Genetic Locus for the Biosynthesis of the Variable Portion of *Neisseria gonorrhoeae* Lipooligosaccharide

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Summary

A locus involved in the biosynthesis of gonococcal lipooligosaccharide (LOS) has been cloned from gonococcal strain F62. The locus contains five open reading frames. The first and second reading frames are homologous, but not identical, to the fourth and fifth reading frames, respectively. Interposed is an additional reading frame which has distant homology to the *Escherichia coli rfaI* and *rfaJ* genes, both glucosyl transferases involved in lipopolysaccharide core biosynthesis. The second and fifth reading frames show strong homology to the *lex-1* or *lic2A* gene of *Haemophilus influenzae*, but do not contain the CAAT repeats found in this gene. Deletions of each of these five genes, of combinations of genes, and of the entire locus were constructed and introduced into parental gonococcal strain F62 by transformation. The LOS phenotypes were then analyzed by SDS-PAGE and reactivity with monoclonal antibodies. Analysis of the gonococcal mutants indicates that four of these genes are the glycosyl transferases that add GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4 to the substrate Glcβ1 → 4Hep → R of the inner core region. The gene with homology to *E. coli rfaI/rfaJ* is involved with the addition of the α-linked galactose residue in the biosynthesis of the alternative LOS structure Galα1 → 4Galβ1 → 4Glcβ1 → 4Hep → R. Since these genes encode LOS glycosyl transferases they have been named *lgtA*, *lgtB*, *lgtC*, *lgtD*, and *lgtE*. The DNA sequence analysis revealed that *lgtA*, *lgtC*, and *lgtD* contained poly-G tracts, which, in strain F62 were, respectively, 17, 10, and 11 bp. Thus, three of the LOS biosynthetic enzymes are potentially susceptible to premature termination by reading frame changes. It is likely that these structural features are responsible for the high-frequency genetic variation of gonococcal LOS.

While *Neisseria* species commonly colonize many mammalian hosts, human beings are the only species subject to invasive disease by members of this species. *Neisseria meningitidis* is the etiologic agent for septicemia and meningitis that may occur in epidemic form. *Neisseria gonorrhoeae* is the causative agent of gonorrhea and its manifold complications. These organisms, particularly the gonococcus, have proved remarkably adept at varying the antigenic array of their surface-exposed molecules, notably their adhesive pili and opacity-related proteins. The genetic mechanisms for the variation of pilus (1–4) and opacity-related (5–7) protein expression are in the main well understood. Like other gram-negative bacteria the *Neisseria* species carry LPS in the external leaflet of their outer membranes (8). In contrast to the high molecular weight LPS molecules with repeating O-chains seen in many enteric bacteria, the LPS of *Neisseria* species is of modest size and therefore is often referred to as lipooligosaccharide (LOS)[1]. Although the molecular size of the LOS is similar to that seen in rough LPS mutants of *Salmonella* species, this substance has considerable antigenic diversity. In the case of the meningococcus, a serological typing scheme has been developed that separates strains into 12 immunotypes (9, 10). A remarkably complete understanding of the structure of meningococcal LPS (for review see reference 11) has resulted from the studies of Jennings et al. (12), Michon et al. (13), Gamian et al. (14), and Pavliak et al. (15). In the case of *N. gonorrhoeae*, antigenic variability is so pronounced that a serological classification scheme has proved elusive. In part this is due to the heterogeneity of LOS synthesized by a particular strain; LOS preparations frequently contain several closely spaced bands by SDS-PAGE (16). Furthermore, studies using monoclonal antibodies indicate that gonococci are able to change the serological characteristics of the LOS they express, and that this antigenic variation occurs at a frequency of 10^{-2}–10^{-3}, indicating that some genetic mechanism must exist to achieve these high frequency variations (17, 18). Because of the molecular heterogeneity and antigenic variation of the LOS produced by gonococci, the determination of the structural chemistry of this antigen has proved to be a difficult problem, and definitive informa-

[1] *Abbreviations used in this paper:* CMP-NANA, *N*-acetyl neuraminic acid cytidine monophosphate; GC, gonococcus; LOS, lipooligosaccharide; wt, wild-type.

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tion based on very sophisticated analyses has only recently become available (19–22). These are summarized in Fig. 1. Of particular interest is the presence of the tetrasaccharide Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4, which is a perfect mimic of lacto-*N*-neotetraose of the sphingolipid paragloboside (23, 24). In LOS, this tetrasaccharide frequently bears an additional *N*-acetylgalactosamine residue (GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4), and then it mimics gangliosides. In some strains of gonococci, an alternative side chain is found that has the structure Galα1 → 4Galβ1 → 4Glcβ1 → 4Hep → R (21). This is a mimic of the saccharide portion of globoglycolipids (25), and is the structure characteristically found in *N. meningitidis* immunotype L1.

The LOS molecules have a number of biological activities. They are potent endotoxic molecules believed to be the toxin responsible for adrenal cortical necrosis seen in severe meningococcal disease. They serve as the target antigen for much of the bactericidal activity present in normal or convalescent human sera (26). Gonococci possess a very unusual sialyl transferase activity: they can use externally supplied *N*-acetyl neuraminic acid cytidine monophosphate (CMP-NANA) and add NANA to the LOS on the surface of the organism (27–29). Group B and C meningococci have the capacity to synthesize CMP-NANA, and frequently sialylate their LOS without requiring exogenous CMP-NANA (30). In *N. meningitidis* strain 6275 immunotype L3, the sialic acid unit is linked α2 → 3 to the terminal Gal residue of the lacto-*N*-neotetraose chain (31). The levels of CMP-NANA found in various host environments is sufficient to support this reaction (32). The sialylation of LOS causes gonococci to become resistant to the antibody complement-dependent bactericidal effect of serum (28). The resistance is not only to the bactericidal effect mediated by antibodies to LOS, but to other surface antigens as well (33). van Putten (34) has demonstrated that exposure of gonococci to CMP-NANA markedly reduces their ability to invade epithelial cells in tissue culture. These findings strongly suggest that the ability of gonococci to vary the chemical nature of LOS provides them with the ability to cope with different host environments (35).

Perhaps most telling, it has been found that LOS variation is selected in vivo in infections of human beings. A well-characterized gonococcal laboratory strain $MS11_{mk}$ variant A was used to inoculate volunteers (36). Over a period of 4–6 d, the population of gonococci recovered in the urine of the two infected individuals increasingly shifted to two variants that expressed antigenically different LOS (37). A structural analysis revealed that the inoculated variant A produced a truncated LOS containing only the β-lactosyl group linked to Hep1, while one of the new variants (variant C) produced a complete LOS (20). This suggests that the addition of the additional sugars GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3 is likely to be under control of a phase variation mechanism.

Little information on the genetics of LOS synthesis in *Neisseria* is available. A major advance has been the creation (38) and biochemical characterization (21) of five pyocin mutants of gonococcal strain 1291, dubbed 1291a–e. Immunological and biochemical data have shown that 1291a, 1291c, 1291d, and 1291e produce LOS with sequential shortening of the lacto-*N*-neotetraose chain, with mutant 1291e lacking the glucose substitution on the heptose. Mutant 1291b synthesizes the alternative LOS structure Galα1 → 4Galβ1 → 4Glc (Fig. 1). Only the genetic basis of the 1291e mutant is now defined. It is a mutation of phosphoglucomutase (*pgm*), which precludes the synthesis of UDP-glucose and hence the addition of the first residue of the lacto-*N*-neotetraose unit (39, 40). It also has been shown that *galE* mutants of meningococcus or gonococcus produce truncated LOS in keeping with the inability to synthesize UDP-galactose (41, 42).

We describe a locus in *N. gonorrhoeae* strain F62 containing five genes. Four of the genes are responsible for the sequential addition of the GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4 to the substrate Glcβ1 → 4Hep → R of the inner core region (19). The fifth gene is involved with the addition of the α-linked galactose residue in the biosynthesis of the alternative LOS structure Galα1 → 4Galβ1 → 4Glcβ1 → 4Hep → R (21). DNA sequence analysis revealed that the first, third, and fourth reading frames contained poly-G tracts, which in strain F62 were, respectively, 17, 10, and 11 bp. Thus, three of the LOS biosynthetic enzymes are potentially susceptible to premature termination by reading frame changes, as has been reported for the gonococcal *pilC* genes (43, 44). It is likely that these structural features are responsible for the high-frequency genetic variation of gonococcal LOS (18).

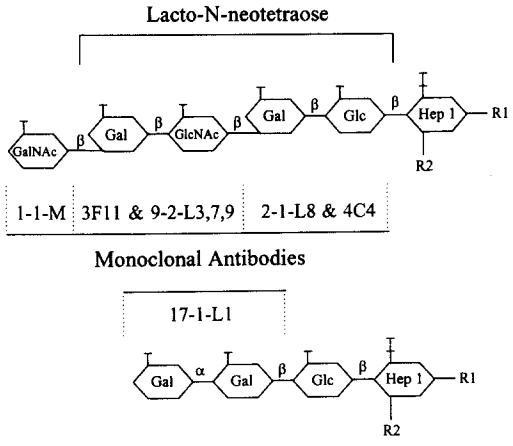

Figure 1. Alternative structures found in gonococcal LOS. R1 refers to the inner core region of LOS, consisting of two keto-deoxy-octulosonic acid residues. These in turn are attached to a lipid A structure. R2 in gonococci is typically GlcNAcβ1 → 2Hepα1 → 3. The structure in the top panel contains a tetrasaccharide identical to lacto-*N*-neotetraose found in paragloboside glycolipids. In many strains, this tetrasaccharide bears a terminal GalNAcβ1 → 3. The lower panel shows an alternative trisaccharide structure with the terminal Gal α1 → 4 linked. This trisaccharide is seen in meningococci of the L1 serotype and in some gonococcal strains. The portions of the two structures recognized by the monoclonal antibodies used in this study are indicated: 4C4 (39), 3F11 (24, 69) 1-1-M (69), 2-1-L8 (21, 38, 72), 9-2-3,7,9, and 17-1-L1 (Zollinger, W. D., personal communication).

Materials and Methods

Reagents and Chemicals. Most laboratory chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Media and Growth Conditions. Escherichia coli strains were grown in solid or liquid Luria-Bertani (LB) medium (45); antibiotics were added as applicable. Carbenicillin was used at 50 μg/ml and erythromycin at 200 μg/ml. *N. gonorrhoeae* strain F62 was grown on gonococcus (GC) agar (46) or GC agar containing 2 μg/ml erythromycin. For isolation of LOS or genomic DNA, gonococci were grown in 1.5% proteose peptone broth (Difco Laboratories, Detroit, MI), 30 mM phosphate, 8.5 mM NaCl supplemented with 1% isovitalex (Becton Dickinson Microbiology Systems, Cockeysville, MD).

Recombinant DNA Methods. Plasmids were purified using either Qiagen columns or the QIAprep spin columns obtained from Qiagen, Inc. (Chatsworth, CA). Digestion with restriction enzymes, gel electrophoresis, ligations with T4 DNA polymerase, and transformation of *E. coli* were done according to the method of Sambrook et al. (45). Southern hybridization was performed on membranes (Hybond N+; Amersham Corp., Arlington Heights, IL) with DNA labeled with an ECL kit (Amersham Corp.). Genomic DNA was isolated as described by Moxon et al. (47).

A gene bank of *N. gonorrhoeae* strain F62 genomic DNA was constructed by ligating ~20-kb fragments obtained by incomplete digestion with Sau3A into BamHI/EcoRI-digested λ2001 (48). The phage library was screened by hybridization with random prime-labeled plasmid pR10PI, and five clones were isolated by plaque purification. The phages from these clones were purified by sedimentation followed by flotation on CsCl (49), and the DNA was isolated. From one of these clones, two ClaI fragments of 4.9 and 3.4 kb were isolated by gel electrophoresis and recovery with Geneclean II (BIO 101, Inc., La Jolla, CA). These were ligated into ClaI-cut pBluescript II SK- (Stratagene, Inc., La Jolla, CA) and called p4900 and p3400, respectively. p4900 contained a PstI site in the insert and was subdivided into two clones containing inserts of 2.1 and 2.8 kb. The clone containing the 2.8-kb insert was called pPstCla. The inserts in p3400 and pPstCla were sequenced by the chain termination method (50) using Sequenase II (United States Biochemical Corp., Cleveland, OH). All of the sequence presented in Fig. 2 was completed in both directions; part of the sequence was determined by LARK Sequencing Technologies (Houston, TX).

The insertion and deletions shown in Fig. 6 were constructed as follows. I1, I3, Δ1 and Δ2 used plasmid pPstCla cut, respectively, with BsaBI, AscI, and StyI and double cut with StyI and BsaBI. I2 and Δ3 used plasmid p3400 cut with AgeI or StyI. The complete locus was assembled by cloning the ClaI–ApaI fragment from p3400 into pPstCla cut with ClaI and ApaI, and the plasmid was called pLOS5. Deletions Δ4 and Δ5 were constructed by use of pLOS5 and digestion with StyI and BbsI or StyI alone. In all instances (except digestion with BsaBI), the cut plasmids were treated with the Klenow fragment of *E. coli* DNA polymerase to blunt the ends, and *ermC'* (erythromycin resistance marker) was inserted. The *ermC'* gene was isolated from plasmid pIM13 (51) as a ClaI–HindIII fragment and cloned into the same sites in plasmid pHSS6 (52). From this plasmid it was excised as a NotI fragment, the ends blunted by treatment with Klenow fragment of DNA polymerase and purified by gel electrophoresis and recovery with Geneclean II (BIO 101 Inc.).

Transformation of piliated *N. gonorrhoeae* strain F62 was performed with plasmids isolated from *E. coli* (53) and the transformants selected on GC agar (46) containing 2 μg/ml erythromycin. The fidelity of the genomic alteration of each of the gonococcal transformants was verified by sequencing the upstream and downstream junctions of the *ermC'* gene in their genomic DNA by use of a PCR technique. Two 5' biotinylated primers (GCCGAGAAAACTATTGGTGGA and AAAACATGCAGGAATTGACGAT) were synthesized; these were based on the *ermC'* sequence near its upstream and downstream ends, respectively. The primers were designed such that their 3' ends pointed outward from the *ermC'* gene. Each of these primers was used together with a suitable primer matching the sequence of the LOS locus near the putative insertion. PCR was performed according to the instructions supplied with the GeneAmp PCR Reagent Kit from Perkin Elmer (Branchburg, NJ) using 25 cycles. In all instances the expected size product was obtained. The DNA sequence of these products was determined by purifying the PCR product on magnetic streptavidin beads from DYNAL, Inc. (Lake Success, NY) and sequencing with the Sequenase II kit according to a protocol provided by DYNAL, Inc., based on the method developed by Hultman et al. (54). The sequences were analyzed by computer programs in the GCG package of Genetics Computer Group Inc. (Madison, WI).

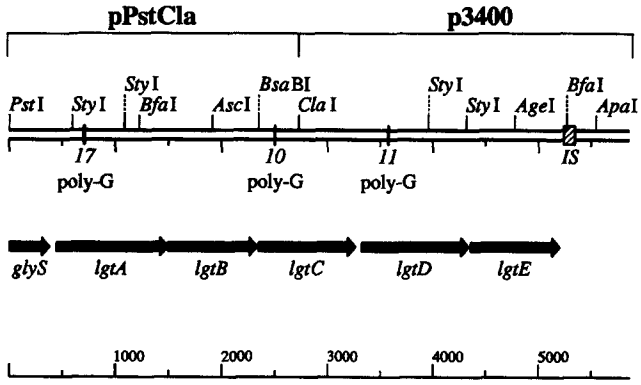

Figure 2. Genetic map of the LOS locus based on the DNA sequence. Sequence information for bp 1–2,725 was obtained from plasmid pPstCla and bp 2,725–5,859 from plasmid p3400 (see Materials and Methods). IS refers to an area of the sequence that has homology to a previously reported neisserial insertion sequence IS1106 (64). The positions of the reading frames of *lgtA–E* are indicated. Three tracts of poly-G were found in *lgtA* (17 bp), *lgtC* (10 bp), and *lgtD* (11 bp) and are indicated by *vertical black bars.*

Immunological Methods. Monoclonal antibodies 17-1-L1 (L1), 9-2-L3,7,9 (L3), 2-1-L8 (L8) were provided as filtered ascites fluids by Dr. Wendell Zollinger (Walter Reed Army Institute for Research, Washington DC). Dr. Michael Apicella (University of Iowa, Iowa City, IA) supplied antibody 1-1-M as ascites fluid and 3F11 and 4C4 as tissue culture supernatants. LOS was extracted from each of the gonococcal mutants by the hot phenol–water method (55) and purified as described (56). The LOS was diluted to 200 μg/ml in the Western blot buffer described by Towbin et al. (57), and 1.5 μl aliquots were spotted on Immobilon-P membrane from Millipore Corp. (Bedford, MA) that was lying on 3MM filter paper (Whatman Ltd., Maidstone, UK) soaked in the blotting buffer. The spots were allowed to absorb into the membrane over a period of 2 min, and the strips were placed in blocking buffer for at least 60 min. The blocking buffer consisted of 3% gelatin dissolved in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 0.02% NaN_3. The strips were washed three times in the same buffer containing 1% gelatin. The strips were treated for 2 h with monoclonal antibodies diluted in blocking buffer. The antibodies

available as ascites fluids were diluted 1:1,000, and antibodies available as tissue culture supernatants were diluted 1:10. The strips were washed, incubated for 60 min with a 1:1,000 dilution of phosphatase-conjugated anti-IgG, IgA, IgM (Organon Teknika Co., Durham, NC), washed, and stained as described previously (58).

Gel Electrophoresis. Gel electrophoresis of LOS samples was performed as described by Lesse et al. (59), and the gels were silver stained (60).

Results

Cloning of the LOS Locus. During attempts to isolate the porin gene of *N. gonorrhoeae*, pBR322 clones containing a 4.9-kb ClaI fragment were repeatedly isolated that reacted by colony blots with a rabbit antiserum to purified porin. An immunoreactive subclone, pR10PI, consisting of a 1,305-bp RsaI–ClaI fragment, was derived and its DNA sequence determined. This sequence had homology to a gene isolated from *Haemophilus influenzae*, called *lex-1* (61) or *lic2A* (62), that is known to be involved in LPS synthesis of that species. Using subclone pR10PI as a probe, Southern blots of *N. gonorrhoeae* genomic DNA digested with ClaI revealed hybridization with two fragments, 4.9 and 3.4 kb, respectively. However, digestion with some other restriction enzymes gave rise to only a single band. Notably, digestion with BfaI gave rise to a single band of 4.1 kb, suggesting that the two copies were closely linked (data not shown).

A λ2001 bank of *N. gonorrhoeae* strain F62 DNA was screened by hybridization with pR10PI, and five clones were isolated. One of these clones, when digested with either ClaI or BfaI and examined by Southern hybridization with pR10PI used as the probe, gave rise to a pattern identical to that seen with genomic DNA. The appropriate ClaI fragments of this λ2001 clone were isolated and cloned into the ClaI site of pBluescript II SK−. The entire sequence of the 3,400-bp ClaI fragment was determined. Mapping of the clone containing the 4,900-bp ClaI fragment indicated that there was a single PstI site in the clone ~2.8 kb from one side, allowing the clone to be divided into two subclones. Partial sequence of the ends of the 2.1-kb subclone indicated that it contained a coding frame homologous to the *E. coli* COOH-terminal portion of the α subunit of glycyl-tRNA synthetase (*glyS*) and the majority of the β subunit of this gene (63). The predicted length of DNA needed to match the *E. coli* sequence was present; this clone was not examined further.

DNA Sequence of the LOS Locus. A summary of the features found by sequencing the two clones is illustrated in Fig. 2. The sequence has been deposited in GenBank with the acquisition number U14554. After the *glyS* gene, we found five closely spaced open reading frames. The last frame has 46 bp downstream of the termination codon a sequence typical of a ρ-independent termination signal. Subsequently, there is an area of ~100-bp that has striking homology to the IS1106 neisserial insertion sequence (64). Work presented below shows that the five open reading frames code for LOS glycosyl transferases, and hence they have been named *lgtA–lgtE*.

Searches for internal homology within this locus indicates that the DNA coding for the first two genes (*lgtA*, *lgtB*) is repeated as the fourth and fifth genes (*lgtD*, *lgtE*), and that interposed is an additional open reading frame, *lgtC*. This is in keeping with the data obtained by Southern hybridization presented above, in which pR10PI probe containing the *lgtB* and a small portion of the *lgtC* gene hybridized with two ClaI fragments, but with only one BfaI fragment (see positions of the BfaI sites in the LOS locus in Fig. 2). In more detail, 16 bp after the stop codon of the tRNA synthetase (*glyS*) is the beginning of a stem loop structure followed closely by a consensus ribosome binding site, and within 6 bp is a TTG believed to be the initiation codon of *lgtA*. 2,871 bp downstream from the beginning of the stem loop (closely following the stop codon of *lgtC*) there is an almost perfect repeat of the stem loop structure, the ribosome binding site, and the TTG initiation codon of *lgtD* with the downstream sequence strongly homologous for ~500 bp. The sequences then diverge to some extent. However, at the beginning of *lgtB* and *lgtE*, the homology again becomes nearly perfect for ~200 bases, then diverges toward the latter part of the orfs. The similarity of the homologous proteins is illustrated in Figs. 3 and 4. It demonstrates the near-perfect conservation of the primary structure in the NH_2-terminal portions of the molecules with increasing divergence toward the COOH termini of the proteins.

The *lgtC* sequence interposed between the repeated portions of the locus is not repeated within the locus or in the *N. gonorrhoeae* genome (data not shown). It appears to be homologous to *E. coli rfaI* or *rfaJ* genes, very closely related genes that serve as glucosyl transferases in core LPS biosynthesis (65). The similarity of *rfaI* with *lgtC* is illustrated in Fig. 5.

We found that three of these genes contained within their

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lgtA   1 LQPLVSVLICAYNVEKYFAQSLAAVVNQTWRNLDILIVDDGSTDGTLAIA 50
         ||||||||||||.||||||||||||.||||||||||||||||||| |||
lgtD   1 LQPLVSVLICAYNAEKYFAQSLAAVVGQTWRNLDILIVDDGSTDGTPAIA 50

lgtA  51 KDFQKRDSRIKILAQAQNSGLIPSLNIGLDELAKSGGGGGEYIARTDADD 100
         :.||..|:||:|:..:.| |:|:||||||||||||  ||||||||||||
lgtD  51 RHFQEQDGRIRIISNPRNLGFIASLNIGLDELAKS..GGGEYIARTDADD 98

lgtA 101 IASPGWIEKIVGEMEKDRSIIAMGAWLEVLSEEKDGNRLARHHKHGKIWK 150
         ||||||||||||||||||||||||||||||||.: . ||   ::| ||.
lgtD  99 IASPGWIEKIVGEMEKDRSIIAMGAWLEVLSEENNKSVLAAIARNGAIWD 148

lgtA 151 KPTRHEDIAAFFPFGNPIHNNTMIMRRSVIDGGLRYDTERDWAEDYQFWY 200
         ||||||||.|.|||||||||||||||||||||:|..   ||||.|||
lgtD 149 KPTRHEDIVAVFPFGNPIHNNTMIMRRSVIDGGLRFDPAYIHAEDYKFWY 198

lgtA 201 DVSKLGRLAYYPEALVKYRLHANQVSSKHSVRQHEIAQGIQKTARNDFLQ 250
         :.:|||||||||||||||:|.:|.|||..:.|: .| |... |.:::.
lgtD 199 EAGKLGRLAYYPEALVKYRFHQDQTSSKYNLQQRRTAWKIKEEIRAGYWK 248

lgtA 251 SMGFKTRFDSLEYRQTKAAAYELPEKDLPEEDFERARRFLYQCFKRTDTP 300
         . |: .  |:|:|   |..||.| ||.|.::|:: | ||||:| . :.
lgtD 249 AAGIAVGADCLNYGLLKSTAYALYEKALSGQDIGCLRLFLYEYFLSLEKY 298

lgtA 301 PSGAWLDFAADGRMRRLFTLRQYFGILYRLIKNRR 335
         . ..:||| .|  ||:||. .|| || :::: ::
lgtD 299 SLTDLLDFLTDRVMRKLFAAPQYRKILKKMLRPWK 333
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Figure 3. Homology of the protein products of *lgtA* and *lgtD*. The primary structure of two proteins is very similar, particularly in the first half of the sequences. The glycine residues starting at position 86 reflect the coding of the poly-G regions in the respective genes. The Bestfit program of the Genetics Computer Group package was used, and the symbols |, :, and . represent degrees of similarity based on the Dayhoff PAM-250 matrix.

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lgtB   1 MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLEQAMAELVP 50
lgtE   1 MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLEQAMAELVP 50

lgtB  51 GLSAHLYLSGVEKACFMSHAVLWEQALDEGLPYIAVFEDDVLLGEGAEQF 100
lgtE  51 GLSAHPYLSGVEKACFMSHAVLWEQALDEGLPYIAVFEDDVLLGEGAEQF 100

lgtB 101 LAEDTWLQERFDPDSAFVVRLETMFMHVLTSPSGVADYGGRAFPLLESEH 150
lgtE 101 LAEDTWLEERFDKDSAFIVRLETMFAKVIVRPDKVLNYENRSFPLLESEH 150

lgtB 151 CGTAGYIISRKAMRFFLDRFAVLPPERLHPVDLMMFGNPDDREGMPVCQL 200
lgtE 151 CGTAGYIISREAMRFFLDRFAVLPPERIKAVDLMMFTYFFDKEGMPVYQV 200

lgtB 201 NPALCAQELHYAKFHDQNSALGSLIEHDRRLNRKQQRRDSPANTFKHRLI 250
lgtE 201 SPALCTQELHYAKFLSQNSMLGSDLEKD....REQGRRHRRSLKVMFDLK 246

lgtB 251 RALTKIGREREKRRKRR.....EQTIGKIIVPFQ 279
lgtE 247 RALGKFGREKKKRMERQRQAELEKVYGRRVILFK 280
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Figure 4. Homology of the protein products of *lgtB* and *lgtE*. The primary structure of two proteins is very similar, particularly in the first half of the sequences. These sequences also have significant homology to *lex-1* (62) and *lic2A* (62) genes of *H. influenzae*. For meaning of symbols, see Fig. 3.

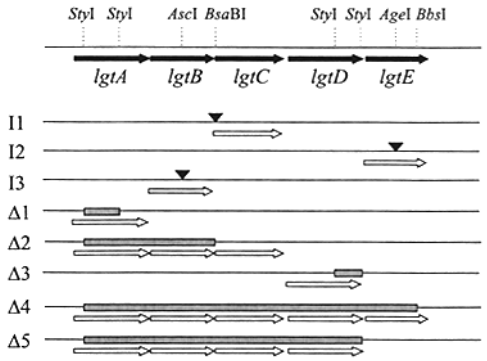

Figure 6. Deletions in the LOS locus. Three insertions and five deletions of the LOS locus were constructed as detailed in Materials and Methods. The restriction sites that were used are indicated. The insertions are marked by triangles and the extent of the deletions by *stippled boxes*. The open arrows indicate the open reading frames disrupted by the construction. In each of the constructs, the erythromycin marker *ermC'* was inserted at the site of the insertion or the deletion.

coding frame runs of guanosines coding for stretches of glycines (Fig. 2). These poly-G regions were found in *lgtA* (17 bp), *lgtC* (10 bp), and *lgtD* (11 bp); in each case, the number of G residues was one that maintained an intact reading frame (Figs. 3 and 5). In each of the three genes, a change of 1 or 2 G bases would cause premature termination of the transcript.

LOS Phenotype of Neisseria gonorrhoea F62 with Deletions of the LOS Locus. To define the function of the *lgt* genes, insertions or deletions of the LOS locus were constructed in plasmids propagated in *E. coli*. The insertions or deletions in each case were marked with the *ermC'* gene, which is an excellent selective marker in *N. gonorrhoeae* (53). The constructions are summarized in Fig. 6. I1, I2, and I3 refer to insertions of the *ermC'* marker into, respectively, a BsaBI, AgeI, and AscI site. Similarly, the deletions were constructed by excising portions of the plasmids and substituting the erythromycin marker. The open arrows indicate the gene or genes disrupted. Each of these plasmids was used to transform *N. gonorrhoeae* strain F62, and transformants were selected on erythromycin-containing plates. The fidelity of the genomic alteration of a prototype of each of the gonococcal transformants was verified by sequencing the upstream and downstream junctions of the *ermC'* gene. To simplify the nomenclature, in this report the gonococcal mutants have been given the same names used to identify the plasmid constructs shown in Fig. 6.

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rfaI  29 LDIAYGTDKNFLFGCGISIASILKYNEGSRLCFHIFTDYFGDDDRKYFDA 78
lgtC   1 MDIVFAADDNYAAYLCVAAKSVEAAHPDTEIRFHVLDAGISEENRAAVAA 50

rfaI  79 LALQYKTRIKIYLINGDRLRSLP.STKNWTHAIYFRFVIADYFINKAPKV 127
lgtC  51 .NLRGGGNIRFIDVNPEDFAGFPLNIRHISITTYARLKLGEY.IADCDKV 98

rfaI 128 LYLDADIICQGGTIEPLINFSFPDDKVAMVV...TEGQADWWEKRAHSLGV 174
lgtC  99 LYLDTDVLVRDGLKPLWDTDLGGNWVGACIDLFVERQEGYKQK....IGM 144

rfaI 175 AGIAKGYFNSGFLLINTAQWAAQQVSARAIAMLNEPEIIKKITHPDQDVL 224
lgtC 145 AD.GEYYFNAGVLLINLKKWRRHDIFKMSCEWVEQYKDVMQ..YQDQDIL 191

rfaI 225 NMLLADKLIFADIKYNTQFSLNYQLKESFINPVTNDTIFI..........  264
lgtC 192 NGLFKGGVCYANSRFNF.MPTNYAFMANGFASRHTDPLYLDRTNTAMPVA 240

rfaI 265 ..HYIGPTKPWHDWAWDYPVSQAFMEAKNASPWKNTALLKPNNSNQLRYS 312
lgtC 241 VSHYCGSAKPWH...RDCTVWGAERFTELAGSL..TTVPEEWRGKLAVPP 285

rfaI 313 AKHMLKKHRYLKGFSNYLFYFI 334
lgtC 286 TKCML..QRWRKKLSARFLRKI 305
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Figure 5. Homology of the protein products of *rfaI* and *lgtC*. The *E. coli rfaI* and *rfaJ* genes are very closely related. They serve as glucosyl transferases of two glucose residues in the LPS core region (65). The glycines at position 54–56 in *lgtC* are encoded by the poly-G tract. For meaning of symbols, see Fig. 3.

The LOS of the mutants were examined by SDS-PAGE and compared with the LOS of strain 1291e. This strain was originally isolated by Dudas and Apicella (38) as a pyocin-resistant mutant of strain 1291 wild-type (wt) and has been extensively characterized, both chemically and genetically. Chemical analysis has shown that this mutant lacks completely the lacto-*N*-neotetraose substitution on heptose 1 (21). The genetic basis of this mutant has been defined (39, 40); it is a mutation of the *pgm* gene coding for phosphoglucomutase. This mutation prohibits the synthesis of UDP-glucose and hence the addition of glucose to the heptose. As seen in Fig. 7, the parental wt F62 strain gives rise to two major LOS bands; their appearance is indistinguishable from SDS-PAGE patterns previously published by other workers (66). The mutants are arranged on the gel according to the size of the major band that they contain. The size decreases from the top band of the F62 wt LOS in four clear steps to the size of the LOS of Δ4 or I2. Since the I2 mutant (with an insertion into *lgtE*, the last gene in the locus) has the same phenotype as Δ4 (which has a complete deletion of the locus), it suggests that the *lgtE* product performs the first biosynthetic step. Thus, the enzymes encoded by *lgtA–D*, although intact, do not have a substrate to act upon. Mutant Δ5 (a deletion of the locus with the exception of *lgtE*) gives rise

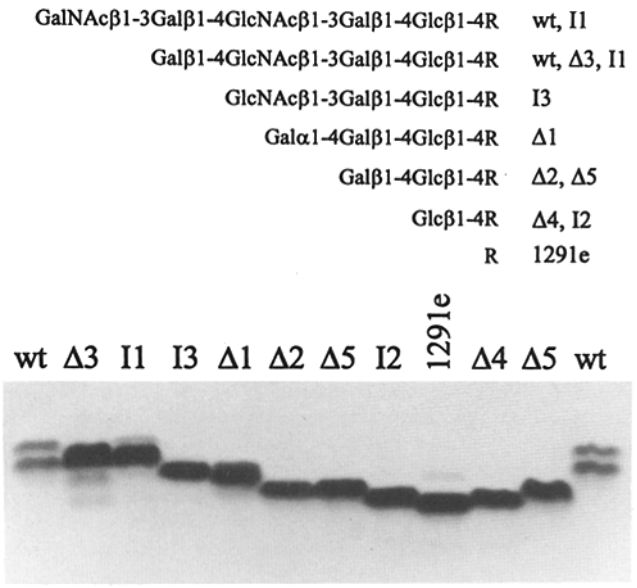

Figure 7. Silver-stained SDS-PAGE of LOS preparations. Gel electrophoresis of purified LOS samples of 375 ng was performed and stained as described in Materials and Methods. Above the gel are indicated the structure of the LOS of the major bands inferred to be present in each of the preparations. These structures are based on the reactivity with monoclonal antibodies shown in Fig. 8, but are presented in this figure to facilitate interpretation of the patterns observed. *R* stands for the inner core region and lipid A. *1291e* is a pyocin-resistant mutant (38).

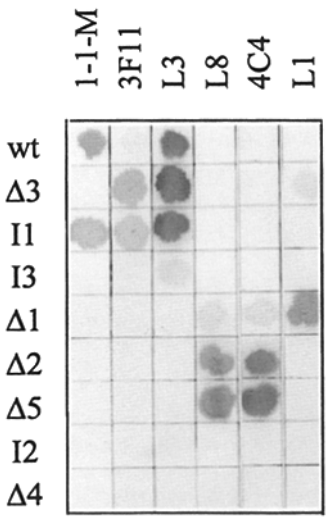

Figure 8. Reactivity of LOS from strain F62 wt and mutants with monoclonal antibodies. The names of the following monoclonal antibodies were abbreviated: 17-1-L1 (*L1*), 9-2-L378 (*L3*), 2-1-L8 (*L8*). Purified LOS was applied to Immobilon-P membranes, allowed to react with the antibodies, and developed as described in Materials and Methods. The specificity of the monoclonal antibodies is summarized in Fig. 1.

to a LOS that is one step larger, supporting the idea that this gene accounts for the initial biosynthetic step. Note that the LOS of both I2 and Δ4 mutants is perceptibly larger than the LOS of strain 1291e, which is known to be unable to add glucose, the first residue in the lacto-*N*-neotetraose chain. These data suggest that *lgtE* encodes the galactosyl transferase enzyme, which adds the first galactose of the lacto-*N*-neotetraose chain.

The LOS preparations were also studied by use of a dot blot technique for their reactivity with monoclonal antibodies. The monoclonal antibodies used and their reported specificities are shown in Fig. 1. The reactions observed with the LOS obtained from the parental strain and the mutants are summarized in Fig. 8. The reactivity of the parental F62 with 1-1-M, 3F11, and L8 was as reported previously by Mandrell et al. (67) and Yamasaki et al. (68). Mutants Δ4 and I2 fail to react with any of the antibodies. However, Δ5 gives a strong reaction with antibodies 4C4 and L8, indicating that the first galactose residue is present. This is in keeping with the SDS-PAGE results (see Fig. 6) and supports the role of *lgtE* as the galactosyl transferase. It also indicates that deletions upstream of *lgtE* do not significantly inactivate its function by polar effects. The LOS of F62 wt parent has strong reactivity with L3 and weak reactivity with 3F11. It is known that reactivity 3F11 is occluded by the addition of the GalNAc residue (37); this is not the case with the L3 antibody. The wt LOS reacts with 1-1-M, the antibody reactive when the terminal GalNAc residue is present. The reactivity with 1-1-M is lost in Δ3, which has a deletion only in *lgtD*. This suggests that this gene encodes the GalNAc transferase. The reactivity with antibody L1 (specific for the alternative LOS structure capped with an α1 → 4Gal) is not seen in wt LOS, is absent in I1, and all deletions that affect *lgtC*. The reactivity is strongest in Δ1, which has a deletion of *lgtA* only. Note that this mutant also has lost reactivity with 3F11 and L3. These two findings suggest that *lgtA* codes for the GlcNAc transferase, and when this residue is not added, the incomplete chain is a substrate for the action of *lgtC* to produce the alternative LOS structure. The sizes of the LOS products seen in Fig. 7 are in accord. This suggests that *lgtC* encodes the α-Gal transferase. This is further supported by the weak reactivity of mutant Δ3 with antibody L1. Mutant Δ3 has a deletion of *lgtD* and fails to add the terminal GalNAc, allowing the α-Gal transferase to modify the lacto-*N*-neotetraose group to produce a P_i-like globoside (25). Mutant I3 (with inactive *lgtB*) has lost reactivity with 1-1-M, 3F11, and L1, and remains only weakly reactive with L3. Together with the size of the product, these observations suggest that *lgtB* encodes the galactosyl transferase–adding Galβ1 → 4 to the GlcNAc residue. *Ricinus* lectin RCA-I is specific for terminal galactose in β linkage (69, 70) and was used to confirm the presence of this structure on the LOS preparations. Using ELISA tests, we found that wt, Δ3, Δ2, and Δ5 LOS, expected to bear a terminal βGal, bound the lectin (Fig. 7), while Δ4, I2, Δ1, and I3 were unreactive (data not shown).

Discussion

We have cloned a locus containing five open reading frames. The effect of eight defined mutations within this locus on the size and serological reactivity of the LOS produced by gonococcal transformants suggests that these genes are the glycosyl transferases responsible for the biosynthesis of most of the lacto-*N*-neotetraose chain. The data obtained allow a tentative identification of the function of each of these genes. It is noteworthy that *lgtB* and *lgtE*, which are structurally very closely related, also perform an apparently very similar

biosynthetic task (i.e., the addition of Galβ1 → 4 to, respectively, GlcNAc or Glc). Similarly, the very related *lgtA* and *lgtD* add, respectively, GalNAc or GlcNAc β1 → 3 to a Gal residue. *lgtC*, which is unrelated to the other genes in the locus, is responsible for the addition of a Galα1 → 4. A full structural analysis of the LOS products of each of the mutants is in progress.

The DNA sequence showed that three of the genes (*lgtA*, *lgtC*, and *lgtD*) contain tracts of guanosines, which code for glycines residues in the proteins. These provide a potential mechanism for high-frequency variation of expression of these genes. Slippage in such poly-G tracts is well documented to control the expression of the gonococcal *pilC* genes, with resultant effects on pilus adhesiveness to human epithelial cells (44). In strain F62, the numbers of bases in each of the three poly-G regions were such that the proteins are in frame; this is in keeping with the ability of F62 wt to produce a complete LOS, including the addition of the terminal GalNAc. Three aspects of LOS biosynthesis would potentially be subject to high-frequency variation. The first is the addition of the terminal GalNAc (*lgtD*). This would cause an alteration of reactivity with monoclonal antibody 1-1-M, and this phase variation has been reported by van Putten (34). Similarly, a change in *lgtA* would cause the failure of the addition of GlcNAc to the growing chain and truncate the LOS at the β-lactosyl level. This is a very common form of LOS in gonococci, with a 3.6-kD molecule that confers resistance to the bactericidal effect of normal human serum (71). It is tempting to speculate that the in vitro variation between variants A and C of $MS11_{mk}$ from the β-lactosyl chain to a complete LOS (which had a selective advantage in vivo in the volunteers) could be explained by regaining functional expression of the GlcNAc transferase *lgtA*. Finally, the variable addition of α1 → 4Gal to either the β-lactosyl (p^k-like globo-triose) or the lacto-*N*-neotetraose group (P_i-like globoside) (25) would be under the control of the expression of *lgtC*. The activity of the *lgtC* transferase appears to compete poorly with the other transferases for precursor, and its activity is evident only if either *lgtA* or *lgtD* is silent. For the Galα1 → 4Galβ1 → 4Glc trisaccharide to be synthesized, the GlcNAc transferase *lgtA* must be inactive, and for expression of the P_i-like globoside Galα1 → 4Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc, the GalNAc transferase *lgtD* must be silent.

Comparable high-frequency antigenic variation of *H. influenzae* LOS has also been noted and has been attributed to changes in translational frame caused by shifts in the number of CAAT repeats in two separate loci, *lic1* (72) and *lic2* (62). Shifts allowing the expression of the *lic2* gene are correlated with the expression of an epitope with the structure Galα1 → 4Galβ1 → . Since the *lic2* gene is homologous to *lgtB* and *lgtE*, the galactosyl transferases that link Galβ1 → 4 to Glc or GlcNAc, respectively, it is likely that this is its function in *H. influenzae* LOS synthesis. It is remarkable that, while both these mucosal pathogens have evolved frame shift mechanisms to cause antigenic variation of the LOS, the gonococcal homologs of *lic2* (*lgtB* and *lgtE*) are not the ones that contain poly-G tracts.

While the frame-shift mechanisms discussed above are suited for on/off regulation of gene expression, the structure of the locus also lends itself to more subtle regulation of the level of expression of the genes. It has been demonstrated that growth rate affects the molecular weight distribution and antigenic character LOS species produced (73). While we have not determined the size of the RNA transcripts, it is very likely that *lgtA*, *lgtB*, and *lgtC* (in the instance where the poly-G tracts are such that the coding frame is maintained) are transcribed together. The termination codon of *lgtA* and the initiation codon of *lgtB* in fact overlap, and the distance between the TAA of *lgtB* and the ATG of *lgtC* is only 11 bp. Similarly, the stop codon of *lgtD* and the start codon of *lgtE* are separated by only 18 bp. Yet the organization is such that if any of the three genes subject to phase variation are in the off configuration, then transcription is able to reinitiate effectively at the beginning of the next gene. This ability to reinitiate transcription was clearly seen with the mutations constructed in this study.

The correlation of LOS structure with function is still in its early stages. The major advances in the field have been the development of an understanding of the structure of the molecules and the ability to relate this, often unambiguously, to the reactivity with a number of well-characterized monoclonal antibodies. Added to this is the realization that, in the in vivo environment, which provides CMP-NANA, the organisms may or may not sialylate the LOS, depending on whether the LOS synthesized is a competent acceptor structure. It is well known that sialylation induces a serum-resistant state in many strains. However, the effect of sialylation in local infection is not as well studied. van Putten (34) has shown that sialylation of LOS has a marked inhibitory effect on epithelial cell invasion, without apparently greatly altering adhesion. His studies suggest that, in the mucosal infection, LOS structures that cannot be sialylated may be important for efficient cell invasion. In the context of this report, such structures could be achieved either by the efficient addition of the terminal GalNAc or by shortening the LOS chain by silencing the GlcNAc transferase. The correlation of LOS chemistry with biological reaction has been complicated by the leakiness of the existing LOS mutants isolated by pyocin selection (38, 74). This is in fact exemplified with mutant 1291e, which shows, in addition to the major low molecular weight band, an additional higher band (Fig. 7). The new insight provided into the genetics of the biosynthesis of gonococcal LOS will allow construction of mutants that are not leaky. For instance, Δ4 and Δ5 should be stable mutants since they no longer contain genes with poly-G tracts. The expression of the genes containing the poly-G tracts could be stabilized by engineering the areas so that glycines are encoded by other codons.

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