

## Relationship between UDP-Glucose 4-Epimerase Activity and Oligoglucose Glycoforms in Two Strains of *Neisseria meningitidis*

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**Sodium dodecyl sulfate-polyacrylamide gel analysis of lipooligosaccharide (LOS) from *Neisseria meningitidis* has demonstrated considerable microheterogeneity in the variable region of LOS due to the presence of novel glycoforms. As a step toward understanding the basis for the expression of these novel glycoforms, we have examined the LOS structures and UDP-glucose 4-epimerase (epimerase) activity levels in two strains (NMB and MA-1) and their respective *galE* mutants. Strain NMB was found to have low epimerase activity and to contain multiple glycoforms, some of which appear to contain only glucose sugars. The *galE* mutant had only the oligoglucose glycoforms. Strain MA-1 had higher epimerase activity at both log and stationary phases (2- and 12.5-fold, respectively) and one glycoform with a putative lactosyl structure. Strain MA-1 *galE* had two glycoforms that contained one or two glucose residues. To understand the molecular basis for the different epimerase activities, we examined the predicted amino acid sequences of the respective *galE* open reading frames and determined the relative amounts of GalE protein. We found no significant differences between the predicted amino acid sequence of the GalE protein in NMB and that in MA-1. We observed no significant differences in the level of GalE protein between MA-1 and NMB at exponential or stationary phase. We also observed an 8.2-fold drop in epimerase activity in NMB between the log and stationary phases that was not due to the GalE protein level or low glucose levels.**

Pathogenic *Neisseria* species are gram-negative obligate human pathogens. *Neisseria meningitidis*, the causative agent of meningococcal meningitis, possesses a number of virulence factors. One of these virulence factors is the lipooligosaccharide (LOS). This molecule is composed of a variable oligosaccharide portion and a conserved core-lipid A structure (1).

The assembly of the LOS molecule is a complex anabolic process involving an array of biosynthetic enzymes including kinases, transferases, and isomerases. One of the isomerases is the UDP-glucose 4-epimerase (galactowaldenase, EC 5.1.3.2). This enzyme carries out the reversible epimerization of UDP-glucose to UDP-galactose, the cognate substrate for galactosyltransferases. The *Escherichia coli* UDP-glucose 4-epimerase has been purified and studied in detail at both the biochemical and the structural level. The holoenzyme is a homodimer held together by hydrophobic interactions and contains one NAD<sup>+</sup> molecule per subunit (4).

The number of unique LOS species expressed by a meningococcal strain can vary widely (24, 29). We have previously reported on the LOS microheterogeneity of serogroup B *N. meningitidis* NMB and its *galE* mutant NMB-SS3 (14). The basis of this microheterogeneity was the presence of three novel glycoforms that contained two to four glucose residues. In the *galE* mutant NMB-SS3, only the oligoglucose glycoforms were detected, and the relative amounts of some of these novel glycoforms were significantly increased compared to those in NMB. Also, in *N. meningitidis* MC58 *galE*, a second

glycoform with two glucose molecules was observed (33). It is not known if the diglucose glycoform was present in the parent strain. Pathogenic *Neisseria* species cannot utilize exogenous sources of galactose, and thus the only source of UDP-galactose is UDP-glucose. This suggests an important role for UDP-glucose 4-epimerase in determining the UDP-glucose and UDP-galactose concentrations and thereby perhaps influencing the activity of cognate glycosyltransferases. In this report, we present the LOS structures of MA-1 and its *galE* mutant MA-1 *galE*, and we compare the UDP-glucose 4-epimerase activity level of strain NMB to that of strain MA-1. We also present evidence demonstrating growth phase-dependent variation of epimerase activity levels in NMB.

### MATERIALS AND METHODS

**Materials.** Chemicals and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs, Promega Co., and Boehringer Mannheim Biochemicals.

**Bacterial strains and plasmids.** The bacteria and plasmids used in this study are described in Table 1.

**Growth of bacteria.** *E. coli* was grown at 37°C in Luria-Bertani medium with or without 1.5% agar and supplemented with antibiotics as needed. Wild-type *N. meningitidis* was grown either on gonococcal agar with 1% IsoVitalX supplement (BBL Laboratories) or in brain heart infusion (BHI) broth supplemented with 2.5% heat-inactivated fetal calf serum and 1% IsoVitalX. Kanamycin-resistant *N. meningitidis* was grown on supplemented BHI agar with 45 µg of kanamycin/ml or in supplemented BHI broth with 5 (for strain MA-1) or 25 (for strain NMB) µg of kanamycin/ml. *N. meningitidis* on agar plates was cultured in the presence of 5% CO<sub>2</sub> at 85% relative humidity.

**Recombinant DNA and transformation methods.** All recombinant DNA techniques were used as outlined elsewhere (23). Transformations of *N. meningitidis* were performed as previously described by Catlin (4) and modified by Stephens et al. (25). Electroporations were carried out by using the GIBCO-BRL Cell-Porator under the recommended conditions.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or selective marker	Source or reference
<b>Strains</b>		
<i>E. coli</i> XL1 BLUE	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> <sup>r</sup> ZΔM15 Tn10(Tet <sup>r</sup> )](Con)	Stratagene
<i>N. meningitidis</i> NMB	Wild-type clinical isolate; serogroup B; L2, L3, L7, L9	14, 22, 25
<i>N. meningitidis</i> MA-1	Wild-type clinical isolate; serogroup A; L8	This study
<i>N. meningitidis</i> MA-1 Δ <i>galEt::npt</i>		This study
<b>Plasmids</b>		
pDEADII	Kanamycin	pZERO (Invitrogen) modified by author
pBluescript II SK(-)	Ampicillin	Stratagene
pBSL14	Ampicillin and kanamycin	1

**DNA sequencing.** The nucleotide sequences of cloned genomic DNA fragments were determined at the DNA Facility at the University of Iowa by using an ABI373A automated sequencer.

**Hybridizations.** Analyses of Southern blots were carried out with radiolabelled random-primed probes at 65°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 20 mM Na<sub>3</sub>P<sub>2</sub>O<sub>7</sub>, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of heterologous DNA/ml. The filters were washed twice at the hybridization temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 15 min, followed by two washes in 0.1× SSC and 0.1% SDS for 15 min. The radioactive blots were exposed to Kodak XAR5 or BioMaxMR film at -70°C.

**Hot-phenol LOS extraction.** LOS was purified from *N. meningitidis* by a modified hot-phenol method (34) as previously described (14). The purified LOS was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining as described elsewhere (30).

**Structural characterization of LOS.** To determine the effects of UDP-glucose 4-epimerase activity on LOS biosynthesis, LOS from the wild-type strain MA1 and a *galE* mutant were partially characterized by mass spectrometry and composition analysis. Crude LOS preparations (~1 mg each) from the two strains were first O-deacylated by treatment with hydrazine under mild conditions (37°C for 30 min), followed by precipitation with chilled acetone (10). For mass spectrometric analysis, O-deacylated LOS was dissolved in a stock water solution (10 μg/μl) and analyzed by matrix-assisted laser desorption ionization (MALDI) (7) and electrospray ionization (ESI) (8) mass spectrometry as previously described. For the MALDI analyses, several dilutions were made of the original stock (1/10 to 1/100), and ~0.1 μg or less of each O-deacylated LOS was added to 1 μl of a matrix solution of 2,5-dihydroxybenzoic acid (DHB) and spotted on a stainless-steel MALDI sample plate. Mass spectra were taken on a PerSeptive Biosystems Voyager MALDI-time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, Mass.) fitted with a N<sub>2</sub> laser operating at 337 nm. Typically, 50 to 100 single laser shots were averaged to obtain a single spectrum by using a 100-ns delay time and a laser power setting of 1,800 to 2,000. Spectra were smoothed (Savitsky-Golay procedure; 2 by 19 point) and externally calibrated with a close proximity standard consisting of the commercial peptides bradykinin ( $M_{avg} = 1,060.2$ ) and ACTH 1-24 ( $M_{avg} = 2,465.7$ ). For ESI analyses, a stock solution of each O-deacylated LOS preparation was diluted fivefold in water-acetonitrile (1:1, vol/vol) containing 1% acetic acid to yield a final O-deacylated LOS concentration of approximately 2 μg/μl. Five microliters of each LOS sample was then injected via a Rheodyne valve and analyzed in the negative-ion mode on a Sciex API 300 triple quadrupole mass spectrometer (Perkin-Elmer SCIEX, Mississauga, Ontario, Canada) with a flow rate of 3 to 4 μl/min. Mass calibration was carried out with an external myoglobin reference by using the commercial software supplied.

Monosaccharide composition analyses were carried out on O-deacylated LOS preparations by two independent methods. For neutral sugar analysis, aliquots of each O-deacylated LOS pool were hydrolyzed in 2 M trifluoroacetic acid for 3 h at 100°C. Amino sugars were analyzed after hydrolysis in 6 N HCl for 3 h at 100°C. In both cases, aliquots of the final hydrolysates were evaporated to dryness, redissolved in 20 μl H<sub>2</sub>O, dried, and then reconstituted in water. A 5% aliquot (1 of 20 μl) of each sample was analyzed by high pH anion-exchange chromatography by using a Dionex high-pressure liquid chromatography system equipped with a PA1 column as previously described (20). To identify and quantify the monosaccharides from each hydrolysate, a standard mixture of monosaccharides containing equimolar amounts of fucose, galactosamine, glucosamine, galactose, glucose, and mannose was analyzed before and after each run.

**Glucose supplement of stationary-phase culture.** A fresh overnight culture of *N. meningitidis* NMB grown in Morse's defined medium with 20 mM glucose (18) was used to inoculate 50 ml of fresh medium and incubated at 37°C with shaking. Aliquots (15 ml) were removed at mid-log and early-stationary phases, and epimerase activities were determined. Glucose was added to the remaining culture

to a final concentration of 20 mM, and it was incubated for one additional hour, after which the epimerase activity was determined.

**Purification of recombinant meningococcal GalE.** The NMB *galE* open reading frame was cloned into plasmid pCYB2 of the IMPACT Protein Purification System (New England Biolabs), and the recombinant meningococcal GalE was purified according to the manufacturer's recommendations. The purified protein was verified by amino-terminal sequencing at the Protein Structure Facility, University of Iowa.

**Generation of polyclonal antibodies.** Mouse polyclonal antibodies to recombinant meningococcal GalE were raised by coinjection with the RIBI Adjuvant System (RIBI ImmunoChem Research, Inc.) according to the manufacturer's recommendations. The ascites fluid was harvested and assayed for reactivity to the purified antigen by enzyme-linked immunosorbent assay.

**SDS-PAGE and Western blot analysis.** Cultures of *N. meningitidis* MA-1 and NMB were harvested and lysed by sonication. Total protein concentrations were determined with the Bio-Rad Protein Assay Reagent system, and equivalent amounts of total protein were fractionated by 12.5% SDS-12.5% PAGE. The resolved proteins were transferred to nitrocellulose under standard conditions and reacted with mouse polyclonal antisera to recombinant meningococcal GalE that had been preabsorbed with paraformaldehyde-fixed MA-1 *galE*. The reacting bands were visualized with a horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (Bio-Rad, Inc.) and the SuperSignal Horseradish Peroxidase Detection System from Pierce Chemicals. The relative intensities of the bands were estimated with Eastman Kodak (Rochester, N.Y.) 1D Image Analysis Software.

**Preparation of cell extract.** *N. meningitidis* strains were grown in supplemented BHI broth with kanamycin as required. For each strain, a 50-ml culture was inoculated with 0.01 volume of fresh overnight culture and incubated overnight at 37°C with shaking. The washed pellets were resuspended in 2 ml of buffer (0.125 M potassium bicarbonate [pH 8.5]-1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The bacterial debris was pelleted by centrifugation at 15,800 × g for 30 min at 4°C. The supernatants were transferred to prechilled microcentrifuge tubes and kept on ice. The total protein contents of the cleared cell extracts were determined by using the Bio-Rad Protein Assay Reagent system following the microassay protocol. Equal amounts of total protein were added to the two-step UDP-glucose 4-epimerase assay as described below.

**Enzyme assays.** The standard UDP-glucose 4-epimerase assays have been published elsewhere (35). We modified the assay slightly to optimize it for meningococcal extracts. The first step of the two-step assay was carried out in a 500-μl reaction volume (0.125 M bicarbonate [pH 8.5]-0.45 mM UDP-galactose) at 37°C for 15 min. The reaction mixture was then placed in a boiling water bath for 90 s, chilled on ice for 5 min, and then centrifuged at 15,800 × g for 10 min at 4°C. A 400-μl aliquot of the supernatant was added to the second step of the assay in 600 μl (0.125 M bicarbonate [pH 8.5]-1.25 mM NAD<sup>+</sup>-0.02 U of UDP-glucose dehydrogenase). The reaction mixture was incubated at room temperature for 3 min in a methacrylate cuvette, and the increase in absorbance was measured at 340 nm at 15-s intervals. We were able to achieve a twofold increase in sensitivity with the modifications compared to that of the standard two-step method using the glycine buffer. All extracts, including appropriate controls, were assayed in triplicate.

**Determination of UDP-glucose 4-epimerase activity levels.** The net absorbance was determined after adjustment for endogenous UDP-galactose and UDP-glucose and for UDP-glucose contamination of exogenous UDP-galactose preparations. The initial velocities of the second reaction (UDP-glucose to UDP-glucuronic acid) were determined over the first 30 s. This initial reaction velocity ( $V_i$ ) is a function of the initial UDP-glucose concentration, which in turn is a function of the UDP-glucose 4-epimerase activity level. These values were converted to nanomoles of NADH generated per minute by using the Beer-Lambert law and the equation  $\epsilon_{NADH} = 6.2 \times 10^3 \cdot M^{-1} \cdot cm^{-1}$ . The assay results were analyzed by paired *t* tests using the Statview program (Abacus Concepts).

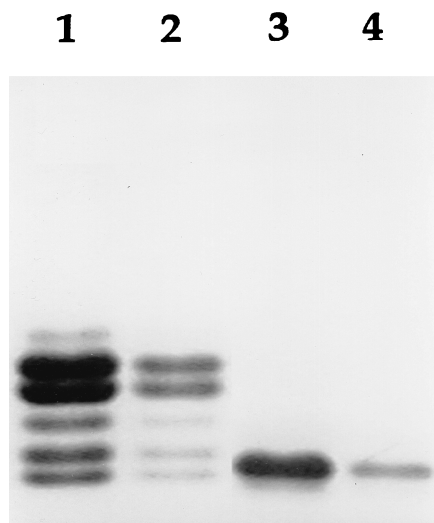


FIG. 1. SDS-PAGE analysis of LOS isolated from *N. meningitidis* MA-1 and NMB. This figure illustrates the multiple glycoforms observed in NMB LOS due to variations in the oligosaccharide or PEA composition and the apparent homogeneity of MA-1 LOS. Lanes 1 and 2, *N. meningitidis* NMB LOS (2.0 and 0.2  $\mu$ g, respectively); lanes 3 and 4, *N. meningitidis* MA-1 LOS (2.0 and 0.2  $\mu$ g, respectively).

**Nucleotide sequence accession number.** The nucleotide sequence the *galE* region of strain MA-1 is listed under accession no. AF083467.

## RESULTS

**Analysis of LOS from MA-1 and MA-1 *galE* by SDS-PAGE.** MA-1 LOS migrated faster than the major NMB LOS species on SDS-PAGE analysis and did not appear to be sialylated (Fig. 1). The MA-1 LOS also migrated as a single band, in contrast to NMB LOS. The MA-1 LOS also reacted with monoclonal antibody 4C4, which recognizes Gal-Glc-Hep or Glc-Hep structures (12) (data not shown).

A 4.2-kb *Dra*I fragment that contained the *galE* gene and three other open reading frames was cloned from strain MA-1. The last three open reading frames were identified as *galE*, *rfbB*, and *rfbA* homologues. The first open reading frame (ORF1) was identical to the recently reported *mynD* (27). A FASTA search of the protein database indicated that ORF1 (*mynD*) had the highest similarity to a putative DNA-binding protein from yeast ( $P = 0.062$  over 201 amino acids) (data not shown).

We placed a kanamycin cassette 647 bp into the *galE* open reading frame and introduced the mutation into the chromosome by allelic replacement. A kanamycin-resistant transformant designated MA-1 *galE* was screened by Southern hybridization (Fig. 2a), SDS-PAGE analysis (Fig. 2b), and epimerase enzyme assay (data not shown). The results confirmed the insertion of the kanamycin cassette into the meningococcal genome through allelic replacement, resulting in a significant decrease in UDP-glucose 4-epimerase activity. SDS-PAGE analysis of isolated MA-1 *galE* LOS indicated the presence of two bands. The majority of the LOS molecules appeared to be the expected truncated form, but there was a minor band which appeared to comigrate with wild-type LOS (Fig. 2b).

**Genetic analysis of the MA-1 *galE* gene.** The organization of the 4.2-kb fragment suggested that the MA-1 *galE* gene was within an operon, perhaps contiguous with the previously described *myn* operon (27). We introduced a kanamycin cassette in polar and nonpolar orientations upstream of the *galE* open reading frame at two different sites (Fig. 3). Analysis of these mutants by epimerase enzyme assay and SDS-PAGE of their

LOS (Table 2) indicated that the presence of polar insertions at *Eco*RI and *Nde*I sites (RKR and NKR, respectively) abrogated the expression of the *galE* gene.

The promoter for the *galE* gene of a serogroup B strain, B1940, has been mapped previously (9). The nucleotide sequence upstream of the NMB *galE* gene is identical to the sequence from B1940 (data not shown).

**ESI mass spectrometric analysis of LOS isolated from *N. meningitidis* MA-1 and MA-1 *galE*.** Mass spectrometric analyses of the O-deacylated LOS preparations from the wild-type strain, MA-1, and the *galE* mutant are shown in Fig. 4 for the corresponding MALDI spectra. For the wild-type strain, *N. meningitidis* MA-1, two prominent, singly deprotonated molecular-ion species were identified in the high-mass range ( $m/z > 2,000$ ) with  $m/z$  values of  $(M - H)^- = 2,428.0$  and  $2,551.1$ , as well as peaks related to these two major species through either loss of water ( $-18$  Da),  $\beta$ -elimination of phosphoric acid ( $-98$  Da), or noncovalent addition of one or more sodium ( $+22$  Da) or magnesium ( $+38$  Da) ions or both. As shown in Table 3, these masses are consistent with a LOS composition of a Hep<sub>2</sub>-HexNAc-2 molecules of 3-deoxy-D-manno-octulosonic acid (Kdo<sub>2</sub>)-lipid A core structure substituted with two hexoses (galactose and glucose) and containing either one (LOS B; M calculated 2,428.2) or two (LOS B'; M calculated 2,551.3) phosphoethanolamine (PEA) groups ( $\Delta M = 123$  Da).

The MALDI spectra of the *galE* mutant LOS were markedly different from those of the wild-type MA-1 LOS. In the former case, the two most abundant molecular-ion species were seen at  $m/z \sim 2,265$  and  $2,388$ , approximately 1 hexose lower in mass (i.e., Hex = 162 Da) than the two base peaks in the wild-type MA-1 MALDI spectra. However, peaks corresponding to the two wild-type molecular ions at  $m/z \sim 2,427$  and  $2,551$  were also present in the mutant LOS preparations, but at considerably lower abundances. Electrospray analysis of the mutant and wild-type LOS preparations (see Table 3) also supported these assignments, containing both doubly and triply charged ions for the LOS A and B glycoforms (with and without the additional PEA) for the mutant, and primarily the two LOS B glycoforms in the wild type.

In addition to the prominent molecular-ion regions of these two spectra, the low-mass regions of the two MALDI spectra ( $m/z < 1,600$ ) contained ions whose relative abundance was dependent on the laser power. As reported previously (7), these ions are "prompt fragments" and are generated primarily from cleavage at the Kdo glycosidic bond to the lipid A moiety. Figure 5 shows the likely fragmentation pathways for these ions for MA-1, which give additional support for the assignment of the major glycoform species. For example, in the MA-1 spectra the peak at  $m/z$  951.7 would correspond to the deprotonated lipid A species containing two phosphate groups. The small peak at  $m/z$  1,031.7 suggests that a triphosphoryl lipid A species is also present, but at a much lower abundance ( $< 10\%$ ) than the dominant diphosphoryl lipid A form. The peaks at  $m/z$  1,597.0 and 1,473.8 can be assigned as originating from the same cleavage but with charge retention on the oligosaccharide fragments, which can undergo further fragmentation due to losses of 44 Da ( $-\text{CO}_2$ ;  $m/z$  1,552.9 and 1,429.8) and 220 Da for the terminal Kdo moiety ( $m/z$  1,376.7 and 1,254.0). An analogous set of assignments can be made for the *galE* strain; although in addition to the fragments arising from the less-abundant LOS B glycoforms as just described, ions are seen for the oligosaccharide fragments of the LOS A glycoforms that are now shifted down in mass by 1 hexose unit ( $-162$  Da).

Composition analysis clearly supported the loss of galactose as underlying the shift in mass (MALDI spectra) and faster

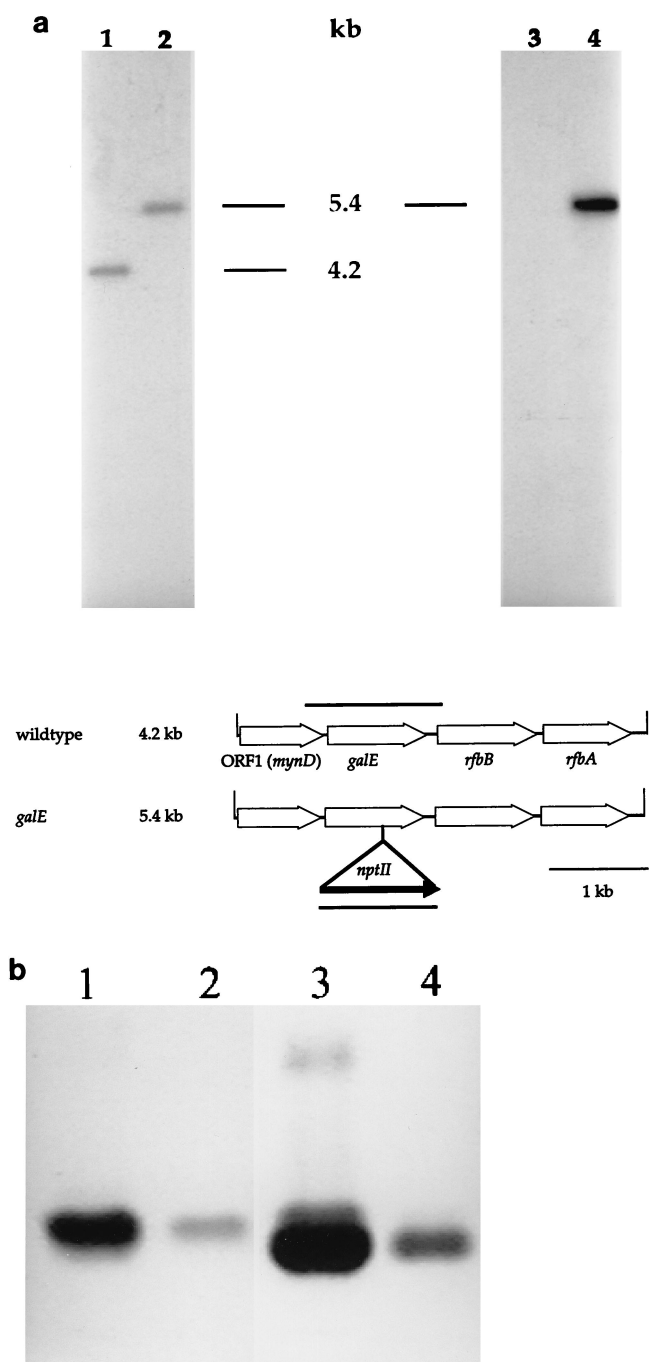


FIG. 2. Analysis of *N. meningitidis* MA-1 *galE*. (a) Southern blot analysis of genomic DNA from *N. meningitidis* MA-1 *galE*. Genomic DNAs isolated from *N. meningitidis* MA-1 and MA-1 *galE* were digested with *Dra*I and transferred to a nylon membrane after electrophoresis. Duplicate filters were probed with either a *galE* gene probe (lanes 1 and 2) or a kanamycin cassette probe (lanes 3 and 4). The hybridization patterns indicated the incorporation of the kanamycin cassette in the *galE* open reading frame. A genetic map of the *N. meningitidis* MA-1 *galE* mutant is shown below. The MA-1 *galE* gene was cloned on a 4.2-kb *Dra*I fragment. This fragment had four open reading frames, as designated. To construct MA-1 *galE*, a kanamycin cassette from pBSL14 was inserted into the unique *Mun*I site 647 bases into the *galE* coding region. This construct was introduced into the chromosome of MA-1 by allelic replacement. Open arrows, meningococcal open reading frames; solid arrow, kanamycin resistance cassette. (b) SDS-PAGE analysis of MA-1 and MA-1 *galE* LOS. The wild-type LOS migrated as a single band. The LOS from the *galE* mutant gave two distinct bands, of which the upper band comigrated with the wild-type LOS molecule. The lower band is the Hex-Hep<sub>2</sub>-GlcNAc-Kdo<sub>2</sub>-lipid A structure. Lanes 1 and 2, MA-1 LOS (2.0 and 0.2  $\mu$ g, respectively); lanes 3 and 4, MA-1 *galE* LOS (2.0 and 0.5  $\mu$ g, respectively).

migration (SDS-PAGE) of *galE* LOS. As shown in Fig. 6, the MA-1 wild-type LOS preparation contained glucosamine (from the lipid A and oligosaccharide regions), galactose, and glucose (oligosaccharide branch). In contrast, the MA-1 *galE* mutant had significantly reduced levels of galactose, which was barely detectable. Both MA-1 and MA-1 *galE* LOS preparations had residual amounts of mannose. The mannose appears to be a contaminant in the LOS, since multiple washes of the LOS result in its removal.

**Comparison of GalE activity in *N. meningitidis* MA-1 and NMB.** The epimerase activity levels in MA-1 and NMB at the exponential and stationary phases were measured by the two-step assay. At the exponential phase, there was a twofold difference in the epimerase activity level between MA-1 and NMB (Table 4). In stationary-phase cultures, there was a 12.5-fold difference in the enzyme activity levels. This difference was not due to an increase in enzyme activity in MA-1, but rather to an 8.2-fold decrease in NMB epimerase activity from the exponential to the stationary phase. *N. meningitidis* MA-1 had similar activity levels at both phases of growth. The addition of 20 mM glucose to a stationary-phase culture of strain NMB did not increase the epimerase activity ( $1.7 \pm 0.3$  versus  $2.7 \pm 0.5$  nmol of NADH generated per min [means  $\pm$  standard errors] for NMB with and without glucose, respective;  $P = 0.0377$ ).

**Comparison of predicted GalE amino acid sequences.** We cloned the *galE* genes from *N. meningitidis* MA-1 and NMB and compared the predicted amino acid sequences of the *galE* open reading frames alongside that of the previously cloned FAM20 *galE* gene (14). There was a high degree of identity among the putative NAD<sup>+</sup> and UDP-sugar binding site residues (28) (mean = 95.65%; 22 of 23 amino acids) and 91% identity over the entire length of the polypeptide. There was one binding site residue change at amino acid 300 (F in MA-1, S in NMB, and F in FAM20) (Fig. 7). The corresponding residue is a tyrosine (position 299) in the *E. coli* GalE polypeptide, and this amino acid is not conserved among the other GalE proteins of gram-negative bacteria. In contrast, other binding site residues are highly conserved between meningococcal GalE and the *E. coli* GalE protein. Protein secondary-structure analysis indicated a high degree of conservation in the first 290 residues between MA-1 and NMB GalE polypeptides. This observation is consistent with the predicted amino acid sequence, where the first 290 residues are highly conserved (97.24% identity) and the last 49 residues are less conserved (75.51% identity). The last 49 residues contain 3 of the 21 active-site residues.

**Analysis of UDP-galactose epimerase activity in *galE* deletion mutants of strains MA-1 and NMB.** The meningococcal genome contains a second partially duplicated copy of the *galE* gene (9, 11, 14, 15) that is not present in the gonococcal genome. This second copy is a duplication of the last 621 bp of the full-length open reading frame and contains one of the two putative hydrophobic domains involved in homodimer formation (3). To determine the contribution of the duplicated *galE* gene to the UDP-glucose 4-epimerase activities in strains MA-1 and NMB, all but the first 20 bp of the second *galE* open reading frame was deleted and replaced with a kanamycin cassette from pBSL14 (1). The deletion was introduced into the chromosomes of *N. meningitidis* MA-1 and NMB by transformation. Kanamycin-resistant transformants were screened by Southern blot hybridization and SDS-PAGE analysis of LOS (data not shown).

Crude cell extracts from cultures of wild-type MA-1 and NMB and their  $\Delta$ *galEt::npt* mutants at exponential and stationary phases were prepared, and epimerase activity levels were measured. For both MA-1 and NMB, the UDP-glucose 4-

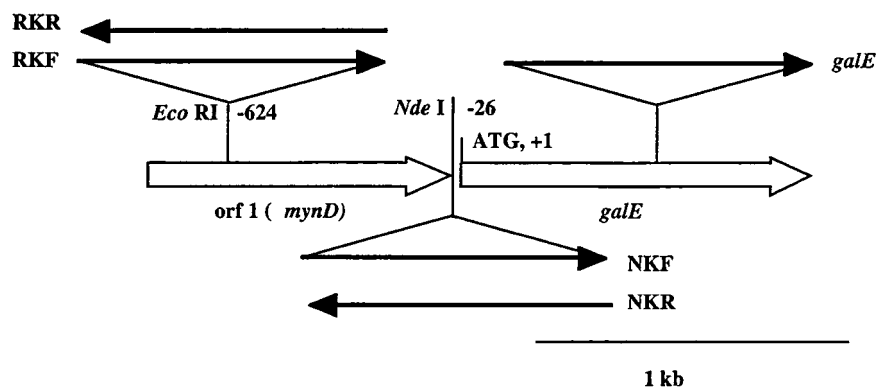


FIG. 3. Positions and orientations of the kanamycin cassette (solid lines with arrows) in the region upstream of the *galE* open reading frame. The positions of the restriction sites are given relative to the *galE* gene start codon. Open arrows, open reading frames. RKR and NKR, polar insertions of the kanamycin cassette at the *EcoRI* and *NdeI* sites, respectively. RKF and NKF, respective nonpolar insertions.

TABLE 2. Genetic analysis of MA-1 *galE* gene

Strain, position <sup>a</sup>	nmol of NADH generated/min ( $\pm$ SE)	LOS migration pattern <sup>b</sup> (kDa)
MA-1	3.5 (0.2)	Wild type (3.4)
RKF, -624	2.7 (0.2) <sup>c</sup>	Wild type (3.4)
RKR, -624	0.4 (0.2) <sup>d</sup>	Truncated (3.2)
NKF, -26	21.3 (0.4) <sup>e</sup>	Wild type (3.4)
NKR, -26	0.0 (0.2) <sup>f</sup>	Truncated (3.2)
MA-1 <i>galE</i>	0.2 (0.0) <sup>g</sup>	Truncated (3.2)

<sup>a</sup> Position of the kanamycin cassette relative to the *galE* gene start codon.

<sup>b</sup> Relative to that of the wild type.

<sup>c</sup>  $P = 0.0527$  for this value versus that obtained with MA-1.

<sup>d</sup>  $P = 0.0153$  for this value versus that obtained with MA-1.

<sup>e</sup>  $P = 0.0008$  for this value versus that obtained with MA-1.

<sup>f</sup>  $P = 0.0091$  for this value versus that obtained with MA-1.

<sup>g</sup>  $P = 0.0034$  for this value versus that obtained with MA-1.

epimerase activity levels were not significantly different in the wild type and the  $\Delta galE::npt$  mutant at exponential phase (Table 4). The growth curves of the wild-type and  $\Delta galE::npt$  strains were identical (data not shown). The deletion of the *galE* gene in MA-1 had no significant effect on epimerase activity at stationary phase. In NMB there was a 2.5-fold increase in enzyme activity in the mutant compared to the wild type at stationary phase (Table 4). The removal of the *galE* gene was not able to restore the 8.2-fold decrease in NMB epimerase activity between the exponential and stationary phases.

#### Western blot analysis of wild-type meningococcal strains.

We analyzed whole-cell extracts from wild-type *N. meningitidis* MA-1 and NMB with polyclonal antisera in order to determine the amounts of GalE protein in the respective strains at the exponential and stationary phases. We detected a band that was specific to the GalE protein which was not present in the extract prepared from MA-1 *galE*. The Western blot analysis indicated that there were equivalent amounts of the GalE protein in *N. meningitidis* MA-1 and NMB at exponential phase. Similarly, at stationary phase, the amounts of GalE protein in MA-1 and NMB appeared to be equivalent. Within NMB, there was a slight increase in the amount of GalE protein at stationary phase compared to exponential phase. A similar observation was made in strain MA-1 (Fig. 8). Compared to the normalized band intensity of NMB GalE at log phase (taken as 1.0), the band intensity of NMB GalE at stationary phase was 1.3 ( $P = 0.0099$ ), that of MA-1 GalE at log phase was 1.0 ( $P =$

0.4639 for MA-1 GalE versus NMB GalE at log phase), and that of MA-1 GalE at stationary phase was 1.8 ( $P = 0.0025$  for MA-1 GalE at log phase versus stationary phase;  $P = 0.1296$  for MA-1 GalE versus NMB GalE at stationary phase). These values are means of three calculations.

## DISCUSSION

To further understand the role of UDP-glucose 4-epimerase in the biosynthesis of oligoglucose glycoforms found in meningococcal LOS, we examined the LOS from strain MA-1 and its *galE* mutant by mass spectrometry. We compared the results from these studies with the structure of LOS from NMB and NMB-SS3, which we had previously characterized (14). We also examined the epimerase activity levels in MA-1 and NMB at the log and stationary phases of growth. The LOS of MA-1 was composed of one glycoform with one or two PEA groups. The MA-1 *galE* LOS had two glycoforms with either one or two glucose residues and PEA substituted for Hep<sup>I</sup>. The UDP-glucose 4-epimerase assay indicated a twofold difference in epimerase activity at log phase and a 12.5-fold difference at stationary phase between MA-1 and NMB (Table 3). Recently, Wakarchuk et al. (33) reported the presence of a glycoform with a diglucose structure in their *galE* strain. Pathogenic *Neisseria* species do not possess the accessory enzymes required for the utilization of exogenous galactose. This implies that UDP-glucose 4-epimerase is involved in controlling the ratio of UDP-galactose to UDP-glucose. The meningococcal epimerase, like the epimerases from yeast and *E. coli*, has an equilibrium constant favoring the formation of UDP-glucose (14). These observations suggest that the oligoglucose glycoforms in strain NMB and the absence of these glycoforms in strain MA-1 may be linked to the level of UDP-glucose 4-epimerase activity in the respective strains. The presence of the oligoglucose glycoforms in NMB-SS3 and the diglucose LOS structure in MA-1 *galE* and MC58 *galE* further support this interpretation.

The oligoglucose glycoforms in NMB-SS3 had up to two additional glucose molecules, while the oligoglucose glycoforms in MA-1 *galE* had one additional glucose. Similarly, MC58 *galE* had a novel glycoform containing an additional glucose (33). The transfer of the additional glucose residue(s) could occur by one of two pathways. The increased UDP-glucose concentration could either activate an unidentified glucosyltransferase or induce its expression. Alternatively, in the absence of UDP-galactose, galactosyltransferase could use UDP-

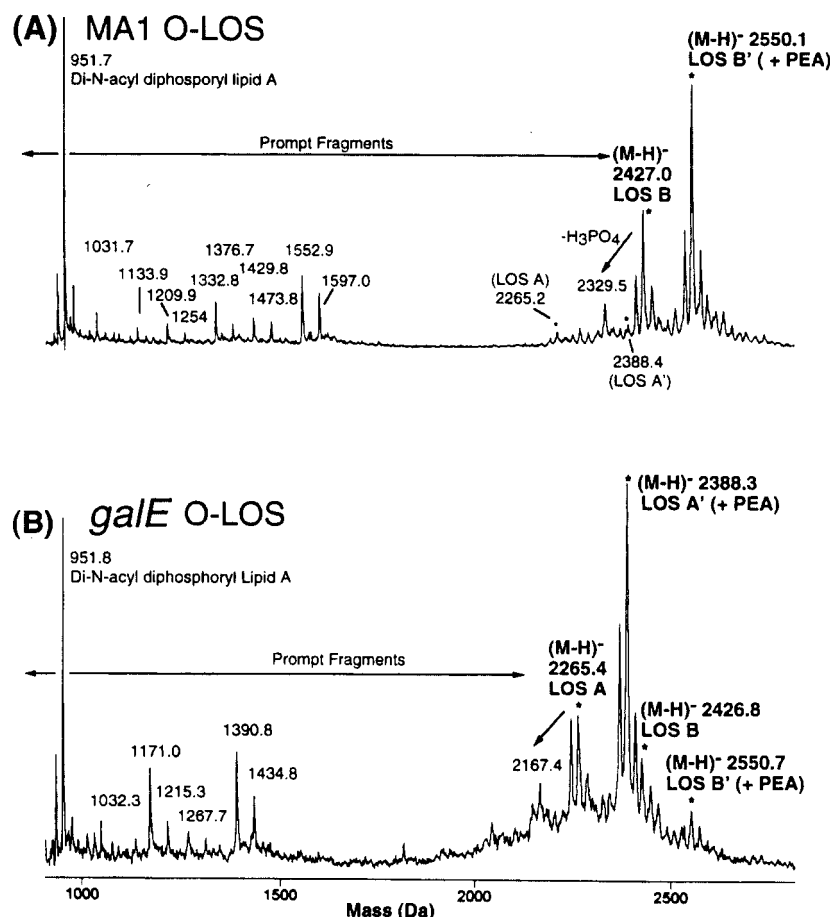


FIG. 4. Negative-ion MALDI-time-of-flight mass spectra of O-deacylated LOS preparations of wild-type MA-1 (A) and MA-1 *galE* (B).

glucose as a substrate. Based on our observations and that of Wakarchuk et al. (33), the first glucose residue after the Glc-Hep<sup>I</sup> core structure could be added by the LgtE protein, a galactosyltransferase. The detection of the tetraglucose glycoform in NMB and NMB-SS3 suggests that the addition of the last two glucose residues to the core Glc-Hep structure in NMB may have been performed by a second glucosyltransferase, perhaps the recently identified glucosyl transferase, LgtG (2). This conclusion is supported by the observation that

NMB LOS has a glucose residue attached to Hep<sup>II</sup> through an  $\alpha$ 1-3 linkage in addition to the conserved *N*-acetylglucosamine (6, 22). These observations suggest that the appearance of the oligoglucose glycoforms in NMB but not in MA-1 may be a combinatorial effect of low UDP-glucose 4-epimerase activity and the presence of a second glucosyltransferase activity. This gene does not appear to be present in the genome of serogroup A meningococci. Recently, Kahler et al. (13) identified the *lgtF* locus in strain NMB. An LgtF<sup>-</sup> strain did not contain any

TABLE 3. Molecular weight analysis of O-deacylated LOS by electrospray MS for MA-1 and MA-1 *galE*

<i>N. meningitidis</i> strain	LOS	Observed (calculated) mol wt <sup>a</sup>	Relative abundance <sup>b</sup> by MALDI (ESI)	Proposed composition <sup>c</sup>
MA-1	LOS B	2,428.0 (2,428.22)	50 (36)	Hex <sub>2</sub> -Hep <sub>2</sub> -GlcNAc-PEA <sub>1</sub> -Kdo <sub>2</sub> -lipid A
	LOS B'	2,551.1 (2,551.27)	100 (100)	Hex <sub>2</sub> -Hep <sub>2</sub> -GlcNAc-PEA <sub>2</sub> -Kdo <sub>2</sub> -lipid A*
MA-1 <i>galE</i>	LOS A	2,266.4 (2,266.07)	32 (43)	Hex-Hep <sub>2</sub> -GlcNAc-PEA <sub>1</sub> -Kdo <sub>2</sub> -lipid A*
	LOS A'	2,389.3 (2,389.12)	100 (100)	Hex-Hep <sub>2</sub> -GlcNAc-PEA <sub>2</sub> -Kdo <sub>2</sub> -lipid A*
	LOS B	2,427.8 (2,428.22)	20 <sup>d</sup> (13)	Hex <sub>2</sub> -Hep <sub>2</sub> -GlcNAc-PEA <sub>1</sub> -Kdo <sub>2</sub> -lipid A*
	LOS B'	2,551.7 (2,551.27)	11 (12)	Hex <sub>2</sub> -Hep <sub>2</sub> -GlcNAc-PEA <sub>2</sub> -Kdo <sub>2</sub> -lipid A*

<sup>a</sup> All molecular weights for O-deacylated LOS are reported from the MALDI data as their average mass values based on singly deprotonated charged molecular ions, (M - H)<sup>-</sup>.

<sup>b</sup> Expressed as a percentage; determined from peak heights of the singly charged molecular ions for MALDI and of the doubly and triply charged ions for ESI.

<sup>c</sup> After O deacylation, the lipid A moiety is converted into diphosphoryl diacyl lipid A, containing two N-linked  $\beta$ -hydroxymyristic acid chains with an average *M<sub>r</sub>* of 953.0.

<sup>d</sup> In the MALDI spectrum, this glycoform peak partially overlaps the potassium adduct of the molecular ion for the more abundant glycoform LOS A' resulting in overestimation of this LOS B glycoform.

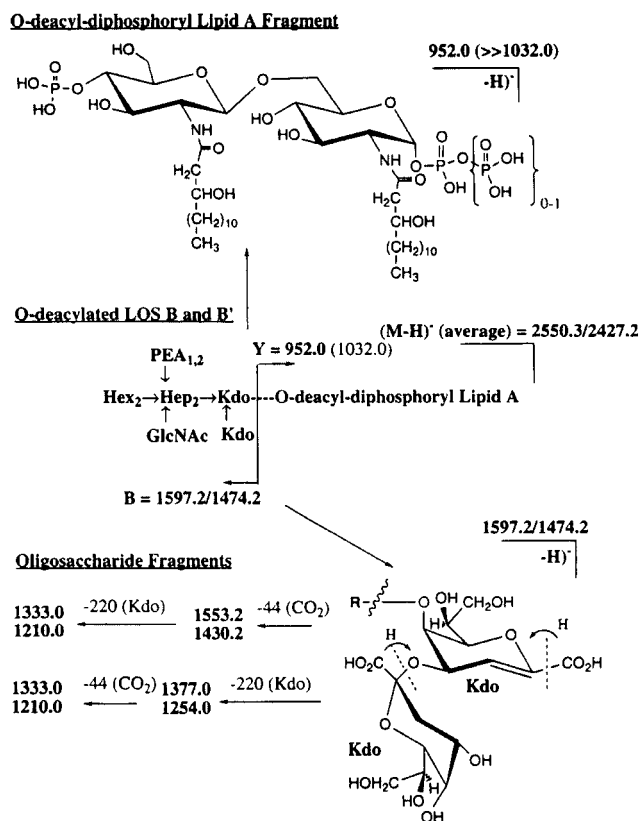


FIG. 5. Fragmentation pathways of the two prominent singly charged ions corresponding to the LOS B and LOS B' glycoforms. All masses are calculated masses. See the spectra (Fig. 4) for the actual experimental masses.

detectable levels of glucose, suggesting that LgtF is responsible for adding the glucose to Hep<sup>I</sup>. The second glucosyltransferase activity (Glc<sup>II</sup> to Hep<sup>II</sup>; LgtG) requires the presence of the first glucose residue on Hep<sup>I</sup>, analogous to the Kdo-dependent acyltransferases in *E. coli* lipid A biosynthesis (21).

Recently, further analysis of LOS from strain NMB was reported by Rahman et al. (22). They were unable to detect oli-

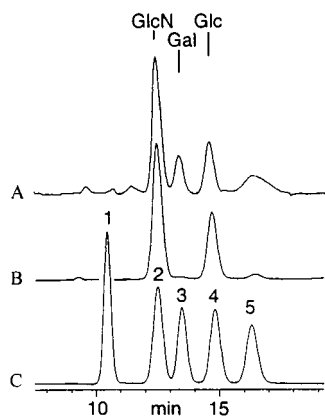


FIG. 6. Composition analysis of MA-1 (A) and MA-1 *galE* (B) under trifluoroacetic acid conditions, compared with standard mixture (profile C) containing galactosamine (peak 1), glucosamine (peak 2), galactose (peak 3), glucose (peak 4), and mannose (peak 5). The conserved core Kdo is destroyed under the hydrolysis conditions; the *L-glycerol-D-manno*-heptose peaks are not shown and elute much later ( $t > 25$  min) under the gradient conditions (see Materials and Methods).

TABLE 4. UDP-glucose 4-epimerase activity levels in *N. meningitidis* MA-1 and NMB and their  $\Delta galE::npt$  mutants

Phase	nmol of NADH generated/min ( $\pm$ SE) in strain:			
	MA-1	MA-1 $\Delta galE::npt$	NMB	NMB $\Delta galE::npt$
Exponential	22.2 (1.4)	21.0 (1.6) <sup>a</sup>	11.3 (0.6) <sup>b</sup>	9.8 (1.0) <sup>c</sup>
Stationary	17.2 (2.0) <sup>d</sup>	12.0 (1.0) <sup>e</sup>	1.4 (0.5) <sup>f,g</sup>	3.4 (1.2) <sup>h</sup>

<sup>a</sup>  $P = 0.4178$  for MA-1 versus MA-1  $\Delta galE::npt$  values in the exponential phase.

<sup>b</sup>  $P = 0.0004$  for MA-1 versus NMB values in the exponential phase.

<sup>c</sup>  $P = 0.1352$  for NMB versus NMB  $\Delta galE::npt$  values in the exponential phase.

<sup>d</sup>  $P = 0.0514$  for MA-1 values in the exponential versus the stationary phase.

<sup>e</sup>  $P = 0.1365$  for MA-1 versus MA-1  $\Delta galE::npt$  values in the stationary phase.

<sup>f</sup>  $P < 0.0001$  for NMB values in the exponential versus the stationary phase.

<sup>g</sup>  $P = 0.0002$  for MA-1 versus NMB values in the stationary phase.

<sup>h</sup>  $P = 0.009$  for NMB versus NMB  $\Delta galE::npt$  values in the stationary phase.

goglucose glycoforms in the LOS prepared from wild-type NMB. The detection of oligoglucose glycoforms in LOS preparations from *galE* strains of NMB, MA-1, and MC58 (14, 15, 33) suggests that they may be present in the wild type, albeit at low levels, perhaps below the detector sensitivity. It is also possible that different culture conditions led to differential expression of various LOS glycoforms. Such observations have been previously reported by other investigators (19, 29).

The putative structure of LOS from strain MA-1 was determined as Gal-Glc-Hep<sub>2</sub>-GlcNAc-Kdo<sub>2</sub>-lipid A based on ESI-mass spectrometric analysis. The absence of a sialylated derivative is consistent with the absence of sialic acid biosynthesis genes in serogroup A meningococci (16). Serogroup A strains are characterized by an ( $\alpha$ 1-6)-linked *N*-acetylmannosamine-1-phosphate capsule (16), rather than the sialic acid-containing capsules associated with other serogroups. It is interesting that the *galE* gene and the genes for production of the *N*-acetylmannosamine-1-phosphate capsule appear to be linked on the same operon. Indeed, polar insertion of a kanamycin marker into our ORF1 resulted in barely detectable levels of epimerase activity. This is not the organization found in serogroup B or C, where the capsule biosynthesis genes and the *galE* gene are separated by more than 1,000 nucleotides (5). Based on comparison of the nucleotide sequences upstream of *galE* genes from strains of serogroup B (B1940) and C (FAM20), it appears that the NMB *galE* gene is transcribed from its own promoter. The significance of this observation is not clear at the moment, but further investigation may yield explanations for the organization of these genes as a potential operon in serogroup A meningococci. The residual epimerase activity detected in MA-1 RKR and MA-1 *galE* was not statistically significant compared to that in MA-1 NKR. The high epimerase activity in MA-1 NKF is likely due to expression from the promoter for the kanamycin cassette.

The UDP-glucose 4-epimerase activity levels varied between MA-1 and NMB by as much as 12.5-fold in stationary cultures and as little as 2-fold in exponential cultures. This is the first report we are aware of documenting strain variability in the activity level of an enzyme important for the virulence of pathogenic *Neisseria* species. The UDP-galactose is not only used in LOS biosynthesis but also serves as the galactose donor in glycosylation of the pilin subunit of *N. meningitidis* (32). Although many LOS biosynthesis genes have been cloned and mutated, the regulation or strain variability of gene expression has not been observed, with the exception of the meningococcal *rfaC* gene (36). Our evidence strongly suggests that UDP-glucose 4-epimerase activity in *N. meningitidis* is different in

Nmn MA-1	MKNILVTGGT	<b>G</b> FIGSHTVVS	LLKSGHQVVI	<b>LDNLCNSSIN</b>	ILPRLKTTITG	50
Nmn NMB	MKKILVTGGT	<b>G</b> FIGSHTVVS	LLKSGHQVVI	<b>LDNLCNSSIN</b>	ILPRLKTTITG	50
Nmn FAM20	MKKILVTGGT	<b>G</b> FIGSHTVVS	LLKSGHQVVI	<b>LDNLCNSSIN</b>	ILPRLKTTITG	50
Nmn B1940	MKKILVTGGT	<b>G</b> FIGSHTVVS	LLKSGHQVVI	<b>LDNLCNSSIN</b>	ILPRLKTTITG	50
Nmn MC58	MT-VLITGGT	<b>G</b> FIGSHTAVS	LVQSGYDAVI	<b>LDNLCNSSAA</b>	VLPRLRQITG	49
<i>H. influ</i>	MA-ILVTGGA	<b>G</b> YIGSHTVVE	LLNVGKEVVV	<b>LDNLCNSSPK</b>	SLERVKQITG	49
<i>E. coli</i>	M-RVLVTGGS	<b>G</b> YIGSHTCVQ	LLQNGHDVII	<b>LDNLCNSKRS</b>	VLPVIERLGG	49
Consensus	MK.IILVTGGT	<b>G</b> FIGSHTVVS	LLKSGHQVVI	<b>LDNLCNSSIN</b>	ILPRLKTTITG	50
Nmn MA-1	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Nmn NMB	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Nmn FAM20	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Nmn B1940	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Nmn MC58	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Ngc MS11	RNIPFYQ <b>G</b> DI	RDCQILRQIF	SEHEIESVIH	<b>FAGL</b> KAVGES	VAEPTKY <b>Y</b> GN	99
<i>H. influ</i>	KEAKFYEGDI	LDRALLQKIF	AENEINSVIH	<b>FAGL</b> KAVGES	VQKPT <b>Y</b> MM	99
<i>E. coli</i>	KHPTFVEGDI	RNEALMTEIL	HDHAIDTVIH	<b>FAGL</b> KAVGES	VQKPLE <b>Y</b> DN	99
Consensus	QEIPFYQ <b>G</b> DI	RDREILRRIF	AENRIDSVIH	<b>FAGL</b> KAVGES	VAEPMKY <b>YD</b> N	100
Nmn MA-1	NVSGSLVLA <b>E</b>	EMARAGVFSI	<b>VFSS</b> SATVYG	DPGKVPYTED	MPLGDTTSP <b>Y</b>	150
Nmn NMB	NVSGSLVLA <b>E</b>	EMARAGVFSI	<b>VFSS</b> SATVYG	DPGKVPYTED	MPLGDTTSP <b>Y</b>	150
Nmn FAM20	NVSGSLVLA <b>E</b>	EMARAGVFKI	<b>VFSS</b> SATVYG	DPGKVPYTED	MQPGDTTSP <b>Y</b>	150
Nmn B1940	NVSGSLVLA <b>E</b>	EMARAGVFSI	<b>VFSS</b> SATVYG	DPGKVPYTED	MPLGDTTSP <b>Y</b>	150
Nmn MC58	NVSGSLVLA <b>E</b>	EMARAGVFSI	<b>VFSS</b> SATVYG	DPGKVPYTED	MPPGDTTSP <b>Y</b>	150
Ngc MS11	NVYGSLLVLA <b>E</b>	EMARAGVLKI	<b>VFSS</b> SATVYG	DAEKVPYTED	MRPGDTAN <b>Y</b>	149
<i>H. influ</i>	NVAGTLVLIQ	EMKKAGVWNF	<b>VFSS</b> SATVYG	DPKIIPITED	CEVGGTT <b>NP</b> Y	149
<i>E. coli</i>	NVNGTLRLIS	AMRAANVKNF	<b>IFSS</b> SATVYG	DQKIPYVES	FPTGT <b>PQ</b> SP <b>Y</b>	149
Consensus	NVSGSLVLA <b>E</b>	EMARAGV.FI	<b>VFSS</b> SATVYG	DPGKVPYTED	MP.GDTTSP <b>Y</b>	150
Nmn MA-1	GTS <b>K</b> SMVERI	LTDIQKADPR	WSMILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Nmn NMB	GTS <b>K</b> SMVERI	LTDIQKADPR	WSVILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Nmn FAM20	GTS <b>K</b> SMVERI	LSDIQKADPR	WSVILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Nmn B1940	GTS <b>K</b> SMVERI	LTDIQKADPR	WSVILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Nmn MC58	GAS <b>K</b> SMVERI	LTDIQKADPR	WSMILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Ngc MS11	GAS <b>K</b> AMVERM	LTDIQKADPR	WSVILLR <b>YFN</b>	PIGAHESGLI	GEQPNGVP <b>NN</b>	199
<i>H. influ</i>	GTS <b>K</b> YMVEQI	LRDTAKAEPK	FSMTILR <b>YFN</b>	PVGAHESGLI	GEDPNGIP <b>NN</b>	199
<i>E. coli</i>	GKS <b>K</b> LMVEQI	LTDLQKAQPD	WSIALLR <b>YFN</b>	PVGAHPSGDM	GED <b>PQ</b> GIP <b>NN</b>	199
Consensus	GTS <b>K</b> SMVERI	LTDIQKADPR	WS.ILLR <b>YFN</b>	PIGAHESGLI	GEQPNGIP <b>NN</b>	200
Nmn MA-1	LLPYICQVAA	GKLPQLAV <b>FG</b>	DDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHVAAMQA	250
Nmn NMB	LLPYICQVAA	GKLPQLAV <b>FG</b>	DDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHIAAMKA	250
Nmn FAM20	LLPYICQVAA	GKLPNWRV <b>FG</b>	GDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHVAAMQA	250
Nmn B1940	LLPYICQVAA	GKLPQLAV <b>FG</b>	DDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHVAAMQA	250
Nmn MC58	LLPYICQVAA	GKLPQLAV <b>FG</b>	DDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHVAAMQA	250
Ngc MS11	LLPYICQVAS	GRLPQLSV <b>FG</b>	GDYTPDGTG	<b>MR</b> DYIHVMDL	AEGHIAAMKA	249
<i>H. influ</i>	LLPYISQVAI	GKLAQLSV <b>FG</b>	SDYDTHDGTG	<b>VR</b> DYIHVVDL	AVGHLKALQR	249
<i>E. coli</i>	LMPYIAQVAV	GRRDSLAI <b>FG</b>	NDYPTDGTG	<b>VR</b> DYIHVMDL	ADGHVVAMEK	249
Consensus	LLPYICQVAA	GKLPQLAV <b>FG</b>	.DYTPDGTG	<b>MR</b> DYIHVMDL	AEGHVAAMQA	250
Nmn MA-1	KSNVAGTHLL	NLGSGRASSV	LEIIRAF <b>EAA</b>	SGLTIPFEIK	<b>PRR</b> AGD <b>LAC</b> F	300
Nmn NMB	KSNVAGTHLL	NLGSGRASSV	LEIIRAF <b>EAA</b>	SGLNIPYQIQ	<b>PRR</b> AGD <b>LAC</b> G	300
Nmn FAM20	KSNVAGTHLL	NLGSGRASSV	LEIIRAF <b>EAA</b>	SGLTIPYEVK	<b>PRR</b> AGD <b>LAC</b> F	300
Nmn B1940	KGGVAGVHLF	NLGSGRAYSV	LEIIRAF <b>EAA</b>	SGLNIPYQIQ	<b>PRR</b> AGD <b>LAC</b> S	300
Nmn MC58	KSNVAGTHLL	NLGSGRASSV	LEIIRAF <b>EAA</b>	SGLTIPYEVK	<b>PRR</b> AGD <b>LAC</b> F	300
Ngc MS11	KGGVAGVHLF	NLGSGRAYSV	LEIIRAF <b>EAA</b>	SGLHIPYRIQ	<b>PRR</b> AGD <b>LAC</b> S	299
<i>H. influ</i>	HENDAGLHIY	NLGTGHGYSV	LDMVKAF <b>EKA</b>	NNITIPYKLV	<b>ERR</b> SGD <b>LAT</b> C	299
<i>E. coli</i>	LANKPGVHIY	NLGAGVGN <b>SV</b>	LDVVNAF <b>SKA</b>	CGKPVNYH <b>FPA</b>	<b>PRR</b> EGD <b>LPA</b> X	299
Consensus	K.NVAG.HL.	NLGSGRA.SV	LEIIRAF <b>EAA</b>	SGL.IPY...	<b>PRR</b> AGD <b>LAC</b> .	300
Nmn MA-1	YADPSYTKAQ	IGWQTQRDLS	QMMEDSWRWV	SNNPNGYDD	339	
Nmn NMB	FADPSHTKQE	TGWETKRSLA	QMMEDSWRWV	SRNPNGYGD	339	
Nmn FAM20	YADPSYAKAQ	IGWQTQRDLT	QMMEDSWRWV	SNNPNGYDD	339	
Nmn B1940	FADPSHTKQE	TGWETKRSLA	QMMEDSWRWV	SRNPDGYGD	339	
Nmn MC58	YADPSYTKAQ	IGWQTQRDLT	QMMEDSWRWV	SNNPNGYDD	339	
Ngc MS11	YADPSHTKQ	TGWETKRGLQ	QMMEDSWRWV	SRNPGRYGD	338	
<i>H. influ</i>	YSDPSLAAKE	LGWAERGLE	KMMQDTWN <b>WQ</b>	KNN <b>S</b> KGYRD	338	
<i>E. coli</i>	WADASKADRE	LNWRVTRTLD	EMAQDT <b>WHWQ</b>	SRHPQGYPD	338	
Consensus	YADPS.TK..	.GW.T.R.L.	QMMEDSWRWV	S.NP.GY.D	339	

FIG. 7. Predicted amino acid alignment of GalE proteins from various gram-negative bacteria. The consensus sequence is shown at the bottom. The meningococcal polypeptide is 1 amino acid longer than the protein from gonococci, *Haemophilus influenzae*, or *E. coli*. According to the structural data from the *E. coli* epimerase (3, 17, 26, 28), the amino acids that form the binding pockets of NAD<sup>+</sup> and UDP-sugar are boldfaced. The amino acids that form hydrogen bonds with NAD<sup>+</sup> are italicized, and those bonding with UDP-sugar are underlined. The residues involved in binding of both the cofactor and the substrate are italicized and underlined. S124 and Y149 of the *E. coli* protein are critical for catalysis, and these residues are conserved in both prokaryotes and eukaryotes. Nmn, *N. meningitidis*; Ngc, *Neisseria gonorrhoeae*; *H. influ*, *H. influenzae*.



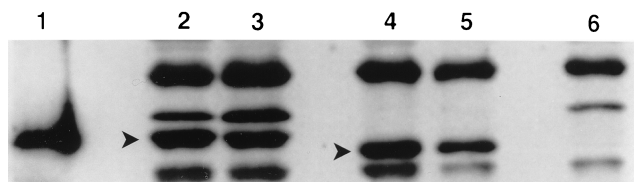


FIG. 8. Western blot analysis of meningococcal whole-cell lysates. Bacteria at either the exponential (lanes 2 and 4) or the stationary (lanes 3 and 5) phase were harvested and lysed by sonication. The protein concentrations were determined, and equal amounts of protein were loaded. The Western blots were incubated with preabsorbed mouse polyclonal antisera to recombinant meningococcal GalE protein. Lane 1, purified recombinant GalE; lanes 2 and 3, *N. meningitidis* NMB; lanes 4 and 5, *N. meningitidis* MA-1; lane 6, MA-1 *galE*. Solid arrowheads denote the band specific to GalE.

different strains. In strain NMB, differences in epimerase activity were also observed between phases of growth.

The strain variability of epimerase activity could be due to a number of factors. The respective epimerases could be structurally different or could have different residues at the binding sites for the cofactor and substrate. This appears unlikely, since the putative active-site amino acids are nearly identical in the meningococcal GalE proteins (Fig. 7). It is also possible that the genes could be transcribed, or that *galE* mRNA could be translated, at different rates. In strain NMB, the *galE* gene appears to be the first gene of an operon containing *rfb* homologues, whereas in MA-1 (Fig. 2a), it is likely the fifth gene of a large operon containing the *myn* genes (27). We had noticed that the region immediately upstream of the NMB *galE* open reading frame start codon is very thymidine rich, whereas the corresponding region in front of the MA-1 *galE* gene has a few more adenines (Fig. 9). The 3' end of the meningococcal 16S rRNA is purine rich, containing mostly guanines. The minor differences in the putative ribosome binding sites of the MA-1 and NMB *galE* genes do not appear to be biologically significant, since the difference in the relative amount of GalE protein between the strains at the exponential or stationary phase was not statistically significant.

In addition to the strain differences in epimerase activity, we observed an 8.2-fold decrease in enzyme activity in NMB between the two sampling time points. This difference was not due to the NMB GalE protein level at stationary phase, which was significantly higher than the protein level at exponential phase. This suggested the presence of a putative inactive enzyme at stationary phase. The putative inactive enzyme did not appear to involve a defective GalE protein from the second partial *galE* gene, since its deletion in NMB, although it resulted in a slight increase in the epimerase activity level, did not restore the stationary-phase epimerase activity to the exponential-phase level. Previously, the expression of the partial *galE* gene was not observed in Northern blot analysis (9). Similarly, our results suggest that the expression of the partial *galE* gene is minimal. These observations suggest a mechanism other than regulation of expression or defective dimer formation as a possible explanation for the low epimerase activity at

<i>N. meningitidis</i> MA-1	5'- ctgat tactt taatt taatt <b>ATG</b>
<i>N. meningitidis</i> NMB	5'- gtatt tttgt atttt attct <b>ATG</b>
<i>N. meningitidis</i> 16S rRNA	3'- ccaag gggau gccga uggaa

FIG. 9. Comparison of the nucleotide sequences of the region upstream of the *galE* open reading frame start codon in MA-1 and NMB. The sequence of the 3' end of the meningococcal 16S rRNA is shown below for comparison.

stationary phase in strain NMB. Such a mechanism may be the formation of abortive enzyme complexes. Abortive UDP-glucose 4-epimerase complexes have been detected in *E. coli* (31). They are homodimers of full-length GalE protein that contain NADH and a uridine nucleotide (usually UTP) instead of NAD<sup>+</sup> and UDP-sugar. It is possible that the physiological conditions in stationary-phase NMB favored abortive epimerase formation. The enzyme activity level was not dependent on the amount of glucose in the culture medium at stationary phase.

In this report we demonstrated that the absence of UDP-glucose 4-epimerase activity resulted in the expression of detectable levels of diglucose glycoforms in strain MA-1. Additionally, the level of epimerase activity may influence the expression of oligoglucose glycoforms in strain NMB. We also demonstrated the strain variability and growth phase-dependent variability of UDP-glucose 4-epimerase activity in meningococci. Based on our investigation, the difference in epimerase enzyme activity at the exponential and stationary phases appears not to be linked to the level of GalE protein. In strain NMB, the low epimerase activity may be due to allosteric inhibition through the formation of abortive epimerase complexes. Further biochemical analysis of UDP-glucose 4-epimerase from stationary-phase cultures of NMB is required to determine the nature of the putative abortive epimerase complexes. We do not know if the oligoglucose glycoforms are assembled throughout growth phase at a constant rate or assembled predominantly at late stages of growth.

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