypersialylated phenotype, inactivation of putative sialyltransferase genes within this strain resulted in some unexpected and variable alterations in the LPS profiles between individual transformants that were seemingly not directly related to sialylation. A degree of complexity in the sialylation phenotype of mutant strains was also reported by Jones *et al.* (9) upon investigation of the putative sialyltransferases, SiaA and LsgB, in strain A2. It could be hypothesized that the changes observed in LPS phenotype between individual transformants in NT*Hi* 375 were due to concomitant phase variation events in other LPS biosynthetic genes, perhaps enhanced by an elevated switching rate of gene expression. A further possibility is that changes in the expression of sialyltransferase enzymes could be exerted through some form of transcriptional control of the relevant gene(s).

In this study a strong correlation was found between incorporation of Neu5Ac on LPS and serum resistance of NT*Hi*, as observed previously (2). It could be envisaged that Neu5Ac would afford protection from human serum in an analogous way to a capsule, potentially explaining the extra investment by NT*Hi* in the presentation of Neu5Ac on their cell surface. This is apparent by the higher percentage of NT*Hi* strains possessing the sialyltransferase gene, *lic3B*, compared with capsular strains of *H. influenzae* and the fact that 25/25 NT*Hi* isolates studied contained Neu5Ac in their LPS (7). The mechanism by which sialic acid contributes to serum resistance remains to be elucidated. Other potential roles of LPS sialylation may be in modulating antigenic mimicry of LPS epitopes or by modulating host interactions either by an indirect charge effect or by steric hindrance. Sialylation of LPS produces a negative charge on the bacterial surface potentially aiding evasion from phagocytic cells in the host environment. It has been proposed that NT*Hi* forms a biofilm *in vivo* associated with increased sialylated LPS glycoforms compared with *in vitro* grown organisms (5, 28–30). Although there has been no formal evidence of an extracellular polysaccharide matrix, one of the cardinal features of a biofilm, the sialylated LPS glycoforms of organisms *in vivo* are clearly implicated in pathogenesis based on studies in a chinchilla model of otitis media (3, 6).

The functional significance of expression of a sialylated LPS phenotype in NT*Hi* isolates may be dependent on the "total package" of LPS glycoforms expressed at any given time in any particular isolate in addition to the contribution of other surface components. Because there are more complex patterns of LPS glycoforms expressed by NT*Hi* than by almost any other mucosal pathogen that has been analyzed, it seems plausible that this complexity might be a key factor in the adaptability of these bacteria between compartments within and between hosts.

Acknowledgments—We thank Marie-France Karwaski and Anna Cunningham for expert technical assistance and Juhani Eskola and the Finnish Otitis Media Study Group for the provision of strains used in this study.

REFERENCES

1. Hood, D. W., Cox, A. D., Gilbert, M., Makepeace, K., Walsh, S., Deadman, M. E., Cody, A., Martin, A., Månsson, M., Schweda, E. K., Brisson, J. R., Richards, J. C., Moxon, E. R., and Wakarchuk, W. W. (2001) *Mol. Microbiol.* **39,** 341–350
2. Hood, D. W., Makepeace, K., Deadman, M. E., Rest, R. F., Thibault, P., Martin, A., Richards, J. C., and Moxon, E. R. (1999) *Mol. Microbiol.* **33,**

679–692
3. Bouchet, V., Hood, D. W., Li, J., Brisson, J. R., Randle, G. A., Martin, A., Li, Z., Goldstein, R., Schweda, E. K., Pelton, S. I., Richards, J. C., and Moxon, E. R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8898–8903
4. Swords, W. E., Moore, M. L., Godzicki, L., Bukofzer, G., Mitten, M. J., and VonCannon, J. (2004) *Infect. Immun.* **72,** 106–113
5. Greiner, L. L., Watanabe, H., Phillips, N. J., Shao, J., Morgan, A., Zaleski, A., Gibson, B. W., and Apicella, M. A. (2004) *Infect. Immun.* **72,** 4249–4260
6. Jurcisek, J., Greiner, L., Watanabe, H., Zaleski, A., Apicella, M. A., and Bakaletz, L. O. (2005) *Infect. Immun.* **73,** 3210–3218
7. Bauer, S. H., Månsson, M., Hood, D. W., Richards, J. C., Moxon, E. R., and Schweda, E. K. (2001) *Carbohydr. Res.* **335,** 251–260
8. Cox, A. D., Hood, D. W., Martin, A., Makepeace, K. M., Deadman, M. E., Li, J., Brisson, J. R., Moxon, E. R., and Richards, J. C. (2002) *Eur. J. Biochem.* **269,** 4009–4019
9. Jones, P. A., Samuels, N. M., Phillips, N. J., Munson, R. S., Jr., Bozue, J. A., Arseneau, J. A., Nichols, W. A., Zaleski, A., Gibson, B. W., and Apicella, M. A. (2002) *J. Biol. Chem.* **277,** 14598–14611
10. Weiser, J. N., Maskell, D. J., Butler, P. D., Lindberg, A. A., and Moxon, E. R. (1990) *J. Bacteriol.* **172,** 3304–3309
11. Hood, D. W., Deadman, M. E., Allen, T., Masoud, H., Martin, A., Brisson, J. R., Fleischmann, R., Venter, J. C., Richards, J. C., and Moxon, E. R. (1996) *Mol. Microbiol.* **22,** 951–965
12. Hood, D. W., Randle, G., Cox, A. D., Makepeace, K., Li, J., Schweda, E. K., Richards, J. C., and Moxon, E. R. (2004) *J. Bacteriol.* **186,** 7429–7439
13. Gilbert, M., Brisson, J. R., Karwaski, M. F., Michniewicz, J., Cunningham, A. M., Wu, Y., Young, N. M., and Wakarchuk, W. W. (2000) *J. Biol. Chem.* **275,** 3896–3906
14. Cody, A. J., Field, D., Feil, E. J., Stringer, S., Deadman, M. E., Tsolaki, A. G., Gratz, B., and Bouchet, V. (2003) *Infect. Genet. Evol.* **3,** 57–66
15. Musser, J. M., Kroll, J. S., Granoff, D. M., Moxon, E. R., Brodeur, B. R., Campos, J., Dabernat, H., Frederiksen, W., Hamel, J., Hammond, G., Hoiby, E. A., Jonsdottir, K. E., Kabeer, M., Kallings, I., Khan, W. N., Kilian, M., Knowles, K., Koornhof, H. J., Law, B., Li, K. I., Montgomery, J., Pattison, P. E., Piffaretti, J. C., Takala, A. K., Thong, M. L., Wall, R. A., Ward, J. I., and Selander, R. K. (1990) *Rev. Infect. Dis.* **12,** 75–111
16. Alexander, H. E. (1965) in *Bacterial and Mycotic Infections of Man* (Dubos, R. J., and Hirsch, J.G., eds) pp. 724–741, Pittman Medical Publishing Co. Ltd., London
17. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Herriott, R. M., Meyer, E. M., and Vogt, M. (1970) *J. Bacteriol.* **101,** 517–524
19. Lesse, A. J., Campagnari, A. A., Bittner, W. E., and Apicella, M. A. (1990) *J. Immunol. Methods* **126,** 109–117
20. Fox, K. L., Yildirim, H. H., Deadman, M. E., Schweda, E. K., Moxon, E. R., and Hood, D. W. (2005) *Mol. Microbiol.* **58,** 207–216
21. Qi, S-.Y., Li, Y., Szyroki, A., Giles, I. G., Moir, A., and O'Connor, C. D. (1995) *Mol. Microbiol.* **17,** 523–531
22. Szabo, M., Maskell, D., Butler, P., Love, J., and Moxon, R. (1992) *J. Bacteriol.* **174,** 7245–7252
23. Månsson, M., Hood, D. W., Li, J., Richards, J. C., Moxon, E. R., and Schweda, E. K. (2002) *Eur. J. Biochem.* **269,** 808–818
24. Vimr, E., Lichtensteiger, C., and Steenbergen, S. (2000) *Mol. Microbiol.* **36,** 1113–1123
25. Wakarchuk, W. W., Watson, D., St. Michael, F., Li, J., Wu, Y., Brisson, J. R., Young, N. M., and Gilbert, M. (2001) *J. Biol. Chem.* **276,** 12785–12790
26. Weiser, J. N., Love, J. M., and Moxon, E. R. (1989) *Cell* **59,** 657–665
27. Srikhanta, Y. N., Maguire, T. L., Stacey, K. J., Grimmond, S. M., and Jennings, M. P. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5547–5551
28. Raynor, M. G., Zhang, Y., Gorry, M. C., Chen, Y., Post, J. C., and Ehrlich, G. D. (1998) *J. Am. Med. Assoc.* **279,** 296–299
29. Post, J. C. (2001) *Laryngoscope* **111,** 2083–2094
30. Ehrlich, G. D., Veeh, R., Wang, X., Costerton, J. W., Hayes, J. D., Hu, F. Z., Daigle, B. L., Ehrlich, M. D., and Post, J. C. (2002) *J. Am. Med. Assoc.* **287,** 1710–1715
31. Wakarchuk, W. W. and Cunningham, A. M. (2003) *Methods Mol. Biol.* **213,** 263–274

Table S1. Primers used in PCR amplification of genes in *H. influenzae*.

Primer name	Primer sequence	Primers used to amplify
052560	5’ gcaggtaatggaacaagttt 3’	*lic3A*
Blic3c	5’ catagaggactttctggc 3’	
lic3f	5’ agtggcaactgtaatggg 3’	
lic3g	5’ atatacaaacttcggcgg 3’	
PB3	5’ cacccgactaaaaattgtt 3’	
L3B1	5' ttttacacgctcaaggga 3'	
6024Q	5’ taaatcaggaaaataagacc 3’	*lgtC*
6024R	5’ attattgtatttattcatcc 3’	
HI0864	5’ tgacgtatttaccttcttgtcc 3’	*lic3B*
HI0865	5’ cgcatataattttctgcacg 3’	
lsgB	5’ aaaataaatgccctacag 3’	*lsgB*
lsgC	5’ tttgttgtacaccattgc 3’	
siaB	5’ taaccgattgtatcatcc 3’	*siaB*
siaC	5’ gagagcgggttctaaagg 3’	
GalEA	5’ caactaatggacatagcc 3’	*galE*
GalEB	5’ tattttagaaatttcgcc 3’	
5’glnA	5’ ttttggtccagagcctgaat 3’	*glnA*
3’glnA	5’ catactctgtcaatacag 3’	

Fig. S1. Amino acid sequence alignments of Lic3A and Lic3B from NT*Hi* isolate 375 (the sequence is shown from the initiation codon (ATG2) just upstream of the $(SINQ)_n$ repeats). The sequences of NT*Hi* 375 Lic3A and Lic3B (GenBank accession numbers DQ447744 and DQ444277 respectively) are the translated sequences correlating to 24 5'CAAT repeats being present. Homology is indicated by the level of shading, no shading indicates non-synonymous changes.

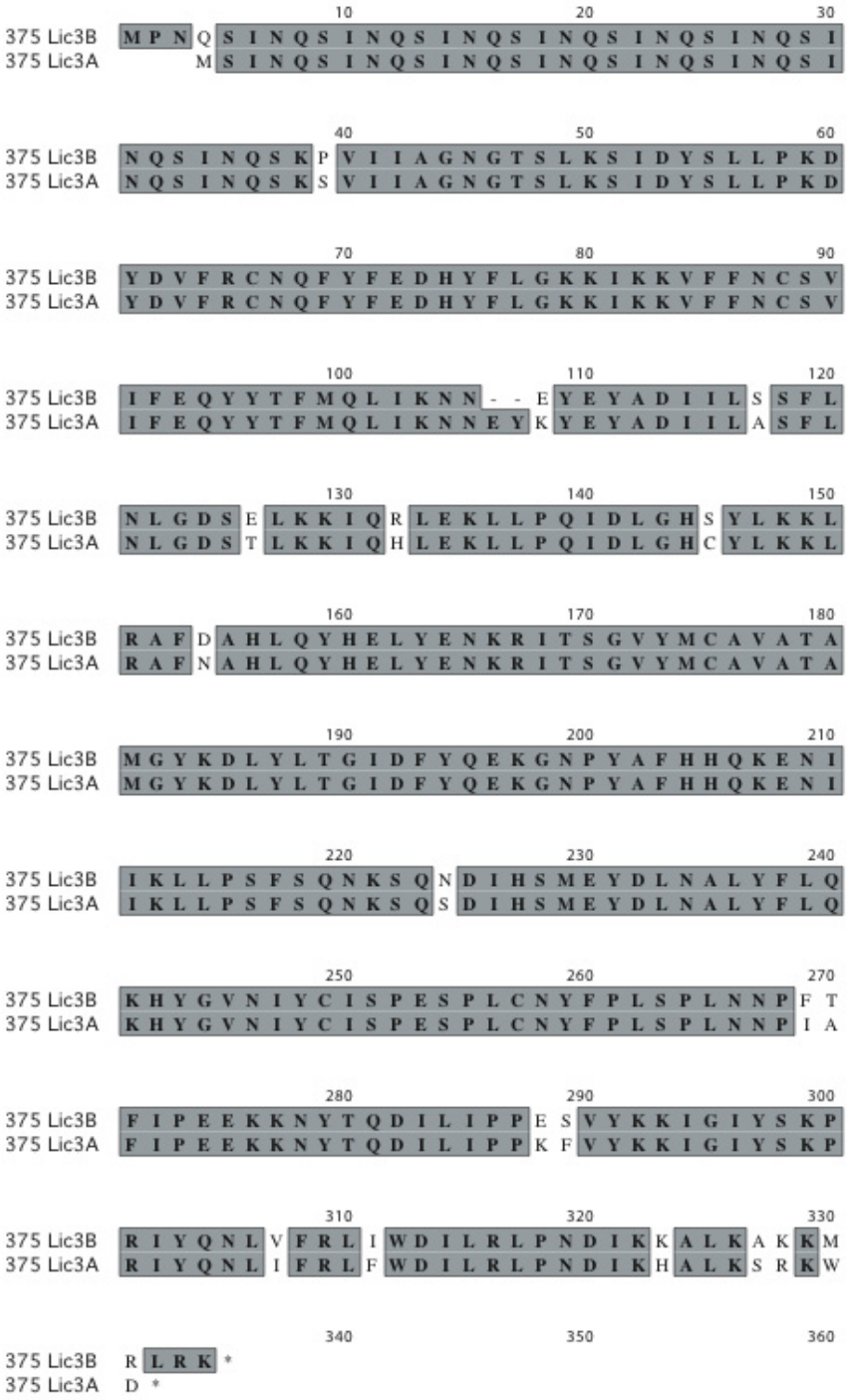
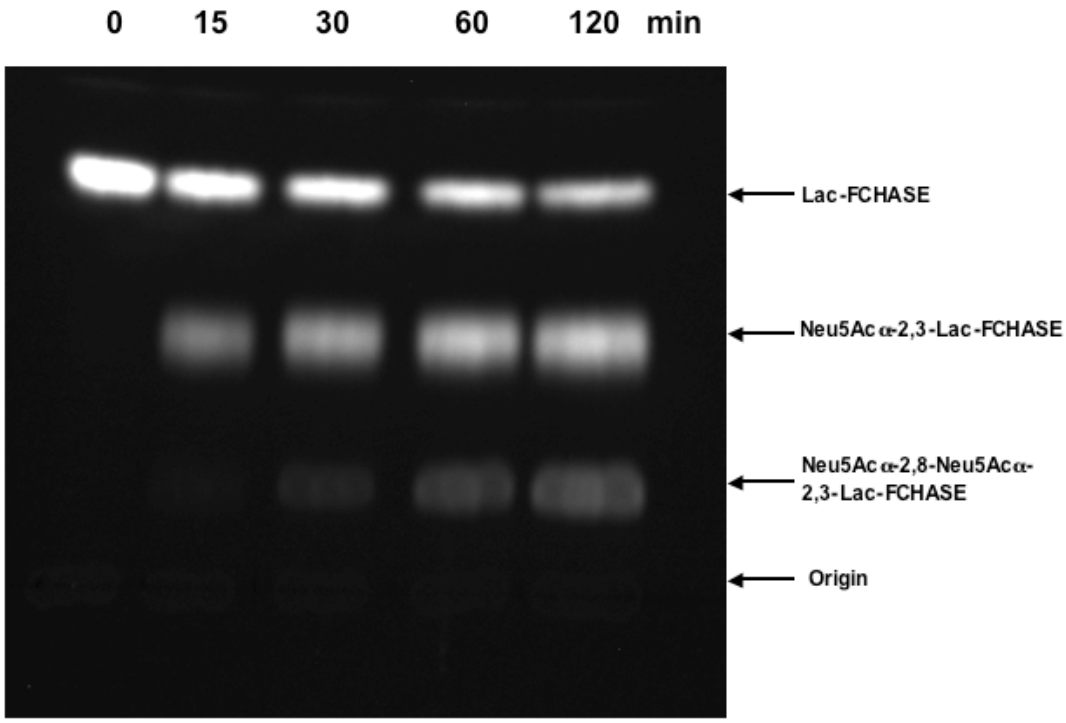


Fig. S2 Sequential addition of two Neu5Ac residues to the terminal galactose residue of Lac-FCHASE. Purified recombinant Lic3B was incubated with a fluorescent lactose derivative and a 2-fold excess of CMP-Neu5Ac. The reaction products were separated on a TLC silica plate. After a 120 min incubation time, we observed a distribution of 24.2% Lac-FCHASE, 65% Neu5Acα-2,3-Lac-FCHASE and 10.8 % Neu5Acα-2,8-Neu5Acα-2,3-Lac-FCHASE.



Identification of a Bifunctional Lipopolysaccharide Sialyltransferase in *Haemophilus influenzae*: INCORPORATION OF DISIALIC ACID

Kate L. Fox, Andrew D. Cox, Michel Gilbert, Warren W. Wakarchuk, Jianjun Li, Katherine Makepeace, James C. Richards, E. Richard Moxon and Derek W. Hood

J. Biol. Chem. 2006, 281:40024-40032.
doi: 10.1074/jbc.M602314200 originally published online October 27, 2006
