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Dependence of the Bi-functional Nature of a Sialyltransferase from *Neisseria meningitidis* on a Single Amino Acid Substitution*

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The L1 immunotype strain 126E of *Neisseria meningitidis* has been shown to have an *N*-acetyl-neuraminic acid-containing lipooligosaccharide in which an α -linked galactose from a P^k epitope is substituted at the O6 position (Wakarchuk, W. W., Gilbert, M., Martin, A., Wu, Y., Brisson, J. R., Thibault, P., and Richards, J. C. (1998) *Eur. J. Biochem.* 254, 626–633). Using a synthetic P^k-epitope containing acceptor in glycosyltransferase reactions, we were able to show by NMR analysis of the reaction product that the 126E(L1)-derived sialyltransferase can make both α -2,3 and α -2,6 linkages to the terminal galactose. Gene disruption experiments showed that the *lst* gene in 126E(L1) was responsible for the *in vivo* addition of the α -2,6-linked *N*-acetyl-neuraminic acid residue. By site-directed mutagenesis it was possible to change the MC58(L3)-derived enzyme into a bifunctional enzyme with a single amino acid change at position 168, where a glycine was changed to an isoleucine. We performed a gene replacement experiment where the 126E(L1) α -2,3/6-sialyltransferase was replaced by allelic exchange with the monofunctional MC58(L3) α -2,3-sialyltransferase and with the mutant MC58(L3) allele G168I. We observed that the level of LOS sialylation with the G168I allele was very similar to that of the wild type 126E(L1), indicating that residue 168 is the critical residue for the α -2,6-sialyltransferase activity *in vitro* as well as *in vivo*.

Mucosal pathogens in the genera *Neisseria*, *Campylobacter*, and *Haemophilus* all possess a major cell surface lipooligosaccharide (LOS)¹ containing *N*-acetylneuraminic acid (Neu5Ac) (2–4). These carbohydrate structures are not a static feature of the bacterium's outer surface but are produced with significant variation depending on the genetic makeup of the strain and the environment in which it is growing (5–7). This phenomenon is thought to play an important role in the pathogenesis of these organisms (8) (9), relating as it does to the structural similarity of the terminal oligosaccharide portion of the LOS to those found on human glycolipids. In *Neisseria* a major structure is a terminal α -2,3-sialyllacto-*N*-neotetraose (Fig. 1), in *Haemophilus influenzae* strain RM118, the LOS structures

include α -2,3-sialyllactose (7), and in the NTHi 375 strain there is a simple disialic acid structure (perhaps related to the ganglioside GD3) (4). In *Campylobacter jejuni* there are structures similar to various gangliosides, from GD3 to GT1a (10). Such oligosaccharides provide these pathogens with a means of evading the host immune response through molecular mimicry as well as providing ligands for binding to receptors on human cells (11), and they are therefore potent virulence factors (12).

The genetic analysis of the biosynthesis of LOS has shown that variation in the oligosaccharide portion of the LOS can arise in several ways. In *H. influenzae*, some LOS biosynthesis genes including glycosyltransferases are inactivated through a mechanism involving a variable number of tandem tetranucleotide repeats, which produces a phase-variable LOS phenotype (13). A related phase variation mechanism is used in *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *C. jejuni*, where changes in the length of polynucleotide tracts in certain glycosyltransferase genes leads to gene inactivation, which in turn allows alternate oligosaccharides to be produced (14–16). In addition to these mechanisms, a new source of structural diversity exists in *C. jejuni*, where the sialyltransferase Cst-II from the OH4384 strain has been shown to be bi-functional, using two different acceptor oligosaccharides and has been shown to be mono-functional in the 0:19 serostrain (17). The existence of these different mechanisms of structural variation makes it impossible to predict the LOS structure based on the gene complement alone.

The *N. meningitidis* α -2,3-sialyltransferase, *Lst*, has a relaxed acceptor specificity, being able to use synthetic acceptors that present terminal *N*-acetylglucosamine, lactose, or galactose (18). Furthermore, the *Lst* from *N. meningitidis* strains MC58(L3) and 126E(L1) can also use a terminal α -D-galactose to make an α -2,3-sialyl-P^k epitope *in vitro* with a synthetic acceptor molecule (19). We have previously demonstrated that the LOS of the *N. meningitidis* 126E(L1) was also sialylated but at the O6 position of the terminal α -D-galactose (1). To our knowledge, this structure has not been reported in any mammalian glycolipid (nor in any other LOS); hence, we cannot conclude that it is used as a form of molecular mimicry as is the case with the sialyllacto-*N*-neotetraose found in the strains with the L3 immunotype. The L1 immunotype has been isolated from disease outbreaks (20), and therefore, this unique LOS structure may play a role in meningococcal disease. We therefore sought to determine the enzyme responsible for its biosynthesis.

In this paper we demonstrate that the *Lst* of 126E(L1) is also responsible for the addition of the Neu5Ac at O6 of the α -galactose in the 126E(L1) LOS. Additionally, the bi-functional nature of the enzyme from the 126E(L1) can be introduced into the mono-functional MC58(L3) enzyme by site-directed mutagenesis of a single residue.

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¹ The abbreviations used are: LOS, lipooligosaccharide; CE, capillary electrophoresis; Neu5Ac, *N*-acetylneuraminic acid; FCHASE, 6-(5-fluoresceincarboxamido)hexanoic acid succinidyl ester.

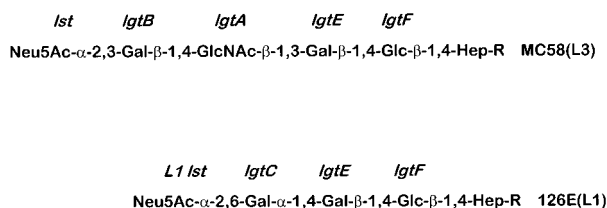


FIG. 1. Outer core oligosaccharides from *N. meningitidis* MC58(L3) and 126E(L1). The genes for the glycosyltransferases used for each addition have been identified, and their names are shown above each sugar residue (14, 18, 23).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The following strains of *N. meningitidis* were used: immunotype L1 strain 126E (NRCC 4010); immunotype L3 strain MC58 (NRCC 4728). The cloning vector pCW was used for expression of the *lst* genes and has been described previously (21). Plasmids were propagated in *Escherichia coli* strain AD202 (CGSC 7297).

Preparation and Analysis of Lipooligosaccharide—*N. meningitidis* 126E(L1) was grown and harvested, and the LOS was extracted by the hot phenol-water method as previously described (1), except that the plates were supplemented with 25 μ g/ml CMP-Neu5Ac. Small scale isolations were performed as described previously (22). Capillary electrophoresis-mass spectrometry of LOS was performed as previously described (1, 22). Methylation analysis to determine linkage positions between residues was performed as previously described (1).

Measurement of Sialyltransferase Activity—For detection of activity in *N. meningitidis* strains, cells were scraped off freshly grown Columbia blood agar plates that had been supplemented with 25 μ g of CMP-Neu5Ac/ml. The cell pellet was then extracted with 0.2% Triton X-100 in 20 mM Tris HCl, pH 8.0. The cell-free extract was then assayed as previously described (19) with acceptor molecules derived from aminophenylglycosides labeled either with FCHASE or FEX fluorophors. Analysis of all of the site-directed mutants was performed with a construct that contained only the transferase gene. The recombinant proteins were assayed in sonicated extracts. The thin layer chromatography conditions for separation of the α -2,3/2,6-sialylated P^k-FCHASE were: isopropanol, *n*-butyl alcohol, 0.1 M HCl (2:1:1) or ethyl acetate, methanol, water, acetic acid (4:2:1:0.1). High performance TLC plates (Whatman) were used for the separations.

Genetic Manipulations of the 126E(L1) and MC58(L3) α -2,3-Sialyltransferase—The construction of the allelic replacement was performed essentially as described previously (23). Briefly, the kanamycin resistance gene from pUC-4K was liberated from the plasmid as a *SalI* fragment. The 5'-upstream region and 3'-downstream regions of the α -2,3-sialyltransferase structural gene were amplified from *N. meningitidis* 126E(L1) by polymerase chain reaction with Pwo polymerase as described by the manufacturer (Roche Molecular Biochemicals). The primers for the 126E(L1) upstream-flanking sequence (5' end) of the coding regions were: 5'-GGGGGGGAATTCCATATTTTGGCGGCTTTGTTCCGCGC-3' and 5'-GGCGGTGGGCATATGATCCCTAAATCCATTCCGACAAATTG-3'. The region is 355 base pairs. The primers for the L1 downstream-flanking sequence (3' end) of the coding sequence were 5'-GGGGGGGTGCGACTCGCATAGCAAATCAAAAATAGAAAATGG-3' and 5'-GGGGGGAAGCTTCCGCGCACTGCCCGCCGTTTGGTCCGG-3'. The region is 350 base pairs. The coding sequence from MC58 L3 was amplified with the following primers: 5'-CTTAGGAGGTCATATGGCTTGAAAAGGCTTGTGACC-3' and 5'-GGGGGGTTCGACTTAAATTTTATCGTCAAATGTCAAATC-3'. The fragment was 1140 base pairs. The plasmid for the gene replacement was assembled in the following steps. pUC19 was digested with *EcoRI* and *HindIII* and purified for use as the cloning vector. A four-fragment ligation was performed with the 5'-flanking sequence of 126E(L1)-*lst* (*EcoRI/NdeI*-digested), the MC58(L3)-*lst* structural gene (*NdeI/SalI*-digested), the 3'-flanking sequence of 126E(L1)-*lst* (*SalI/HindIII*), and the pUC19 vector (*EcoRI/HindIII*). Once this construct was verified, we then inserted a Kan^R marker between the structural gene and the 3'-flanking sequence. The recombinant plasmids were introduced into *N. meningitidis* by electroporation.

The gene disruption vector was constructed in a different fashion. The sialyltransferase gene (*NdeI-SalI* fragment as described above) was restricted with *Sau3A-I* to generate three fragments. The middle 22-base pair fragment was discarded. The two larger fragments were ligated with the kanamycin resistance marker that had been liberated from pUC-4K with *BamHI*. These fragments were then ligated into

pUC19 which had been digested with *EcoRI* and *SalI*. These plasmids were then introduced into the *N. meningitidis* strain by electroporation.

Site-directed Mutagenesis—The MC58(L3)-*lst* allele was mutagenized using the U-DNA method of Kunkel (24). All mutant genes were completely sequenced to ensure no other mutations were introduced.

Capillary Zone Electrophoresis-Electrospray Mass Spectrometry—A crystal model 310 CE instrument (AYI Unicam, Madison, WI) was coupled to an API 3000 mass spectrometer (PerkinElmer Life Sciences/Sciex, Concord, Canada) via a microionspray interface. A sheath solution (isopropanol, methanol, 2:1) was delivered at a flow rate of 1 μ l/min to a low dead volume tee (250 μ m internal diameter, Chromatographic Specialties, Brockville, Canada). The separations were obtained on a 90-cm-length bare-fused silica capillary using 30 mM morpholine in deionized water, pH 9.0, containing 5% methanol. A voltage of 25 kV was typically applied for the separation. The outlet of the capillary was tapered to \sim 15 μ m internal diameter using a laser puller (Sutter Instruments, Novato, CA). Mass spectra were acquired with dwell times of 3.0 ms/step of 1 *m/z* unit in full-mass scan mode. 20 nl of sample was typically injected by using 150 mbar for a duration of 0.1 min.

NMR Spectroscopy—NMR experiments were performed on a Bruker AMX-600 and Varian INOVA 600 NMR spectrometers. Experiments on P^k-FCHASE compound were done using an inverse broadband detection probe at 27 °C with the mono-deuterated water resonance at 4.756 ppm. Experiments on α -2,6-sialyl-P^k-FCHASE compound were done using a 5-mm Z gradient triple resonance probe at 25 °C with the mono-deuterated water resonance at 4.774 ppm. NMR samples were prepared by dissolving 0.1–0.3 mg of material in 600 μ l of D₂O after freeze-drying. All NMR experiments were performed as previously described (17). For the proton chemical shift reference, the methyl resonance of internal or external acetone was set at 2.225 ppm (¹H). For the ¹³C chemical shift reference, the methyl resonance of internal or external acetone was set at 31.07 ppm relative to external dioxane at 67.40 ppm.

RESULTS

Detection of α -2,6-Sialyltransferase Activity—When we analyzed reactions performed with the 126E(L1)-*Lst* and the P^k-FCHASE acceptor, we noticed a second product peak (slightly faster migrating) in the CE electropherograms (Fig. 2), which was sensitive to sialidase treatment (data not shown). This second product is only seen with P^k-FCHASE acceptor. The major product (\sim 90% of the product) had the same migration time as α -2,3-Neu5Ac-P^k-FCHASE that we had previously described (19). We were able to resolve these two products by TLC, but we could only see a second product in reactions performed with the 126E(L1)-*Lst* and not MC58(L3)-*Lst*. We were able to obtain enough of the unique secondary product using preparative TLC, and this material was subjected to analysis by NMR spectroscopy (see below).

NMR Analysis—To assess the linkage specificity of the 126E(L1)-*Lst*, the unique product formed with P^k-FCHASE as an acceptor was analyzed by NMR spectroscopy on nanomole amounts. We have previously published the NMR spectrum for α -2,3-Neu5Ac-P^k made with the MC58(L3)-*Lst* (19), and we found the NMR spectrum of the major sialylated product made with 126E(L1)-*Lst* was identical to our previously published one. We present here only the spectra from a nonsialylated acceptor, P^k-FCHASE = compound I, and the unique product formed in a reaction with the 126E(L1)-*Lst*, α -2,6-sialyl-P^k-FEX = compound II. The comparison of these two spectra clearly shows the site of sialylation of the terminal α -galactose in the acceptor. At the time the preparative sialylation reaction was performed, we were using the FEX aglycon, but we have seen that during storage that the FCHASE aglycon is more stable. The nonsialylated P^k-FCHASE spectra was collected on material made at a later time.

Both compounds were soluble and gave sharp resonances with line widths of a few Hz since the H-1 anomeric doublets ($J_{1,2} = 4$ and 8 Hz) are well resolved (data not shown). For compound I, the mono-deuterated water resonance was very broad and overlapped with one of the anomeric signals. In

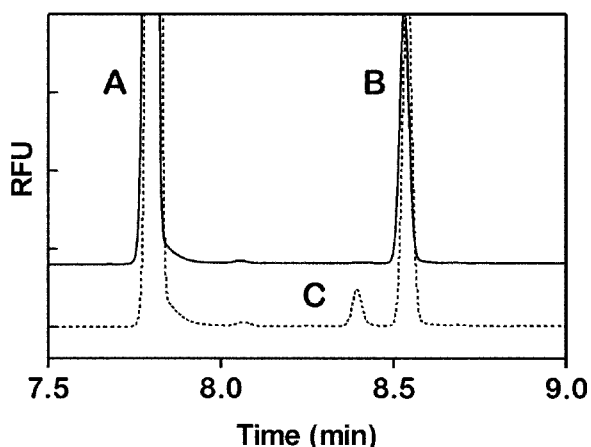


FIG. 2. Capillary electrophoresis analysis of MC58(L3)-Lst and 126E(L1)-Lst. Sialyltransferase reactions with both versions of the Lst enzyme were analyzed by capillary electrophoresis. The top electropherogram shows the products from the MC58(L3)-Lst reaction (solid line), and the lower electropherogram shows the products from the 126E(L1)-Lst reaction (dashed line). Peak A is the starting material P^k-FCHASE, peak B is the α -2,3-Neu5Ac-containing product, and peak C is the α -2,6-Neu5Ac-containing product. RFU, relative fluorescence units.

Table I, the proton assignments were obtained from standard homonuclear experiments, correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), one-dimensional NOESY, one-dimensional TOCSY, and the ¹³C assignments from a heteronuclear multiple quantum coherence (HMQC) experiment, which detects C-H correlations (17).

The linkage site on the aglycon was determined from a comparison of the ¹³C chemical shifts of the enzymatic product with those of the precursor to determine glycosidation shifts, as done previously (17). Although a different fluorescent tag was used, it only influenced the proton chemical shift of the Glc residue. For compound I, the proton and ¹³C chemical shifts were in accord with those reported for Gal- α 1,4-Gal- β 1,4-Glc- β -1-O-2-(trimethylsilyl)ethanol (25). Compound II was identified as Neu5Ac- α 2,6-Gal- α 1,4-Gal- β 1,4-Glc- β -1-FEX. From a comparison of the heteronuclear multiple quantum coherence spectra of compound I and II, it is obvious that the linkage site is at C-6 of α Gal due to the large downfield shift of -2.6 ppm for α Gal C-6 upon sialylation (Fig. 3). Large proton chemical shifts differences of 0.18 and -0.17 ppm were also observed for the H-6 and H-6' resonances of α Gal upon sialylation. An upfield shift of 1.3 ppm for the α Gal C-5 was also observed (Table I), typical of 2–6 sialyloligosaccharides (26). The ¹³C chemical shifts of compound II are similar to those reported for the NeuN- α 2,6-Gal- α 1,4-Gal- β 1,4-Glc moiety present in the 126E(L1) LOS from *N. meningitidis*, except for the chemical shift of NeuN C-4, which is obviously wrong in the latter (1). In Table I and Fig. 3, the proton chemical shift differences for the Glc unit between compounds I and II are attributed to different fluorescent aglycons.

Site-directed Mutagenesis of MC58(L3)-Lst—A sequence comparison of the 126E(L1)-Lst (GenBankTM accession U60662) and MC58(L3)-Lst (GenBankTM accession U60660) proteins showed only 6 amino acid changes. We sequentially changed all of the residues in MC58(L3)-Lst that differed from 126E(L1)-Lst to the corresponding residue from 126E(L1)-Lst (E40D, R102W, S129A, G168I, T242A, K273N). Only the G168I mutation resulted in an enzyme with α -2,6-sialyltransferase activity. We then performed additional mutagenesis to put all possible amino acids at the position Gly-168 in MC58(L3)-Lst to determine whether other amino acids also permit the second

enzyme activity. The results of these experiments were analyzed by first measuring the activity of the mutants on β -N-acetyllactosamine-FCHASE, the preferred acceptor for the MC58(L3)-Lst, to ensure the mutants were functional. Both Lst enzymes make only a single product, the α -2,3-linked Neu5Ac, with β -N-acetyllactosamine-FCHASE. Only one of the mutations resulted in an inactive enzyme, G168P, and this was not due to lack of protein production (data not shown). These mutants were then analyzed for activity on the P^k-FCHASE acceptor using equivalent amounts of enzyme as measured on the β -N-acetyllactosamine-FCHASE acceptor. Only a few of these had significant α -2,6-sialyltransferase activity, and these data is shown in Fig. 4. The mutants G168I and G168L showed levels of α -2,6-sialyltransferase activity equivalent to the 126E(L1)-Lst. The G168V mutant showed 40% of the α -2,6 activity level of 126E(L1)-Lst, and the G168M mutant showed 25% of the level of α -2,6 activity of 126E(L1)-Lst.

We also noted that certain mutants were less active than the wild type on P^k-FCHASE in general but basically unaffected for activity on β -N-acetyllactosamine-FCHASE. These mutants were G168F, G168Y, and G168W, and using the standard assay described here, they showed a higher specificity for β -N-acetyllactosamine-FCHASE than they did for P^k-FCHASE.

Production of the 126E(L1)-lst Knock-out—An insertional inactivation mutant of the *lst* gene was made in the 126E(L1) strain, and then the LOS was analyzed by mass spectrometry. When the *lst* gene has been inactivated, no Neu5Ac could be detected in the LOS (data not shown). The mutation should not cause any polar effects as the next gene downstream of *lst*, a cytochrome *c'* homologue, is transcribed in the opposite direction and is not involved in sialic acid metabolism (18).

Allelic Exchange of the 126E(L1)-lst Gene with That from MC58(L3) and with the G168I Mutant Allele—To assess the bi-functional nature of the *lst* gene from 126E(L1) *in vivo*, we constructed isogenic mutants that contained either the MC58(L3)-derived *lst* gene or the G168I mutation. After obtaining transformants, they were screened for sialyltransferase activity, and then the resident *lst* gene was amplified by polymerase chain reaction and sequenced to ensure the correct gene had been inserted. Both gene replacement mutants produced similar levels of sialyltransferase activity as measured with the *in vitro* assay on synthetic acceptors. The activity was similar to what we had seen *in vitro*; the MC58(L3)-derived enzyme did not make a significant amount of the α -2,6-linked Neu5Ac, whereas the G168I mutant showed the same ratio of α -2,3 to α -2,6 product (data not shown). LOS was prepared from these strains and was analyzed by CE-electrospray mass spectrometry (Fig. 5). The level of Neu5Ac incorporation in the 126E(L1) mutant strain with the MC58(L3)-*lst* gene was very low, but certainly detectable. The level of Neu5Ac incorporation from the G168I mutant gene was comparable with the wild-type 126E(L1) strain. Methylation analysis of these LOS samples (Table II) showed 6-substituted Gal, indicating that the Neu5Ac in both cases was α -2,6-linked and that no α -2,3-linked Neu5Ac was present.

DISCUSSION

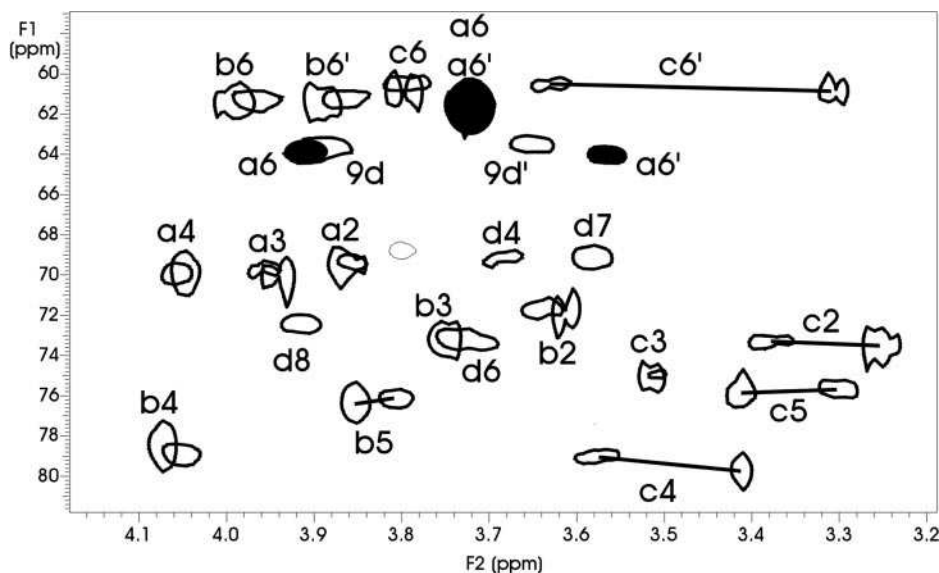
The proteins involved in the addition of the sialic acid to the outer core oligosaccharide portion of the LOS from *C. jejuni*, *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae* have been identified (7, 17, 18). These proteins have a variety of acceptor specificities, and only the *C. jejuni* and *H. influenzae* enzymes share some sequence identity with each other. No structure/function analysis for any of these enzymes has so far been reported. Since there is no conserved sequence in the group of enzymes as a whole, it has been difficult to predict which

TABLE I
Proton NMR chemical shifts for the fluorescent glycosides synthesized using the cloned glycosyltransferases

Data are in ppm from heteronuclear multiple quantum coherence (HMQC) spectra. The error is ± 0.02 ppm for ^1H chemical shifts and ± 0.2 ppm for ^{13}C chemical shifts.

Residue	Atom	I		II	
		^1H	^{13}C	^1H	^{13}C
α -Gal	1	4.98	101.4	4.96	101.3
	2	3.87	69.6	3.86	69.3
	3	3.94	70.2	3.96	69.8
	4	4.05	69.9	4.06	69.9
	5	4.38	71.8	4.44	70.5
β -Gal	6	3.73, 3.73	61.4	3.56, 3.90	64.0
	1	4.39	104.5	4.45	104.4
	2	3.62	71.9	3.64	71.7
	3	3.75	73.2	3.73	73.2
	4	4.08	78.4	4.05	78.9
β -Glc	5	3.86	76.4	3.80	76.1
	61	3.90, 4.00	61.4	3.87, 3.97	61.3
	1	4.65	100.5	4.61	101.0
	2	3.26	73.6	3.37	73.3
	3	3.52	75.1	3.51	75.0
α -Neu5Ac	4	3.42	79.8	3.58	79.0
	5	3.42	75.7	3.30	75.6
	61	3.31, 3.80	60.8	3.63, 3.79	60.4
	3			1.76, 2.73	40.9
	4			3.68	69.1
	5			3.85	52.6
	6			3.71	73.4
	7			3.59	69.1
	8			3.91	72.4
	9			3.65, 3.88	63.6
	NAc			2.04	22.8

FIG. 3. Identification of the glycosidation site from a comparison of the heteronuclear multiple quantum coherence spectra of precursor and product glycosides. The two spectra of the precursor and enzymatic product different by one sugar are overlaid. Only one contour is drawn for each spectrum. The two cross-peaks for the same common atom in the two compounds that do not overlap are joined by a solid line. The linkage site is identified from the large ^{13}C downfield glycosidation shift, indicated by filled cross-peaks. The proton chemical shift axis is F2, and the ^{13}C chemical shift axis is F1. Cross-peaks are labeled using the letter *a* for α -Gal, *b* for β -Gal, *c* for Glc, *d* for Neu5Ac, and by the atom number as in Table I for compound I and II.



residues might be involved in donor/acceptor recognition or catalysis.

Sialic acid transfer to the LOS of *N. meningitidis* species has been shown to be performed by the product of the *lst* gene (18). The terminal oligosaccharide produced in most strains is a mimic of the human glycolipid oligosaccharide sialyllacto-*N*-neotetraose. An alternate sialylated LOS structure with the terminal oligosaccharide α -2,6-Neu5Ac- P^k has been shown to be produced in the 126E(L1) strain (1, 20), but the enzyme for producing this novel α -2,6-sialylated structure was not reported. We have now determined the enzyme responsible for the addition of the α -2,6-linked Neu5Ac in the 126E(L1) strain and have shown it to be a bi-functional version of the *Lst* enzyme that previously had been shown to transfer the α -2,3-linked Neu5Ac in *N. meningitidis* MC58(L3) strain.

The first reported multifunctional enzyme involved in lipopo-

lysaccharide biosynthesis was the 3-deoxy- α -D-mannooctulosonic acid (KDO) from *Chlamydia pneumoniae*, which adds KDO to the lipid A core through a α -2,6 linkage to GlcNAc and then in an α -2,4 linkage to the first KDO residue, and then in an α -2,8 linkage to the second KDO (27). Recently a bi-functional sialyltransferase from *C. jejuni* was described where the enzyme could also use two very different acceptors, namely a β -linked Gal or a α -2,3-linked Neu5Ac (17). The sialyltransferase enzyme from the 126E(L1) strain of *N. meningitidis* described in this paper is also bi-functional but uses the same terminal sugar acceptor for the reaction and makes an alternate linkage (α -2, 6) in addition to the more usual α -2,3-linkage. This activity is also found only with terminal Gal residues that are in an α -linkage with the previous galactose residue. We have never seen any second (α -2,6) product formed during *in vitro* reactions on β -linked galactose residues. We detected

FIG. 4. Sialyltransferase activity of site-directed mutants of the MC58(L3)-Lst on P^k-FCHASE. The mutants were examined along with both the 126E(L1) and MC58(L3) controls. The top panel shows a thin layer chromatography analysis of all of the strains. The arrowheads indicate the migration of α -2,3-sialyl-P^k-FCHASE and α -2,6-sialyl-P^k-FCHASE. These reactions were also quantitated by capillary electrophoresis, and the percentage of the reaction product that is α -2,6-linked is plotted versus the mutation at position 168 of the MC58(L3) gene.

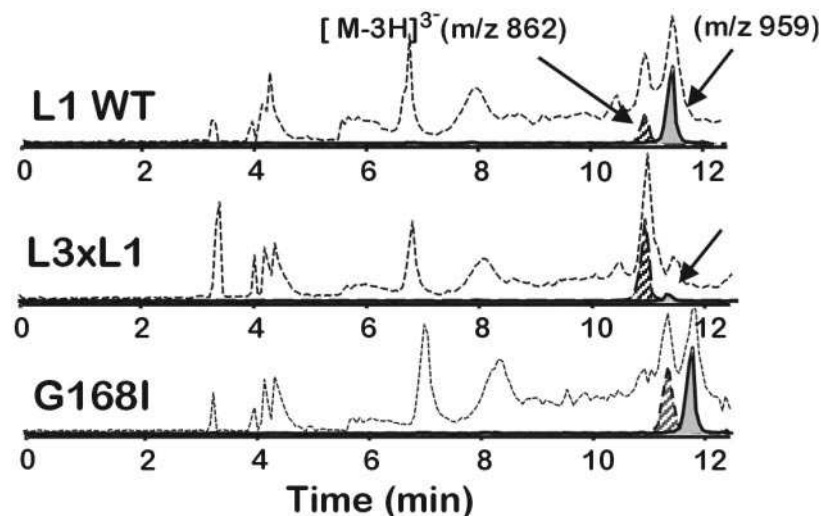
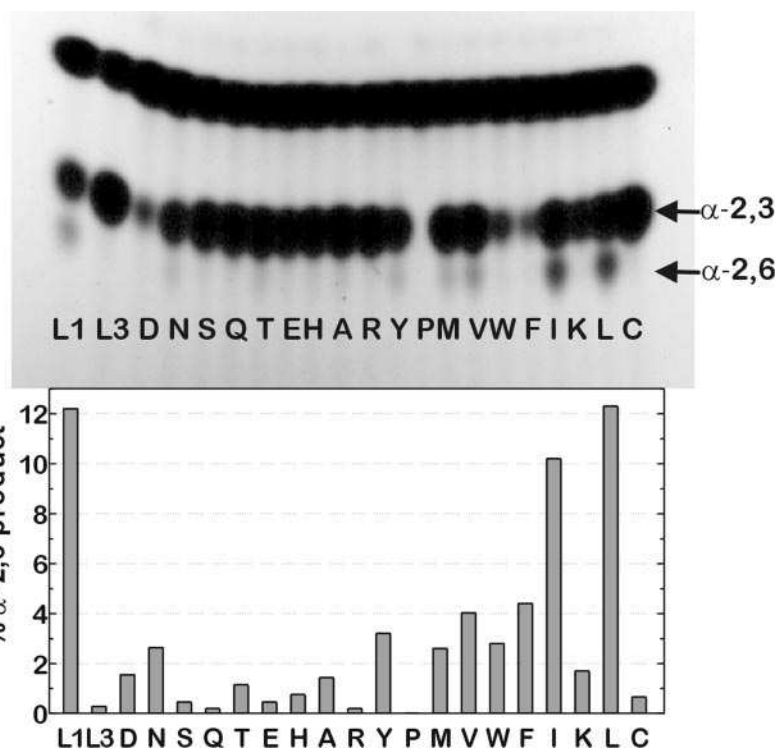


FIG. 5. Capillary zone electrophoresis-electrospray mass spectrometry analysis of mixtures of *O*-deacylated LOS from different strains: total ion electropherograms (m/z 600–1500) and reconstructed ion electropherograms for triply-deprotonated ions at m/z 862.0 (nonsialylated) and m/z 959.0 (sialylated). a, 126E(L1) wild type (WT); b, MC58(L3) \times 126E(L1); c, MC58(L3)::G168I. Separation conditions: 20- μ l injection of 1 mg/ml *O*-deacylated LOS, bare fused-silica (90 cm \times 50 μ m internal diameter, 190- μ m outer diameter), 5% methanol in 30 mM morpholine, pH 9.0, +25 kV.

TABLE II
Methylation analysis of LOS

Bold values indicate the level of Gal-substituted galactose in the various strains of LOS preparations.

Methylated sugar (alditol acetate)	L1 wild type	L3 replacement	G168I replacement
2,3,4,6-Me ₄ -Gal	0.42	0.62	0.55
2,3,6-Me ₃ -Gal	1.00	1.00	1.00
2,3,6-Me ₃ -Glc	1.19	0.60	0.71
2,3,4-Me ₃ -Gal	0.78	0.05	0.29
2,6,7-Me ₃ -Hep	0.46	0.22	0.27
4,6,7-Me ₃ -Hep	0.14	0.14	0.12

the α -2,6 product produced during *in vitro* enzyme reactions, but only about 10% of the synthetic P^k-FCHASE acceptor is converted to the α -2,6 linkage product, whereas the majority of the product formed *in vitro* is the α -2,3-sialyl-P^k structure. The MC58(L3)-Lst enzyme does produce detectable α -2,6-linked Neu5Ac, but the amount of α -2,6-linked Neu5Ac was about 45-fold less than was made by 126E(L1)-Lst, and this could only be seen using the ultra-sensitive laser-induced fluores-

cence detector on the CE. The isomeric α -2,3-sialyl-P^k structure has not been detected *in vivo* in the *N. meningitidis* 126E(L1) strain.

The ability of the enzyme to make these two products *in vitro* may not be too surprising since side reactions are possible with a synthetic acceptor molecule, but what is most important to bear in mind is that *in vivo*, only the α -2,6-Neu5Ac linkage is formed in the 126E(L1) strain. In our examination of the enzyme, we were able to pinpoint a single residue in the MC58(L3)-Lst, Gly-168, which when mutated to Ile (the residue at position 168 in 126E(L1)), permits the enzyme to form the same level of α -2,6-sialylated product either *in vitro* on the synthetic P^k acceptor or *in vivo* on the LOS. What was surprising, however, was the fact that *in vivo* the MC58(L3)-Lst could form a very small amount of sialylated LOS in the 126E(L1) strain and that this material was also α -2,6-linked. The amount of Neu5Ac in this 126E(L1) mutant (MC58(L3)-Lst) was too small to analyze by NMR, but methylation analysis showed around 6% of the wild type level of the 6-substituted Gal. This level of Neu5Ac incorporation is much higher than

what was expected, given the 45-fold lower α -2,6-sialyltransferase activity of the MC58(L3)-Lst enzyme measured *in vitro*. These data suggest that some factor *in vivo* completely blocks the O3 position of the terminal α -galactose and that something also must promote the formation of the α -2,6-linked product since this is the linkage formed exclusively in L1. At present we do not know if this factor is another protein that may bind either Lst or the LOS, the conformation of the LOS during biosynthesis, or another molecule present at the site of biosynthesis. Molecular modeling of the 126E(L1) LOS suggests the conformation of the terminal trisaccharide could produce a sterically hindered product where the O3 of the terminal α -Gal is effectively blocked by the phosphoethanolamine substituent on Hep-2 of the inner core.²

Consequently the Lst variants with very low α -2,6 activity would be inactive in L1 strains when LgtC is functional. Since a relatively subtle change (Gly to Ile/Leu) results in the α -2,6-sialyltransferase activity, we speculate that the 126E(L1) strain maintained this version of Lst because it was advantageous for its survival in the human host, and as this oligosaccharide structure has not been described in man, it may or may not be molecular mimicry. We do not yet know if the specificity of the 126E(L1)-Lst is in all L1 immunotype strains. Data by Griffiss *et al.* (20) would suggest the structure we described in strain 126E(L1) and the *lst* gene variant occurs in other L1 immunotype strains.

Our mutagenesis of position 168 in the MC58(L3)-Lst revealed that all amino acid side chains are tolerated there except for proline. Surprisingly, the activity level of all of the mutants was similar on the acceptor β -N-acetyllactosamine-FCHASE, except G168P, which was not active at all likely due to distortion of the enzyme active site. The mutants G168Y, G168F, and G168W maintained activity on β -N-acetyllactosamine-FCHASE but have very reduced activity on the P^k-FCHASE compared with the MC58(L3)-Lst or 126E(L1)-Lst. When this residue is Ile or Leu, it then directs the specificity of the reaction to allow the formation of the α -2,6 linkage as well as the 126E(L1)-Lst. The presence of the aromatic amino acid side chains reduced the activity of the enzyme toward the P^k-FCHASE acceptor significantly. With the CE-based assay, the overall activity of the G168W/G168F/G168Y mutants were 4–9-fold less active on this acceptor (data not shown), whereas their activity on β -N-acetyllactosamine-FCHASE appeared very similar to the wild type MC58(L3)-Lst protein. This suggests that the steric hindrance from the large inflexible ring containing side chains specifically block the enzyme from utilizing the α -linked terminal Gal residue.

We postulate that the position 168 in this enzyme must be in a cavity that has enough space to accommodate both the α and β anomers of the terminal Gal. It will be essential to obtain structural information about this enzyme that will show us

what the active site looks like to understand how these two different acceptor conformations are accommodated such that the product of the 126E(L1)-Lst reaction is α -2,6-linked Neu5Ac, whereas the MC58(L3)-Lst produces α -2,3-linked Neu5Ac. Other factors in the synthesis of the 126E(L1)-LOS influence the Neu5Ac linkage formation so it will also be important to identify what these are to fully understand the formation of this novel glycolipid structure.

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² J. R. Brisson, unpublished data.

Dependence of the Bi-functional Nature of a Sialyltransferase from *Neisseria meningitidis* on a Single Amino Acid Substitution

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