Biochemical properties of *Neisseria gonorrhoeae* LgtE

Andrzej Piekarowicz[1] and Daniel C. Stein[2]

[1]Institute of Microbiology, University of Warsaw, 02-096 Warsaw, Poland and [2]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742

Corresponding Author: Dr. Daniel C. Stein

Phone: 301-405-5448

Fax: 301-314-9489

Email: DS64@UMAIL.UMD.EDU

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Summary

A fragment of chromosomal DNA encoding the *lgtE* gene of *Neisseria gonorrhoeae* strain F62 was amplified by the polymerase chain reaction and cloned into the expression vector pET15b. Functional LgtE was purified and its biochemical properties determined. The purified enzyme was maximally active in buffer containing manganese; minimal activity was obtained in buffer containing other divalent cations. LgtE was only able to mediate the addition of Uridine diphosphate-galactose into Neisserial lipooligosaccharides (LOSs). We used a variety of genetically defined and chemically verified LOS structures to determine acceptor specificity. LgtE was able to mediate the addition of galactose into a variety of LOS structures, indicating the this enzyme possesses broad acceptor specificity. Furthermore, it was able to add multiple galactose residues onto LOS. We also determined that this enzyme was capable of adding galactose onto both the ? and ? chains of Neisserial LOS.

Introduction

Lipooligosaccharide (LOS) is an important virulence determinant of the pathogenic Neisseria (12, 16). It consists of an oligosaccharide component that is attached to lipid A via a KDO linkage. The genes involved in the synthesis of the oligosaccharide portion of this molecule have been identified and characterized from a variety of species (1, 2, 11, 13, 17, 28). A common feature of LOS expression in all of these species is the expression of multiple phase-variable LOS structures. Most of this variability is attributed to changes in the carbohydrate composition of the molecule. The genetic basis for this variation has been well characterized. Key genes in the biosynthetic pathway contain homopolymeric runs of guanine (2, 11). Changes in the number of guanines result in reading frame shifts, with the end result being the truncation or elongation of a particular LOS molecule, depending on the nature of the starting reading frame (4, 5, 37).

The data presented in Figure 1 summarizes the genetic potential and reported carbohydrate structures that have been identified in *Neisseria gonorrhoeae* (7, 9, 10, 15, 18, 34-36). The genes responsible for the addition of most of these sugars have been defined genetically; loss of gene function results in the truncation of an LOS structure. Biochemical characterization of several of these gene products has been performed by measuring the incorporation of sugars from various UDP-sugars to a variety of synthetic carbohydrates. LgtA possessed broad substrate specificity towards ? and ? galactosides. Depending on the acceptor, this enzyme could mediate the transfer of GlcNAc from UDP-GlcNAc and GalNAc from UDP-GalNAc (3). However, this broad specificity was not seen *in vivo* (27). The biochemical properties of LgtB and LgtC have similarly been examined (21, 30, 31). Both enzyme possessed the predicted galactosyltransferase activities.

Genetic evidence supporting the function of *lgtE* as encoding a glycosyl transferase responsible for the addition of galactose ?-1, 4 to glucose has been reported in a variety of publications (6, 11, 14, 26). However, purified LgtE was unable to mediate the transfer of galactose to synthetic LOS biosynthetic intermediates (30). Erwin et al. (6) showed that when *lgtE* was nonfunctional, galactose was not added onto the ? chain. However,

no direct biochemical evidence was presented to implicate LgtE directly in this addition. We purified functional LgtE from *Escherichia coli* strains containing recombinant plasmids expressing LgtE, and then used this recombinant enzyme to demonstrate its ability to modify various Neisserial LOSs that possessed defined structures.

Experimental Procedures

Bacterial strains, plasmids, oligonucleotides and culture conditions. *N.* gonorrhoeae strain F62 was obtained from Dr. P. F. Sparling, University of North Carolina, Chapel Hill. *N. subflava 44* and F62? lgtA? lgtFG+ have been previously characterized in this laboratory (24, 28). *Escherichia coli* strain DH5? MCR was obtained from Life Technologies (Rockville, MD); Strain ER2566 (F^- ? $^-$ *fhuA2 [lon] ompT lacZ::T7 geneI gal sulA11 ? (mcrC-mrr)114::IS10 R(mcr-73::*miniTN10)2 *R(zgb-210::*TN10)1(tet ^{s})*endA1 [Dcm])* was obtained from New England Biolabs, (Beverly, MA). Plasmid pET15b was obtained from Novagen (Madison, WI). Neisseria strains were grown in standard gonococcal medium {designated GCP if broth, GCK if agar} (Difco laboratories) plus growth supplements (33) and 0.042% sodium bicarbonate if in broth or in a 37 ^{o}C CO_2 incubator. *E. coli* strains were grown on LB plates (23). Ampicillin was used at 50 ? g/ml, spectinomycin at 50 ? g/ml and X-gal at 35 ? g/ml when the selection or colorimetric detection were applied.

N. gonorrhoeae strain F62? lgtA? lgtF? rfaKlgtG+ was constructed by PCR amplification of rfaK region using primers RFAK147 and RFAK3780, and cloning this fragment in the SmaI-HindIII sites of pK18up (25), giving pRFAK. A 458 bp DraI fragment, located within the coding sequence of *rfaK*, was deleted from this plasmid, giving pRFAK? 2-1. This deletion was introduced into F62? lgtA? lgtF? lgtG+, giving rise to F62? lgtA? lgtF? rfaKlgtG+. All constructs were verified by PCR amplification of the desired region.

Chemicals, reagents and enzymes. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). All chemicals used for this study were reagent grade or better and were

purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Tris-tricine gels (16.5%) and running buffer were obtained from Bio-Rad Laboratories (Richmond, CA). The Mab 3G9 was graciously provided by Dr. Peter Rice, Boston University, Boston, MA.

LOS purification and analysis. LOSs were purified from broth grown cells using acetone-powdered organisms by the hot phenol-water method (32). LOS was extracted with hot phenol-water and concentrated by lyophilization. Extractions were continued until the purified LOS gave a minimal absorbance when measured at 200 nm.

SDS-PAGE analysis. Approximately 0.1 ? g of LOS was subjected to SDS-PAGE on a 16.5% Tris-tricine gel (from Bio-Rad) in Tris-tricine running buffer following the protocol suggested by the manufacturer. The gel was fixed overnight in 40% ethanol 5% acetic acid and the LOS visualized by silver staining (29).

Western Blot and colony blot analysis. After SDS-PAGE, LOSs were electrotransferred onto Immobilon-P membrane (Millipore Corp., MA) in a Tris-tricine-methanol buffer (10 mM Tris, pH 8.3, 10 mM Tricine, 0.01% SDS, 20% methanol) at a constant voltage of 100V for 1h following the protocol provided by Bio-Rad Corp. After air drying for 1h, the membrane was processed by the same procedure as "Colony Blot"

For colony blot analysis, overnight colonies were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH), incubated in buffer (20 mM Tris, 150 mM NaCl, 2% milk powder) to block all non-specific binding sites, and screened for reactivity to the appropriate Mab. Bound Mabs were detected by reacting the nitrocellulose filter with Mab, and visualizing the bound antibody by reacting the blot with Goat-anti-mouse Horseradish peroxidase-labeled IgG.

Transformation. Recombinant DNA transformation into *E. coli* were done according to standard protocols (23). Recombinant DNA transformation into *N. gonorrhoeae* were done by resuspending T1 cells to a density of approximately 1 x 10^8 cells/ml in GCP broth containing 1X Kellogg's solution, 0.042% $NaHCO_3$, 10 mM $MgCl_2$ and 1 ? g of the DNA of interest. Cells were incubated for about 5 hrs with shaking at 37 ^oC. Cells were plated onto GCK plates containing spectinomycin.

Polymerase Chain Reaction. The PCR was used to generate the DNA fragments employed in gene cloning experiments and for mutant *N. gonorrhoeae* strain verification. Primers were made by Bioserve Biotechnologies (Laurel, MD). DNA amplifications were performed by using a PCR supermix kit (from Life Technologies, Grand Island, NY) following the procedure provided by the company. Purified chromosome DNA or plasmid DNA was used as template. For strain construction verifications, DNA was isolated directly from colonies by the following procedure. A small colony was added to 5 ? l of 0.5 M NaOH, the cell mixture allowed to incubate at RT for 10 min and the solution was neutralized with 5 ? l 1M Tris-HCl, pH 7.5. After adding 90 ? l H_2O, 3 ? l of this solution was used for PCR amplifications.

Cloning of *lgtE* and purification of LgtE. A fragment of chromosomal DNA encoding the *lgtE* gene was obtained by PCR amplification of F62 chromosomal DNA using the following primers: LGTE1; 5' TTCCAACATATGCAAAACCACGTTATCAGC 3' (The *Nde*I site that was used for cloning is underlined) and LGTE2; 5' ATGCATGGATCCCGCGGGAATGACAGTGTGTCCA 3' (The *Bam*HI site that was used for cloning is underlined). The PCR product was cleaved with *Nde*I and *Bam*HI and ligated into the expression vector pET15b, that had been cleaved with the same enzymes. The ligation mixture was used to transform *E. coli* DH5? MCR, and individual transformants were screened for the presence of the appropriate recombinant plasmid. Plasmid pET15b-lgtE was transformed into *E. coli* ER2566 strain and a single colony was used to inoculate 25 ml of Luria broth containing ampicillin. Cells were incubated with moderate shaking at 37 ^oC until the OD_{600} reached a value of 0.6. IPTG was added to a final concentration of 1 mM and incubations were

continued at 25 °C for 10 hr. The cells were collected by centrifugation and resuspended in 1 ml of binding buffer (Novagen). Lysozyme was added to a final concentration of 100 ? g/ml and the mixture incubated on ice for 60 min. Samples were frozen at - 70 °C, thawed and sonicated (three times for 15 sec). The cell extract was clarified by centrifugation for 30 min at 15,000 rpm in a Sorvall SS34 rotor. While the majority of LgtE protein was present as an insoluble fraction, soluble proteins were purified on a Nickel column according to the protocols of the manufacturers (Novagen) resulting in the pure LgtE protein. The enzyme was dialyzed against Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 50 % of glycerol and stored at -20 °C.

Galactosyltransferase assays. The standard reaction volume was 30 ? l and contained 50 mM MES buffer, pH 7.3, 10 mM $MnCl_2$, 10 ? g of purified LOS or 5 x 10^6 whole cells, and 1.5 ? g of purified LgtE protein. When radioactive substrates were employed, 0.2 nmol were added UDP-[^{3}H]Gal (17.8 Ci/mmol), UDP-[^{3}H]Glc (25 Ci/mmol) or UDP-[^{14}C]GlcNAc (266 Ci/mmol)]; when non radioactive substrates were employed, compounds were added to a final concentration of 1 mM. The reactions were incubated for at least 2 h at 30 °C. The reactions were terminated by the addition of 10 ? l of 20 % SDS and heating at 70 °C for 3 min. For the separation of unincorporated radioactive material from LOS, reactions were loaded onto 3 ml Sephadex G-100 columns (made from a Pasteur pipette and equilibrated with water). The columns were washed with water and fractions of three drops were collected (The first 3 fractions contained 6 drops). LOS eluted at fractions 5 - 12, while the radioactive LOS starting from fractions 18 -20. Samples of fractions 5 to 15 (10 ? l) were spotted on 1 cm x 1cm filter paper, dried and the radioactivity was assayed by liquid scintillation counting. The total volume of all fractions containing radioactive LOS was measured and used to calculate the total transfer of radioactive substrate into the acceptor LOS structures.

Galactosyltransferase activity was also determined by the autoradiography. LOS samples were radiolabeled and purified as above and then run in parallel on the same SDS-Tricine gel as described above. Half of the gel was stained for visualization of the LOS using a standard silver-staining procedure while the

second half of the gel was soaked in sodium salicylate (1 M, pH 6.0) for 30 min. The gel was placed on 3 MM Whatman paper, dried for a 10 minutes on air, covered with a saran paper and subjected to autoradiography.

Results

Cloning and expression of the *N. gonorrhoeae* F62 *lgtE* gene in *E. coli*

Through the use of PCR amplification, a fragment of chromosomal DNA encoding the *lgtE* gene was obtained and cloned into the expression vector pET15b. While we were able to isolate numerous clones that contained the desired amplicon, most of these clones failed to express LgtE after growth in *E. coli* ER2566 strain and induction with IPTG. Furthermore, in those strains that did express LgtE, the majority of the protein was present as an insoluble fraction. However, we were able to purify the soluble proteins on a Nickel column, using the protocol of the manufacturer (Novagen); we obtained a significant amount of pure LgtE protein (Figure 2). The presence of precipitated proteins both before and after purification prevented us from calculating the specific activity and total yield of the enzyme. The molecular mass of LgtE as determined by SDS-PAGE (35 kDa) agreed with the predicted mass for this protein, as translated from the cloned chromosomal fragment encoding this gene. Since *E. coli* strain ER2566 possesses as mutation in the OmpT protease, we did not observe the problem of proteolytic degradation of expressed proteins that was described by Wakarchuk et al. (31).

The biochemical properties of the enzyme.

Optimal conditions for assaying galactosyl-transferase activity of LgtE were determined, using [^{3}H]-UDP-galactose as a substrate and purified F62? LgtA? LgtE LOS as a acceptor. Strain F62? LgtA? LgtE expresses a single LOS molecule that possesses a single glucose on the ?-chain and a phosphate as the ?-chain (28). The purified enzyme showed an absolute dependence on the presence of the Mn^{2+}. In the presence of the Mg^{2+} less then 10 % of the maximal activity was observed; in the presence of other divalent ions, minimal activity was observed (Table 1). The maximum of activity was obtained at pH 7.5 (Figure 3) and 30 ^{o}C (data not presented). Wakarchuk et al. (31) reported that LgtC required free thiols for enzymatic activity, while LgtB did

not. We tested our preparation of LgtE to determine if it required free thiols for activity. The data presented in figure 4 indicate that purified LgtE showed a linear rate of activity up to 90 min.; we concluded that this enzyme did not possess a thiol requirement.

Substrate specificity.

Since some of the other gonococcal glycosyl transferases possessed broad substrate specificity, we used various radiolabeled compounds to test for their ability to act as a substrate for LgtE. The data indicate that the enzyme was only able to efficiently use UDP-Gal as a substrate (Table 2). Incorporation of radioactivity into purified LOS using UDP-GalNAc and UDP-Glc occurred at an efficiency of less then 1 % of the level seen for UDP-Gal. This is in contrast to LgtA from *N. meningiditis* that can use both UDP-GlcNAc and UDP-GalNAc as a substrate at almost the same efficiency (3).

Acceptor specificity.

The genetic data indicate that the natural acceptor for LgtE should be the LOS structure present in strain F62? lgtA? lgtE and the activity of this enzyme should be the addition of a galactose onto the ?-chain glucose. The LOS structure present in the wild-type F62 strain lacks a free ? or ? -chain glucose; hence it should not be a acceptor for LgtE. To test this prediction we used the LOS isolated from several derivatives of *N. gonorrhoeae* F62 strain and from *N. subflava* 44. The data presented in Table 3 show that LgtE could mediate the addition of radiolabeled Gal onto LOS isolated from a variety of strains in addition to the one that possessed an LOS structure that was predicted to be the biosynthetic intermediate. The efficiency of transfer of Gal residues to F62? lgtA? lgtE and F62 showed the same efficiency in the reactions containing decreased concentrations of acceptor LOS structures (Fig. 4). To prove that the LOS from both strains possessed the same acceptor specificity, an autoradiography experiment was performed. After radiolabeling, LOSs were analyzed by electrophoresis on a SDS-Tris-Tricine gel and then subjected to autoradiography. While the data clearly

indicated that radioactivity was incorporated into the LOS samples, the width of the signal on the autoradiogram prevented us from determining which LOS structure was serving as an acceptor (Data not shown)

As an alternate approach to demonstrating the addition of galactose onto Neisserial LOS's, we utilized unlabeled UDP-galactose as a substrate, and various LOS acceptors as recipients. Strain F62? lgtA? lgtE expresses an LOS structure that is the natural acceptor for LgtE. The data presented in figure 6A (Lanes 4 and 6) indicate that LgtE is able to modify LOS expressed by F62? lgtA? lgtE. These data indicate that under the experimental conditions we employed, LgtE appeared to mediate the addition of multiple galactose residues.

The data presented in table 3 indicate that strain LOS F62? lgtA? RfaK? LgtF LOS was able to serve as a acceptor for galactose from UDP-galactose. Since this strain's LOS is truncated and contain only two heptoses, it should not possess the acceptor structure for LgtE. In an effort to visualize this addition, whole cells were incubated with purified LgtE as described above. The data presented in Fig. 6, panel B, lane 2, clearly shows that incubation of whole cells with LgtE results in the appearance of a new LOS band. Since the mobility of this band is slower than that of LOS isolated from LOS F62? lgtA, it suggests that this LOS has had three or more galactose moieties added to it. However, only a small percentage of the molecules were acted upon by LgtE. While the addition of galactose onto this acceptor was unexpected, the addition of multiple galactose moieties is consisted with the data seen in figure 6, panel A.

In order to determine if LgtE mediates the addition of galactose onto both the ? and ? chains of gonococcal LOS, we tested whether purified LgtE was able to modify LOS isolated from *N. subflava* strain 44. This strain makes two LOS structures, with the smaller LOS structure possessing a glucose on both the ? and ? chains (28). LOS isolated from this strain fails to bind the Mab 3G9; reactivity with this Mab requires that the ? and ? chains consist of lactose (35). The data presented in Fig. 6, panel C indicate that LgtE is able to modify the LOS expressed by *N. subflava* 44.

In order to determine if LgtE added galactose onto both chains of LOS expressed by *N. subflava* 44, we used our *in vitro* assay to modify LOS isolated from this strain, and then tested the reaction product for its acquisition of reactivity with various Mabs. The data presented in Figure 7, panel A indicates that while F62? lgtA? lgtE LOS could be modified by purified LgtE, and that these reaction products now bind Mab 2-1-L8, the resulting reactants failed to bind Mab 3G9 (Figure 7, Panel B). *N. subflava* 44 LOS, when modified *in vitro*, clearly acquired the ability to bind both Mabs. From these data, we concluded that LgtE is able to mediate the addition of galactose onto both the ? and ? chain glucose moieties.

Discussion

Many genes have been identified that are involved in LOS expression in the *Neisseria*. However, few biochemical studies have been performed that demonstrate a clear structure/function relationship between the gene product and its predicted biochemical function. A set of linked genes (*lgtA-E*) has been identified that seem to encode the majority of glycosyl transferases needed to synthesize the carbohydrate portion of the ?-chain (11). The biochemical functions of LgtA-D have been demonstrated, and correspond to the functions inferred from analysis of mutations in these genes (3, 30, 31). While there is significant genetic evidence supporting the function of LgtE as the glycosyl transferase responsible for the addition of galactose ?-1, 4 to glucose (6, 11, 14, 26), biochemical data supporting this assignment has been lacking. Furthermore, since the addition of galactose ?-1, 4 to glucose can occur on both the ? and ? chains, it is unclear if LgtE is responsible for both of these biosynthetic processes.

Erwin et al. (6) demonstrated that in *lgtE* mutants, galactose was not added to either the ? or ? chains. While their data clearly indicated that LgtE activity is required for galactose addition, the results could not rule out the possibility that the ?-chain addition of galactose was mediated by an additional unlinked enzyme, whose activity required before the addition of the ?-chain galactose could occur. As a first step in characterizing the *lgtE* gene product, we used a gene cloning strategy to isolate a functional LgtE protein. While we were readily

able to isolate seemingly intact DNA fragments into a *lac*-regulated expression vector, most of the recombinant clones failed to express detectable levels of protein, even after induction with IPTG. Furthermore, expressing clones seemed to lose the ability to express the protein after prolonged incubation, or storage. While we did not investigate the reason for this instability, we believe that it is probably related to the fact that the LgtE protein is able to modify LPS biosynthetic intermediates in *E. coli*, and the accumulation of these modified intermediates are toxic.

Strain F62? lgtA? lgtE produces an LOS that contains a single glucose on the ?-chain and a single phosphate as the ?-chain (28). As such, this strain produces an LOS with the predicted acceptor structure for LgtE. The data shown in Table 3 and Fig. 6 demonstrate that this LOS can serve as an acceptor for galactose from UDP-Galactose. Surprisingly, many other LOS structures were also able to serve as acceptor molecules for LgtE. Furthermore, in our *in vitro* experiments, multiple LOS bands were obtained after incubation of F62? lgtA? lgtE LOS with purified LgtE and UDP-galactose. The SDS-PAGE profile of the elongation product suggests that two galactose residues are added onto F62? lgtA? lgtE LOS.

The ability of LgtE to add galactose onto a variety of LOS structures was unexpected. Most surprising perhaps was its ability to add sugars onto LOS isolated from LOS F62? lgtA? RfaK? LgtF. The OS of this LOS consists of two heptose molecules, and elongation of this LOS by LgtE *in vivo* has not been reported. Furthermore, when galactose is added to the base OS, the resulting band has an SDS-PAGE mobility consistent with the addition of three galactose residues. These data indicate that depending on the nature of the starting LOS, it appears that 2 or 3 galactose residues were added. By varying the amount of LgtE added to the reaction mixture, we could change the relative ratio of the elongation product; more enzyme increased the intensity of the higher molecular weight components (data not shown). Since the aberrant additions only occurred during *in vitro* reactions, and were influenced by the amount of exogenous LgtE added, this suggests that *in vivo*, the

amount of enzyme expressed is quite low. Additional studies from our laboratory indicate that this hypothesis is correct (DCS, unpublished observations).

Wakarchuk and coworkers demonstrated that purified LgtE was unable to add galactose onto synthetic ?-Glc acceptors (30). In light of our observation that LgtE is able to mediate the addition to a variety of molecules that possess a lipid base, indicates that the failure observed by Wakarchuk et al. was most likely due to the inability of the synthetic intermediates to form a stable interaction with LgtE.

Certain LOS immunotypes of *N. meningitidis* possess LOS with variations in the structure of the ?-chain. The observation that excess amount of LgtE in a reaction results in homopolymer additions suggests a mechanism that can explain how the L5 LOS immunotype might arise. If LgtF were overexpressed , it might result in the addition of a second glucose residue at the base of the ?-chain. It is interesting to note that the SDS-PAGE mobility of LOS isolated from L5 strains possess a significant amount of a truncated biosynthetic product. This may reflect the limitation in the ability of LgtE to add galactose onto the diglucosyl structure.

The ability of purified LgtE to modify LOS isolated from *N. Subflava* 44 to reactivity with Mab 3G9 clearly indicates that LgtE is able to mediate the addition of galactose onto the ?-chain. Since LgtG expressing strains that express LgtA (*N. gonorrhoeae* 15253 or F62LgtG+) do not add sugars onto the ?-chain beyond the galactose, this indicates that LgtA possesses a structural requirement that biases its addition to the ?-chain. It is possible that overexpression of LgtA could result in the elongation of both the ? and ? chains, similar to what we have seen with LgtE. Since the level of expression of the various glycosyl-transferases in the gonococcus is quite low (DCS, unpublished observations), it further suggests an additional mechanism of phenotypic modulation, where subtle changes in the growth rate of the organism would modulate the absolute level of the various proteins, and these results would be translated into small differences in LOS expression. It has been

shown by several investigators that alteration in growth conditions effects LOS expression (8, 19, 20, 22), and we believe that this modulation is due to small changes in the level of the various glycosyltransferases.

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Figures Legends

Fig. 1. Possible LOS structures seen in *N. gonorrhoeae*. The data in the figure are a compilation of published LOS structures. The question marks in the figure indicate structures that have been identified, but the gene responsible for the addition and the exact chemical composition of the sugar remain unknown. The labels above the structures indicate various monoclonal antibodies that have been used as markers for the presence of various components that terminate with te indicated sugars. The genes predicted to be responsible for the various sugar additions are indicated in italic.

Fig. 2. SDS-PAGE of purified LgtE. *E. coli* strains were grown to mid log phase and LgtE expression was induced by the addition of IPTG. Recombinant proteins were purified on a Nickel column. The lanes represent: Lane 1, molecular weight markers; lane 2 and 3, purified enzyme (two consecutive fractions obtained after elution from the column. The sizes of the molecular mass markers are given in kDa. A single protein of 35 kDa is present in the preparation.

Fig. 3. Effect of the pH on the activity of recombinant LgtE. The activity was assayed using 1.5 ? g of LOS purified from *N. gonorrhoeae* F62? LgtA? LgtE strain as an acceptor using standard incubation conditions except for the variation in buffer pH.

Fig. 4. Linearity with time of the reaction catalyzed by recombinant LgtE. Standard assay conditions were employed using 1.5 ? g of LOS purified from *N. gonorrhoeae* FA62? LgtA? LgtE strain.

Fig. 5. Dependence of the efficiency of transfer of donor UDP-Gal residue to different concentrations of the LOS structures isolated from F62? LgtA? LgtE and F62 strains. The ability of the LgtE protein to transfer galactose to various concentrations of LOS was determined by incubating in 30 ? l of reaction buffer (50 mM MES buffer, pH 7.3, 10 mM $MnCl_2$, and 1 mM UDP-galactose) different concentrations of LOS. The

reactions were incubated for 2 hr. at 30 °C and terminated by the addition of 10 ? l of 20 % SDS and heating at 70 °C for 3 min. (triangles denotes LOS from F62, squares from F62? LgtA? LgtE)

Fig. 6. Transfer of galactose into Neisserial LOS. The ability of the LgtE protein to transfer galactose to various LOS structures was determined by incubating in 30 ? l of reaction buffer (50 mM MES buffer, pH 7.3, 10 mM $MnCl_2$, 5 x 10^6 whole cells, and 1 mM UDP-galactose). The reactions were incubated overnight at 30 °C and terminated by the addition of 10 ? l of 20 % SDS and heating at 70 °C for 3 min. A 10 ? l of each reaction was analyzed on a 16.5%Tris-tricine gel. Panel A. The lanes represent LOS isolated from: 1) F62? LgtA; 2) F62? LgtA? LgtE; 3) F62? LgtA? LgtE incubated with reagents, minus LgtE; 4) F62? LgtA? LgtE, plus 1 ? l LgtE preparation; 5) F62? LgtA? LgtE incubated with reagents, minus LgtE; 6) F62? LgtA? LgtE, plus 7 ? l LgtE preparation. Panel B. The lanes represent LOS isolated from: 1) F62? rfaKA; 2) F62? rfaK incubated with reagents, plus 7 ? l LgtE preparation; 3) F62? LgtA; and 4) F62? LgtA? LgtE. Panel C. The lanes represent: 1) F62? LgtA; 2) *N. subflava* 44; 3) *N. subflava* 44 incubated with LgtE; 4) F62? LgtA? LgtE.

Fig. 7. Reactivity of in vitro modified LOS with various Mabs. Cells were incubated with purified LgtE (1 or 7 ? l) plus UDP-galactose in standard reaction buffer, and an aliquot was spotted onto a nitrocellulose filter. Reactivity to various Mabs was determined using our colony blotting procedure. Panel A represents samples that had been exposed to Mab 2-1-L8; Panel B represents samples that had been exposed to Mab 3G9.

Table 1. Effect of divalent cations on the activity of recombinant LgtE

Cation added (15 mM)	Relative activity (%)
None	< 1
Mn^{2+}	100
Mg^{2+}	8
Ca^{2+}	< 1
Zn^{2+}	< 1

Standard assay conditions were employed using 1.5 ? g of LOS isolated from *N. gonorrhoeae* F62? LgtA? LgtE.

Table 2. Nucleotide-sugar substrate specificity of recombinant LgtE

Substrate (0.18 mM)	Relative enzyme activity (%)
UDP-Gal	100
UDP-GalNAc	< 1
UDP-Glc	< 1

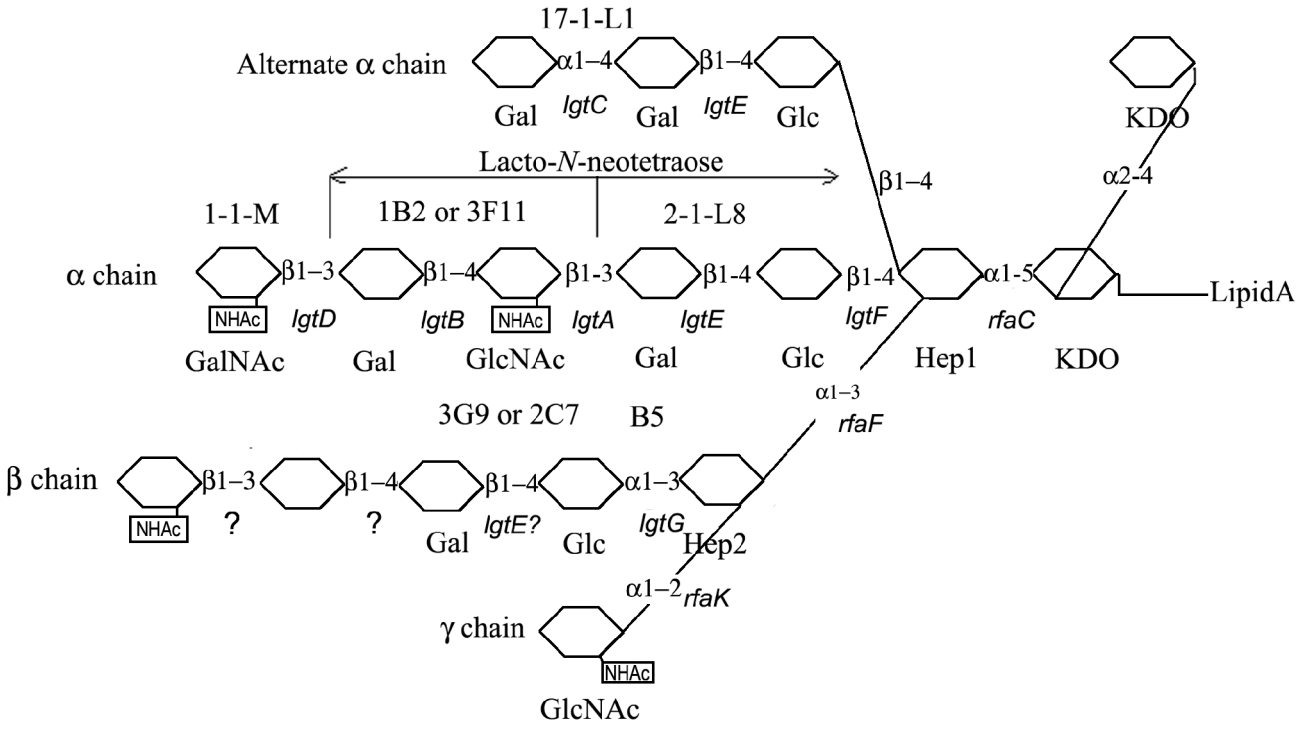
Standard assay conditions were employed using 1.5 ? g of LOS isolated from *N. gonorrhoeae* F62? LgtA? LgtE.

Table 3. LOS acceptor specificity of recombinant LgtE protein

Acceptor (5 ? g)	Relative activity (%)
LOS F62? LgtA? LgtE	100
LOS F62	80
LOS F62? LgtA? RfaK? LgtF	70
LOS F62? LgtA? LgtG	50
LOS *N. subflava* 44	50

Standard assay conditions were used. Reactions differed only in the LOS that was employed.

Figure 1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α1–4
β1–4
Gal
lgtC
Gal
lgtE
Glc
KDO
Lacto-N-neotetraose
β1–4
α2-4
1-1-M
1B2 or 3F11
2-1-L8
α chain
β1–3
β1–4
β1-3
β1-4
β1-4
α1-5
LipidA
NHAc
lgtD
lgtB
NHAc
lgtA
lgtE
lgtF
rfaC
GalNAc
Gal
GlcNAc
Gal
Glc
Hep1
KDO
α1–3
rfaF
3G9 or 2C7
B5
β chain
β1–3
β1–4
β1–4
α1–3
NHAc
?
?
Gal
lgtE?
Glc
lgtG
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α1–2
rfaK
γ chain
NHAc
GlcNAc

Figure 2

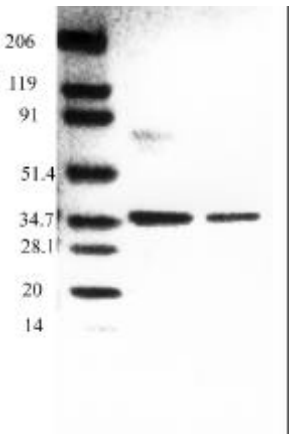

Figure 3

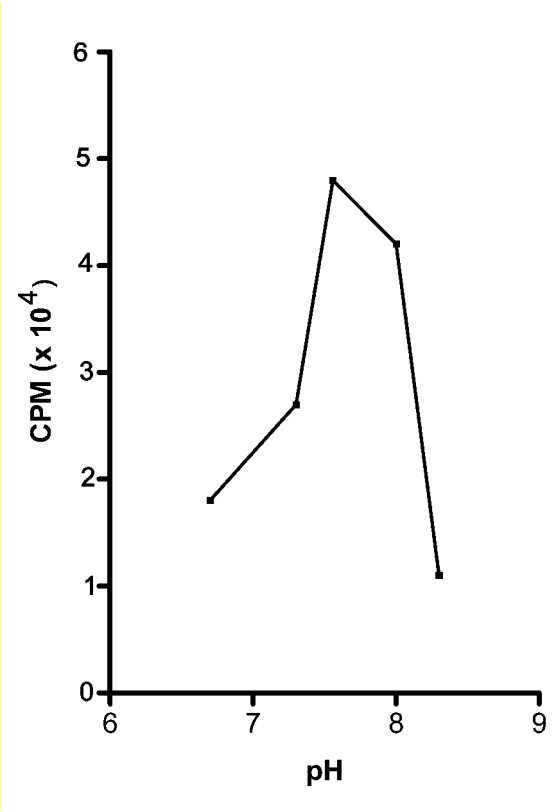

Figure 4

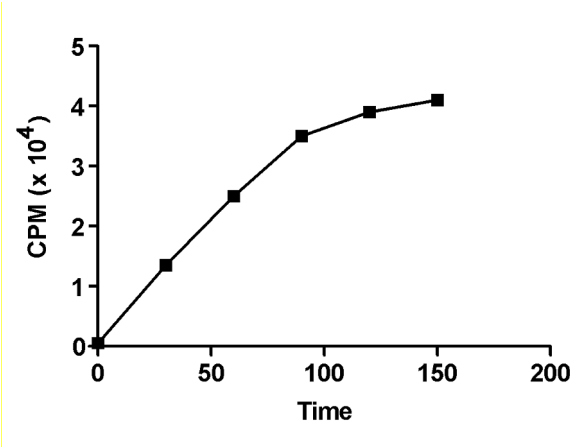

Figure 5

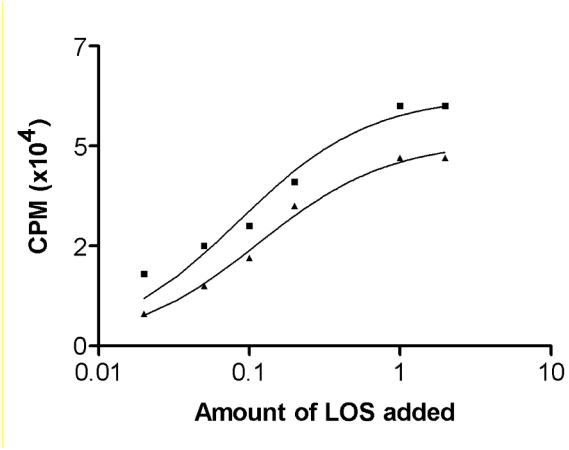

Figure 6

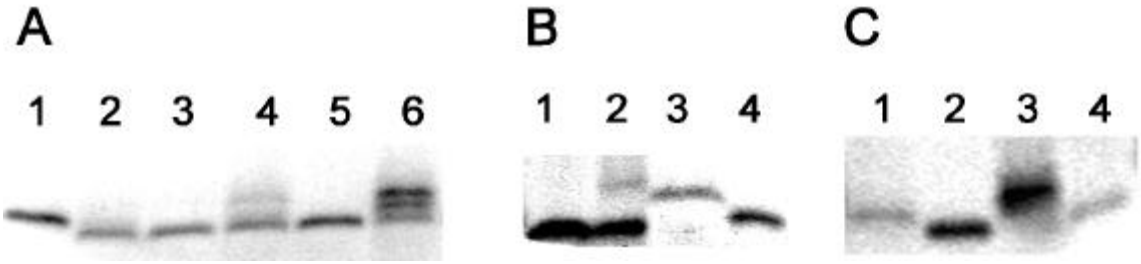

Figure 7