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The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in *Neisseria meningitidis*

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Neisseria meningitidis **strains express a diverse range of lipopolysaccharide (LPS) structures that have been classified into 12 immunotypes. A feature of meningococcal LPS is the reversible, high-frequency switching of expression (phase variation) of terminal LPS structures. A number of studies are strongly suggestive of a key role for these terminal structures, and their phase-variable expression, in pathogenesis. In a previous study, a locus of three LPS biosynthetic genes, *lgtABE*, involved in the biosynthesis of one of these terminal structures, lacto-*N*-neotetraose, was described. The molecular mechanism of phase-variable expression of this structure is by high-frequency mutation in a homopolymeric tract of G residues in the *lgtA* gene. To investigate the genetic basis of the structural differences between the immunotypes, and the potential for strains to express alternative immunotypes, this locus was examined in all of the immunotype strains. Initially, the *lgt* locus of strain 126E, an L1 immunotype strain, was cloned and sequenced, revealing two active genes, *lgtC* and *lgtE*. The remnants of the *lgtA* and *lgtB* genes and an inactive *lgtD* gene were also present, indicating that the locus may have once contained five active genes, similar to a locus previously reported in *Neisseria gonorrhoeae* strain F62. Probes based on each of the *lgt* genes (*ABCDE*), and the recently reported *lgtG* gene, were used to determine the presence or absence of *lgt* genes within individual strains, allowing the prediction of the phase variation repertoire of these strains. Sequencing to determine the nature of homopolymeric tract regions within the *lgt* genes was carried out to establish the potential for LPS switching. In general, the set of strains examined could be sorted into two distinct groups: one group which phase-vary the α-chain extension via *lgtA* or *lgtC* but cannot make β-chain; the second group phase-vary the β-chain extension via *lgtG* but do not vary α-chain (lacto-*N*-neotetraose).**

Keywords: phase variation, *lgt* genes, lipopolysaccharide, lipooligosaccharide, *Neisseria meningitidis*

INTRODUCTION

Structural studies and the use of mAbs have demonstrated the heterogeneity and complexity of meningococcal LPS (reviewed by Verheul *et al*., 1993), which has been divided into 12 immunotypes (Scholten *et al*., 1994). A feature of meningococcal LPS is the reversible, high-frequency switching of expression (phase variation) of terminal LPS structures (i.e. switching between immunotypes). A limitation of the current immunotyping system is that a particular strain is typed on the basis of the immunotype being expressed at the time of isolation. The potential for an individual strain to switch between immunotypes (its phase variation rep-

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ertoire) cannot be determined by this immunological assay (Scholten *et al*., 1994).

The type of LPS structure expressed is a key factor in interactions with the host. For example, strains expressing the adhesin Opc and the L3 immunotype LPS structure (lacto-*N*-neotetraose, containing a terminal sialic acid) are non-invasive, relative to bacteria expressing the L8 immunotype, non-sialylated, LPS structure, which are invasive (Virji *et al*., 1995). The L3, 7 and 9 immunotypes are relatively serum resistant compared to the L8 immunotype (Moran *et al*., 1994). Studies in the mouse model of infection reveal that the L8 immunotype predominates in the nasopharynx while the L3, 7 and 9 immunotypes are most commonly found in the blood of the infected mice (Mackinnon *et al*., 1993). These studies are strongly suggestive of a key role for these terminal LPS structures, and the phase variation of their expression, in pathogenesis.

Over recent years there has been significant progress in the genetics of LPS biosynthesis (recently reviewed by Kahler & Stephens, 1998). A locus containing three genes, *lgtABE*, required for the biosynthesis of the terminal LPS structure lacto-*N*-neotetraose in *Neisseria meningitidis* strain MC58 has previously been described (Jennings *et al*., 1995). This study also describes the mechanism that controls the phase-variable expression of this structure, which operates via high-frequency mutation in a homopolymeric tract of 14 G residues in the first gene of the locus, *lgtA*. Structural studies of LPS from *lgt* mutant strains and enzyme assays have confirmed that these three genes encode glycosyltransferases for the biosynthesis of lacto-*N*-neotetraose (Wakarchuk *et al*., 1996). Prior to this work, Gotschlich (1994) described a similar locus in *Neisseria gonorrhoeae* strain F62 which contained five LPS biosynthetic genes, *lgtABCDE*. The *N. meningitidis* genes described above are present in the same orientation and order as those in the *N. gonorrhoeae* locus, so that the major difference is the absence of *lgtC* and *lgtD* in the *N. meningitidis* locus. The *lgtC* and *lgtD* genes are involved in the biosynthesis of LPS structures that are not expressed by *N. meningitidis* strain MC58, which can express only the L3 or L8 immunotype (see Fig. 2).

In order to determine whether MC58 was representative of *N. meningitidis* strains, in terms of the limited genetic potential for LPS structural variation compared to the *N. gonorrhoeae* F62 example, we carried out a study of *lgt* loci in a set of *N. meningitidis* immunotype typing strains. Here we present the results of this survey, which has revealed the phase variation repertoire of this set of strains based on the *lgt* genes present and the potential of these genes for phase variation.

METHODS

Media and growth conditions. Meningococcal strains were grown at 37 °C in 5% CO_2 on either brain heart infusion (BHI; Oxoid) or GC-agar (Difco) plates. BHI plates were made with 1% agar and supplemented with 10% Levinthial base (Alexander, 1965). *Escherichia coli* was cultured in LB broth or on LB plates containing 1·5% bacteriological agar (Difco; Sambrook *et al*., 1989). Ampicillin was used at a final concentration of 100 μg ml^{-1}.

Reagents and enzymes. Reagents were supplied by BDH with the exception of Tris (Boehringer Mannheim) and antibiotics (Sigma). Restriction and modification enzymes were obtained from Boehringer Mannheim.

Recombinant DNA techniques and nucleotide sequence analysis. Most recombinant DNA techniques were as described in Sambrook *et al*. (1989). Using methods described previously (Jennings *et al*., 1993), a DNA fragment size enriched library was made to clone the *lgt* locus of strain 126E on a 6 kbp *Xba*I–*Cla*I fragment. The probe used was a PCR product specific for the *lgtE* gene (see below). Conditions for colony screening were the same as described below for Southern blotting. Nucleotide sequence analysis was performed using the PRISM Dye Terminator Sequencing Kit with AmpliTaq DNA polymerase FS (Perkin Elmer) in conjunction with a model 373a automated sequencer (Applied Biosystems). PCR was essentially as described by Saiki *et al*. (1988). Nucleotide sequence analysis was done using the GCG sequence analysis package (Devereux *et al*., 1984). Oligonucleotide primers used for PCR and sequencing are listed in Table 1. Sequence analysis of the number of residues in the homopolymeric tracts of the *lgtA*, *lgtC*, *lgtD* and *lgtG* genes was determined in a set of immunotype typing strains. The *lgtD* and *lgtC* homopolymeric tracts were sequenced from the cloned 126E *lgt* locus (this work). The *lgtC* gene of M978 was amplified with lgtCF and lgtCR, and sequenced with primers lgtCF and R99. The homopolymeric tract region of *lgtA* was amplified using lic31ext and lic16ext, and sequenced with the same primers. The *lgtG* homopolymeric tract region was amplified using primers LG1 and LG2, and sequenced with LG3 and LG4. All sequences reported were determined by nucleotide sequence analysis of pooled triplicate independent PCR products on both strands.

Southern blotting and hybridization. Restriction-endonuclease-digested genomic DNA was separated on 0·7% agarose gels and transferred to Hybond-N nylon membrane (Amersham International), essentially as described in Sambrook *et al*. (1989). DNA fragments were labelled with [α-^{32}P]dCTP (Amersham International), using a random primed labelling kit (Promega). Probes for each of the *lgtA* genes were made by PCR amplification using either p1b11 (Jennings *et al*., 1995) or p126E as templates (this work). The primer pairs (see Table 1) and probe sizes were as follows: *lgtA* probe, primers lic2 and lic23, 550 bp; *lgtB* probe, primers lic13 and lic1, 804 bp; *lgtC* probe, primers lgtCF and lgtCR, 945 bp; *lgtD* probe, primers lic28 and Xmore37, 622 bp; *lgtE* probe, primers X1356ext and HS135ext, 589 bp; *lgtG* probe, primers LG2 and LG3, 603 bp. Filters were prehybridized at 65 °C in a mixture of 6 × SSC, 0·5% SDS, 5 × Denhardt's solution and 100 μg denatured salmon sperm DNA ml^{-1}, for 4–6 h. Hybridizations were performed overnight in the same solution and filters were subsequently washed, in 0·1 × SDS, 0·1% SSC for 3 × 15 min at 65 °C, prior to autoradiography.

Colony-immunoblots. To examine the potential for phase variation between immunotypes, colony-immunoblots were performed as described previously (Jennings *et al*., 1995). For each strain tested at least 10000 individual colonies were plated and examined for switching. mAbs Mn14F20-11, Mn4A8B2 and 17–1-L1 are described by Scholten *et al*. (1994). mAb Mn42F12.32 was isolated after immunization of mice

Table 1. Oligonucleotide primers

Primer	Sequence 5′–3′	Position*	Reference sequence
lic2	atgcagacgatattgccg	614–631	U25839
lic23	ttccggcaaatgtttctcca	1144–1164 rc	U25839
lic31ext	cctttagtcagcgtattgatttgcg	334–358	U25839
lic16ext	cgatgatgctgcggtctttttccat	664–688 rc	U25839
lic13	gttatcagcttagcttcc	1383–1400	U25839
lic1	ggcacaatgaactgttcg	2171–2188 rc	U25839
lgtCF	ggagaaaagatggacatcgtattttgcgg	652–679	U65788
lgtCR	gtcaataaatcttgcgtaagaatct	1573–1597 rc	U65788
R99	cccaaatcggtatcccataa	1003–1022	U65788
lic28	ttatggcctctttaaaacat	1867–1885	U65788
Xmore37	cctgtccggacaaggctttt	2470–2489 rc	U65788
X135ext	ccatgttatcagcttag	2355–2371	U25839
HS135ext	aggcgggattaacctg	2929–2944 rc	U25839
LG1	atgaagctcaaaatagacattg	209–230	AF076919
LG2	ttatacggatgccagcatgtc	1244–1264 rc	AF076919
LG3	atacggcgttccccccgaaa	661–680	AF076919
LG4	atgggaataggaaaacggtt	718–800 rc	AF076919

*Position in relation to the GenBank sequence quoted in the last column. rc, Reverse complement.

with outer-membrane complexes of the L2 strain 3006 (B. Kuipers & P. van der Ley, unpublished).

RESULTS

Cloning and sequencing of the *lgt* locus from *N. meningitidis* strain 126E

In order to examine the *lgt* locus of a strain known to express a different LPS structure to the previously described locus of strain MC58 (L3 immunotype; Jennings *et al*., 1995), we chose the L1 immunotype type strain 126E. This strain expresses a distinct, di-galactoside structure that defines the L1 immunotype (Scholten *et al*., 1994; see Fig. 2). Chromosomal DNA from this strain was digested with a range of restriction endonucleases and probed in a Southern hybridization with an *lgtE* probe derived from *N. meningitidis* strain MC58 (Methods and Fig. 1). A 6 kbp *Xba*I–*Cla*I band which hybridized with the probe was chosen for cloning (result not shown). A plasmid containing the desired fragment, identified above, was isolated by colony hybridization using the same probe. A map of this plasmid, designated pMJ126E, is shown in Fig. 1. Nucleotide sequence analysis of the cloned fragment confirmed that it contained the complete *lgt* locus (the sequenced region is indicated in Fig. 1). The locus contains four ORFs. The first of these begins with TTG at position 296 and terminates at a TAA codon at position 659. The first half of this ORF is homologous to the *lgtA* gene of *N. meningitidis* strain MC58 (Jennings *et al*., 1995). Between positions 486 and 487 this homology ends and the remainder of the ORF is homologous to the *lgtB* gene of MC58. Comparison with the same region in strain

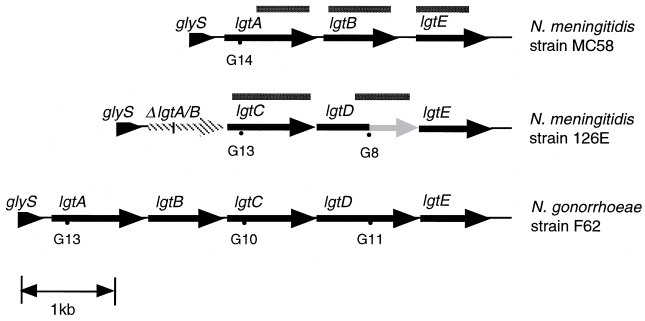

Fig. 1. Diagrammatic representation of the *lgt* loci of *N. meningitidis* strain MC58 (Jennings *et al*., 1995) and *N. gonorrhoeae* strain F62 (Gotschlich, 1994) and of the cloned *Xba*I–*Cla*I fragment in plasmid pMJ126E from *N. meningitidis* strain 126E (this work). The *lgt* genes are indicated by arrowed lines. A frameshift mutation in the homopolymeric tract of *lgtD*, which puts the gene out of frame for expression, is indicated by transition from a black to a grey arrowed line. An ORF composed of the remnants of the *lgtA* and *lgtB* genes, which is presumed to be inactive, is indicated with a striped, arrowed line. Where a homopolymeric tract of G residues is present in a gene its position and the number of residues in the tract are indicated below the gene. The horizontal bars above the genes indicate the probes used in Southern blot experiments.

MC58 revealed that there is a 1·5 kbp deletion in this locus between the *lgtA* and *lgtB* genes. This deletion has removed 81·5 % of the *lgtA* coding region and 82·6 % of the *lgtB* coding region (see Fig. 1 for comparison), so that these genes are presumably inactive. Twelve base pairs downstream of this ORF is an ATG codon for the

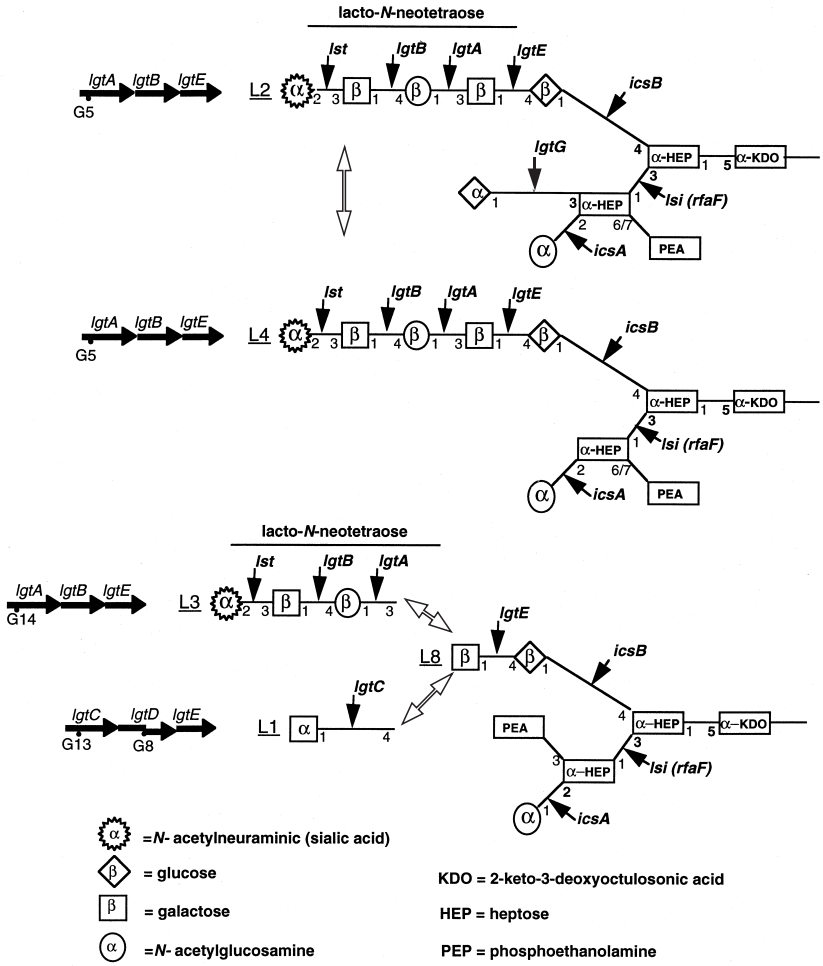

Fig. 2. Primary structure of a selection of meningococcal oligosaccharides of immunotypes L1, L2, L3, L4 and L8. Immunotypes are labelled to the left of each structure. Open arrows show phase variation between structures. Only the terminal structures of L1 and L3 are shown, as they are extensions of the L8 basal structure. The structure of immunotype L6 has the same basal structure as L4, but the terminal two sugars [α-Neu5Ac-(2–3)-β-D-Gal-] are missing. The enzyme activities of the *lgtA*, *lgtB*, *lgtC*, *lgtE* and *lgtG* gene products are indicated with arrows. The *lgt* genes present in each of the type strains are indicated with arrowed lines on the left of the figure. The arabic numbers indicate the position of the binding site; α and β indicate the anomeric configuration. Based on Scholten *et al.* (1994).

beginning of the next ORF which terminates with a TGA codon at 1596. This ORF is homologous to the *lgtC* gene of *N. gonorrhoeae* strain F62 (Gotschlich, 1994). Notable in the sequence is a homopolymeric tract of 13 G residues, since, in contrast, the *N. gonorrhoeae* homologue of strain F62 has 10. Alterations in the number of Gs in this region of the *lgtC* gene of *N. gonorrhoeae* have been shown to mediate phase variation (Yang & Gotschlich, 1996). The next ORF has a possible TGG initiation codon at 1656 and terminates at 2105. This ORF is homologous to the *lgtD* gene of *N. gonorrhoeae* (Gotschlich, 1994). The *N. meningitidis* 126E *lgtD* gene contains a short homopolymeric tract of eight G residues which places sequence located 3′ of the homopolymeric tract out of reading frame. By comparison, *N. gonorrhoeae* strain F62 has a homopolymeric tract of 11 Gs which is in-frame, due to an extra A residue adjacent to the tract (in comparison to the 126E *lgtD* gene). The activity of *lgtD* in *N. gonorrhoeae* is to add a terminal *N*-acetylgalactosamine to lacto-*N*-neotetraose. As *lgtA* and *lgtB* are inactive in strain 126E, and lacto-*N*-neotetraose cannot be made, the *lgtD* gene product has no acceptor molecule for its transferase activity. The final ORF begins at 2684 and ends at 3526, and is homologous to the *lgtE* gene of *N. meningitidis* strain MC58 (Jennings *et al.*, 1995).

A survey of the *lgt* loci of immunotype typing strains by Southern hybridization

To determine the distribution of the different *lgt* genes in a set of immunotype strains, and thereby the genetic potential for structural variation, all of the immunotype typing strains were probed for the presence of the *lgtA*, *lgtB*, *lgtC*, *lgtD* and *lgtE* genes. Fragments to be used as probes were amplified by PCR from strain MC58 or strain 126E chromosomal DNA (see Methods and Table 1). The positions of these probes are indicated in Fig. 1. The result displayed in Fig. 3 is of chromosomal DNA from a set of immunotype type strains digested with *Cla*I. The same *Cla*I band is identified by each of the different probes within a particular strain, indicating that all of the *lgt* genes identified by these probes are present on the same *Cla*I fragment, presumably within a locus similar to those previously described (*N. meningitidis* strain MC58, Jennings *et al.*, 1995; *N. meningitidis* strain 126E, this work; and *N. gonorrhoeae* strain F62, Gotschlich, 1994). The size of the *Cla*I fragment varies

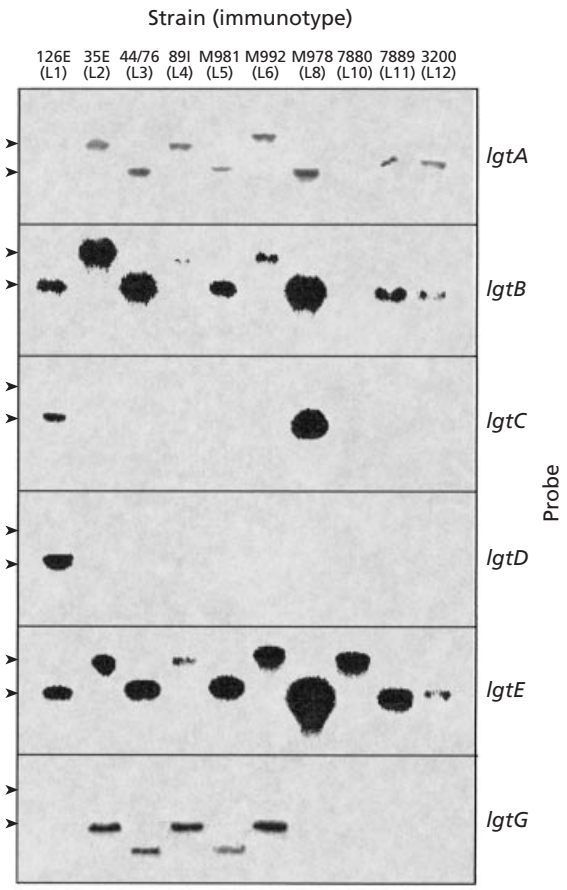

Fig. 3. Panels show the results of Southern hybridization experiments with probes specific for *lgt* genes (see Methods). The probe used in each experiment is indicated at the right of the figure. The meningococcal strain that was the source of the DNA preparation is indicated at the top of the figure with the immunotype of the strain indicated below the strain name in parentheses. Arrowheads to the left of each panel indicate the position of molecular mass markers; the lower corresponds to 6 kbp and the higher to 9 kbp.

from strain to strain, and it is noteworthy that there is a trend for strains expressing a common basal structure (e.g. L1, L3 and L8), or immunotypes that are restricted to occurring in the same serogroup (e.g. L10 and L11; serogroup A) to have *Cla*I bands of the same size.

During the preparation of this manuscript the sequence of a new LPS biosynthetic gene, *lgtG*, from *N. gonorrhoeae* was reported (Banerjee *et al*., 1998). The activity of this gene product is the transfer of a glucose residue α1–3-linked to the second heptose of the core (β-chain extension). This structure is also found in the L2 (see Fig. 2) and L5 immunotypes. The authors also reported a homopolymeric tract of C residues that controls phase-variable expression of this gene. Using primers based on the published *lgtG* sequence we amplified this gene from the L2 type strain, 35E (see Methods). Nucleotide sequence analysis revealed that the PCR product was highly homologous to that reported for *N. gonorrhoeae* (data not shown). The same set of *N. meningitidis* immunotype typing strains was probed with an *lgtG* probe. The gene was detected in L2, L3, L4, L5 and L6 strains only. The size of the *Cla*I fragment detected with the *lgtG* probe was different to that detected with the other *lgt* probes, indicating that the *lgtG* gene is not closely linked.

Variation in the poly-G regions of the *lgtA* gene and poly-C region of the *lgtG* gene in the immunotype typing strains

In previous work, we have described the mechanism of phase variation between the immunotypes, which is based on high-frequency frameshift mutations which occur in a homopolymeric tract of G residues in the *lgtA* gene (Jennings *et al*., 1995). This mechanism has been confirmed in independent studies in *lgt* genes of *N. gonorrhoeae* (Danaher *et al*., 1995; Yang & Gotschlich, 1996; Banerjee *et al*., 1998). Variation between the L3 and L8 immunotypes, mediated by *lgtA* expression, results in the loss and gain of the lacto-*N*-neotetraose structure. Such variation has not been observed in the L2 and L4 immunotypes, i.e. a switch between either L2 or L4, and a structure lacking lacto-*N*-neotetraose (see Fig. 2). To investigate whether this was due to differences in the homopolymeric tract of the *lgtA* gene, we determined the number of G residues in the *lgtA* genes of each of the immunotype strains. This was achieved by PCR amplification of the region followed by nucleotide sequence analysis of the homopolymeric tract region (Methods and Fig. 4). As expected, MC58 and another L3 type strain, H44/76, contained 14 G residues consistent with *lgtA* expression. The L8 strain, M978, contains 13 G residues resulting with an (currently) inactive *lgtA* gene, consistent with the absence of the lacto-*N*-neotetraose moiety in the L8 immunotype structure (see Fig. 2). Immunotypes L10, L11 and L12 contained 9, 12 and 10 G residues, respectively, all currently out of frame for expression. L11 and L12, which have *lgtA*, *lgtB* and *lgtE* (see above), presumably only required a single nucleotide change to express lacto-*N*-neotetraose. L10 was negative for *lgtA* in the Southern blot experiment, and presumably contains only the 5′-end of *lgtA*, outside the region of probe binding.

In contrast to the immunotypes listed above, immunotypes L2, L4, L5 and L6 do not contain a long homopolymeric tract in *lgtA*. Instead they have a tract of only five G residues, preceded by the sequence 5′-AGGAAT-3′ (see Fig. 4). This sequence is in-frame for expression of the *lgtA* gene and, consequently, expression of lacto-*N*-neotetraose by all of these immunotypes (or equivalent in the case of L6). Phase variation to a truncated structure that lacks lacto-*N*-neotetraose (similar to the L8 structure) has not been reported in these immunotypes. The reduced number of G residues, presumably not subject to high-frequency mutation, is consistent with constitutive expression of lacto-*N*-neotetraose by these strains.

Strain	IT	Nucleotide sequence of the *lgtA* poly G region	#G	in/out of frame
35E	L2	TTGGCAAAGTCAGGAAT---GGGGGAATATATTGCACGCACCGATGCAGACGATATTGCC	5	in
MC58	L3	TTGGCAAAGTCGGGGGGGGGGGGGGAATATATTGCGCGCACCGATGCAGACGATATTGCC	14	in
H44/76	L3	TTGGCAAAGTCGGGGGGGGGGGGGGAATATATTGCGCGCACCGATGCAGACGATATTGCC	14	in
89I	L4	TTGGCAAAGTCAGGAAT---GGGGGAATATATTGCACGCACCGATGCCGACGATATTGCC	5	in
M981	L5	TTGGCAAAGTCAGGAAT---GGGGGAATATATTGCACGCACCGATGCCGACGATATTGCC	5	in
M992	L6	TTGGCAAAGTCAGGAAT---GGGGGAATATATTGCACGCACCGATGCCGACGATATTGCC	5	in
M978	L8	TTGGCAAAGTC-GGGGGGGGGGGGGGAATATATTGCACGCACCGATGCCGACGATATTGCC	13	out
7880	L10	TTGGCAAAGTC-----GGGGGGGGGGAATATATTGCACGCACCGATGCCGACGATATTGCC	9	out
7889	L11	TTGGCAAAGTC--GGGGGGGGGGGGGAATATATTGCGCGCACCGATGCAGACGATATTGCC	12	out
3200	L12	TTGGCAAAGTC----GGGGGGGGGGGAATATATTGCGCGCACCGATGCAGACGATATTGCC	10	out

LeuAlaLysSer.....GluTyrIleAlaCysThrAspAlaAspAspIleAla ..*lgtA* gene product.

Variable region

Strain	IT	Nucleotide sequence of the *lgtG* poly C region	#C	in/out of frame
35E	L2	AATCCAAGTCGCCCCCCCCCCC---GCAGATACGGAACGC	11	in
H44/76	L3	AATCCAAGTCGCCCCCCCCCC----GCAGATACGGAACGC	10	out
H44/76L2	L2	AATCCAAGTCGCCCCCCCCCCC---GCAGATACGGAACGC	11	in
89I	L4	AATCCAAGTCGCCCCCCCCCC----GCAGATACGGAACGC	10	out
M981	L5	AATCCAAGTCGCCCCCCCCCCC---GCAGATACGGAACGC	11	in
M992	L6	AATCCAAGTCGCCCCCCCCCCCCCCCGCAGATACGGAACGC	14	in

IleGlnValAla.....AlaAspThrGluArg ..*lgtG* gene product.

Variable region

Fig. 4. Nucleotide sequence of the homopolymeric tract region of the *lgtA* and *lgtG* genes from a set of immunotype type strains. The region shown for *lgtA* corresponds to 1261–1313 in the sequence deposited under accession number U73942, derived from strain MC58. The region shown for *lgtG* corresponds to 682–718 in the sequence deposited under accession number AF076919, derived from *N. gonorrhoeae* strain 15253. The strain name and immunotype from which the sequence was determined are indicated to the left of the sequence. The deduced amino acid sequence is indicated below each set of sequences. The number of residues in the homopolymeric tract is indicated to the right of the sequences along with an indication of whether this number of residues puts the gene in or out of reading frame.

In order to investigate the size of the homopolymeric tract of the *lgtG* gene of each strain, the *lgtG* gene was amplified by PCR and the tract region sequenced. The result, shown in Fig. 4, revealed that the L2 and L5 immunotypes contained 11 C residues, like the *N. gonorrhoeae* example (Banerjee *et al.*, 1998), which is in-frame for expression. Strain M992 (L6) had 14 C residues, which is also in-frame for expression of *lgtG*. These findings are consistent with the published structures for L2 and L5 immunotypes, which express the α1–3-linked glucose structure, but not the L6 strain, which is not reported to express this structure. The L4 and L3 immunotype strains were out of frame for expression, consistent with the absence of the α1-3-linked glucose extension in the structures of these immunotypes.

Colony-immunoblot analysis of the phase variation repertoire

To confirm the potential for structural variation that was predicted by the Southern blot data, the phase variation repertoire of several strains was tested for immunotype switching by colony-immunoblot experiments. The selection of strains that could be tested was restricted as only a subset of the mAbs used in immunotyping ELISA (Scholten *et al.*, 1994) are suitable for colony-immunoblot experiments. The results of the colony-immunoblot experiments are displayed in Table 2. Southern blots (see above) suggested that strain M978 (L8 immunotype) could potentially switch between three alternate immunotypes, L8, L1 and L3, as it contained the *lgtA*, *lgtB*, *lgtC* and *lgtE* genes (see Figs 2 and 3). Using mAb Mn4A8B2, switching from $-$ to $+$ was observed, so from L8 to L3. Using mAb 17-1-L1 there was switching from $-$ to $+$, so from L8 to L1. These results confirm the phase variation repertoire predicted for strain M978 from the Southern blot data. Strain 126E is predicted to be limited to variation between L1 and L8 as it contains only active *lgtC* and *lgtE* genes. With mAb 17-1-L1 there was a switch from $+$ to $-$, so from L1 to L8. With mAb Mn4A8B2 all colonies were negative, so there was no switch from L1 to L3 – consistent with absence of the *lgtA* gene in the Southern blot result, which was confirmed by the deletion of most of the *lgtA* and *lgtB* genes seen in the nucleotide sequence of strain 126E. Strain M992 (L6 immunotype) hybridized with the *lgtB* probe, suggesting the potential for extension of the L6 structure to the L4-like structure (see Figs 2 and 3). With mAb Mn15A8-1 all colonies are negative, therefore no switch was detectable from L6 to L4. With mAb Mn4C1B all colonies remain positive. This result

Table 2. Phase variation experiments using colony-immunoblots

Strain tested for phase variation	mAb used in colony-immunoblots (immunotype specificity)				
	17-1-L1 (L1)	**Mn4A8B2 (L3)**	**Mn42F12.32 (L2)**	**Mn15A8-1 (L4)**	**Mn4C1B (L6)**
M978 (L8 type strain)	− to + (L8 to L1)	− to + (L8 to L3)	ND	ND	ND
126E (L1 type strain)	+ to − (L1 to L8)	− to + (not seen)	ND	ND	ND
H44/76	ND	ND	− to + (L3 to L2)*	ND	ND
M992	ND	ND	ND	− to + (not seen)	+ to − (not seen)

ND, Not done.

* Upon switching of H44/76 − to + with Mn42F12.32 (L2), switched colonies also become Mn4A8B2 (L3) negative. Rate of variation estimated at 1 in 1000–5000.

suggests that although strain M992 hybridizes with the *lgtB* probe, the gene may be inactive.

The data from the Southern blot and sequencing suggest that the L3 strain H44/76 has an *lgtG* gene and that this gene is out of frame for expression (see Figs 3 and 4). Experiments using mAb Mn42F12.32 revealed a switch from L3 and L2. Nucleotide sequence analysis of the *lgtG* homopolymeric region of a switched colony, H44/76L2, revealed an alteration in the number of C residues from 10 to 11. These results are consistent with the phase variation of *lgtG* mediating the L3 to L2 switch.

DISCUSSION

Comparison of the *lgt* loci of *N. gonorrhoeae* strain F62 and *N. meningitidis* strain MC58 reveals a stark contrast between the simple phase variation between two possible terminal structures from the first heptose in the case of *N. meningitidis* ('α-chain extension'; see Fig. 2) and the complex situation in *N. gonorrhoeae*, where four alternate α-chain extensions are possible within one strain (Gotschlich, 1994). A recent analysis of *N. gonorrhoeae* strain 1291 has revealed that this strain also has a locus containing *lgtABCDE*, suggesting the F62 example is not atypical of *N. gonorrhoeae* strains (M. P. Jennings & M. Apicella, unpublished). In this study we sought to determine whether strain MC58 was typical of meningococcal strains in the repertoire of phase variation in this region of the LPS molecule. Analysis of the nucleotide sequence of *N. meningitidis* 126E revealed that, apart from a large deletion event which has inactivated the *lgtA* and *lgtB* genes, this locus clearly derived from a locus similar to that reported in *N. gonorrhoeae* F62. However, the deletion event has limited the strain to active *lgtC* and *lgtE* genes, resulting in a variation between only the L8 and L1 immunotype structures.

Analysis of the set of immunotype type strains with probes based on the five *lgt* genes involved in α-chain extension demonstrates that most strains contain only three genes: *lgtA*, *lgtB* and *lgtE*. This indicates that these strains can only make the lacto-*N*-neotetraose extension from the terminal glucose which extends from the first heptose (see Fig. 2). The exceptions to this trend are the L1 type strain, 126E (discussed above), the L10 type strain, 7880, which contains only *lgtE*, and the L8 type strain, M978, which contains *lgtA*, *lgtB*, *lgtC* and *lgtE*. Colony-immunoblot experiments confirmed that the latter strain can make the L1, L3 and L8 immunotype structures. These data are consistent with our original observation of a restricted repertoire of terminal structures in *N. meningitidis* with only one of the 10 strains examined (M978) capable of more than one alternative α-chain extension.

The *lgtG* gene of *N. gonorrhoeae* which encodes a transferase for an α1-3-linked glucose extension from the second heptose (β-chain extension) was reported during the preparation of this manuscript (Banerjee *et al.*, 1998). This structure is also present in the L2 and L5 immunotype structures, and our additional Southern blot experiments revealed that, as expected, the *lgtG* gene was present in the L2 and L5 type strains, and also in the L4, L6 and L3 type strains.

Southern blot analysis is limited to an indication of gene content, suggesting structures that may be expressed by an individual strain. To examine the current state of expression (in or out of reading frame) and the potential for switching between structures, we looked at the homopolymeric tract regions of *lgtA* and *lgtG* genes, in which the high-frequency mutation events occur that mediate phase variation. In this study, we determined that the L2, L4, L5 and L6 immunotype strains contain only five G residues in the homopolymeric tract of their *lgtA* genes. This suggests that in these strains, the expression of this gene is not subject to phase variation,

and that the terminal lacto-*N*-neotetraose is constitutively expressed (see Fig. 2). The set of strains examined fell into two main groups with respect to phase variation: one group phase-vary α-chain extension via *lgtA* or *lgtC* but cannot make β-chain (immunotype type strains for L1, L8, L11 and L12); the second group phase-vary the β-chain extension via *lgtG* but presumably cannot vary α-chain, lacto-*N*-neotetraose, expression (immunotype type strains for L2, L4, L5 and L6). The exception to this grouping is the L3 strain, H44/76, which appears to have the capability of making extensions from both heptose molecules possessing both *lgtA* and *lgtG*, although the *lgtG* gene is out of frame for expression. We have demonstrated that H44/76 can switch from L3 to L2, and that this correlates with a change in predicted *lgtG* expression via an alteration in the poly-C tract. This finding is consistent with a recently published structural analysis of strain NMB (Rahman *et al*., 1998), which reported that both the L2 and L3 immunotype structures are expressed by this strain. The rate of phase variation from L3 to L2 in H44/76 was observed to be 1 in 1000–5000. This is an order of magnitude lower than has been observed for other LPS phase variations, such as the L3 to L8 transition in strain MC58 which is 1 in 200 (M. P. Jennings, unpublished). This lower rate of variation may be due to the characteristics of the homopolymeric tract of *lgtG* or may indicate that other, *lgtG*-independent events are required prior to the addition of the α1-3-linked glucose structure.

As described above, the switching between L3 and L8 immunotypes in strain MC58, which is mediated by high-frequency mutation in the homopolymeric tract of 14 G residues in the *lgtA* gene, results in modulation of LPS sialylation: L3 immunotype LPS is sialylated; L8 immunotype LPS is not. This difference in LPS sialylation has been demonstrated to have profound effects on adherence and invasion in *in vitro* model systems (Virji *et al*., 1995). The level of surface sialylation has also been associated with serum resistance in both *N. meningitidis* and *N. gonorrhoeae*. As structural studies have reported that the strains which constitutively express lacto-*N*-neotetraose do contain sialic acid (Kogan *et al*., 1997; Gamian *et al*., 1992), the question of how these strains regulate the level of LPS sialylation is raised. Two likely possibilities are either environmental regulation of the *lst* gene, which encodes the sialyltransferase, or an alternative phase variation event which may modify the LPS acceptor molecule.

As described above, the advantage of examining LPS expression in *N. meningitidis* genetically rather than immunologically is the ability to classify strains by their phase variation repertoire, based on a combination of gene content and phase variation potential (homopolymeric tract presence and/or length). This is far more informative than determining the immunotype which happens to be expressed by a single colony picked at the time of isolation, and has the potential to reveal new relationships between aspects of meningococcal disease and this important virulence factor.

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