Glycobiology vol. 12 no. 9 pp. 523–533, 2002

Neisseria gonorrhoeae strain PID2 simultaneously expresses six chemically related lipooligosaccharide structures

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Received on November 21, 2001; revised on February 28, 2002; accepted on February 28, 2002

Neisseria gonorrhoeae **strain PID2 was isolated from a woman suffering from pelvic inflammatory disease. When LOS expressed by this strain is analyzed on SDS–PAGE gels, at least six different lipooligosaccharide (LOS) components are visualized. We characterized the LOSs made by this strain by exoglycosidase digestion, sugar composition analysis, mass spectrometry, and analysis of the genes needed for its synthesis. DNA sequence analysis showed that the *lgt* gene cluster in this strain has undergone a rearrangement and that it possesses two copies of *lgtA*, one copy of *lgtB* and *lgtC*, and a hybrid gene containing sequences from *lgtB* and *lgtE*. We determined that the hybrid *lgtB/E* gene retained the *lgtE* gene function. DNA sequence analysis of the gene organization suggested that an intramolecular recombination between *lgtA* and *lgtD* and *lgtB* and *lgtE* had occurred via homologous recombination between similar sequences. Our studies demonstrated that fluorophore-assisted carbohydrate electrophoresis can be utilized to rapidly determine the composition of LOS. By combining exoglycosidase digestion, in combination with mass spectrometry analysis and compositional analysis, the data indicate that all of the LOS components produced by PID2 extend off of the α chain. The longest α chain oligosaccharide structure is Gal–GlcNAc–Gal–GlcNAc–Gal–Glc–Heptose I, and the six LOS components are built up by sequentially adding sugars onto the first heptose. PID2 LOS is the first Neisserial LOS to be shown to be devoid of phosphoethanolamine modifications. Because PID2 can surface express its LOS, it indicates that the addition of phosphoethanolamine is not required for LOS surface expression.**

Key words: carbohydrate epitopes/FACE analysis/ lipooligosaccharide structure/LOS/mass spectrometry

Introduction

Neisseria gonorrhoeae causes a variety of diseases. Lipooligosaccharide (LOS), one of its major surface structures, is an important factor in determining the clinical outcome of infection (Apicella and Mandrell, 1989; Apicella *et al.*, 1986; Estabrook *et al.*, 1997; Griffiss, 1995; Gulati *et al.*, 1996; John *et al.*, 1999; Schneider *et al.*, 1991). LOS is a family of complex glycolipid molecules (Apicella and Mandrell, 1989; Apicella *et al.*, 1986; Estabrook *et al.*, 1997; Gibson *et al.*, 1989; Griffiss, 1995; Gulati *et al.*, 1996; John *et al.*, 1991; Schneider *et al.*, 1991; Yamasaki *et al.*, 1991) that possesses many antigenic determinants that are important in natural and acquired immunity (Joiner *et al.*, 1985; Rice and Kasper, 1977; Yamasaki *et al.*, 1994, 1999).

Neisserial LOS has been examined by chemical (Gibson *et al.*, 1993; Griffiss *et al.*, 1987; John *et al.*, 1991), biological (Griffiss *et al.*, 1994), and immunological techniques (Mandrell *et al.*, 1988; Schneider *et al.*, 1985), as well as through visualization by silver staining of samples analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Kim *et al.*, 1988; Schneider *et al.*, 1984). It is an amphipathic molecule that consists of a hydrophilic carbohydrate moiety and a hydrophobic lipid A moiety. These domains are linked together through the acidic sugar 3-deoxy-D-manno-2-octulosonic acid (KDO).

The oligosaccharide (OS) is multiantennaeary with chain extensions from each of the two heptose residues, forming three elongation centers defined as the α, β, and γ chains (Gibson *et al.*, 1993; John *et al.*, 1991; Phillips *et al.*, 1990; Yamasaki *et al.*, 1991a,b). The α chain elongates from the first heptose (Hep I) and may contain several structures that are mimics of human carbohydrate epitopes (Apicella and Mandrell, 1989; Moran *et al.*, 1996). Its synthesis is directed by a gene cluster (*lgtA-E*) (Gotschlich, 1994), with structural variations being regulated by changes in the number of guanines contained in the polyguanine tract of three of the genes (*lgtA*, *C*, and *D*) (Burch *et al.*, 1997; Danaher *et al.*, 1995; Yang and Gotschlich, 1996). β chain (extending from the second heptose [Hep II]) expression is modulated by the expression of *lgtG* (Banerjee *et al.*, 1998) and may be composed of single glucose, lactose, or additional sugars added onto Glc (Gibson *et al.*, 1989; Yamasaki *et al.*, 1994). The γ chain has been found in all strains examined and consists of a GlcNAc or GlcNAc (OAc) linked to Hep II. Occasionally, this chain is elongated by the addition of galactose (Griffiss *et al.*, 2000). Some positions of Hep I and Hep II are also available for phosphoethanolamine (PEA) or phosphate addition. Most of the genes that mediate gonococcal and meningococcal LOS biosynthesis have been cloned and characterized (Banerjee *et al.*, 1998; Drazek *et al.*, 1995; Gilbert *et al.*, 1996; Gotschlich, 1994; Kahler *et al.*, 1996; Levin and Stein, 1996; Sandlin *et al.*, 1993; Sandlin and Stein, 1994; Stojiljkovic *et al.*, 1997; Zhou *et al.*, 1994).

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N. gonorrhoeae strain PID2, isolated from a women suffering from pelvic inflammatory disease, produces at least six different LOS components when these molecules are separated on an SDS–PAGE gel. The mobility of the four slowest-migrating components are similar in mobility to LOS components isolated from strain MS11mkC, however, LOS isolated from MS11mkC binds mAbs 2-1-L8 and B5, whereas LOS isolated from PID2 fails to bind these monoclonal antibodies (mAbs). This indicates that there are a structural differences between LOS isolated from PID2 and MS11mkC. This study was undertaken to determine the structure of the various LOS components isolated from PID2 and to identify key genes needed for its synthesis.

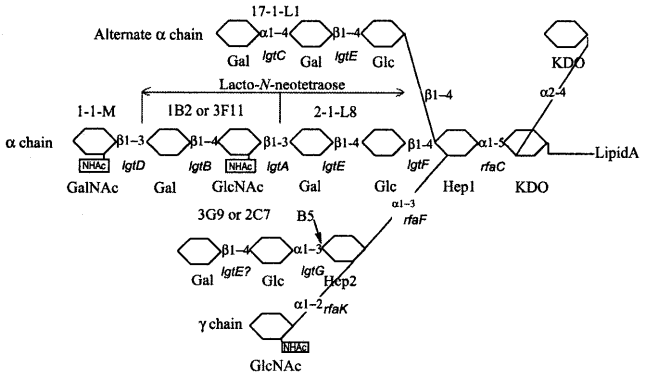

Fig. 1. Schematic representation of Neisserial LOS structures. The structures presented represent the various glycoforms that have been identified in *N. gonorrhoeae* strain F62. The alternate α chain structure is made when *lgtC* encodes a functional protein. The β chain is added when *lgtG* encodes a functional protein. The names of the genes whose product mediate the various additions are as indicated. 17-1-L1, 1-1-M, 1B2 or 3F11, 2-1-L8, 3G9, 2C7, and B5 represent the names of various monoclonal antibodies. The sugars needed for antibody recognition are as shown (see text for details).

Results

Structure and mAb reactivity of LOS

The structure of LOS isolated from *N. gonorrhoeae* strain F62 has been previously determined (Yamasaki *et al.*, 1991a,b), and a schematic of the various LOS isoforms expressed by this strain, the identification of genes needed for its synthesis, and the structures recognized by various mAbs used in this study is shown in Figure 1. (The structure LOS isolated from MS11mkC differs from that of F62 in that is has been shown to express an additional component that adds an additional lactosamine onto the α chain.) The mAb 4C4 recognizes LOS with only one glucose on the α chain (Noda *et al.*, 2000). The mAb 2-1-L8 binds to an epitope that is formed when a lactosyl group is attached to Hep I and PEA is attached to Hep II of the basal OS (Banerjee *et al.*, 1998; Schneider *et al.*, 1984). The mAb 1B2 recognizes an epitope on LOS where the lactosamine (Galβ1→4GlcNAc) is attached to the lactosyl structure needed for mAb 2-1-L8 recognition (Kim *et al.*, 1988; Schneider *et al.*, 1985). This tetrasaccharide is a perfect mimic of lacto-*N*-neotetraose of the sphingolipid paragloboside (Mandrell *et al.*, 1988). In LOS, this tetrasaccharide frequently bears an additional GalNAc at its terminus, and this structure is recognized by mAb 1-1-M. In some strains, an alternative α chain is found that has the structure Gal→Gal→Glc→Hep I, and this structure is recognized by mAb 17-1-L1. The mAbs 2C7 and 3G9 react with LOS that contain a lactosyl group on the β chain (Yamasaki *et al.*, 1999). The mAb B5 (Plested *et al.*, 1999) binds to LOS that contains a PEA on carbon 3 of Hep II and mAb 25-1-LC1 (Tong *et al.*, 2001) binds to LOS that terminates with glucose on the β chain, when the α chain is a either a lactose or a glucose.

Analysis of LOS biosynthetic genes of PID2

In F62, the proteins encoded by *lgtF* and *rfaK* are needed to make the core LOS structure, the LgtG protein is required for β chain extensions, and the proteins encoded by the *lgt* gene cluster (including *lgtA*, *B*, *C*, *D*, and *E*) are needed to make the various forms of the α chain. Southern hybridization experiments demonstrated that PID2 possessed DNA sequences homologous to all of these genes (data not shown). To characterize the *lgt* gene cluster of PID2, a 4.8-kb polymerase chain reaction (PCR) fragment containing this region was amplified and cloned into pGEM-7Zf(-) (named pPID2*lgt*). Restriction analysis of the amplicon indicated that it contained a *Cla*I site, located in the middle of the amplicon in a region that should encode *lgtC*. Because Southern hybridization experiments with the probe specific for *lgtA* suggested that this clone had a duplicated *lgtA* gene, we separated the two duplicated genes to determine the entire DNA sequence. Plasmid pPID2*lgt* was digested with *Eco*RI and *Cla*I or *Bam*HI and *Cla*I and the resulting fragments subcloned into pGEM-7Zf(-), generating pPID2*lgt*α and pPID2*lgt*β respectively. DNA sequence analysis of these two subclones showed that the PID2 *lgt* gene cluster contained five open reading frames, corresponding to *lgtA1*, *lgtB*, *lgtC*, *lgtA*2, and *lgtB/E* (data not shown).

The DNA sequence of *lgtB* indicated that it should encode a functional protein. The polyguanine tract contained in *lgtC* was composed of nine guanines and should produce a frame shift mutation in LgtC. Additionally, at the 3′ end of the *lgtC* coding sequence, there were some differences relative the DNA sequence seen for *lgtC* of F62 (Gotschlich, 1994). The DNA sequence obtained from the cloned fragment indicate that *lgtA1* and *lgtA2* have more than 99% identity with each other and would encode nonfunctional proteins (*lgtA1* contained a polyguanine tract of 14 bp and *lgtA2* contained a polyguanine tract of 9 bp). However, if *lgtA1* contained a polyguanine tract of 13 guanines, it would produce an in-frame LgtA protein; *lgtA2* would need a polyguanine tract of 10 guanines to produce a functional protein. The last gene in the cluster is a hybrid between *lgtB* and *lgtE*; the first 560 bp are identical to its own *lgtB* gene, and the last 300 bp would encode a peptide identical to F62*lgtE* at the amino acid sequence level.

PID2 SDS–PAGE profile and mAb binding

N. gonorrhoeae PID2 expresses at least six LOS components that can be separated on Tris-tricine gels. As shown in Figure 2, from top to bottom we have numbered them as 1 to 6. Bands 1 and band 3 react with mAb 1B2 (Figure 2b, lane 5). Others have shown that band 6 can bind mAb 4C4, which recognizes LOS with only one glucose on the α chain (Noda *et al.*, 2000). None of the other bands were able to bind the mAbs 2-1-L8,

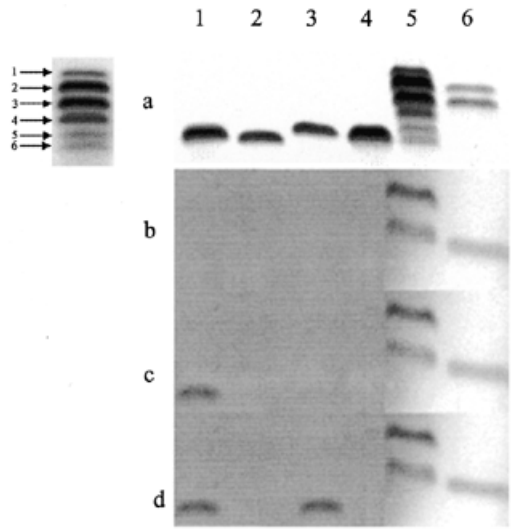

Fig. 2. SDS–PAGE and western blot analysis of LOS isolated from *N. gonorrhoeae* PID2 and its transformation mutants. SDS–PAGE profile of *N. gonorrhoeae* PID2. The six bands of PID2 LOSs are labeled as 1–6 from top to bottom. (**a**) A silver stain of a SDS–PAGE gel. (**b**, **c**, and **d**) Western blot of a duplicated gel obtained with mAb 1B2, B5, and 25-1-LC1 sequentially. The lanes represent: (1) mAb B5$^+$ control: *N. gonorrhoeae* F62Δ*lgtA*Δ*lgtF*; (2) PID2FΩ; (3) PID2EΩ#13; (4) PID2EΩ#1; (5) PID2; (6) mAb 1B2$^+$ control: *N. gonorrhoeae* F62.

17-1-L1, 3G9, B5, and 25-1-LC1 (Figure 2b and data not shown).

Insertion omega cassette into PID2 lgtF *and* lgtB/E

To determine if PID2 LOS contained α or β chain extensions, we generated a PID2 mutant with a nonfunctional *lgtF* (deficient in adding the first glucose onto the α chain) and a mutant with a nonfunctional *lgtE* (deficient in adding the heptose-proximal galactose of lacto-*N*-neotetraose). If all of the LOS components expressed by PID2 contained extended α chains, we expected strains with a nonfunctional *lgtF* or *lgtE* to express only truncated LOS that would have SDS–PAGE mobility and mAb binding profiles similar to structurally defined derivatives of F62.

The insertional disruptions of these genes, contained on plasmids pF62*lgtF*Ω and pF62*lgtDE*Ω, were introduced into the PID2 chromosome by transformation. Because these plasmids cannot replicate in the gonococcus, spectinomycin-resistant colonies can only arise via homologous recombination between the plasmid DNA and the chromosome. Because pF62*lgtF*Ω has the omega cassette inserted into the *lgtF* gene without transcription termination signals, this construct should have no polar effects; thus only the *lgtF* gene is nonfunctional because of the insertion and *rfaK* (necessary for γ chain biosynthesis) retains its function. The plasmid pF62*lgtDE*Ω has the complete omega cassette (including the transcription termination signals) inserted between the *lgtD* and *lgtE* genes; thus the *lgtE* gene is not transcribed, due to the polar effect of the insertion. (We have shown that the insertion of this cassette at this point in a variety of gonococcal strains completely oblates the expression of *lgtE*; unpublished data).

After introducing the desired insertion into the gonococcal chromosome by DNA-mediated transformation, several transformants were isolated; each was screened for reactivity with various mAbs. All transformants generated by pF62*lgtF*Ω possessed the same SDS–PAGE profile and no longer reacted with any of the mAbs specific for the parental LOS. One mutant was named PID2FΩ (see Figure 2b, lane 2). When we tested transformants generated by pF62*lgtDE*Ω for reactivity with mAb 25-1-LC1, of the hundreds of spectinomycin-resistant transformants we obtained, we identified a single transformant that had acquired the ability to bind this mAb (see Figure 2b, lane 3). The acquisition of mAb 25-1-LC1 reactivity by this mutant, named PID2EΩ#13, probably arose via a spontaneous phase shift in *lgtG*. One mutant, representing the class that failed to bind mAb 25-1-LC1 was named as PID2EΩ#1 (see Figure 2b, lane 4). The SDS–PAGE and western blot analysis of LOS isolated from these strains demonstrate that with a nonfunctional *lgtF*, as seen in strain PID2FΩ (Figure 2b, lane 2), only a single LOS component is expressed, and it possesses a mobility similar to F62Δ*lgtA*Δ*lgtF* LOS (Figure 2b, lane 1). This LOS did not bind mAb B5, indicating that this LOS does not contain a PEA on the 3-carbon of Hep II. Because it did not bind mAb 25-1-LC1, it indicated that this strain also lacked a glucose on the β chain.

Of the two kinds of mutants generated when the omega cassette was inserted before the *lgtE* gene, only one of them, PID2EΩ#13, was mAb 25-1-LC1 reactive; the others (represented by the strain PID2EΩ#1) all possessed the same SDS–PAGE profile. Because the β chain glucose is necessary for binding by mAb 25-1-LC1 (Tong *et al.*, 2001), we concluded that *lgtG* is out of frame in PID2, and the one transformant we isolated that acquired the ability to bind mAb 25-1-LC1 represented a spontaneous frame shift in the polycytosine tract of *lgtG*.

Exoglycosidase digestion of PID2 LOS

To further characterize the LOS made by PID2, two exoglycosidases were used to digest purified PID2 LOS. Jack bean meal β-galactosidase can readily hydrolyze nonreducing terminal Gal β1-6 GlcNAc and Gal β1-4 GlcNAc. Jack bean meal β-*N*-acetylhexosaminidase can cleave nonreducing terminal β1-2, 1-3, 1-4, and 1-6 linked *N*-acetylglucosamine and *N*-acetylgalactosamine residues. However, this enzyme has different pH optima for the hydrolysis of *p*-nitrophenol (pNP) *N*-acetyl-β-D-glucosaminide (pH 5.0–6.0) and pNP *N*-acetyl-β-D-galactosaminide (pH 3.5–4.0). Thus, by controlling the digestion conditions, we could differentiate between terminal GlcNAc and GalNAc residues. *Streptomyces plicatus* β-*N*-acetylhexosaminidase cleaves terminal GlcNAc and GalNAc with equal efficiency.

Purified LOS from different strains were digested by these exoglycosidases and analyzed on a SDS–PAGE gel. The results shown in Figure 3A show that β-*N*-acetylhexosaminidase (from jack bean meal, pH 5.0) could not excise the terminal GalNAc from F62 LOS (Figure 3A, lane 7), whereas *S. plicatus* β-*N*-acetylhexosaminidase could (Figure 3A, lane 6). Jack bean meal β-galactosidase was able to remove Gal from F62Δ*lgtD* LOS (Figure 3A, lane 4), however, this β-galactosidase could not cleave Gal from F62Δ*lgtA* (Figure 3A, lane 3). Figure 3B shows the digestion results from PID2 LOS. When treated with β-galactosidase, PID2 LOS bands 1 and 3 disappeared and more of bands 2 and 4 appeared (Figure 3B, lane 4). When digested with jack bean meal β-*N*-acetylhexosaminidase, bands 2 and 4 were degraded, and more of band 3 and 5 appeared on the gel (Figure 3B, lane 3). When treated

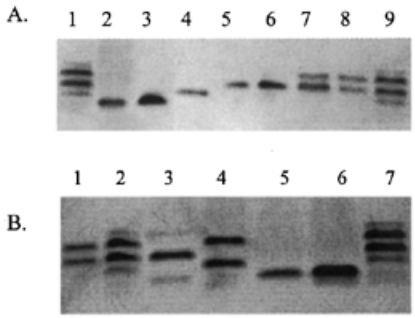

Fig. 3. Exoglycosidases digestion of LOS. (**A**) The lanes represent (1) PID2; (2) F62Δ*lgtA*; (3) F62Δ*lgtA* + β-galactosidase; (4) F62Δ*lgtD* + β-galactosidase; (5) F62Δ*lgtD*; (6) F62 + *Streptomyces plicatus* β-*N*-acetylhexosaminidase; (7) F62 + jack bean meal β-*N*-acetylhexosaminidase; (8) F62; (9) PID2. (**B**) The lanes represent (1) F62; (2) PID2; (3) PID2 + jack bean meal β-*N*-acetylhexosaminidase; (4) PID2 + β-galactosidase; (5) PID2 + β-galactosidase then jack bean meal β-*N*-acetylhexosaminidase; (6) PID2 + jack bean meal β-*N*-acetylhexosaminidase then β-galactosidase; (7) PID2.

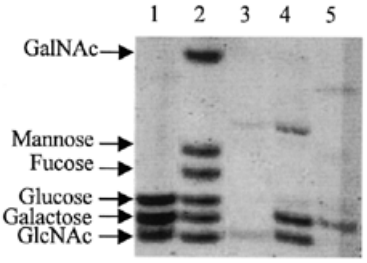

Fig. 4. FACE analysis of PID2 LOS components. The lanes represent: (1) PID2 LOS hydrolysis with 4 N HCl for 2 h; (2) MONO ladder standard; (3) PID2 LOS digested with jack bean meal β-*N*-acetylhexosaminidase; (4) PID2 LOS digested with jack bean meal β-*N*-acetylhexosaminidase and β-galactosidase; (5) PID2 LOS digested with β-galactosidase.

with β-galactosidase first and then β-*N*-acetylhexosaminidase or vice versa, only bands 5 and 6 were seen on the gel (lane 5 and 6). Because PID2 LOS was degraded with jack bean meal β-*N*-acetylhexosaminidase, but F62 LOS was not, we concluded that the PID2 band 2 and the larger F62 band have different terminal sugars, with the PID2 band terminating in GlcNAc and the F62 band terminating with GalNAc.

FACE monosaccharide composition analysis of PID2 LOS

As an alternate means of demonstrating the nature of the sugars contained in PID2 LOS, its LOS was subjected to fluorophore-assisted carbohydrate electrophoresis (FACE) monosaccharide composition analysis. The data shown in Figure 4, lane 1 demonstrate that only three kinds of sugars exist in PID2 LOSs: Glc, Gal, and GlcNAc. FACE analysis was also used to confirm the results of the exoglycosidase digestions. The data presented in Figure 4 also show that β-galactosidase excised Gal from PID2 (Figure 4, lane 5) and that jack bean meal β-*N*-acetylhexosaminidase released GlcNAc from PID2 (Figure 4, lane 3). Because the γ chain GlcNAc is found in an α linkage to Hep II, the GlcNAc released by β-*N*-acetyl-hexosaminidase was only from the α chain. Treatment with both exoglycosidases released Gal and GlcNAc (Figure 4, lane 4).

Structural validation of PID2 OSs

From the staining pattern observed on SDS–PAGE gels, enzymatic digestion, sugar composition analysis, and manipulations of genes involved in LOS biosynthesis, a specific set of LOS structures was anticipated. To confirm these expectations, mass spectrometry (MS) was performed on total extracts following mild hydrolysis and methylation. These procedures have provided a simple and direct cleanup for improved detailed characterization of *N. gonorrhoeae* OSs by MS^n (Muhlecker *et al.*, 1999). Unfortunately, the basic conditions of methylation degrades the reducing-end KDO (peeling), adding complexity to the mass spectrum (Tong *et al.*, 2001). These modified reducing-end structures are readily discerned in the MS profile, however, and their presence provides alternative structures to confirm the OS (Figure 5, *m/z* 1400 to 1800). The indigenous OS structures were easily identified as a series of monomer extensions (Hex, HexNAc), frequently referred to as glycoforms. Thus the major ions in the spectrum can be identified with one of three sets; KDO at the reducing terminus; *m/z* 1290.7, 1494.8, 1739.8, and 1943.9; and two additional ion sets *m/z* 1174.6, 1379.7, 1623.8, 1827.9 and *m/z* 1014.4, 1218.6, 1463.6, 1667.8 (Figure 5A), which can be accounted for as a modified KDO (KDO_f), and the total loss of KDO from the reducing-end terminus. The mass increments within each set of structures are equal to a Hex, Hex, HexNAc, and Hex residue.

To confirm these expectations, individual parent ions shown in Figure 5A were studied in detail by MS^N. Detailed analyses of two components are presented from the KDO_f ion series (*m/z* 1174.6, 1379.7; Figure 5A, 5B, 5C). Immediately identified in each spectrum was a branched OS structure characterized by the independent loss of two nonreducing termini from each parent, $-_t$Hex, $-_t$HexNAc. Such information is available only from methylated samples, which increments remote site losses by 14 amu (identified with the subscript $_T$). Further examination of these spectra differentiates antennal fragments, allowing a positioning of monomer increments to specific antennae. These features were observed with the Hep I–Hep II rupture (*m/z* 530.2/667.3, Figure 5B). As an example, compare the ion *m/z* 667.3 (Figure 5B) to *m/z* 871.4 in Figure 5C, which was an increment of 204 amu, indicating a hexose extension to the α chain. In contrast, the *m/z* 530.2 ion in both spectra remains unchanged. The ladder profile observed with the SDS–PAGE gels, the data presented in Figure 5A, and these results position the mass increments (Figure 5B) on the α chain. This observation was further supported by activation and collision-induced dissociation analysis of the highest-molecular-weight OS detected, *m/z* 967.5^{2+} (Figure 5D). This spectrum supports a sequence, HexNAc→Hex→HexNAc→Hex→Hex→(HexNAc→Hep)Hep, and the proposed structure.

The remote neutral losses that provide the α chain sequence, *m/z* 1654.6/1449.9/1204.5, were unusual considering the presence of GlcNAc glycosidic linkages, which usually rupture to dominate a spectrum. To confirm the proposed sequence and structure of this ion (*m/z* 967.5^{2+}), an alternative product ion (*m/z* 838.5^{+2}) was selected and activated, MS^3 (Figure 5E). This ion represents the loss of a single HexNAc residue ($_t$HexNAc), and the product ion spectrum clearly indicates this residue originates from the α chain. Subsequent glycosidic rupture proximal to the GlcNAc moiety, *m/z* 472.2/1204.5, dominates the product ion spectrum and confirms the α chain sequence.

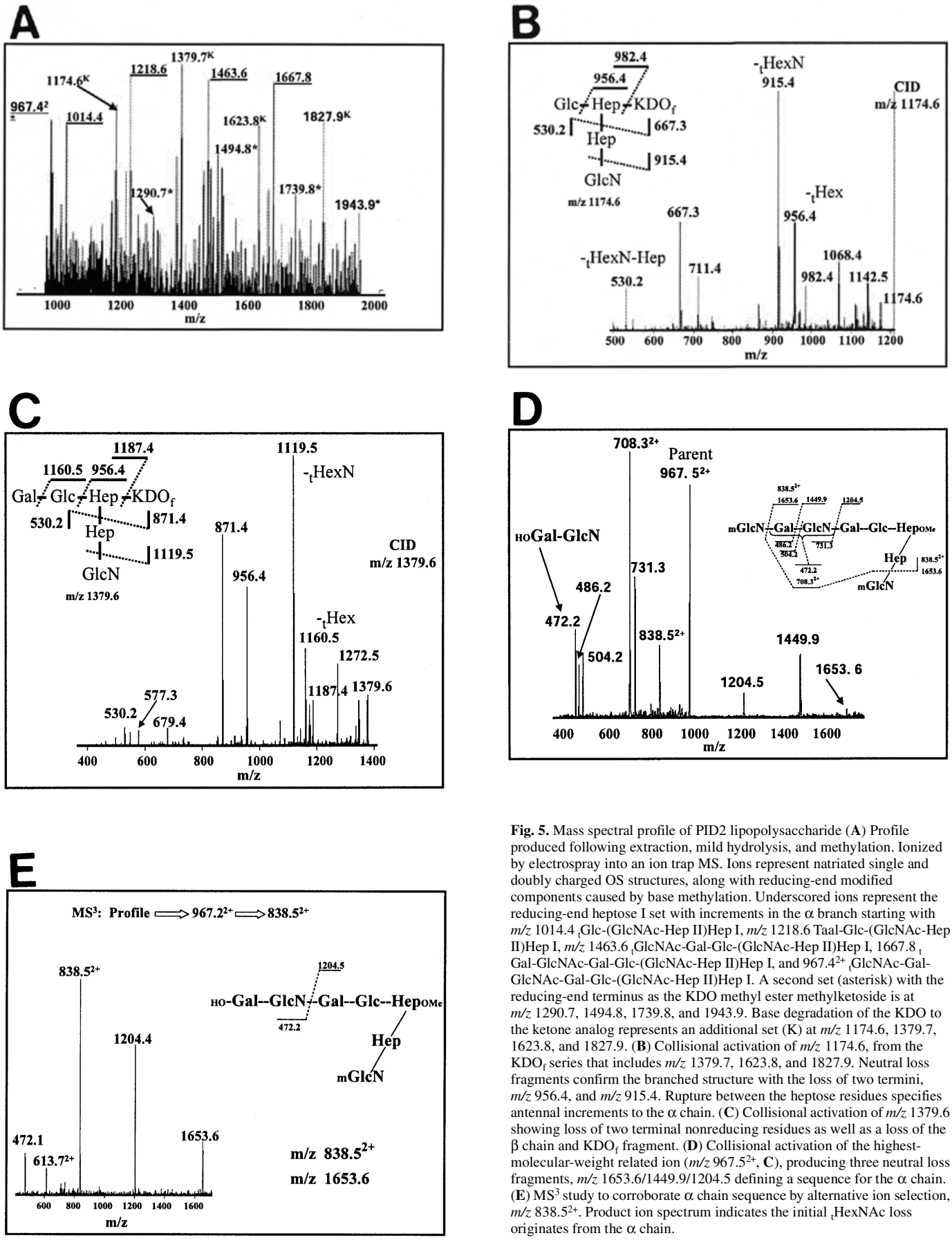

Fig. 5. Mass spectral profile of PID2 lipopolysaccharide (**A**) Profile produced following extraction, mild hydrolysis, and methylation. Ionized by electrospray into an ion trap MS. Ions represent natriated single and doubly charged OS structures, along with reducing-end modified components caused by base methylation. Underscored ions represent the reducing-end heptose I set with increments in the α branch starting with *m/z* 1014.4 $_t$Glc-(GlcNAc-Hep II)Hep I, *m/z* 1218.6 Taal-Glc-(GlcNAc-Hep II)Hep I, *m/z* 1463.6 $_t$GlcNAc-Gal-Glc-(GlcNAc-Hep II)Hep I, 1667.8 $_t$ Gal-GlcNAc-Gal-Glc-(GlcNAc-Hep II)Hep I, and 967.4^{2+} $_t$GlcNAc-Gal-GlcNAc-Gal-Glc-(GlcNAc-Hep II)Hep I. A second set (asterisk) with the reducing-end terminus as the KDO methyl ester methylketoside is at *m/z* 1290.7, 1494.8, 1739.8, and 1943.9. Base degradation of the KDO to the ketone analog represents an additional set (K) at *m/z* 1174.6, 1379.7, 1623.8, and 1827.9. (**B**) Collisional activation of *m/z* 1174.6, from the KDO_f series that includes *m/z* 1379.7, 1623.8, and 1827.9. Neutral loss fragments confirm the branched structure with the loss of two termini, *m/z* 956.4, and *m/z* 915.4. Rupture between the heptose residues specifies antennal increments to the α chain. (**C**) Collisional activation of *m/z* 1379.6 showing loss of two terminal nonreducing residues as well as a loss of the β chain and KDO_f fragment. (**D**) Collisional activation of the highest-molecular-weight related ion (*m/z* 967.5^{2+}, **C**), producing three neutral loss fragments, *m/z* 1653.6/1449.9/1204.5 defining a sequence for the α chain. (**E**) MS^3 study to corroborate α chain sequence by alternative ion selection, *m/z* 838.5^{2+}. Product ion spectrum indicates the initial $_t$HexNAc loss originates from the α chain.

Discussion

N. gonorrhoeae strain PID2 produces six different LOS components. Because the four largest LOS components of PID2 have the same SDS–PAGE mobility as the four LOS components expressed by strain MS11mkC, it suggested that the structure of these comigrating components of PID2 LOS might be the same as those found in MS11mkC. The terminal lactosamine made by MS11mkC LOS is added onto a core that can bind mAb 2-1-L8 (John *et al.*, 1999). Because strain PID2 possessed a LOS component with the same mobility as the LOS component that can bind mAb 2-1-L8, yet failed to bind this mAb, we hypothesized that the mAb 1B2-reactive component might be added onto a structurally different biosynthetic intermediate. By using gene characterization, exoglycosidase digestion, and sugar composition analysis to characterize the LOS structures found in PID2, we were able to define the structure of each of the LOS molecules made by this strain. We found that although PID2 LOS shares many of the structural features found in MS11mkC, its LOS had some fundamental differences: PID2 LOS is devoid of PEA decorations and the α chain of PID2 LOS is devoid of terminal N-acetyl-galactosamine residues. Furthermore, this strain has lost the genetic capability to add N-acetylgalactosamine to its α chain.

DNA sequence analysis of a PCR-amplified chromosomal DNA fragment demonstrated that the PID2 *lgt* gene cluster consisted of *lgtA1*, *lgtB*, *lgtC*, *lgtA2*, and *lgtB/E*; there was no *lgtD* gene. Although the DNA sequence obtained indicated that both copies of *lgtA* would be nonfunctional due to the length of the polyguanine tracts contained within their coding sequences, we believe that this is an artifact of PCR amplification, because the LOS expressed by this strain appears to have been modified by LgtA. Others have shown that PCR amplification of these polyguanine tracts can produce spontaneous frame shifts (Jennings *et al.*, 1995).

In *N. gonorrhoeae* strain F62, the genes *lgtA* and *lgtD* are more than 70% identical and have long stretches of sequence identity, especially at the 5′ end of the genes. Likewise, the genes *lgtB* and *lgtE* share greater than 70% identity (Gotschlich, 1994). The data presented in Figure 6 depict a model that shows how the *lgt* gene cluster is organized in F62 and how the organization found in PID2 might arise. This model involves homologous recombination between conserved DNA sequences of *lgtA* and *lgtD* and *lgtB* and *lgtE*. This intramolecular recombination provides another mechanism that could allow for LOS antigenic variation to occur. However, the net result of this mechanism would be the loss of genetic information. In *N. meningitidis*, deletions within the *lgt* gene cluster are common (Jennings *et al.*, 1995, 1999; Tettelin *et al.*, 2000).

Because *lgtD* is responsible for the addition of a terminal GalNAc (Wakarchuk *et al.*, 1996), PID2 should lack this terminal sugar in its LOS because it lacks this gene. Exoglycosidase digestion and sugar composition analysis allowed us to determine that the only sugars found in the various PID2 LOS components were Gal, Glc, and GlcNAc, and that GalNAc was not present as a terminal sugar. The structural relationship of each of the LOS components made by PID2 are shown in Figure 7. The linear relationship between each of these bands was demonstrated by sequential exoglycosidase digestion and

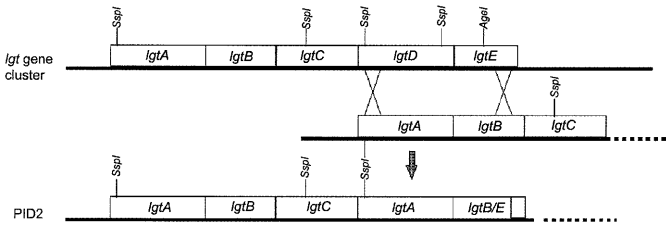

Fig. 6. Proposed model for intragenomic recombination within the *lgt* gene cluster in strain PID2. By use of Southern hybridization experiments and analysis of PCR amplification products, It is postulated that the *lgt* gene cluster in strain PID2 exists as described in the bottom line of the panel. The top two lines show how this recombination might occur.

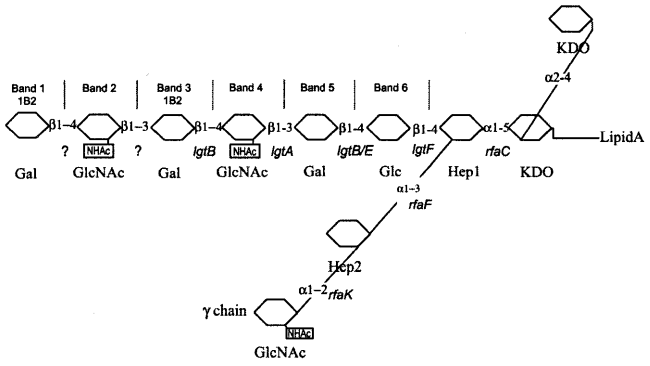

Fig. 7. Structural depiction of various LOS components expressed by PID2.

demonstrated that all the higher bands (bands 1–4) are built from core lactose (band 5).

lgtE encodes the first enzyme needed for the synthesis of the lacto-*N*-neotetraose component seen in F62. In PID2, this gene is a hybrid between sequences found in *lgtB* and *lgtE*. Because PID2 is able to make high-molecular-mass LOS components, it indicates that the hybrid gene has retained some function. Both of these genes add a Gal onto a growing OS. These two enzymes differ in the acceptor molecule, with LgtB adding a Gal onto GlcNAc and LgtE adding a galactose onto Glc. In comparing F62 LgtB and LgtE amino acid sequences and taking into account the functionality of the hybrid protein in PID2, it is suggested that the amino terminus of the protein contains that part of the protein is responsible for substrate specificity and that the carboxy terminus of the protein is responsible for recognizing the acceptor molecule.

The structure of LOS isolated from *N. gonorrhoeae* strain 15253 has been determined. In addition to the lacto-N-neotetraose on the α chain, this strain also contains a β chain consisting of lactose (Yamasaki *et al.*, 1999). Mutations in *lgtE* result in the production of an LOS that consists of a glucose on both the α and β chains (Banerjee *et al.*, 1998), and this LOS binds the mAb 25-1-LC1 (Tong *et al.*, 2001). We generated a nonfunctional *lgtE* mutant of PID2. Because only truncated LOS molecules were expressed in the mutant strain and this LOS was not able to bind mAb 25-1-LC1, a mAb that requires a single β-chain glucose and an α chain consisting of glucose or lactose to bind (Tong *et al.*, 2001), it indicated that this LOS did not possess a glucose on the β chain. This LOS also failed to bind mAb B5, an antibody that requires the presence of PEA

on C3 of Hep II for binding (Plested *et al.*, 1999). These data indicate that the structural differences between PID2 and MS11mkC are seen as differences in the addition of PEA. They further suggest that all of the LOS components found in PID2 are extended off of α chain. MS studies were readily able to identify the presence of a five-sugar OS extending from Hep I. These studies also clearly showed that these OS molecules lack PEA. Though these studies were unable to confirm the presence of a six-sugar oligosaccharide, we believe that this failure is due to the limitations of the methodology employed and the low abundance of the high-molecular-mass ion, relative to the large amount of the smaller ions.

We still do not understand the mechanism that allows for the surface expression of LOS biosynthetic intermediates, as seen in strains like PID2 and MS11mkC. Burch *et al.* (1997) demonstrated that limiting the amount of the LgtA allowed strain FA19 to surface express two related LOS components, one that was able to bind mAb 2-1-L8 and one that was able to bind mAb 1B2. They suggested that the transcription/trasnslation of each of the proteins encoded by the *lgt* gene cluster is highly regulated and that small shifts in the amount a enzyme made can dramatically effect the surface expressed phenotype. If the hybrid LgtB/E had a lower K_{cat} than the wild-type LgtE, the amount of LgtE activity would be limiting in PID2. This would allow for the surface expression of small LOS biosynthetic intermediates. This same mechanism could explain how the gonococcus expresses the lactosamine repeat. In PID2, *lgtA* is duplicated. In theory, this could double the amount of this enzyme made, relative to the other enzymes. Because this enzyme mediates the addition of GlcNAc to Gal, if it were in excess, it could act to add sugars onto the terminal Gal of the lacto-*N*-neotetraose, which could then be further elongated by the *lgtB* gene product. This would produce the lactosamine repeat seen in PID2 and MS11mkC. However, we do not favor this hypothesis because if it is true, we would expect to see limited amounts of lactosamine repeats in all strains. We believe that additional as yet uncharacterized genes mediate the addition of polylactosamine.

The absence of phosphoethanolamine in neisserial LOS has not been reported. PID2 could surface express high-molecular-mass LOS components in the absence of this modification, and this indicates that this decoration is added later in biosynthesis and plays no role in the surface localization of LOS. The LOS components that lack this modification possess the same electrophoretic mobility as those that contain it. Because mAb 1B2 is able to bind LOS, irrespective of the presence of the PEA modification, whereas mAb 2-1-L8 is only able to bind when this modification is present, it serves as a further reminder of the dangers of ascribing structural features to an LOS molecule based solely on the binding of a mAb or an SDS–PAGE profile.

Though a systematic study of LOS structures found on strains causing PID has not yet been performed, strains associated with PID are generally serum-sensitive and possess an LOS that can be modified by gonococcal sialytransferase (Rice *et al.*, 1994). Sialyltransferase competes with LgtD for the terminal galactose found on the lacto-N-neotetraose. It is possible that the increased virulence associated with PID2 may be due to this loss in ability to cap the lacto-N-neotetraose structure through the action of LgtD. The data presented herein serve as a starting point for this line of investigation because they demonstrate that FACE analysis, combined with enzymatic digestion of LOS, can be used to rapidly define the nature of the sugars that are found at the termini of surface expressed LOS.

The data reported here represent the first application of FACE technology to the identification of sugar components in lipopolysaccharides. This technology will be invaluable in future studies because, in combination with enzymatic digestions, it will allow one to rapidly identify the nature of terminal sugars in gonococci. This will allow for the systematic study of LOSs isolated from strains associated with PID. The loss of *lgtD* in PID2 results in the loss of the ability to cap an LOS molecule with N-acetylgalactosamine. *N. meningitidis* has increased virulence, relative to the gonococcus. All of these strains characterized to date lack the ability to add this modification (Jennings *et al.*, 1999), suggesting that this modification reduces virulence.

Materials and methods

Bacterial strains, plasmids, oligonucleotides, and culture conditions

All bacterial strains, plasmids, and oligonucleotide primers used in this study are listed in Table I. All *Neisseria* strains were grown in standard gonococcal medium (designated GCP if broth, GCK if agar) (Difco Laboratories) plus growth supplements (White and Kellogg, 1965) and 0.042% sodium bicarbonate if in broth or in a 37°C CO_2 incubator if on agar. *Escherichia coli* strains were grown on Luria Bertani plates (Sambrook *et al.*, 1989). Ampicillin was used at 50 μg/ml, spectinomycin at 50 μg/ml, and X-gal at 35 μg/ml when the selections were applied.

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 (St. Louis, MO) unless otherwise specified. Tris-tricine gels (16.5%) and running buffer were obtained from Bio-Rad (Richmond, CA). FACE monosaccharide composition analysis kit was purchased from Glyko (Novato, CA). Acetic acid was from Fisher Scientific (Silver Spring, MD). The enzymes β-galactosidase and β-*N*-acetylhexosaminidase (both from jack bean meal) were purchased from Glyko. β-*N*-Acetylhexosaminidase (from *Streptomyces plicatus*) was from New England Biolabs. mAb 1B2 was a gift from Dr. J. McLeod Griffiss (UCSF). The mAbs 25-1-LC1, 17-1-L1, and 2-1-L8 were generously provided by Dr. Wendell Zollinger (Walter Reed Army Institute of Research, Washington, DC). The mAb B5 was a gift from Dr. Margaret A. Gidney (Institute for Biological Sciences, National Research Council, Ottawa, Canada). mAb 3G9 was graciously provided by Dr. Peter A. Rice (Maxwell Finland Laboratory for Infectious Disease, Boston University, Boston, MA).

LOS purification

LOSs were purified from broth-grown cells using acetone-powdered organisms by the hot phenol–water method (Westphal and Jann, 1972). Methylation of LOS for use in MS experiments was achieved by dissolving samples in a NaOH/dimethyl sulfoxide suspension, prepared by vortex mixing

Table I. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Reference(s) or source
Strains		
N. gonorrhoeae		
F62	full α chain of LOS, no β chain, $1B2^+$ and $1\text{-}1\text{-}M^+$	Yamasaki *et al.*, 1991a,b; West and Clark, 1989
F62Δ*lgtA*	239-bp *Apo*I *deletion in lgtA*, truncated lactosyl α chain, $2\text{-}1\text{-}L8^+$	Banerjee *et al.*, 1998
F62Δ*lgtD*	*lgtD* fixed out of frame, only express lacto-*N*-neotetraose LOS	Song *et al.*, 2000
PID2		H. Shneider (Walter Reed Army Institute for Research, Washington DC)
PID2FΩ		This work
PID2EΩ		This work
F62Δ*lgtA*Δ*lgtE*	622-bp *BspE*I and *Age*I *deletion in lgtE* of F62Δ*lgtA*, only Glc in α chain, $25\text{-}1\text{-}LC1^+$	Tong *et al.*, 2001
F62Δ*lgtA*Δ*lgtF*	240-bp *BsiW*I and *BsrG*I deletion in *lgtF* of F62Δ*lgtA*, without α chain and β chain, $B5^+$	Tong *et al.*, 2001
E. coli		
DH5αMCR	cloning host strain, F^- mcrAΔ (mrr-hsdRMS-mcrBC)	
Plasmids		
pGEM-7Zf(-)		Promega
pPID2*lgt*	PID2 *lgt* gene cluster cloned into pGEM-7Zf(-)	This work
pPID2*lgt*α	first 2.4kb fragment of PID2 *lgt* gene cluster cloned into pGEM-7Zf(-)	This work
pPID2*lgt*β	the second 2.4kb fragment of PID2 *lgt* gene cluster cloned into pGEM-7Zf(-)	This work
pRFAK2-1	F62 *lgtF* and *rfaK* fragment cloned into pK18-up	Tong *et al.*, 2001
pF62*lgtDE*Ω	omega cassette inserted just before the start codon of F62 *lgtE*	This work
pF62*lgtF*Ω	omega cassette inserted in the middle of F62 *lgtF*	This work
pHP45Ω	source of the omega cassette	Prentki and Krisch, 1984
Primers		
rfaK-147F	5′AAGCCCGGGCGTATGTTTGGGCTTTTTTGC3′, 5′ end of *lgtF*	Tong *et al.*, 2001
rfaK-3780R	5′GTGAAGCTTATATTGCATCCAATAATTTGTCG3′, 3′ end of *rfaK*	Tong *et al.*, 2001
JL51	5′ATGGATCCGGGGCGATTTTACCTAGCAGATGAA3′, 200 bp downstream of stop codon of F62 *lgtE*	Tong *et al.*, 2001
JL50	5′CTGAATTCGGCCGACATCGCGCTTTTGGGCG3′, 5′ end of F62 *lgtA*	Tong *et al.*, 2001
Got5220R	5′GAATGACAGTGGATCCATTTCTGATTTTA3′, 5¢ end of F62 *lgtE*	Tong *et al.*, 2001
Got3240R	5′TGCGCCATCTTTGAAGCATACA3′	This work
JL12	5′AGCGGCCCATCCCGATAC3′	This work
lgtF-PstIF	5′CAACTGCAGACAATATTTCAACAAGTTCAACAA3′	This work
lgtF-PstIR	5′TGTCTGCAGTTGTCGTACGTATAATGGTAC3′	This work
omega-F	5′CCACTGCAGCAATTCCCCTGCTCGCGCAGG3′	This work
omega-R	5′CCACTGCAGCAGCTTAGTAAAGCCCTCGCT3′	This work
lgtDE-F	5′GGAAATACCGCAGCTATTGAATTCCGA3′	This work
lgtDE-R	5′GCATGATTTATCCTGTTCGAATTCAAT3′	This work

dimethyl sulfoxide and powdered NaOH (Ciucanu and Kerek, 1984). After 1 h incubation at room temperature, 50 µg of methyliodide was added and the suspension incubated for an additional hour at room temperature with occasional vortexing (Reinhold *et al.*, 1996). The methylated product was back-extracted by adding 1 ml chloroform, and the suspensions were back washed four times with 2–3 ml 30% acetic acid. The chloroform layer was taken to dryness and stored at –20°C. Methylated samples were redissolved to a concentration of 10 µM in a 1 mM solution of sodium acetate in 70:30 methanol/water just prior to analysis.

FACE monosaccharide composition analysis

Purified LOS (~5 g) was hydrolyzed in 1% acetic acid for 2 h at 80°C. The hydrolysate was centrifuged (12,000 × *g*, 20 min) and the supernatant containing the OS collected. For sugar

composition analysis, the OS was treated following the procedure provided by Glyko, with the only difference being that the OS was hydrolyzed with 4 N HCl for 2 h instead of with 2 N trifluoracetic acid for 5 h. For exoglycosidase digestion, the samples were labeled after digestion.

SDS–PAGE analysis

Proteinase K–treated whole cell lysates were prepared from 18 to 20 h cultures by the procedure of Hitchcock and Brown (1983). Approximately 0.1 g LOS was subjected to SDS–PAGE on a 16.5% Tris-tricine gel (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 (Tsai and Frasch, 1982).

Western blot

After separation on an SDS–PAGE gel, LOSs were electro-transferred onto Immobilon-P membrane (Millipore, MA) in a Tris-glycine-methanol buffer (0.025 M Tris, 0.192 M glycine, 20% methanol) at a constant voltage of 100 V for 1 h. After air-drying for 10 min, membranes were blocked with a filler solution (2% casein, 0.2% NaN_3, and 0.002% phenol red, in 100 mM Na_2HPO_4 pH 7.5) for 30 min. The membranes were incubated in primary antibody (mAb 25-1-LC1, B5, 3G9, 17-1-L1, or 1B2) with gentle shaking for at least 2 h. The membranes were washed with 100 mM Na_2HPO_4, pH 8.0, three times (10 min each) and incubated in filler solution containing secondary antibody (goat anti-mouse IgG conjugated with horseradish peroxidase; goat anti-mouse IgM conjugated with horseradish peroxidase when mAb 1B2 was used) for at least 2 h. After three washes, antibody binding on the membranes was visualized by incubating the membranes in developing solution (50 mM Tris–HCl, pH 8.0, 1% 4-chloro-1-napthol, 0.86% H_2O_2).

Transformation

Recombinant DNA transformation into *E. coli* DH5αMCR was done according to the standard protocols (Sambrook *et al.*, 1989). Recombinant DNA transformation into *N. gonorrhoeae* were done by resuspending piliated cells to a density of approximately 1×10^8 cells/ml in GCP broth containing growth supplements, 0.042% $NaHCO_3$, 10 mM $MgCl_2$ and 1 μg of the DNA of interest. Cells were incubated for about 5 h with shaking at 37°C. Cells were plated onto GCK plates containing spectinomycin.

PCR

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

DNA sequencing

All DNA sequencing was done using nested overlapping primers. The DNA sequence of the *lgt* gene region for PID2 has been submitted to GenBank under accession number AF313394.

Insertion of the omega cassette into various genes

To insert the omega cassette into F62*lgtF*, primer lgtF-PstIF and lgtF-PstIR were designed to amplify pRFAK2-1, inserting *Pst*I site into the F62*lgtF* gene. Primer omega-F and omega-R, containing terminal of *Pst*I sites, were used to amplify the omega cassette from pHP45Ω (Prentki and Krisch, 1984). After digestion with *Pst*I, the two fragments were ligated and transformed into *E. coli* DH5α giving pF62*lgtF*Ω. To insert the omega cassette into the *lgt* gene cluster, primer lgtDE-F and lgtDE-R were designed to amplify pF62*lgt*, inserting an *Eco*RI site just before *lgtE* start codon. The omega cassette was inserted into this site, giving pF62*lgtDE*Ω.

Electrospray ionization MS

Mass measurements were performed on an ion trap mass spectrometer (LCQ, Finnigan-MAT, San Jose, CA) equipped with electrospray ionization. Samples were dissolved in methanol:water solutions (6:4, v/v) containing 0.25 mM NaOH and analyzed by syringe pump flow injection (1.5 μl/min directly into the electrospray chamber. Ions were injected axially into the ion trap by a gate lens and a trapping field was established with a 100–1100 kHz radio frequency applied to the ring electrode.

Acknowledgments

This work was supported by grants from the National Institutes of Health to D.C.S. (AI24452) and V.N.R. (GM54045). Dan Arking was supported by a grant from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program. The authors thank Dr. Paul Rick at the Uniformed Services University for allowing us to use his FACE system.

Abbreviations

FACE, fluorophore-assisted carbohydrate electrophoresis; KDO, 3-deoxy-D-manno-2-octulosonic acid; LOS, lipo-oligosaccharide; mAb, monoclonal antibody; MS, mass spectro-metry; OS, oligosaccharide; PCR, polymerase chain reaction; PEA, phoshoethanolamine; pNP, *p*-nitrophenol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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