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Two Glycosyltransferase Genes, *lgtF* and *rfaK*, Constitute the Lipooligosaccharide *ice* (Inner Core Extension) Biosynthesis Operon of *Neisseria meningitidis*

CHARLENE M. KAHLER,[1,2] RUSSELL W. CARLSON,[3] M. MAHBUBUR RAHMAN,[3] LARRY E. MARTIN,[1,2] AND DAVID S. STEPHENS[1,2]*

Departments of Medicine and Microbiology and Immunology, Emory University School of Medicine,[1] and Department of Veterans Affairs Medical Center,[2] Atlanta, and The Complex Carbohydrate Research Center, University of Georgia, Athens,[3] Georgia

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We have characterized an operon required for inner-core biosynthesis of the lipooligosaccharide (LOS) of *Neisseria meningitidis*. Using Tn*916* mutagenesis, we recently identified the α-1,2-*N*-acetylglucosamine (GlcNAc) transferase gene (*rfaK*), which when inactivated prevents the addition of GlcNAc and α chain to the meningococcal LOS inner core (C. M. Kahler, R. W. Carlson, M. M. Rahman, L. E. Martin, and D. S. Stephens, J. Bacteriol. 178:1265–1273, 1996). During the study of *rfaK*, a second open reading frame (*lgtF*) of 720 bp was found upstream of *rfaK*. An amino acid sequence homology search of the GenBank and EMBL databases revealed that the amino terminus of LgtF has significant homology with a family of β-glycosyltransferases involved in the biosynthesis of polysaccharides and O antigen of lipopolysaccharides. The chromosomal copy of *lgtF* was mutagenized with a nonpolar antibiotic resistance cassette to minimize potential polar effects on *rfaK*. Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and composition analysis of the LOS from the nonpolar *lgtF* mutant showed that this strain produced a truncated LOS structure which contained a LOS inner core of $GlcNAc_1Hep_2KDO_2$lipid A but without the addition of lacto-*N*-neotetraose to HepI or glucose to HepII. These results and the amino acid homology with β-glycosyltransferases suggest that *lgtF* encodes the UDP-glucose:LOS-β-1,4-glucosyltransferase which attaches the first glucose residue to HepI of LOS. Reverse transcriptase PCR and primer extension analysis indicate that both *lgtF* and *rfaK* are cotranscribed as a polycistronic message from a promoter upstream of *lgtF*. This arrangement suggests that completion of the LOS inner core and the initiation of the α chain addition are tightly coregulated in *N. meningitidis*.

Neisseria meningitidis is an obligate human pathogen that causes fulminant sepsis and meningitis but is also capable of persisting in the human nasopharynx in an asymptomatic state in 5 to 10% of the adult population during nonepidemic periods (45). Lipooligosaccharide (LOS) is an outer membrane glycolipid which is involved in meningococcal attachment and colonization of the human nasopharynx (47), influences resistance to complement-mediated killing in the bloodstream (26) and is a potent inducer of the inflammatory response, as seen in meningococcal sepsis and meningitis (3, 43). LOS consists of a short oligosaccharide chain of variable composition (termed the α chain) attached to a branched inner core which is linked in turn to lipid A. The composition of the α chain of LOS is variable but in all forms mimics human glycosphingolipids (11, 22), thereby decreasing the recognition by and production of antibodies to this structure (34). The composition of the α chain of meningococcal LOS is subject to high-frequency, reversible phase variation which involves switching between immunologically distinct LOS structures (44). Many of these immunotypes (L2, -3, -4, -5, -7, -9, -10, -11) contain a common epitope, lacto-*N*-neotetraose, in the α chain, whereas the α chains of immunotypes L8 and L1 contain mono- and digalactoside epitopes, respectively (42, 46).

Epidemiological studies of meningococcal outbreaks have revealed that encapsulated bloodstream isolates express L3,7,9 LOS, whereas carriage isolates are predominately acapsulate and express L1,8 immunotype LOS (15). In vitro epithelial-cell invasion studies have demonstrated that the expression of L3,7,9 LOS in combination with capsule inhibit Opc-mediated attachment and invasion (47). However, the L3,7,9 LOS structure and capsule appear to be necessary for survival in the bloodstream by enhancing resistance to complement-mediated killing (26). Recently, an operon, *lgtABCDE*, encoding the sugar transferases required for the biosynthesis of the α chain were identified and a slipped-strand transcription mechanism was proposed for α chain phase variation (10, 14). However, the β-1,4-glucosyltransferase which is required to attach the α chain to the LOS inner core was not encoded in this operon.

We have used Tn*916* mutagenesis to isolate LOS biosynthesis mutants in *N. meningitidis* (18, 39), and with this approach have identified *galE* (21) and *pgm* (50). Further, we recently identified the α-1,2-*N*-acetylglucosamine transferase gene, *rfaK*, which adds GlcNAc to the LOS inner core and found that inactivation of this gene resulted in a LOS structure that did not contain the α chain lacto-*N*-neotetraose (17). We describe here a meningococcal LOS biosynthesis gene upstream of *rfaK* which appears to encode the UDP-glucose:LOS-β-1,4-glucosyltransferase, and we propose that it initiates α chain extension by catalyzing the addition of the first residue, glucose, of the lacto-*N*-neotetraose structure to HepI of the LOS inner core. The organization of *rfaK* and *lgtF* and analysis of the mRNAs from these two genes suggest that they form an operon, which we have named the *ice* (inner-core extension) operon.

* Corresponding author. Mailing address: Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, 69 Butler St., SE, Atlanta, GA 30303. Phone: (404) 728-7688. Fax: (404) 329-2210. Electronic mail address: dstep01@emory.edu.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence[a]	Reference(s) or source
Strains		
N. meningitidis		
NMB	B:2B:P1.2,5:L2 (CDC8201085)	40
44/76	B:15:P1.16:L3,7,8	26
44/76*lgtF*::*aphA-3*	Nonpolar insertion in *lgtF*, Km^r	This study
M981	B:4:NT:L5	25, 51
M981*lgtF*::*aphA-3*	Nonpolar insertion in *lgtF*, Km^r	This study
NMB*rfaK*::*aphA-3*	Nonpolar insertion in *rfaK*, Km^r	17
NMB*rfaK*::Ω	Polar insertion in *rfaK*, Sp^r	17
E. coli JM109	JM107 *rec*A1	48
Plasmids		
pCR2	TA cloning, Ap^r Km^r	Stratagene
pUC18K	*aphA-3* (Km^r) Ap^r	24
pHP45	Ω (Sp^r) Ap^r	31
pPR510	Cm^r	32
pHSG298	Km^r	41
pCK15	pCR2 containing the cloned internal 320-bp fragment of *lgtF*, PCR amplified with primers CK23 and CK24	This study
pCK16	320-bp *Eco*RI insert from pCK15 in pPR510	This study
pCK17	*aphA-3* cassette cloned into *Sna*BI site of *lgtF* insert in pCK16	This study
pCK18	1.12-kb *Eco*RI insert (*lgtF*::*aphA-3*) from pCK17 in pHSG298	This study
pCK25	Ω cassette cloned into *Sna*BI site of *lgtF* insert in pCK16	This study
pCK26	2.32-kb *Eco*RI insert (*lgtF*::Ω) from pCK25 in pHSG298	This study
Primers		
CK12	5′GAAGCCTGTGTGATATTGCATCG3′	This study
CK13	5′GACCTTGATAAAGTGTGATAAGTCC3′	17
CK23	5′CGTGTAATAGCTGTGGTTGACGG3′	This study
CK24	5′GACAGCGTATGCCGTTTGATGC3′	This study
CK37	5′GGTTTAAGACGAGGCGTTCG3′	This study
CK38A	5′GACTCATAATCCCTTGGTCGTGG3′	This study
LgtF1	5′CAATACATTCCCGAATGTGG3′	This study

[a] The typing scheme of *N. meningitidis* is as follows: serogroup of capsule, serotype of class 2/3 proteins, serotype of class 1 proteins, and immunotype of LOS (47). NT, nontypeable by current techniques. Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The conditions for the maintenance of *N. meningitidis* serogroup B strains has been described previously (17, 28). *N. meningitidis* serogroup B strains 44/76 and M981 (Table 1) were kindly provided by Wendell Zollinger (Walter Reed Army Institute of Research). The following antibiotic concentrations were used to maintain meningococcal mutants in GC (Difco) and brain heart infusion media supplemented with 2.5% fetal calf serum: 5 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml and 80 μg of spectinomycin (chloride salt) per ml. Kanamycin selection was performed in brain heart infusion medium at 60 μg/ml (sulfate salt).

Construction of polar and nonpolar insertions into *lgtF*. The methods used for the preparation and manipulation of DNA have been described previously (17). An internal fragment of *lgtF* was PCR amplified with primers CK23 and CK24 (Table 1), purified with a QIAquick Spin PCR purification kit (QIAGEN), and subsequently cloned into a pCR2 vector (Stratagene) to form pCK15 (Table 1). The insert from pCK15 was excised with flanking *Eco*RI sites and ligated into the Cm^r vector pPR510 (pCK16) to allow positive selection in later steps. The nonpolar kanamycin resistance cassette, *aphA-3*, was released from pUC18K with *Eco*RI and *Bam*HI, and the overhangs were filled with Klenow DNA polymerase (23). The polar spectinomycin resistance (Ω) cassette was released from pPH45 with *Sma*I. Both cassettes were cloned into a unique *Sna*BI site within the *lgtF* insert of pCK16. Combinations of recipient vector and cassette antibiotic resistance markers were used to select transformants harboring the correct recombinant plasmid. The junctions of pCK17 (*lgtF*::*aphA-3*) were sequenced to confirm that the translational start codon downstream of the *aphA-3* gene of the nonpolar cassette and the carboxy-terminal coding region of *lgtF* were in frame. The orientation of the polar cassette in pCK25 (*lgtF*::Ω) was determined by partial restriction analysis. The flanking *Eco*RI sites were used to transfer both nonpolar and polar constructs from pCK17 and pCK25 into pHSG298 to form pCK18 (*lgtF*::*aphA-3*) and pCK26 (*lgtF*::Ω), respectively.

Preparation of total RNA from *N. meningitidis*. Total RNA was prepared from NMB, NMB*rfaK*::Ω, NMB*rfaK*::*aphA-3*, NMB*lgtF*::Ω, and NMB*lgtF*::*aphA-3* by the modified method of Baker and Yanofsky (1). To remove contaminating RNase, glassware and plasticware were baked at 180°C overnight and autoclaved. Plasticware which could not be baked was treated with RNaseZAP (Ambion) and autoclaved before use. Solutions were prepared by aseptic techniques and treated with diethylpyrocarbonate overnight at 37°C. *N. meningitidis* strains were grown to early logarithmic phase (optical density at 560 nm = 0.2 to 0.3) in 1-liter broths with the appropriate antibiotic selections. Bacteria were retrieved by centrifugation, and the pellet was resuspended in ice-cold killing buffer (20% [wt/vol] sucrose, 20 mM Tris HCl [pH 7.3], 5 mM $MgCl_2$, 20 mM NaN_3, 400 μg of chloramphenicol per ml) by vigorous vortexing. Bacteria were pelleted by centrifugation at 10,000 × *g* for 10 min and resuspended as before in killing buffer without sucrose. Cells were lysed by the addition of 0.32 ml of freshly prepared 6-mg/ml chicken egg white lysozyme (Sigma) and 100 μl of 1-mg/ml RNase-free DNase (Promega), followed by three freeze-thaw cycles of a dry ice-ethanol bath and a 45 to 50°C water bath. After the last thaw, 162 μl of 3 M sodium acetate (pH 5.2) and 1 ml of 5% sodium dodecyl sulfate (SDS) were added to complete cell lysis. Proteins were extracted from the lysis mixture by the addition of 12 ml of acid phenol (pH 4.0; Ambion). After being mixed, the phases were separated by centrifugation at 10,000 × *g* for 10 min and the aqueous phase was saved to a sterile tube. Total RNA was precipitated overnight from the aqueous phase by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol and pelleted by centrifugation at 10,000 × *g* for 15 min. The pellet was washed with 95% ethanol, air dried, and resuspended in diethylpyrocarbonate-treated sterile water for storage at −70°C. The concentration of the sample was determined by spectrophotometry by the method of Maniatis et al. (23). The quality of the RNA sample was determined by formaldehyde gel electrophoresis (23).

RNA analysis techniques. Before using RNA in reverse transcriptase PCR (RT-PCR) and primer extension assays, contaminating DNA was removed by digestion with RQ1 DNase (Promega) in the presence of RNase block (Stratagene) at 37°C for 1 h. The reaction was extracted with acid phenol, and the RNA was recovered by ethanol precipitation and centrifugation as described above. The air-dried pellet was dissolved in diethylpyrocarbonate-treated water, and the RNA concentration was determined by spectrophotometry. A two-step RT-PCR protocol was used to amplify cDNA synthesized from total RNA. cDNA was transcribed from 1 μg of total RNA with the appropriate primer and SuperScript II RNase H^- RT (GibcoBRL) according to the manufacturer's instructions. The product of this reaction without further purification was used as a template for standard PCR using the GeneAmp PCR System 9600 (Perkin-Elmer). To check for the presence of contaminating DNA in the RNA preparation, control reaction mixtures consisted of annealed primer and RNA without the addition of RT.

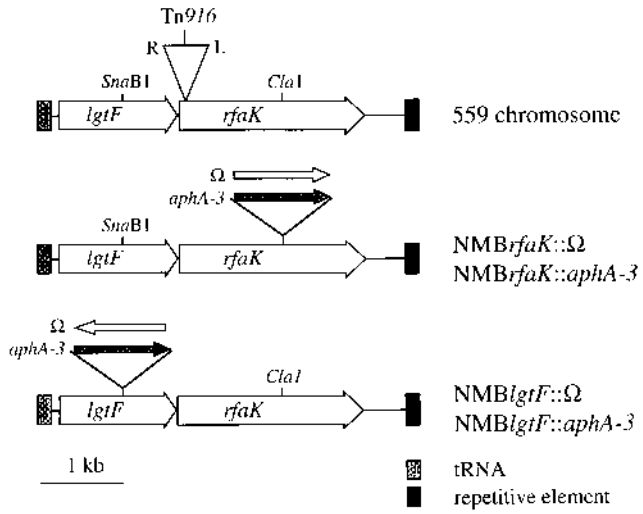

FIG. 1. A schematic diagram showing the arrangement of the *ice* operon. In the top panel, the location and orientation of the Tn*916* insertion (L, left arm; R, right arm) in the chromosome of the LOS mutant 559 are shown. In the middle and bottom panels, the designs of insertion mutations using the *aphA-3* and Ω cassettes and the resulting strain designations are shown. The locations of the tRNA and repetitive element which flank *lgtF* and *rfaK* are also shown. Orientation of arrows indicates the direction of transcription through these genes.

Primer extension protocol. Primer extension analysis was performed according to the manufacturer's instructions for the avian myeloblastosis virus RT primer extension system (Promega). The primer extension reaction was performed with 40 μg of total RNA in a final volume of 20 μl. To reduce the loading volume, the RNA and labelled cDNA were precipitated by the addition of 2 μl of RNase-free 3 M sodium acetate (pH 5.2) and 60 μl of 100% ethanol and incubated in a dry ice bath for 10 min. The precipitate was collected by centrifugation for 5 min at 12,000 × *g* in a benchtop centrifuge. The pellet was washed twice with 70% ethanol, air dried, and resuspended in 5 μl of loading buffer.

Glycosyl composition analysis of LOS. Purified LOS was prepared from 5 g (dry weight) of bacteria by a modified version of the hot-phenol extraction procedure previously described (8, 17). Glycosyl composition analysis was performed by the preparation and analysis of alditol acetates as described by York et al. (49). Briefly, LOS samples were hydrolyzed in 2 M trifluoroacetic acid at 121°C for 2 h. After the samples were dried with a stream of air, the resulting glycoses were reduced with sodium borohydride. Excess borohydride was destroyed by a few drops of glacial acetic acid, and the borate was removed as methyl borate esters by repeated evaporation (four times) with methanol-acetic acid (10:1) followed by evaporation with methanol. The resulting alditols were acetylated with acetic anhydride in pyridine, extracted into methylene chloride, and analyzed by gas-liquid chromatography and combined gas-liquid chromatography–mass spectrometry. The gas-liquid chromatography analysis was performed with a 30-m DB-1 column from J&W Scientific. The various glycosyl residues were identified and quantified by comparison with authentic standards.

Nucleotide sequence accession number. The DNA sequence of *lgtF* has been deposited in the EMBL and GenBank nucleotide sequence databases under accession no. U58765. Sequence analysis was performed by using the GAP amino acid sequence comparison program of the Genetics Computer Group sequence analysis software package version 7.3.1-UNIX (30). Amino acid sequence alignments were performed by using PILEUP (6).

RESULTS

Organization of the *ice* operon. Tn*916* mutagenesis and monoclonal antibodies were used to detect LOS mutants of the meningococcal strain NMB (17, 39). The Tn*916* mutant, 559, which expresses a truncated LOS of 3.0 kDa was isolated by using this strategy. The single Tn*916* insertion in this mutant was in a 1,065-bp open reading frame (ORF) subsequently identified as the α-1,2-*N*-acetylglucosamine transferase gene, designated *rfaK* (17). A second ORF of 720 bp, designated *lgtF*, was found upstream of the transposon (Fig. 1). An amino acid sequence homology search of the GenBank and EMBL databases revealed that this ORF has significant homology to the following three ORFs of unknown function: 32.9% identity and 54.2% similarity with the *Haemophilus influenzae* HI0653 coding region (7), 25.6% identity and 53.2% similarity with ORF 1 found upstream of the tetrapyrrole biosynthesis genes in *Anacyctis nidulans* (16), and 28.9% identity and 52.8% similarity with an ORF from *Synechocystis* sp. strain slr0072 (accession no. D64004).

The amino termini of these proteins contain a highly conserved domain of unknown function which is proposed to be

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Rhmel ExoO   1 .......mnp paidkvpdVt fVVaaYNsAd tivraIeSa. LaQegvtvEV VVVDDcSaD.
Rhmel ExoU   1 .......... mtaaaptdVc IIIsakNaAd tiaraVaSa. La.epeaaEV VVIDDGSTDd
Anab HetA   31 npqspipnpq spipnpmkIs VIIsnYNyAr ylsraInSv. LaQthsdiEI VIVDDGSTDn
Yent TrsB    1 .......... ..mdnkklls lIIpvFNaeq yldaclsSv. FvQwdntlEI IVInDGSTDn
Ecoli YibD   1 .......... ....mramIs alV..... .....wkSl. itQtwtalEI IIInDGSTDn
Hinf HI0868  1 .......... ...mnmplIs IImpvYNaec ylnqgIlSc. LnQsyqniEl IlIDDGSTDk
Yent TrsC    1 .......... .....misVt VftptFNrAh vlkrcylSi. LeQdrddiEw lIVDDGSTDn
Nmen LgtF    1 .......... .....mkkVs VlIvakNeAn hirgcIeSc. ....rfdkEV IVIDDhSTDn
Rhmel ExoA   1 .......ms sdeltstssl IVIpcLNeAs hiealIeklr psltplnarV VIaDgGSTDg
Consensus      ---------- --------V- -II--YN-A- -----I-S-- L-Q-----EV --IDDGSTD-

Rhmel ExoO      .atpalvaai pdprvrlial drNrGpggAR NagIg..aAr GrWIavLDsD D  99
Rhmel ExoU      sasVaraadd gtgrlnvvrf eeNrGpaaAR NhaIa..ish splIgvLDAD D  97
Anab HetA       srDVitqLqe qapdkikpif qaNqGqggAF Nagfa..aAt GEvVafLDAD D 138
Yent TrsB       sanIiqkYse kydfv..Yis qeNtGisvvR Nmgls..vst GEYItfLDAD D  94
Ecoli YibD      svEIakyYae nyphvr.Llh qaNaGasvAg NrgIe..vAt GkYVafvDAD D  81
Hinf HI0868     siEIinniid kdkrvklFft ptNqGpaaAR Nigle..kAq GDYItfLDsD D  95
Yent TrsC       taEVvdsFki enklnikYiy qdNsGkqaAW NkaVe..nAs GEYfigLDsD D  93
Nmen LgtF       taEIaegLga k.....vFqr hlNgdfgaqk tfaIe..qAg GEWVfliDAD E  84
Rhmel ExoA      trEIarrLat edprv.lFld npkriqsaAv NraVaelgAg sDYliriDAh g 102
Consensus       --EI---L-- -------F-- --N-G---AR N--I----A- GEYI--LDAD D
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FIG. 2. Alignment of the N-terminal domain of LgtF (Nmen LgtF) with those of members of the β-glycosyltransferase family (35) as well as homologous sequences of unknown function. They are the hypothetical protein from *E. coli* (Ecoli YibD; accession no. X06690), *H. influenzae* potential ORF HI0868 (Hinf HI0868; accession no. L45506), *Y. enterocolitica* TrsB and TrsC (Yent TrsB and Yent TrsC, respectively; accession no. Z47767), *Anabaena* sp. HetA (Anab HetA; accession no. M31722), and *R. meliloti* ExoU, ExoA, and ExoO (Rhmel ExoU, Rhmel ExoA, and Rhmel ExoO, respectively; accession no. L20758). This alignment was constructed by using PILEUP (6) with a plurality of six to construct the consensus sequence. Capital letters indicate identical residues and conserved replacements of the consensus sequence. The numbers indicate the first and last residues of the amino acid sequences used in this alignment. Gaps introduced into the alignment and consensus sequences are indicated by dots and dashes, respectively.

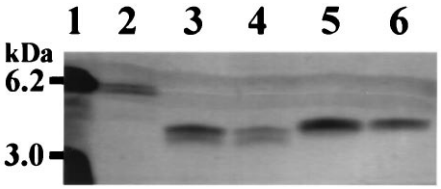

FIG. 3. Comparison of the LOS profiles of the *lgtF* and *rfaK* insertion mutants with that of the parent strain, NMB. The LOS preparation and tricine SDS-PAGE conditions have been previously described (17). Lane 1, prestained low-molecular-mass standards; lane 2, NMB; lane 3, NMB*rfaK*::Ω; lane 4, NMB*rfaK*::*aphA-3*; lane 5, NMB*lgtF*::Ω; and lane 6, NMB*lgtF*::*aphA-3*.

characteristic of β-glucosyltransferases involved in polysaccharide and O antigen biosynthesis (9, 27, 35). The sequences of eight proteins that have N-terminal homology with LgtF are shown in Fig. 2. *Yersinia enterocolitica* TrsB and TrsC are postulated to be β-glucosyltransferases involved in O antigen biosynthesis (38), whereas *Anabaena* sp. HetA (12) and *Rhizobium meliloti* ExoU, ExoA, and ExoO (9) are involved in capsule polysaccharide biosynthesis. *R. meliloti* ExoU, ExoA, and ExoO have been enzymatically characterized and are β1-6, β1-3, and β1-6 glucosyltransferases, respectively, involved in the production of succinoglycan exopolysaccharide capsule. These analyses suggested that LgtF could be a β-glycosyltransferase involved in LOS biosynthesis in *N. meningitidis*.

Upstream of *lgtF* is a region that has 81% nucleotide sequence identity with the *Escherichia coli ileX* gene encoding tRNA-Ile (29). A similar analysis of the region downstream of *rfaK* revealed a 150-bp region that is 93% identical to the reiterated sequences surrounding *frpB*, an iron-regulated outer membrane protein from *N. gonorrhoeae* (2), and 92% identical to the direct repeats, DR 1L and DR 1R, of IS*1106* (20).

Insertional inactivation of *lgtF*. To assess the potential role of *lgtF* in meningococcal LOS biosynthesis, we introduced mutations into the *lgtF* ORF (Fig. 3). Plasmids containing nonpolar (pCK18) and polar (pCK26) mutations in *lgtF* were introduced into the parent strain, NMB, by transformation and homologous recombination (13). Since there was no intergenic space between the potential translational start codon for *rfaK* and the translational stop codon for *lgtF*, both types of mutation were introduced to determine whether interruption of *lgtF* had polar influences upon *rfaK* expression. The site of insertion into *lgtF* and the integrity of the region were confirmed by PCR and sequence analysis (data not shown). LOSs prepared from both NMB*lgtF*::Ω (polar) and NMB*lgtF*::*aphA-3* (nonpolar) and analyzed by tricine SDS-polyacrylamide gel electrophoresis (PAGE) were shown to be truncated compared with parent NMB LOS (Fig. 3). The relative mobilities of the LOSs from these strains were compared with those from the *rfaK* mutants NMB*rfaK*::*aphA-3* (nonpolar) and NMB*rfaK*::Ω (polar) (17). Composition and structural analyses showed that the *rfaK* mutants produced an inner core of two heptoses without any additional saccharides. The NMB*lgtF*::*aphA-3* LOS migrated slightly more slowly than did the *rfaK* mutant LOS samples, which is consistent with the normal expression of *rfaK* and the addition of an *N*-acetylglucosamine (GlcNAc) to the LOS core. However, the LOS from the polar *lgtF* mutant, NMB*lgtF*::Ω, which was expected to have the same mobility as NMB*rfaK* mutants, migrated as a wide band or doublet which spanned the molecular-weight range between the NMB*lgtF*::*aphA-3* and NMB*rfaK* mutant LOSs. This may be interpreted as either an incomplete polar effect on *rfaK* expression or heterogeneous phosphorylation which has resulted in multiple LOS structures with different mobilities.

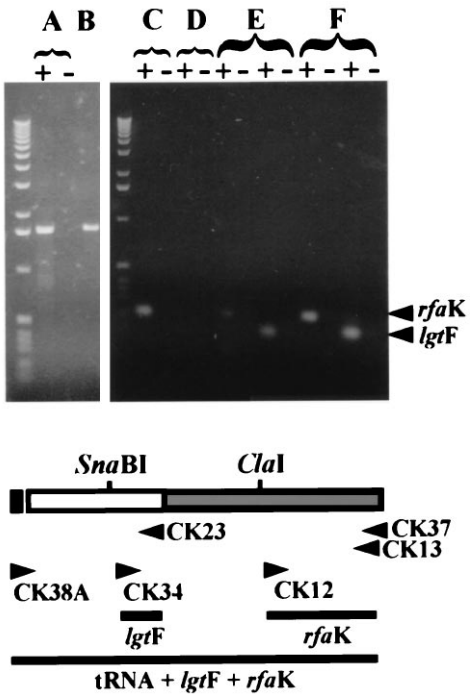

FIG. 4. Detection of *lgtF* and *rfaK* mRNA transcripts by RT-PCR in NMB and LOS mutants. In these assays, the cDNA was synthesized from total RNA with the CK37 primer which anneals to the 3′ terminus of *rfaK* (+). A duplicate reaction without the addition of RT was performed as a negative control (−). (A) A polycistronic mRNA encoding the tRNA, *lgtF*, and *rfaK* was amplified by PCR primers CK38A and CK13 from total NMB RNA; (B) PCR amplification of chromosomal NMB DNA with these same primers produced a product of the same size. mRNA transcripts corresponding to *rfaK* and *lgtF* were detected with primer pair CK12 and CK13 and primer pair CK23 and CK34, respectively. Transcription through *rfaK* was detected in NMB*rfaK*::*aphA-3* (C) but not NMB*rfaK*::Ω (D), therefore confirming the nonpolar and polar effects of these insertions. Both *lgtF*- and *rfaK*-specific mRNAs were detected in NMB*lgtF*::*aphA-3* (E) and NMB*lgtF*::Ω (F), suggesting that neither of these mutations was polar. Amplification products were not detected in the negative controls of these assays, indicating that there was no DNA contamination of the RNA preparations.

Transcription of *rfaK* in the presence of *lgtF* mutations. To confirm that the nonpolar and polar mutations in *lgtF* had the predicted influences upon *rfaK* expression, RT-PCR was used to detect the transcription of *rfaK* in the presence of *lgtF* mutations. An initial analysis of NMB total RNA detected cDNA which corresponded to a polycistronic mRNA transcript encoding tRNA, *lgtF*, and *rfaK*, confirming that these genes form an operon (Fig. 4). Transcription of *rfaK* in the presence of *lgtF* insertion mutations was conducted in a similar manner (Fig. 4). RT-PCR detected an *rfaK* transcript downstream of the insertion site in NMB*rfaK*::*aphA-3* but not in NMB*rfaK*::Ω, indicating that these insertions were nonpolar and polar, respectively. However, *lgtF*- and *rfaK*-specific transcripts were detected in both NMB*lgtF*::*aphA-3* and NMB*lgtF*::Ω, indicating that the *lgtF*::Ω mutation did not completely eliminate *rfaK* transcription. These transcripts could have originated from a weak promoter within the 3′ terminus of *lgtF*, from other promoters within the Ω cassette, or from the inability of the Ω cassette to completely block transcription from the *lgtF* promoter.

We had previously postulated that the addition of α-1,2-linked GlcNAc by *rfaK* to the LOS inner core was a necessary prerequisite for α chain extension from HepI (17). Therefore, although these results suggested that the NMB*lgtF*::*aphA-3*

TABLE 2. Glycosyl compositions of the oligosaccharide components of purified LOSs from *N. meningitidis* NMB and isogenic *rfaK* and *lgtF* mutants

Glycosyl residue	Relative mol% in:			
	NMB	NMB*rfaK*::Ω	NMB*lgtF*::Ω	NMB*lgtF*::*aphA-3*
Glucose	34	0	0	0
Galactose	38	0	0	0
DL-Heptose	14	100	84	68
N-Acetylglucosamine	14	0	16	32
KDO	+	+	+	+

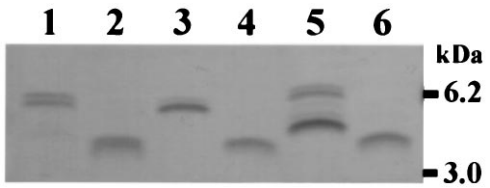

FIG. 5. LOS profiles of *N. meningitidis* strains and corresponding *lgtF*::*aphA-3* mutants analyzed by tricine SDS-PAGE and visualized by silver staining. Lane 1, *N. meningitidis* NMB; lane 2, NMB*lgtF*::*aphA-3*; lane 3, *N. meningitidis* 44/76 (L3,7,8); lane 4, 44/76*lgtF*::*aphA-3*; lane 5, *N. meningitidis* M981 (L5); lane 6, M981*lgtF*::*aphA-3*. The positions of molecular mass markers are shown on the right.

mutant produced a LOS inner core containing GlcNAc, the addition of α chain was still blocked by a mutation in this gene.

Composition analysis of the *lgtF* mutant LOS. The oligosaccharide compositions of LOSs from the *lgtF* mutants were analyzed and are compared in Table 2 with the previously determined compositions of NMB*rfaK*::Ω mutant and NMB parent LOSs (17). As previously reported, *rfaK*::Ω mutant LOS consists of the incomplete LOS inner core, Hep_2KDO_2lipid A, without GlcNAc or the additional sugars of the α chain or inner core. The oligosaccharide from the NMB parent LOS had a molar glucose/galactose/GlcNAc/Hep ratio of 2:2:1:1. As previously reported, heptose and GlcNAc are underestimated in this analysis because of the difficulty in detecting phosphorylated residues (17). The LOS from NMB*lgtF*::*aphA-3* (nonpolar *lgtF* mutant) had a GlcNAc/Hep ratio of 1:2.125, with no other hexoses detected, indicating that the structure $GlcNAc_1Hep_2KDO_2$lipid A was present. This confirmed that the insertion of *aphA-3* into *lgtF* had no effect on the expression of *rfaK*, since GlcNAc was present in the LOS structure, but that the addition of the β-1,4-linked glucose to HepI, the first step in adding α chain sugars to the inner core, and α-1,3-linked glucose to HepII was blocked. In contrast, the oligosaccharide from the NMB*lgtF*::Ω LOS did not contain any hexose and had a GlcNAc/Hep ratio of 5.25:1. If we assume that the GlcNAc present in NMB*lgtF*::*aphA-3* is terminally linked, then the LOS from NMB*lgtF*::Ω contained less α-1,2-linked GlcNAc than did the oligosaccharide from the nonpolar mutant NMB*lgtF*::*aphA-3*. Therefore, it is proposed that NMB*lgtF*::Ω expresses both $GlcNAcHep_2KDO_2$lipid A and Hep_2KDO_2lipid A structures. This result is consistent with the RT-PCR analysis of NMB*lgtF*::Ω which showed that the Ω insertion into *lgtF* did not completely eliminate *rfaK* expression. Since no glucose or galactose was detected in either of the *lgtF* mutants, they lacked both the β-1,4-linked glucose to HepI and α-1,3-linked glucose to HepII.

Inactivation of *lgtF* affects a glucosyl linkage that is common to L2, L3, and L5 immunotype LOSs. Our previous hypothesis predicted that the addition of GlcNAc to the neisserial LOS inner core was a prerequisite for the initiation of α chain extension from HepI (17). Since the LOS of NMB*lgtF*::*aphA-3* met this requirement but was still unable to initiate the addition of the α chain, it was deduced that *lgtF* was also involved in the completion of the LOS inner core, i.e., the addition of glucose to the inner core. However, the wild-type NMB parent strain expresses an L2 LOS structure that contains both a β-1,4-linked Glu to HepI and an α-1,3-linked Glu to HepII. To distinguish which of these linkages were catalyzed by *lgtF*, we analyzed the effects of an *lgtF* mutation in *N. meningitidis* strains producing different LOS structures. *N. meningitidis* 44/76 produces an L3 LOS structure that does not contain the α-1,3-linked Glu to HepII but which is otherwise identical to L2 LOS. *N. meningitidis* M981, which expresses L5 LOS, i.e., L2 LOS with an additional β-1,4-linked glucose in the α chain (25), was used as a control. If *lgtF* catalyzes the addition of the α-1,3-linked Glu to HepII in NMB L2 LOS, we would predict that introduction of the nonpolar *lgtF* mutation into *N. meningitidis* 44/76 would not affect α chain addition to the inner core. Conversely, if *lgtF* catalyzes the addition of the β-1,4-linked Glu to HepI, we would predict that an *lgtF* mutation in this strain would block α chain addition.

To test this hypothesis, *N. meningitidis* 44/76 and M981 were transformed with pCK18 (*lgtF*::*aphA-3*) and allelic replacement of *lgtF* was confirmed by the acquisition of Km^r and by PCR of the *lgtF* locus (data not shown). As expected, both *N. meningitidis* 44/76*lgtF*::*aphA-3* and *N. meningitidis* M981*lgtF*::*aphA-3* expressed a truncated LOS structure with mobility similar to that of NMB*lgtF*::*aphA-3* LOS (Fig. 5). These data further support the hypothesis that *lgtF* encodes the β-1,4-glucosyltransferase required for the addition of β-1,4-linked glucose to HepI to complete the LOS inner core before the initiation of α chain extension.

Primer extension analysis of the promoter regions of *lgtF* and *rfaK*. RT-PCR analysis of NMB mRNA revealed that *lgtF* and *rfaK* were transcribed as a polycistronic message (Fig. 4). Therefore, we used primer extension analysis of *lgtF* transcripts in total RNA from NMB to map the 5′ terminus of the transcript for this operon. cDNA synthesis was performed with ^{32}P-radiolabelled LgtF1 primer which anneals to the 5′ region of *lgtF*. This experiment detected three start points 47 bp upstream of the potential *lgtF* start codon (Fig. 6A). An analysis of this region did not reveal any consensus promoter sequences. However, a consensus σ70 promoter (five of six matches in both the −10 and −35 hexamers and an optimal spacing of 17 bp) was found upstream of the tRNA sequence (Fig. 6B).

DISCUSSION

In *E. coli* and *Salmonella typhimurium*, many of the genes involved in lipopolysaccharide (LPS) synthesis are organized into large complex gene clusters termed *rfa* and *rfb* (33, 36). The *rfa* cluster, which contains 14 genes, is organized into two convergent operons which encode all of the enzymes necessary for inner- and outer-core LPS biosynthesis, whereas the *rfb* operon is essentially dedicated to O antigen biosynthesis. In contrast, many of the genes involved in LOS biosynthesis in *Neisseria* spp. are dispersed around the chromosome (5). A notable exception is the glycosyltransferase gene cluster, *lgtABCDE*, which encodes the five genes necessary for the construction of the lactoside and digalactoside oligosaccharide α chains of LOS immunotypes L3,7,9 and L1,8, respectively (4, 10, 14) (Fig. 7). However, the *lgt* cluster does not contain the UDP-

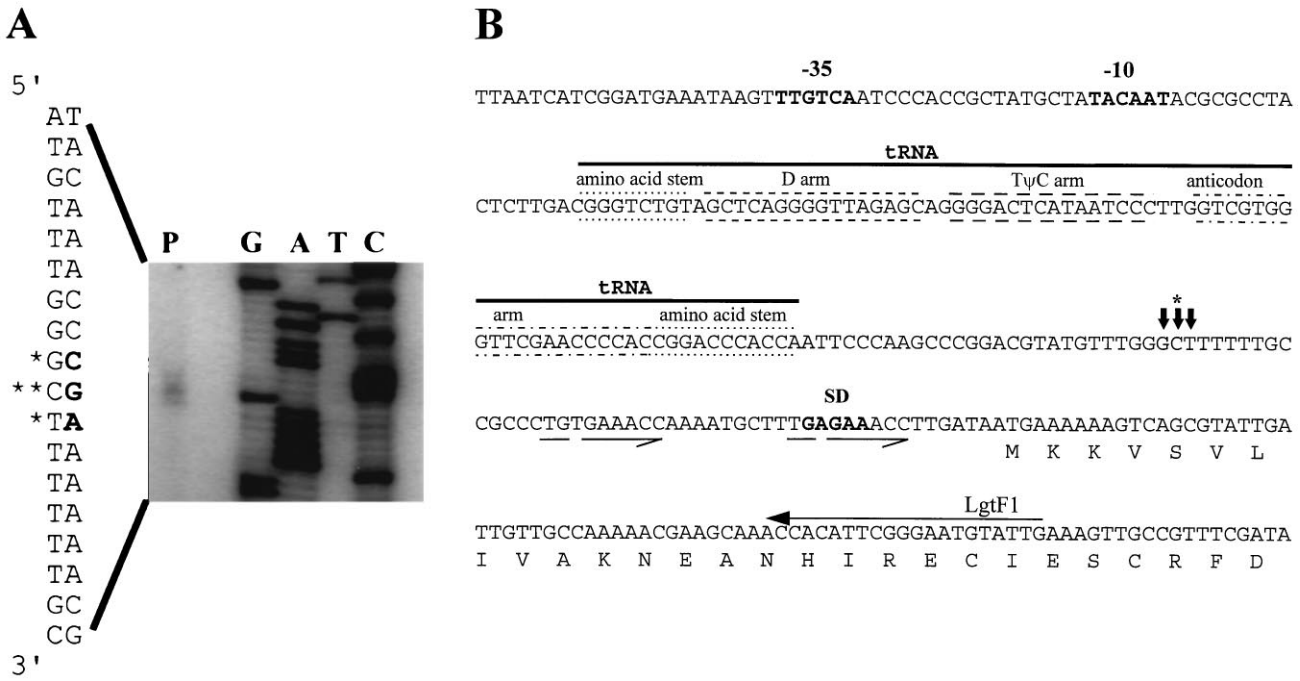

FIG. 6. Primer extension analysis of the mRNA transcripts of *lgtF* in NMB. (A) Total RNA was prepared from *N. meningitidis* NMB as described in Materials and Methods and annealed to the *lgtF*-specific primer, LgtF1. The products of the primer extension reaction (lane P) were compared with a DNA sequence reaction generated from the LgtF1 primer and a DNA template (lanes G, A, T, and C). (B) The sequence upstream of *lgtF* with proposed transcriptional start points (vertical arrows) and the position of the LgtF1 primer (horizontal arrow) shown. The most intense primer extension product is indicated by the asterisk. The location of the tRNA is indicated by the bold line above the sequence, and the proposed stem-loop regions are marked by broken lines. The putative σ70 promoter for the tRNA and the putative ribosome binding site for *lgtF* are in bold. An imperfect direct repeat between *lgtF* and the tRNA is underlined with a half arrow. SD, Shine-Dalgarno sequence.

glucose:LOS-β-1,4-glucosyltransferase which adds the β-1,4-linked glucose residue of lacto-*N*-neotetraose to HepI of the LOS inner core.

By analogy to enterobacterial LPS, we have defined the inner core of meningococcal LOS as the invariant structure conserved in all LOS immunotypes, i.e., Glcβ1,4Hep$_2$GlcNAc$_1$ KDO$_2$lipid A (45). We recently located and characterized the α-1,2-*N*-acetylglucosamine transferase gene (*rfaK*), which adds α-1,2-linked GlcNAc to HepII (17), and noted that this step is a necessary prerequisite for the addition of the α chain to the LOS inner core. Upstream of *rfaK* we have found a second ORF, which has amino acid homology to a family of β-glycosyltransferases (35). Insertional inactivation of this gene, designated *lgtF*, confirmed that it was involved in LOS biosynthesis. Composition analysis of the truncated LOS structure produced by the nonpolar *lgtF* mutant indicated that this structure consists of an incomplete inner core, GlcNAc$_1$Hep$_2$KDO$_2$lipid A, without the β-1,4-linked glucose

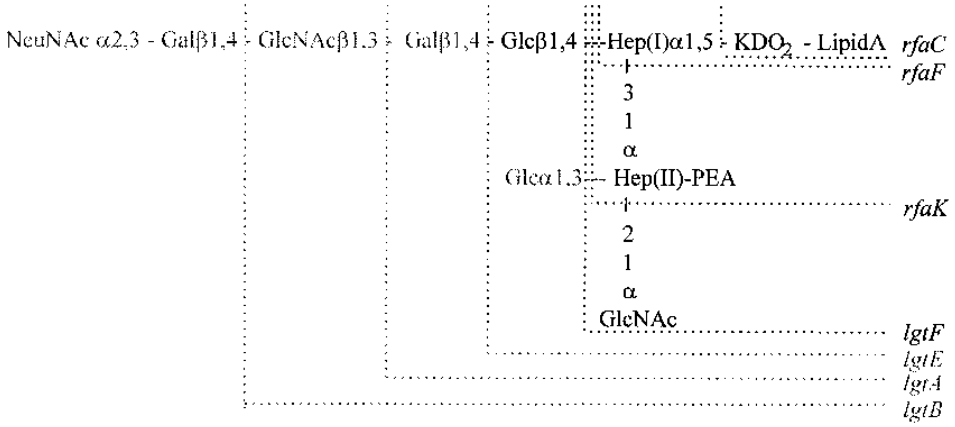

FIG. 7. Schematic diagram of L2 immunotype LOS with the genes involved in the biosynthesis of this structure (inner-core structure is in bold type). The lines encompass the proposed truncated LOS structures produced by the inactivation of each of the specified genes. The following genes are shown: *rfaC*, the α-1,5-heptosyltransferase gene (50); *rfaF*, the α-1,3-heptosyltransferase gene (37); *rfaK*, the α-1,2-*N*-acetylglucosamine transferase gene (17); *lgtF*, the β-1,4-glucosyltransferase gene; *lgtE*, the β-1,4-galactosyltransferase gene (14); the *lgtA*, the β-1,3-*N*-acetylglucosamine transferase gene (14); and *lgtB*, the β-1,4-galactosyltransferase gene (14). The proposed meningococcal inner core is shown in black.

to HepI or α-1,3-linked glucose to HepII. Therefore, the activities of both *rfaK* and *lgtF* are required for the completion of the LOS inner core before α chain extension from HepI of the LOS inner core can occur.

Although the LOS purified from our *lgtF* mutant, NMB*lgtF*::*aphA-3*, lacked both the β-1,4-linked glucose on HepI and α-1,3-linked glucose on HepII, we propose that *lgtF* is the β-1,4-glucosyltransferase which adds glucose to HepI of the neisserial LOS (Fig. 7). This analysis is supported by the amino acid homology of LgtF with the β-glucosyltransferase family described by Saxena et al. (35). Further, introduction of the nonpolar *lgtF* mutation into *N. meningitidis* 44/76, which expresses L3 LOS and contains the β-1,4-linked glucose to HepI but not the α-1,3-linked glucose to HepII, resulted in a truncated LOS structure similar in mobility to those of NMB*lgtF*::*aphA-3* (L2) and *N. meningitidis* M981*lgtF*::*aphA-3* (L5) LOSs. These results imply that the addition of glucose to HepI by *lgtF* precedes the modification of the inner core by the α-1,3-glucosyltransferase found in L2- and L5-expressing strains but not in L3-expressing strains.

The genetic arrangement of the tRNA, *lgtF*, and *rfaK* suggested that these genes form an operon. Composition analysis of the LOS produced by the polar mutant NMB*lgtF*::Ω revealed that the amount of terminally linked GlcNAc was decreased when compared with that of the LOS of the nonpolar *lgtF* mutant. This result corroborates our proposed function for *rfaK* by showing that we can modulate the amount of terminal α-1,2-linked *N*-acetylglucosamine by affecting *rfaK* expression. This experiment also indicates that *rfaK* expression is dependent on transcription through *lgtF*. RT-PCR analysis detected a polycistronic message which encoded the tRNA, *lgtF*, and *rfaK*, suggesting that these genes were coordinately transcribed. Primer extensions of *lgtF* mRNA mapped the 5′ terminus of the transcript upstream of the potential methionine start codon of this gene, yet no consensus σ70 or σ54 promoter sequences were noted in this region, which also contains the tRNA sequence. Therefore, the primer extension products that were detected may represent an mRNA processing site. A putative σ70 consensus sequence was found upstream of the tRNA, which is characteristic of the growth rate-regulated tRNA sequences in *E. coli* (19). This arrangement suggests that *lgtF* and *rfaK* are also transcribed from this promoter and are perhaps regulated by the conditions that control tRNA expression.

In summary, we have identified a second glycosyltransferase operon, called *ice* (inner-core extension), that contains the β-1,4-glucosyltransferase gene, *lgtF*, and the α-1,2-*N*-acetylglucosamine transferase gene, *rfaK*, which are necessary for the completion of the neisserial LOS inner core and the addition of the α chain. The first reported neisserial glycosyltransferase operon, *lgtABCDE* (10), contains the necessary genes for LOS α chain biosynthesis which proceeds after the inner core is completed by *ice*. Both of these operons are similar in that they are preceded by genes involved in tRNA synthesis, an arrangement which may result in coordinated regulation and expression.

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