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Antigenic Variation in *Neisseria gonorrhoeae*: Production of Multiple Lipooligosaccharides

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Individual cells of *Neisseria gonorrhoeae* may express a single lipooligosaccharide (LOS) component on their cell surfaces, or they may simultaneously express multiple LOS structures. Strain FA19 expresses LOS components that react with monoclonal antibodies (MAbs) 2-1-L8 and 1B2. The genetic locus responsible for this phenotype in FA19 was identified by isolating a clone that is able to impart the ability to simultaneously express both LOS molecules to strain 1291, a strain expressing only the MAb 1B2-reactive LOS. This clone, pCLB1, was characterized, and the gene responsible for the expression of both LOS components was determined to be *lsi2*. DNA sequence analysis of $lsi2_{FA19}$ indicates that there are several differences between the DNA sequences of $lsi2_{FA19}$ and $lsi2_{1291}$. The region responsible for the LOS-specific phenotype change in $lsi2_{FA19}$ was identified by deletion and transformation analysis, mapping to a polyguanine tract within *lsi2* where $lsi2_{FA19}$ possesses a +2 frameshift relative to $lsi2_{1291}$. The polyguanine tract in $lsi2_{FA19}$ was modified by site-directed mutagenesis to change the sequence to GGGAGGTGGCGGA to prevent frameshifting during DNA replication, transcription, and/or translation. Transformants of strain 1291 containing this DNA sequence express a single MAb 2-1-L8-reactive LOS component, the same phenotype exhibited by *lsi2*-defective strains. These data indicate that FA19 is able to generate a small amount of functional Lsi2 protein via transcriptional and/or translational frameshifting, and this limited amount of protein allows for the expression of MAb 1B2-reactive LOS molecules.

Neisseria gonorrhoeae, the etiologic agent of the sexually transmitted disease gonorrhea, causes disease through contact with mucosal surfaces such as the urethra, endocervix, pharynx, conjunctiva, and rectum. Because it is capable of proliferating in different physiological environments, it has developed a variety of mechanisms for evading the local host immune response; growth in different environments requires special phenotypes, especially with respect to outer membrane components such as pili (27), protein II (5), and lipooligosaccharide (LOS) (23).

LOS is an outer membrane component that mediates many aspects of disease. It is responsible for damage caused to human fallopian tubes (11), it is a target for bactericidal antibodies found in normal human serum (3, 12), and it plays a role in the attachment of bacteria to epithelial tissues (28). LOS consists of a branched oligosaccharide structure which is anchored to the membrane via lipid A. Variations in its structure are observed within as well as between strains and result in the production of LOSs that differ in the length and/or presence of any of three oligosaccharide chains, the number of LOS components expressed, and/or the relative concentrations of the various components (2, 13, 16, 24, 28). Heterogeneity may result from truncation of the oligosaccharide portion of the molecule at various points in the α-chain or by addition of alternate sugar residues (8, 15, 17, 29). The genes needed to synthesize the α-chain are encoded by a polycistronic operon (10).

The gonococcus is able to express a variety of related LOS structures on its cell surface, and the presence of individual structures can be demonstrated by reactivity with LOS-specific monoclonal antibodies (MAbs). The interconversion between cells making MAb 1B2-reactive and MAb 2-1-L8-reactive LOS has been observed to occur at a rate on the order of 10^{-3} conversion per cell per generation (25). This change in MAb reactivity represents a two-sugar truncation of the LOS α-chain. This interconversion is due to a change in the number of guanine residues in a polyguanine tract located in the middle of one of these genes (*lsi2* [6] = *lgtA* [30]).

N. gonorrhoeae FA19 was clinically isolated by A. Reyn (18) and characterized by Eisenstein et al. (7). It expresses several LOS epitopes, including those that bind MAbs 2-1-L8 and 1B2. The 3.8-kDa LOS terminates with Galβ1 → 4Glc and binds MAb 2-1-L8, while the 4.5-kDa LOS contains lactosamine added to this structure (Galβ1 → 4 GlcNAcβ1 → 3 Galβ1 → 4Glc) and binds MAb 1B2. The implication that production of both of these epitopes may be necessary during some stages of infection (23) underlies the need to understand the genetic mechanism which makes this possible. Since individual *N. gonorrhoeae* cells may express single or multiple LOS components on their surfaces (2), we wished to determine the genetic basis underlying the ability of strain FA19 to produce multiple LOSs, specifically those which bind MAbs 2-1-L8 and 1B2.

Since it is possible that the simultaneous expression of LOS isolated from FA19 that reacts with MAb 2-1-L8 and MAb 1B2 could represent two genetically distinct subpopulations within a single colony (one that reacts with MAb 2-1-L8 and one that reacts with MAb 1B2), we used immunoelectron microscopy to determine if all of the FA19 cells within a colony were producing both LOS components. Microscopy was performed by using a procedure derived from that of Schneider et al. (25). Cells to be examined by electron microscopy were placed on a collodion-coated carbon-stabilized nickel grid that was glow discharged. MAb 2-1-L8 or MAb 1B2 was added to the grids

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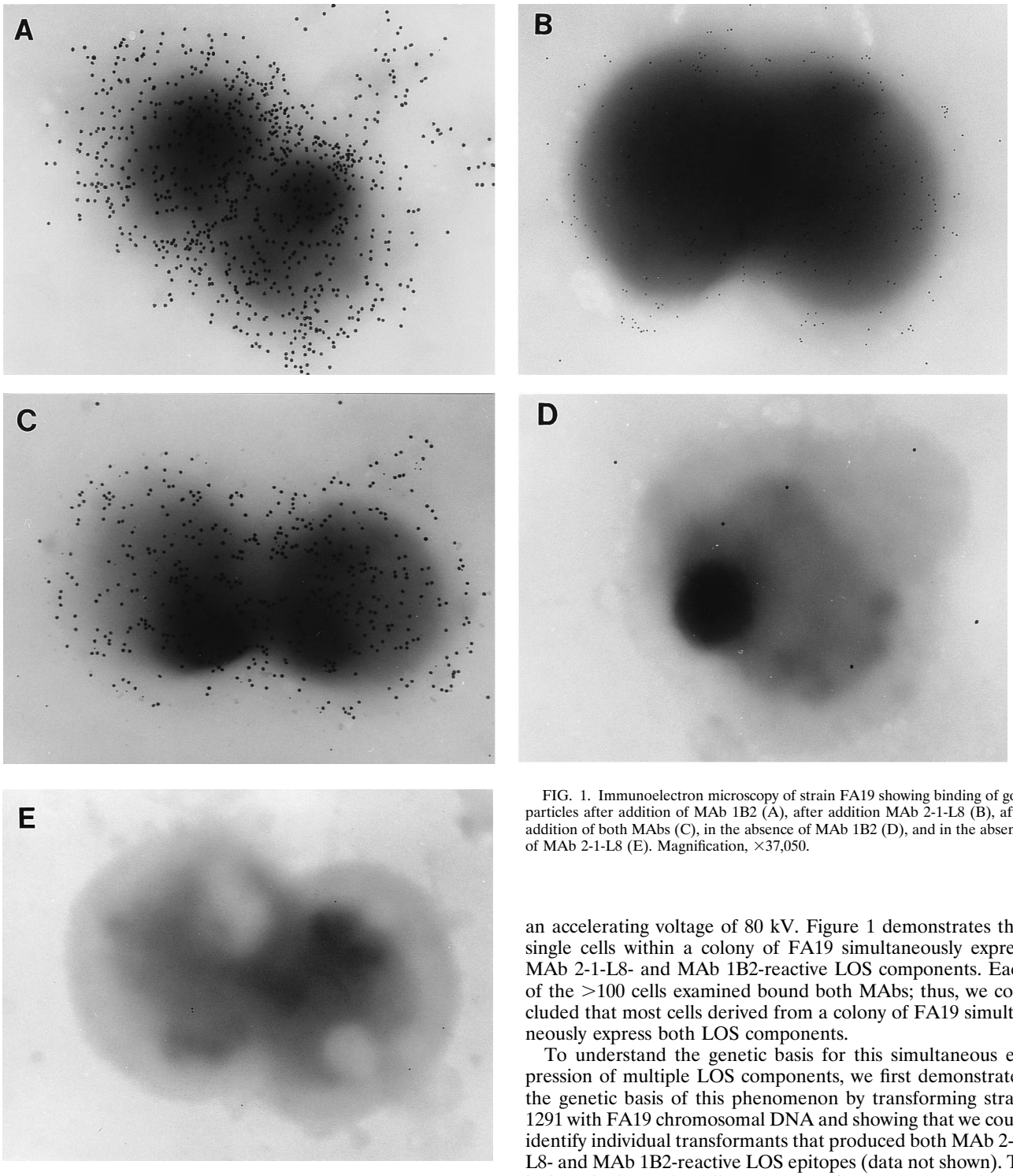

FIG. 1. Immunoelectron microscopy of strain FA19 showing binding of gold particles after addition of MAb 1B2 (A), after addition MAb 2-1-L8 (B), after addition of both MAbs (C), in the absence of MAb 1B2 (D), and in the absence of MAb 2-1-L8 (E). Magnification, ×37,050.

and allowed to bind to the cells, the grids were rinsed with 5% goat serum in phosphate-buffered saline, and gold particles coated with either anti-mouse immunoglobulin G (5-μm particles for the detection of binding of MAb 2-1-L8) or anti-mouse immunoglobulin M (10-μm particles for the detection of binding of MAb 1B2) were added. Grids were viewed in a JEOL 100CX transmission electron microscope operating at an accelerating voltage of 80 kV. Figure 1 demonstrates that single cells within a colony of FA19 simultaneously express MAb 2-1-L8- and MAb 1B2-reactive LOS components. Each of the >100 cells examined bound both MAbs; thus, we concluded that most cells derived from a colony of FA19 simultaneously express both LOS components.

To understand the genetic basis for this simultaneous expression of multiple LOS components, we first demonstrated the genetic basis of this phenomenon by transforming strain 1291 with FA19 chromosomal DNA and showing that we could identify individual transformants that produced both MAb 2-1-L8- and MAb 1B2-reactive LOS epitopes (data not shown). To isolate the locus that allows *N. gonorrhoeae* 1291 to make the MAb 2-1-L8-reactive LOS while retaining the ability to make the MAb 1B2-reactive LOS, a gene bank representing the FA19 chromosome was constructed in the vector pKan18UP (21) with a partial *Hin*PI-*Taq*$^{\alpha}$I-*Msp*I digest of FA19 chromosomal DNA, prepared as described by Rodriguez and Tait (19). Fragments of 3 to 5 kb were isolated by using a sucrose gradient and were ligated into the *Acc*I site of pKan18UP, which was isolated by the alkaline lysis method of Birnboim and Doly (4). This gene bank was introduced into *Escherichia*

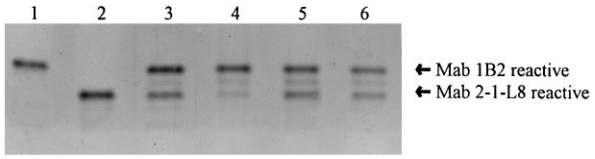

FIG. 2. SDS-PAGE analysis of transformants of *N. gonorrhoeae* 1291. LOS was isolated from each of the strains, and approximately 1 μg of LOS was analyzed on a 13% acrylamide gel. The lanes represent LOS isolated from the following strains: lane 1, 1291; lane 2, 1291_c; lane 3, FA19; lane 4, 1291 transformed with pCLB1; lane 5, 1291 transformed with pCLB5; lane 6, 1291 transformed with pCLB6.

coli DH5α MCR by the $CaCl_2$ transformation procedure (20), the transformants were identified, and pools of 100 transformants were isolated. *N. gonorrhoeae* transformations were performed by the spot transformation method of Gunn and Stein (13a). This allelic replacement procedure allows the incorporation of the desired sequence into the homologous region of the chromosome due to the use of vectors incapable of replicating in the gonococcus. After overnight growth, colonies were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) and screened for reactivity to the appropriate MAb (21). Plasmids that were able to transform strain 1291 to MAb 2-1-L8 reactivity were screened further until a single such plasmid, pCLB1, was isolated.

Transformants of *N. gonorrhoeae* 1291, generated by transformation with pCLB1, were isolated, and the LOSs that they express were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by using the procedure of Hitchcock and Brown (14). LOS was visualized by the staining procedure of Tsai and Frasch (26). The data presented in Fig. 2, lane 4, indicate that when pCLB1 was used as the source of transforming DNA, colonies that acquired the ability to react with MAb 2-1-L8 expressed an LOS phenotype similar to that seen in strain FA19, in that both the MAb 2-1-L8- and MAb 1B2-reactive epitopes were expressed. Since the transformants identified above could represent a mixed population of cells, with some cells expressing the MAb 1B2-reactive LOS and others expressing the MAb 2-1-L8-reactive LOS, immunoelectron microscopy was performed. The data obtained were identical to those presented in Fig. 1 and demonstrate that the transformants expressed both LOS types on the same cell (data not shown). These data indicate that the gene contained in pCLB1 is responsible for the FA19-like LOS expression in the strain 1291 transformants.

Plasmid pCLB1 contains an insert of approximately 3.8 kb of gonococcal DNA. To localize the region responsible for transformation of 1291 to MAb 2-1-L8 reactivity, unidirectional deletion derivatives of pCLB1 were constructed. Because pCLB1 was generated by using pKan18UP, loss of the ability to transform *N. gonorrhoeae* 1291 could not be due to the deletion of a DNA uptake sequence (9) but must correlate with the loss of the DNA sequence needed to mediate the change in LOS expression. As shown in Fig. 3, transforming ability was localized to one end of the insert contained in pCLB1. The DNA sequence of this region was determined by the method of Sanger et al. (22), and BLAST searches were performed (1) with the NCBI program available on the World Wide Web. The sequence was found to be almost identical to a DNA sequence previously identified in strains 1291 (*lsi2*) (6) and F62 (*lgtA*) (10). *lsi2* encodes a glycosyltransferase which adds a

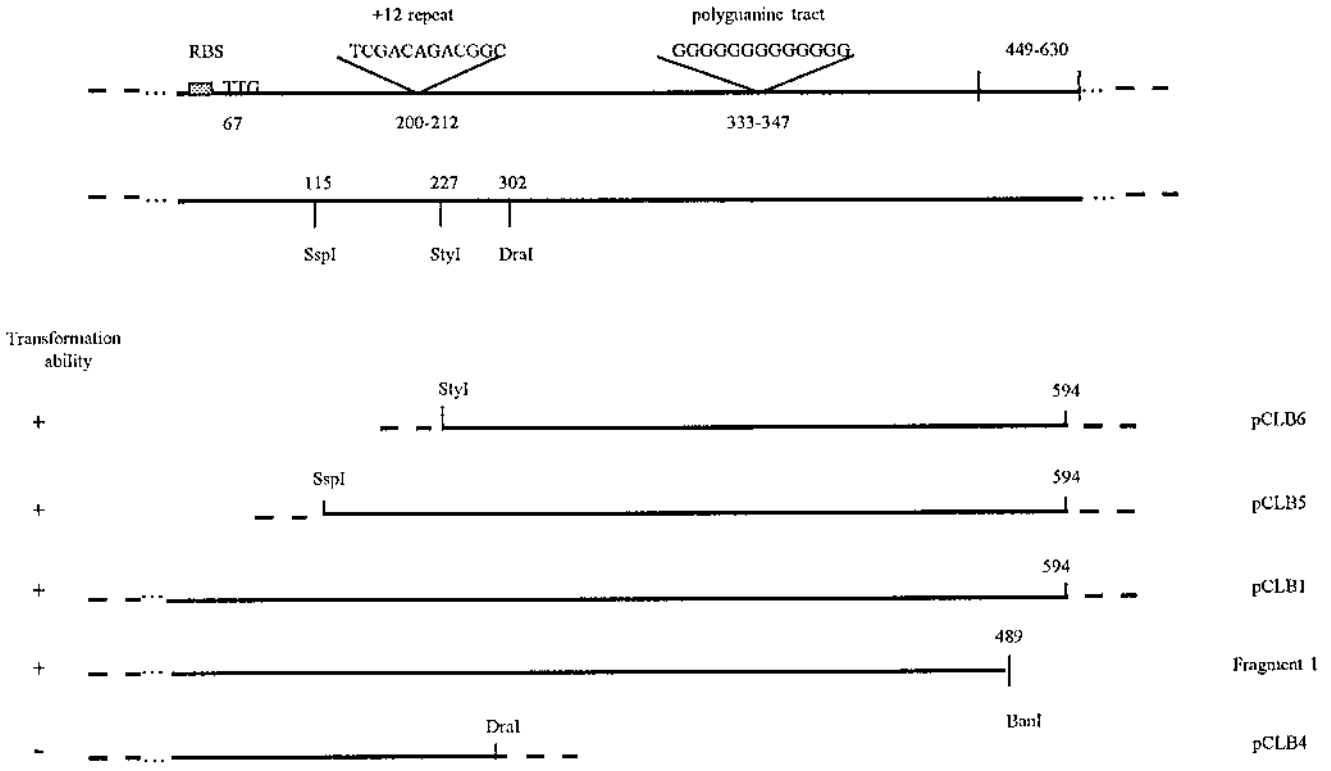

FIG. 3. Transformability of various plasmid constructs, showing the genetic basis of the FA19 phenotype. The column labeled "transformation ability" indicates whether the construct was able to convert strain 1291 to reactivity with MAb 2-1-L8 in a spot transformation assay. The dots in the figure represent additional gonococcal DNA sequences. The dashes indicate vector sequences. RBS, ribosome binding site.

glucosamine to the internal galactose of the α-chain of the LOS molecule. In the 1291 background, the presence of functional Lsi2 allows the addition of lactosamine to an LOS structure that would otherwise react with MAb 2-1-L8, creating the LOS structure that reacts with MAb 1B2.

Since pCLB1 possessed only a partial copy of the *lsi2* gene, we cloned the entire gene from *N. gonorrhoeae* FA19. FA19 chromosomal DNA was digested with *Pst*I and *Age*I, and the resulting DNA fragments were ligated into pGEM2, which had been cut with *Pst*I and *Ngo*MI. This gene bank was used to transform *E. coli* DH5α MCR, and pools of transformants were screened for the ability to transform strain 1291 to MAb 2-1-L8 reactivity; further screenings yielded plasmid pCLB2, which possessed this ability. This allowed us to determine the complete DNA sequence of *lsi2* in FA19.

$lsi2_{FA19}$ shows >98% sequence homology with $lsi2_{1291}$ and $lgtA_{F62}$. Four regions within this sequence that could be responsible for the ability of FA19 to produce multiple LOS moieties were identified: (i) a series of deletions and substitutions directly preceding the *lsi2*/*lgtA* translational start site; (ii) an insertion of a 12-bp direct repeat, 136 bases from the TTG start codon; (iii) a +2 frameshift within the polyguanine tract, beginning at bp 332; or (iv) a series of point mutations between bases 449 and 630.

To identify which sequence difference(s) is responsible for the phenotypic difference between FA19 and 1291, subclones were constructed (Fig. 3). Immunoblotting following spot transformations of 1291 with these subclones as well as SDS-PAGE analysis (Fig. 2) of 1291 transformants showed that a change in the number of guanines in the polyguanine tract is both necessary and sufficient for conferring the MAb 2-1-L8- and MAb 1B2-reactive phenotype on 1291. Thus, the +2 frameshift within the polyguanine tract allows strain FA19 to simultaneously produce MAb 2-1-L8- and MAb 1B2-reactive LOSs.

Gotschlich (10) suggested that the gene $lgtA_{F62}$ encodes a glycosyltransferase that adds *N*-acetylglucosamine to the MAb 2-1-L8-reactive epitope. If this gene product had reduced function due to its truncation caused by the frameshift, its substrate (3.8-kDa LOS that reacts with MAb 2-1-L8) would be present in excess and could be externalized as an incomplete LOS. If the out-of-frame Lsi2 protein had residual function, it is possible that the truncated sugar transferase might add *N*-acetylglucosamine to a limited number of 3.8-kDa LOS molecules. These molecules could then serve as a substrate for the *lgtB* product, thereby producing an LOS component that reacts with MAb 1B2.

From the data presented in Fig. 3, we concluded that the presence of the extra base in the polyguanine tract was responsible for the altered LOS phenotype. This could be due to the fact that the truncated, out-of-frame protein possesses a small amount of *lsi2* activity, thereby allowing the expression of a limited amount of full-length LOS, or it could result from the production of a small amount of functional protein via strand slippage during transcription and/or translation. In order to determine if the truncated LOS protein was functional or if the phenotypic expression was due to a small degree of transcriptional and/or translational frameshifting, we modified the DNA sequence in the polyguanine tract in pCLB1 by changing every third residue to another nucleotide. We modified the polyguanine tract of pCLB1 by PCR using the primers 5′ TATTGCGCGTACGGATGCCGACGA 3′ and 5′ CATCCG TACGCGCAATATATCTCCGCCACCTCCCGACTTTG 3′. The PCR product was digested with *Bsi*WI and ligated to form a plasmid which differs from pCLB1 in that the string of Gs has been replaced with GGGAGGTGGCGGA. This plasmid

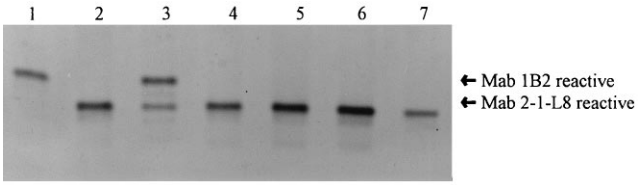

FIG. 4. SDS-PAGE analysis of *N. gonorrhoeae* 1291 transformants generated by pCLB100. LOS was purified, and approximately 1 μg of LOS was loaded per lane. Lane 1, 1291; lane 2, 1291_c; lane 3, FA19; lane 4, 1291 transformant 1 generated with pCLB100; lane 5, 1291 transformant 2 generated with pCLB100; lane 6, 1291 transformant 3 generated with pCLB100; lane 7, 1291 transformant 4 generated with pCLB100.

(pCLB100) was used to transform strain 1291. Transformants that reacted with MAb 2-1-L8 were identified and confirmed by amplifying the *lsi2* region via PCR and digesting this product with *Bsi*WI. Transformants were isolated, and their LOSs were analyzed on SDS-PAGE gels. The data in Fig. 4 indicate that these transformants express a single LOS that reacts with MAb 2-1-L8. All transformants lost the ability to produce the MAb 1B2-reactive LOS. Thus, when the ability to frameshift is removed, the phenotype mediated by Lsi2 expression is not observed.

We have shown previously (6) that MAb 2-1-L8 reactivity results when *lsi2* is nonfunctional. When frameshifting during transcription and/or translation is prevented by changing the polyguanine tract, Lsi2 function is prevented, as is observed in the pCLB100 transformants. From these data, we conclude that the polyguanine tract itself, not just the +2 frameshift, is necessary for the simultaneous expression of LOS components that react with MAbs 2-1-L8 and 1B2 and that transcriptional and/or translational frameshifting results in the production of some functional Lsi2. The inability of pCLB100 transformants to produce MAb 1B2-reactive LOS also allows us to conclude that the truncated frameshifted Lsi2 protein is nonfunctional.

Schneider et al. (23) have shown that after intraurethral inoculation with a piliated LOS variant expressing MAb 2-1-L8-reactive LOS, it is possible to recover variants expressing only MAb 1B2-reactive LOS. Those authors also recovered variants that simultaneously express both molecules and postulated that these variants represent a necessary intermediate stage in the infection process. The data presented in this paper show that the polyguanine tract found in *lsi2* is responsible for the simultaneous expression of both LOS components. Our immunoelectron microscopy experiments demonstrate that these components are simultaneously expressed on the surfaces of individual cells.

The length of the polyguanine tract in *lsi2*/*lgtA* varies among the different strains analyzed. In F62 it can be as long as 17 bases, while in MS11 Var C it is as short as 11 bases (30). Both of these strains produce LOS that reacts with MAb 1B2 in the absence of LOS that reacts with MAb 2-1-L8. Strain MS11 Var A, which contains 12 guanines, is in the +1 reading frame. This strain produces only the MAb 2-1-L8-reactive LOS. Strain 1291 contains 12 guanines and should be out of frame, but it has lost one of the adenine bases immediately 3′ of the polyguanine tract and therefore maintains the correct reading frame. Strain FA19 contains 13 guanines, resulting in a +2 frameshift within *lsi2*. Since FA19 can surface-express both LOSs when it contains a polyguanine tract, but strains with a +1 frameshift express only the MAb 2-1-L8-reactive LOS, there must be a bias towards transcriptional and/or translational frameshifting of +1 nucleotide rather than of −1 nucleotide. If all three phenotypes are necessary during some stage

of the infection process, this bias is essential for replicational frameshifting to be an effective mechanism for creating LOS antigenic variation.

As suggested by Schneider et al. (23), the ability to produce both LOS moieties is probably important during some stages of infection as an intermediary between the phenotypes necessary for attachment and for invasion. The importance of *lsi2* in LOS expression can be summarized as follows. When this gene is in frame, the organism produces a fully functional Lsi2 protein. This allows the gonococcus to make the higher-molecular-weight LOSs. When this gene is in the +1 reading frame, the gene is nonfunctional and the surface-expressed molecule reacts with MAb 2-1-L8. When this gene is in the +2 reading frame, transcriptional and/or translational frameshifting results in the production of a small amount of functional Lsi2 protein. This allows the surface expression of both the MAb 2-1-L8- and MAb 1B2-reactive LOS molecules.

Nucleotide sequence accession number. The DNA sequence of $lsi2_{FA19}$ has been submitted to GenBank under accession number U15992.

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