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A Mutation in the *Neisseria gonorrhoeae rfaD* Homolog Results in Altered Lipooligosaccharide Expression

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The gonococcal *lsi-6* locus was cloned and shown by DNA sequence analysis to have homology with the *E. coli rfaD* gene, which encodes ADP-L-glycero-D-mannoheptose epimerase. This enzyme is involved in the biosynthesis of the lipopolysaccharide precursor ADP-L-glycero-D-mannoheptose. A site-directed frameshift mutation in *lsi-6* was constructed by PCR amplification and introduced into the chromosome of *Neisseria gonorrhoeae* MS11 P^+ by transformation. The lipooligosaccharides (LOS) of mutant and parental strains were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The *lsi-6* mutant produced LOS components with apparent molecular masses of 2.6 and 3.6 kDa as compared with a 3.6-kDa band of the MS11 P^+ strain. The parental LOS phenotype was expressed when a revertant was constructed by transformation of the cloned wild-type gene into the *lsi-6* mutant. The immunoreactivity of LOS from parental and constructed strains was examined by SDS-PAGE and Western blotting. Only the parental and reconstructed wild-type strains produced a 3.6-kDa LOS component that reacted with monoclonal antibody 2-1-L8. These results suggest that the *lsi-6* locus is involved in gonococcal LOS biosynthesis and that the nonreactive mutant 3.6-kDa LOS component contains a conformational change or altered saccharide composition that interferes with immunoreactivity.

Bacteria that colonize mucosal surfaces, including *Neisseria*, *Haemophilus*, and *Bordetella* species, produce short glycolipids lacking the repeating subunits present in the lipopolysaccharides of enteric bacteria (17, 27, 38). Lipooligosaccharides (LOS) of single strains undergo phase variation, demonstrating loss and gain of oligosaccharide components. These LOS variants differ in their electrophoretic mobility and antigenicity (14, 38). The structures of LOS from several gonococcal strains have been determined by physical and immunochemical analyses (13, 20, 21). The LOS may be composed as follows, starting with the lipid A membrane anchor and proceeding to the distal saccharide: 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) (branch KDO), L-glycero-D-mannoheptose (branch L-glycero-D-mannoheptose), glucose, galactose, *N*-acetyl-glucosamine, galactose, and *N*-acetyl-glucosamine (35). LOS contribute to gonococcal attachment to epithelial cells (47), antibody-mediated complement activation (15, 37), and cell damage in fallopian tube organ culture (8) and may be associated with resistance to bactericidal antibody (36).

Several genes that affect LOS expression in *Neisseria gonorrhoeae* have been cloned. The *lsi-1* gene (*lsi* stands for lipooligosaccharide involved) complements an LOS mutant of *N. gonorrhoeae* FA5100 (28). This gene has extensive sequence homology with the *rfaF* genes of *Escherichia coli* and *Salmonella typhimurium*. The *lsi-1* clone complements *rfaF* mutations in these bacteria, demonstrating that *lsi-1* is the gonococcal homolog of *rfaF* (34). The gonococcal phosphoglucomutase gene *lsi-4* was cloned by complementing the LOS mutations in *N. gonorrhoeae* 1291_d and 1291_e (33). This gene was able to complement a *pgm* mutant of *E. coli*. The gonococcal *galE* homolog also was cloned. Insertional inactivation of this gene generates mutants expressing LOS that lack galactose (30). Palermo et al. (25) cloned a piece of DNA that alters lipopolysaccharide expression in *E. coli*, but this gene remains uncharacterized.

In this paper we describe the cloning of a gene that has significant sequence homology with *E. coli* ADP-L-glycero-D-mannoheptose epimerase. We have determined that this gene is involved in LOS biosynthesis in the gonococcus. The construction and analysis of a defined genetic mutation resulting in a stable altered LOS phenotype is described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *N. gonorrhoeae* MS11mk P^+, its nonpiliated derivative MS11mk P^- (43), nonpiliated MS11ms B2 (39), and FA5100 (40) were previously described. Gonococci were cultured at 37°C in gonococcal medium in 5% CO_2 as previously described (42). *E. coli* DH5αMCR was purchased from Bethesda Research Laboratories (Bethesda, Md.) and grown in Luria-Bertani medium supplemented with antibiotics as necessary (23). We constructed a partial *Sau*3AI chromosomal library of *N. gonorrhoeae* MS11 B2 in pUC18 (46). We searched the library by Southern hybridization with degenerate oligonucleotides based on the amino acid sequencing of an outer membrane protein potentially involved in the adherence of nonpiliated *N. gonorrhoeae* to cultured endocervical cells (ATCC HTB 33). Clone pB37 appeared to confer adherent capability on the *E. coli* cloning host. Further study revealed that the cloned genes had sequence homology to *E. coli* LOS biosynthetic genes (12a).

Chemicals, reagents, and enzymes. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Pittsburgh, Pa.) unless otherwise stated. Molecular biology grade reagents for polyacrylamide gel electrophoresis and nitrocellulose membrane were from Bio-Rad Laboratories (Richmond, Calif.). The nylon membrane (Nytran) was from Schleicher and Schuell (Keene, N.H.). Buffers for Southern analysis were from Digene (Silver Spring, Md.). Enzymes were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or GIBCO BRL (Gaithersburg, Md.) unless otherwise stated. DNA amplifications were conducted in 50-μl reaction mixtures, using the PCR kit as specified (Perkin-Elmer Corp., Norwalk, Conn.). The Sequenase DNA sequencing kit was from United States Biochemical Corp. (Cleveland, Ohio). The Genius DNA labeling kit was from Boehringer Mannheim Biochemicals. Chemiluminescent DNA detection reagents were from Tropix, Inc. (Bedford, Mass.). All

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kits were used according to the manufacturer's specifications. Primers were synthesized on a PCR-MATE 391 (Applied Biosystems, Inc., Foster City, Calif.). Mouse monoclonal antibody (MAb) 2-1-L8, prepared by immunization with live group B *Neisseria meningitidis*, strain P355, was provided by Wendell Zollinger (Walter Reed Army Institute of Research, Washington, D.C.). The MAb 2-1-L8 epitope is directed toward the phosphorylated *N*-acetylglucosamine-di(L-glycero-D-manno-heptose) residue found in the 3.6-kDa LOS of *N. gonorrhoeae* MS11mk P^+ (21). The proteinase K-treated lysate (PK lysate) of *N. gonorrhoeae* MS11D was provided by Herman Schneider (Walter Reed Army Institute of Research). Strain MS11D arose during in vitro passage of variant C, which appeared in urine sediments with the onset of urethritis and discharge after experimental infection of male volunteers with strain MS11mk. Strain MS11D produced all five LOS found in any of the recovered variants. Strain MS11D was used as a marker to estimate LOS molecular mass and to reference MAb binding to LOS (35).

Sequencing the cloned gonococcal DNA. Both strands of the gonococcal insert in pB37 were sequenced, using the Sequenase protocol. Initial sequences were determined with the forward (F) and reverse (R) pUC primers. Subsequent primers were based on the obtained sequence. The DNA sequence was compiled and analyzed with MacVector (International Biotechnologies, Inc., New Haven, Conn.) and Genetics Computer Group, Inc., (11) programs.

Southern hybridization. *N. gonorrhoeae* MS11 B2 chromosomal DNA, prepared by the method of Davis et al. (9), was digested with *Sau*3AI, *Dra*I, or both restriction enzymes and separated by gel electrophoresis in a 1% agarose gel in Tris-borate-EDTA buffer for 1.5 h at 100 V (23). Digoxigenin-labeled lambda DNA, *Hin*dIII digested, was included as a molecular mass marker (Boehringer Mannheim Biochemicals). DNA was passively transferred to a Nytran membrane according to the manufacturer's instructions, UV cross-linked, and incubated overnight at 65°C in hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% blocking reagent, 0.1% sodium *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS]) as directed by the Genius kit instructions. Probe DNA was made by PCR amplification of pB37 (Fig. 1, bp 141 to 2544) with primers H and L and labeled with digoxigenin-dUTP. The membranes were washed three times at 65°C in 0.1× SSC–0.1% SDS to remove nonspecific probes. To establish whether the cloned fragment corresponded to the chromosomal region in the *N. gonorrhoeae* strain, the membranes were developed with chemiluminescent reagents and exposed to X-Omat AR5 film for 5 min.

Site-directed mutagenesis. *lsi-6* was mutated by introducing a *Pst*I site 309 bp 3′ to the predicted start of the gene. Oligonucleotide primers based on the pB37 sequence were synthesized with *Eco*RI or *Bam*HI restriction enzyme sites on the 5′ ends. The DNA positions (Fig. 1) of F and R primers are indicated following the primer sequences: primer 1b, TTCAGACGGCATGACCATCCAATGC CGTCTGAAACCC, F-461; primer 27, CCTTATATCCGGCTTCGCGC, R-1455; primer H, GATCGGATCCGACCGAAAAAAGCGCAAAACCTGCGCC, F-141; primer J, GATCCTGCAGCCGTCGTGGTTCATCGTATCGGAACA CGC, R-781; primer K, GATCCTGCAGATGATGGAAAACAACTACCAGT ACACGC, F-789; and primer L, GATCGAATTCGATCAAGGTGGGGTCGT CGCTGCC, R-2544. Primers combinations (H-J and K-L) were used to PCR amplify two fragments of *lsi-6* (96°C for 1 min, 55°C for 2 min, 72°C for 3 min; 30 cycles). The 640-bp H-J product was purified from a low-melting-point agarose gel (4). The H-J and K-L (1768 bp) fragments were digested with *Pst*I and ligated with T4 DNA ligase. The 2.4-kb ligation product was gel purified, digested with *Eco*RI and *Bam*HI, and ligated with pUC18, which had been previously digested with *Eco*RI, *Bam*HI, and calf intestinal alkaline phosphatase. *E. coli* DH5αMCR was electroporated (2.5 KV, 25 μF, 600 Ω) with 25 ng of the ligated DNA and plated on Luria-Bertani–ampicillin (100 μg/ml) agar. pMut*lsi*6 plasmids were prepared by the one-tube method (10) and treated with RNase A (23). The plasmids were screened for a 2.4-kb insert by *Eco*RI-*Bam*HI digestion and for a 650-bp fragment generated by digestion at the *Pst*I mutation site and the single *Pst*I site in the vector. The site-specific mutation in pMut*lsi*6 was confirmed by DNA sequencing. *N. gonorrhoeae* MS11 P^+ was transformed with pMut*lsi*6 by a spot dilution method (15a). Serial dilutions of piliated gonococci were plated on gonococcal agar and allowed to dry. Prewarmed pMut*lsi*6 DNA (1 μg) was spotted on the same area and incubated overnight. Isolated colonies that grew within the zone where the DNA was spotted were examined.

Screening for the site-directed mutation. Twenty isolated *N. gonorrhoeae* colonies, representing independent transformation events, were screened for the *lsi-6* mutation. The whole-cell lysate (WCL) was made by suspension of half of a colony in 5 μl of 0.5 N NaOH, incubation at room temperature for 20 to 30 min, neutralization with 5 μl of 1.0 M Tris (pH 7.5), and dilution to 100 μl with sterile dH_2O (34a). Fifteen microliters of the WCL was amplified by PCR with primers 1b and 27 (96°C for 1 min, 59°C for 2 min, 72°C for 2 min; 25 cycles). DNA from the PCR was digested with *Pst*I and analyzed by agarose gel electrophoresis.

Reconstruction of wild-type epimerase gene. The *lsi-6* mutant *N. gonorrhoeae* was transformed, as described above, with plasmid p*lsi*6 containing the wild-type gene. Backcrosses were screened and purified on the basis of the inability of *Pst*I to digest the 1b-27 PCR product.

SDS-PAGE analysis of LOS. PK lysates were prepared on the equivalent number of cells by the method of Hitchcock and Brown (16). PK lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 13.1% bisacrylamide gels in buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 0.1% SDS). The LOS were visualized by silver staining by the method of Tsai and Frasch (45).

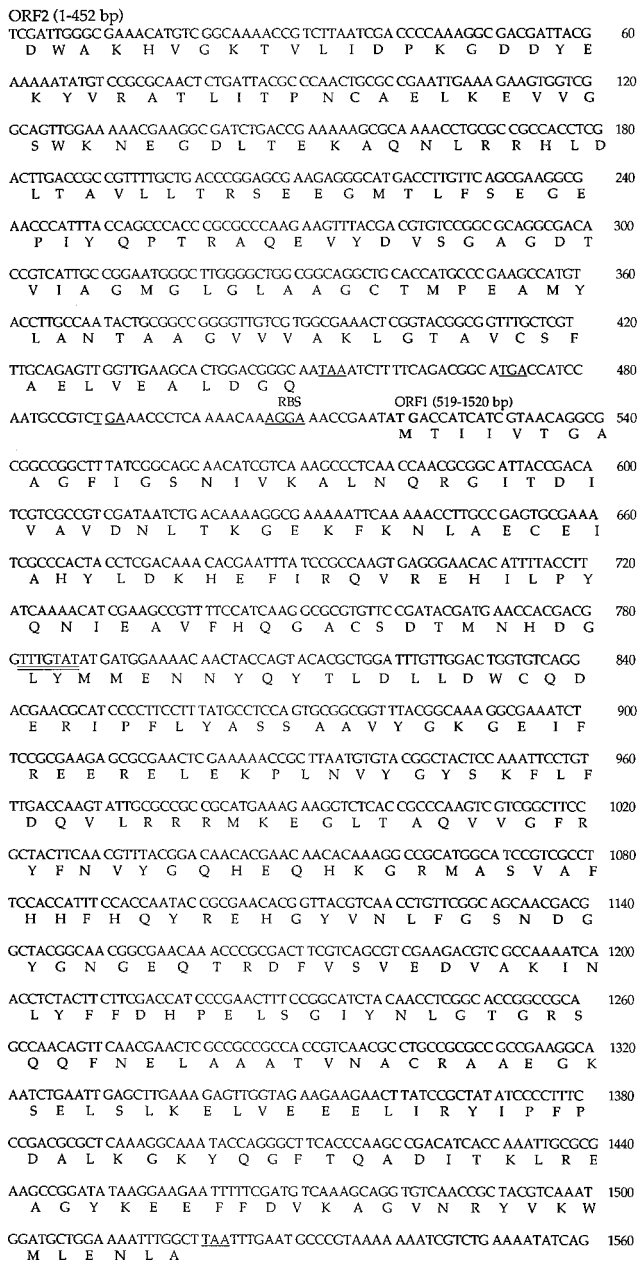

FIG. 1. DNA sequence of the region containing the gonococcal *lsi-6* (bp 519 to 1521) and ORF2 (bp 1 to 452) genes. The start codon is in bold type. Stop codons and the ribosome binding site (RBS) for *lsi-6* are underlined. The target sequence for site-directed mutagenesis is double underlined.

Immunologic detection. SDS-PAGE-separated WCLs were transferred to a nitrocellulose membrane (44), incubated overnight with mouse MAb 2-1-L8, and washed in TS buffer (100 mM Tris [pH 7.5], 150 mM NaCl). Bound antibodies were detected with alkaline phosphatase-linked goat anti-mouse immunoglobulin G and a colorimetric developer (0.1% Naphthol AS-MX phosphate–0.2% Fast Red in TS).

Nucleotide sequence accession number. The DNA sequence of the *lsi-6* region has been submitted to GenBank under the accession number L07845.

RESULTS

DNA sequence analysis of pB37. The sequence of the 2.4-kb *Sau*3AI insert in pB37 was determined by the Sanger dideoxy

TABLE 1. Predicted dinucleotide-binding fold of Lsi-6

Protein	NH_2-terminal sequence	Reference
Lsi-6	MTIIVTGAAGFIGSNIVKALNDKG	
RfaD[a]	MIIVTGGAGFIGSNIVKALNQRG	26
RfaD[b]	MIIVTGGAGFIGSNIVKALNDKG	41
GalE[c]	MTVLITGGTGFIGSHTAVSLVQSG	30
GalE[d]	MAILVLGGAGYIGSHMVDRLVEKG	29
GalE[e]	MSGKYLVTGGAGTVGSVVAQHLVEAG	1
ExoB[f]	MQNNNILVVGGAGYIGSHTCLQLAAKG	5
RfbJ[g]	MTFLKEYVIVSGASGFIGKHLLEALKKSG	19
Conserved residues[h]	*+ * * * *	

[a] *E. coli* ADP-L-glycero-D-mannoheptose-6-epimerase.
[b] *S. typhimurium* ADP-L-glycero-D-mannoheptose-6-epimerase.
[c] *N. gonorrhoeae* UDP-4-glucose epimerase.
[d] *Streptococcus thermophilus* UDP-4-glucose epimerase.
[e] *Streptomyces lividans* UDP-4-glucose epimerase.
[f] *Rhizobium meliloti* UDP-4-glucose epimerase.
[g] *S. typhimurium* CDP-abequose synthase.
[h] *, conserved among all proteins; +, glycine or alanine in the βαβ motif.

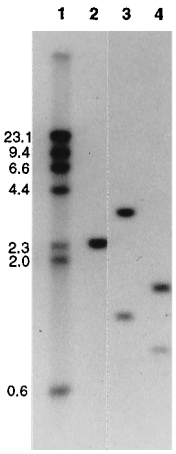

FIG. 2. Southern hybridization of *N. gonorrhoeae* MS11 B2 DNA, using a digoxigenin-dUTP-labeled probe for bp 141 to 2544, PCR amplified from pB37. Chromosomal DNA was digested with *Sau*3AI (lane 2), *Dra*I (lane 3), and *Dra*I plus *Sau*3AI (lane 4). The molecular sizes of digoxigenin-labeled *Hin*dIII-digested λ DNA (lane 1) (in base pairs) are indicated.

method, using primers based on data from sequential gels. An additional 140 bases was determined from a 5′ overlapping cloned fragment. Computer analysis identified two open reading frames, *lsi-6*, spanning bp 519 to 1520, and *lsi-7*, spanning bp 1 to 452. Database searches indicated that *lsi-6* shared sequence homology with *E. coli rfaD*, which encodes ADP-L-glycero-D-mannoheptose epimerase (at the protein level, these gene products had 51.8% identity and 78.4% similarity) (26). *lsi-7* shared homology with a variety of sugar kinase genes, with the highest homology to the *E. coli* ribokinase gene (*rbsK*). *lsi-6* and *lsi-7* were oriented in the same 5′ to 3′ direction and were separated by three stop codons (Fig. 1).

Since *lsi-6* encoded a putative nucleotide-binding protein, we compared its amino acid sequence with the ADP-binding region of several of these proteins. The proposed binding site for the ADP moiety was based on amino acid sequence analysis of the purified *rfaD* gene product of *E. coli* K-12. It is composed of a particular βαβ fold, located near the amino terminus, and contains a Gly-X-Gly-X-X-Gly motif (12, 26). Two proteins in this family have an alanine substituted for one of the glycine residues (48). The analogous region of *lsi-6*, several epimerases, and a sugar synthase contained a Gly/Ala-X-Gly-X-X-Gly sequence (Table 1). All of these enzymes, which participate in the biosynthesis of sugar precursors of LOS or lipopolysaccharide had an additional glycine residue prior to the βαβ motif found in other dinucleotide-binding proteins (26).

Southern hybridization. In *E. coli*, the gene 5′ to *rfaD* is *yibB* (32). The homology data described above indicated that this is not the case for *N. gonorrhoeae*. In order to verify that the cloned fragment had not undergone a deletion, the chromosomal organization of the 2.4-kb fragment in pB37 was examined by restriction endonuclease mapping and Southern hybridization. The 2.4-kb fragment, synthesized by PCR amplification with primers 1b and 27, was used to probe digests of chromosomal DNA from *N. gonorrhoeae* MS11 B2 (Fig. 2). Digoxigenin-labeled *Hin*dIII fragments of λ DNA were included as molecular mass markers (Fig. 2, lane 1). The probe hybridized to the expected 2.4-kb fragment in the *Sau*3AI digest (Fig. 2, lane 2) and to the 1.5- and 0.9-kb bands predicted for the *Sau*3AI-*Dra*I double digest (Fig. 2, lane 4). The probe hybridized to both the 1.2- and 3.7-kb fragments in a *Dra*I digest (Fig. 2, lane 3). These results indicate that there is a single copy of the *lsi-6* locus in MS11 B2 and that the cloned fragment is the same size as the native fragment.

Site-directed mutagenesis of the cloned *lsi-6* gene. To examine the role of *lsi-6* in gonococcal LOS biosynthesis, a mutant gene was constructed. The mutation was constructed by replacing 7 bases with a 6-base restriction site not found in the gene. The strategy used for constructing this site-specific mutation is shown in Fig. 3. External primers H and L were designed for

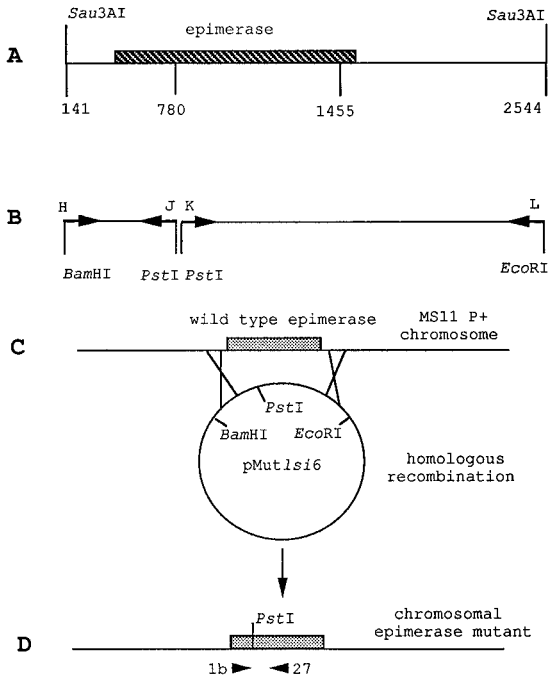

FIG. 3. Strategy for construction of a site-directed mutation in the *lsi-6* gene. *lsi-6* (A [shaded box]) was made in two parts by PCR amplification with primers H plus J and K plus L (B). The amplified fragments were digested with *Pst*I and ligated. The product was digested with *Eco*RI and *Bam*HI and cloned into pUC18. (C) Piliated gonococci were transformed with pMut*lsi*6, and the wild-type gene was replaced by homologous recombination. (D) PCR amplification with primers 1b and 27 produced a 985-bp fragment containing a unique *Pst*I site used for screening.

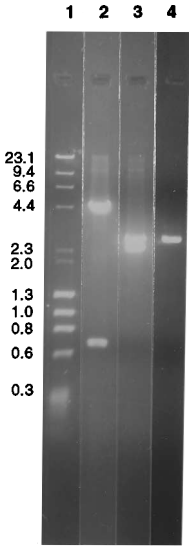

FIG. 4. Verification of a site-directed mutation in pMut*lsi*6. The mutant plasmid, made by introducing a unique *Pst*I site in *lsi-6*, was analyzed by gel electrophoresis. Lane 1, *Hin*dIII-digested λ DNA and *Hae*III-digested ϕX174 DNA; lane 2, pMut*lsi*6 digested with *Pst*I; lane 3, pMut*lsi*6 digested with *Eco*RI plus *Bam*HI; lane 4, pUC18 digested with *Eco*RI. The numbers indicate the sizes (in base pairs) of the λ and ϕX174 bands.

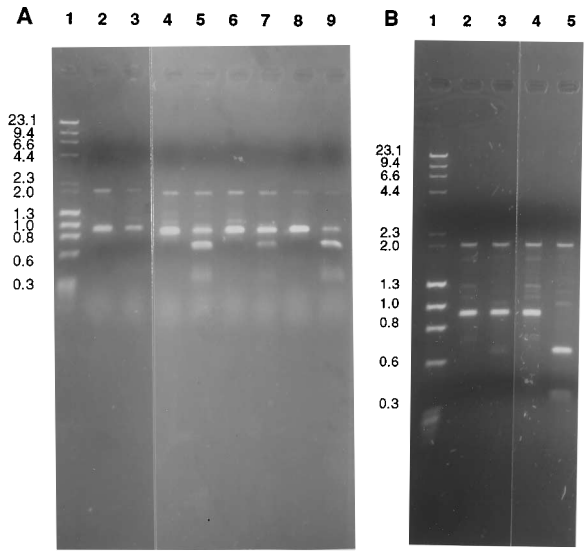

FIG. 5. Identification of a site-directed mutation in the gonococcal chromosomal *lsi-6*. WCLs of potential pMut*lsi*6 transformants were PCR amplified with primers 1b and 27, and the products were analyzed by gel electrophoresis. Lanes 1, *Hin*dIII-digested λ DNA and *Hae*III-digested ϕX174 DNA. The numbers indicate the sizes (in base pairs) of the λ and ϕX174 bands. (A) Wild-type amplimers, undigested (lane 2) and digested (lane 3), were both approximately 985 bp. All transformants produced the 985-bp fragment (lanes 4, 6, and 8) but were incompletely digested by *Pst*I (lanes 5, 7, and 9). (B) Transformant isolation and PCR screening was repeated until pure mutants were obtained. Shown are the wild-type amplimer, undigested (lane 2) and digested (lane 3), and the *lsi-6* mutant undigested (lane 4) and digested (lane 5) with *Pst*I.

directional cloning (Fig. 3A and B). The *lsi-6* gene was PCR amplified in two pieces, using two internal primers (J and K) with *Pst*I sites on the 5′ ends (Fig. 3B). The location of the internal primers was chosen to replace bases 782 to 788 with a *Pst*I site (TTTGTAT → CTGCAG), generating a −1 frameshift after base 782. While the first amino acid encoded by the mutated sequence would not be altered since GGT → GGC is a conserved codon change, the next two amino acids would be altered (LY → CR) and these changes would be followed by a stop codon. The predicted protein would be prematurely truncated at 90 amino acids.

E. coli transformants were screened for a plasmid containing the mutant gene. Purified plasmids digested with *Eco*RI and *Bam*HI produced a 2.4-kb fragment (Fig. 4, lane 3). The introduction of the unique *Pst*I site into *lsi-6*, together with the vector *Pst*I site, was indicated by the appearance of a 650-bp *Pst*I fragment (Fig. 4, lane 2). The site-directed mutation in pMut*lsi*6 was confirmed by DNA sequencing (data not shown).

Construction of a gonococcal *lsi-6* mutant. Plasmid pMut*lsi*6 was used to construct a chromosomal mutation in *N. gonorrhoeae* MS11 P^+ (Fig. 3C). Since pUC18-derived plasmids do not replicate in *N. gonorrhoeae*, the *lsi-6* mutants arise from homologous recombination events. Because no antibiotic resistance marker was used in the construction of the mutated gene, a PCR method was used to identify the gonococcal *lsi-6* mutants. Gonococci were transformed with pMut*lsi*6 DNA. After overnight incubation, multiple colonies were examined by PCR. WCLs of independent MS11 P^+ transformants were PCR amplified with primers 1b and 27 (Fig. 3D), producing a 985-bp fragment (Fig. 5A, lanes 4, 6, and 8) seen in wild-type *N. gonorrhoeae* (Fig. 5A, lane 2). The predicted mutant PCR product, digested with *Pst*I, would generate 315- and 670-bp fragments. The wild-type PCR product was not digested (Fig. 5A, lane 3). All of the gonococcal colonies screened contained transformants mixed with wild-type bacteria, as demonstrated by the presence of both the full-sized PCR product and *Pst*I digestion products (Fig. 5A, lanes 5, 7, 9). Colonies containing the desired mutation were restreaked, and individual colonies were examined by the same PCR screening protocol. This process was continued until no parental PCR product was obtained. The screens from these colonies are shown in Fig. 5B (lanes 4 and 5).

Characterization of *N. gonorrhoeae lsi-6* mutants. We predicted that a gonococcal epimerase mutant would produce altered LOS. LOS from equivalent numbers of wild-type and mutant *N. gonorrhoeae* were examined by SDS-PAGE of PK lysates (Fig. 6). The molecular masses of LOS produced by the *N. gonorrhoeae* strains MS11D and FA5100 (Fig. 6, lane 5 and 9, respectively) were used to estimate the molecular mass of mutant LOS. Piliated MS11 P^+ (Fig. 6, lane 6) and nonpiliated MS11 P^- (Fig. 6, lane 8) produced equal amounts of LOS with an apparent molecular mass of 3.6 kDa. All of the eight independent *lsi-6* mutants had an altered LOS profile (Fig. 6, lanes 1 through 4, 7, and 10 through 12), compared with that of the

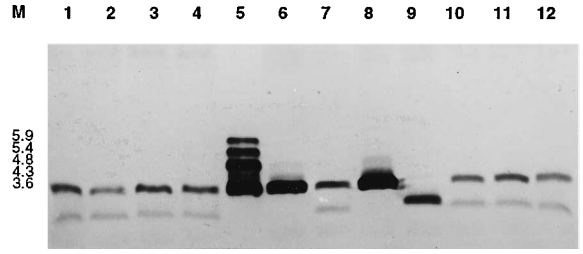

FIG. 6. SDS-PAGE analysis of *N. gonorrhoeae* LOS in PK lysates. Independent *lsi-6* mutants (lanes 1 through 4, 7, and 10 through 12) produced LOS with apparent molecular masses of 2.6 and 3.6 kDa. Parental MS11 P^+ produced the 3.6-kDa band (lane 6) as did nonpiliated MS11 P^- (lane 8). *N. gonorrhoeae* FA5100 produced a 2.9-kDa band (lane 9). *N. gonorrhoeae* MS11D LOS (lane 5), for which the approximate molecular masses (M) are shown on the left, was used to estimate the molecular mass of the mutant LOS.

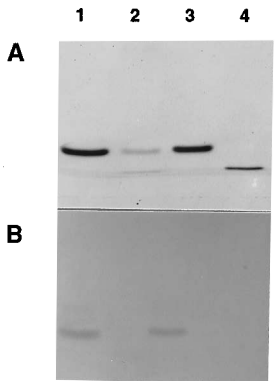

FIG. 7. SDS-PAGE and Western blot analysis of *N. gonorrhoeae* LOS in PK lysates. (A) Parental MS11 P^+ (lane 1), *lsi-6* mutant (lane 2), and the reconstituted wild-type *lsi-6* strain (lane 3) all produced a 3.6-kDa LOS band. The *lsi-6* mutant also produced a novel 2.6-kDa band (lane 2). FA5100 produced a 2.9-kDa band (lane 4). (B) An epitope on the wild-type (lane 1) and reconstituted (lane 3) 3.6-kDa band was recognized by MAb 2-1-L8. Neither band produced by the *lsi-6* mutant retained MAb 2-1-L8 immunoreactivity (lane 3). FA5100 is MAb 2-1-L8 negative (lane 4).

parent strain (Fig. 6, lane 6). The LOS of each mutant had the 3.6-kDa band, present in smaller amounts than in the parental LOS, and a novel 2.6-kDa band. The LOS of *N. gonorrhoeae* FA5100, which contains a single heptose (14), had a single band with a molecular mass of approximately 2.9 kDa (Fig. 6, lane 9).

Characterization of a reconstituted wild-type *lsi-6 N. gonorrhoeae*. The effect of repairing the mutant *lsi-6* gene with the wild-type gene was examined. The gonococcal *lsi-6* mutant was transformed with plasmid containing the wild-type *lsi-6* gene. After overnight incubation, multiple colonies were examined. Since the wild-type gene did not contain a selective marker, a PCR-based screening method was used. WCLs of independent transformants were PCR amplified with primers 1b and 27, producing a 985-bp fragment. The PCR products were digested with *Pst*I. All of the colonies examined contained both digested (mutant) and intact (wild-type) fragments. Colonies were restreaked, and individual subclones were examined by the same PCR screening protocol. This process was continued until no mutant PCR product was obtained. By identifying transformants on the basis of the acquisition of the nonmutated DNA, our selection was not biased toward recovering potential revertants to MAb 2-1-L8 reactivity. PK lysates of equivalent numbers of cells from these colonies were examined by SDS-PAGE and Western blot (immunoblot) analysis. The LOS phenotype of the backcross *N. gonorrhoeae* (Fig. 7A, lane 3) was identical to that seen for the parental strain (Fig. 7, lane 1) and was totally lacking the 2.6-kDa mutant band (Fig. 7, lane 2).

Immunologic analysis of LOS. The immunoreactivity of LOS components produced by the wild-type, *lsi-6* mutant, and reconstructed strains was examined by Western blotting (Fig. 7B) of an SDS-PAGE gel identical to that shown in Fig. 7A. The MAb 2-1-L8 epitope contains the phosphorylated *N*-acetylglucosamine-heptose-heptose residue of LOS (21). This epitope is present on the 3.6-kDa LOS component in *N. gonorrhoeae* MS11 P^+ (Fig. 7B, lane 1) and the backcross *N. gonorrhoeae* LOS (Fig. 7B, lane 3). Neither the 3.6- nor the 2.6-kDa LOS band of the mutant reacted with MAb 2-1-L8 (Fig. 7B, lane 2). MAb 2-1-L8 did not react with the 2.9-kDa LOS band of *N. gonorrhoeae* FA5100 (Fig. 7B, lane 4).

DISCUSSION

In this paper we describe the identification of a gene involved in the biosynthesis of LOS in *N. gonorrhoeae*. DNA sequence analysis of the cloned insert revealed an open reading frame with extensive homology to the *rfaD* gene of *E. coli* (6, 26), suggesting that it encodes the gonococcal ADP-L-glycero-D-mannoheptose-6-epimerase. Analysis of the amino-terminal region of the coding sequence indicated a possible ADP-binding site (G/A-X-G-X-X-G) common to dinucleotide-binding proteins (26, 48). A site-directed mutagenesis procedure allowed for the construction of a small deletion in *lsi-6*, resulting in a frameshift that would direct premature termination of the *lsi-6* product. The altered gene was introduced into the chromosome of *N. gonorrhoeae*. Gonococcal mutants were identified by PCR amplification and by alteration of the restriction endonuclease digestion pattern in the amplified DNA.

Since L-glycero-D-mannoheptose is the first sugar added to KDO in gonococcal LOS, we predicted that a mutation in *lsi-6* would produce a truncated LOS in *N. gonorrhoeae*. Epimerase mutants would be unable to synthesize ADP-L-glycero-D-mannoheptose. Either the heptosyl transferase would be unable to add the D-form to the LOS core or the addition would be inefficient. Subsequent steps in LOS biosynthesis, such as transfer of the next saccharide, the branch heptose, or phosphoethanolamine, might be affected. SDS-PAGE analysis of several independent, defined *N. gonorrhoeae* MS11 P^+ epimerase mutants revealed that all had the identical truncated-LOS profile.

In addition to the truncated LOS, the mutants produced LOS that migrated in SDS-PAGE with the same mobility as parental LOS. The source of this LOS may be explained by the following model. In the absence of ADP-L-glycero-D-mannoheptose epimerase, only the D-form of the sugar would be produced. The L-glycero-D-mannoheptose transferase may have a low affinity for the D-form of the saccharide and would inefficiently catalyze its addition to the KDO-lipid A core. The 2.6-kDa LOS component may be the predicted truncated KDO-lipid A structure. The 3.6-kDa band may be composed of the full outer core oligosaccharide linked to D-glycero-D-mannoheptose in the inner core. The reduced amount of the mutant 3.6-kDa component, compared with the wild-type LOS, may arise from the inefficient addition of the outer core into LOS as a result of sugar transferase substrate specificity.

The potential importance of glycero-D-mannoheptose in LOS conformation is demonstrated by the inability of MAb 2-1-L8 to recognize the mutant 3.6-kDa LOS, which may contain the D-form of the saccharide. Structural analysis has shown that the target of this MAb contains the phosphorylated heptose region of LOS (21). Additional evidence for the importance of the L-glycero-D-mannoheptose conformation in the mutants was observed. Although the *N. gonorrhoeae* cells used for transformation with pMut*lsi*6 formed transparent colonies, all of the recovered mutants had an opaque colony morphology (data not shown). Since the opacity of gonococcal colonies is directly related to the types of proteins found in the outer membrane, this suggested that alterations in LOS composition may have an effect on outer membrane composition. D-Glycero-D-mannoheptose is found as a minor component of enteric lipopolysaccharide (2); however, *rfaD* mutants which contain only the D-form or no heptose have alterations in their outer-membrane-protein profiles and membrane barrier function (3, 7, 22). The gonococcal opacity protein PII binds to LOS, functions as an autoagglutinin (24), and enhances attachment to various human cell types (18). The gonococcal *lsi-6* mutants

may require expression of additional outer membrane proteins such as PII for viability.

The site-directed mutation in the chromosomal MS11 P^+ *lsi-6* gene was replaced by transformation with the cloned wild-type gene. SDS-PAGE and Western blot analysis revealed that the reconstituted strain expressed a single LOS component with the same mobility and immunoreactivity as that of the parent strain. Since the mutant 2.6-kDa LOS band was not apparent, this verifies that the *lsi-6* mutation was involved in the production of the component of that size. Genetic manipulations did not lead to phenotypic variation in the LOS produced by the MS11 P^+ backcross.

Analysis of the cloned DNA revealed that *lsi-6* is preceded by a gene with homology to the *E. coli* ribokinase gene, *rbsK*. In contrast, in *E. coli* the gene 5′ to *rfaD* is *yibB* (32). The *E. coli rfaD*, *rfaF*, and *rfaC* genes, encoding enzymes involved in synthesis of the heptose core, are organized in an operon with that 5′ to 3′ order (31). The DNA sequence 3′ to *lsi-6* did not exhibit homology to *rfaF*. Likewise, DNA sequences 5′ to *rfaF* from *N. gonorrhoeae* do not share homology with *rfaD* (34). This is further evidence that the gonococcal LOS biosynthetic genes are not organized in the same fashion as in *E. coli*. When introduced into *rfaD* and *rfaE* mutants of *S. typhimurium*, the *lsi-6* and *lsi-7* genes, respectively, complement the mutations (22a). Therefore, we have cloned the gonococcal homolog of *rfaD*. We are currently investigating the DNA regions flanking the *lsi-6* gene to determine whether other genes involved in LOS biosynthesis are closely linked, and we are pursuing studies to determine the structure of LOS from the *lsi-6* mutant and LOS parental-type strains.

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