

Lex2B, a Phase-Variable Glycosyltransferase, Adds either a Glucose or a Galactose to *Haemophilus influenzae* Lipopolysaccharide[∇]

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Nontypeable *Haemophilus influenzae* is a commensal that frequently causes otitis media and respiratory tract infections. The *lex2* locus encodes a glycosyltransferase that is phase variably expressed and contributes to the significant intrastrain heterogeneity of lipopolysaccharide (LPS) composition in *H. influenzae*. In serotype b strains, Lex2B adds the second β -glucose in the oligosaccharide extension from the proximal heptose of the triheptose inner core backbone; this extension includes a digalactoside that plays a role in resistance of the bacteria to the killing effect of serum. As part of our studies of the structure and genetics of LPS in nontypeable *H. influenzae*, we show here that there are allelic polymorphisms in the *lex2B* sequence that correlate with addition of either a glucose or a galactose to the same position in the LPS molecule across strains. Through exchange of *lex2* alleles between strains we show that alteration of a single amino acid at position 157 in Lex2B appears to be sufficient to direct the alternative glucosyl- or galactosyltransferase activities. Allelic exchange strains express LPS with altered structure and biological properties compared to the wild-type LPS. Thus, Lex2B contributes to both inter- and intrastrain LPS heterogeneity through its polymorphic sequences and phase-variable expression.

Haemophilus influenzae is a gram-negative bacterium that is an obligate commensal in the human respiratory tract. This organism, however, has a propensity to spread contiguously, and the predominant unencapsulated (nontypeable *H. influenzae* [NTHi]) strains can cause diseases such as respiratory tract infections and otitis media (OM). Lipopolysaccharide (LPS), the major cell surface glycolipid, significantly influences host-microbe interactions and is a virulence determinant. From the well-conserved triheptose-containing inner core backbone, oligosaccharides (OS) comprising mainly hexose sugars and various nonsugar substituents extend, and these OS vary in composition between strains (31, 32, 36). The number of sugars and substituent groups comprising these OS can vary at a high frequency within individual strains, and the reversible loss and gain of epitopes is called phase variation (30). Phase variation of LPS in *H. influenzae* is mediated by switching in the expression of LPS-specific loci that contain tetranucleotide repeats (microsatellites) in the 5' end of the reading frame (17, 19, 21, 38). These repeat tracts undergo mutation via DNA slippage during replication (25), leading to loss or gain of repeat units that place the reading frame in or out of frame with the translational initiation codon. Thus, depending on the number of tetranucleotide units in the tract, each phase-variable gene can be correctly translated ("on") or not correctly translated ("off"). For any *H. influenzae* strain having multiple phase-variable loci, a population of cells can simultaneously generate

a range of LPS glycoforms, allowing the organism to adapt to different microenvironments of the host and helping it escape from host immune responses.

The *lex2* locus, comprising *lex2A* and *lex2B*, was first identified as an *H. influenzae* phase-variable LPS biosynthetic locus in a serotype b strain, strain DL42 (21). A tract of multiple, tandem 5'-GCAA repeats is located in the 5' end of the *lex2A* reading frame (21). Disruption of *lex2B* in strain DL42 led to a loss of reactivity of this strain with an LPS-specific monoclonal antibody (MAb), MAb 5G8 (21). Structural analysis of LPS isolated from mutant *H. influenzae* serotype b strains revealed that Lex2B functions as a glucosyltransferase that adds a second glucose in a β -(1-4) linkage to the first β -glucose linked to the first heptose of the LPS of *H. influenzae* (14) (Fig. 1). Both reading frames, *lex2A* and *lex2B*, are required for Lex2B activity (14). The *lex2* locus permits further OS extensions that include a digalactoside that binds MAb 5G8 (13) (Fig. 1) and that play a role in resistance of the bacteria to the killing effect of serum (12).

H. influenzae exhibits significant LPS structural variation both within and between strains. In addition to phase variation, factors that contribute to LPS heterogeneity are differences in the repertoire of LPS biosynthesis genes between strains and gene sequence polymorphisms that direct alternative transferase functions and additions to the LPS molecule. Depending on the allele present, the *lic1D* gene can direct addition of phosphocholine to one of several locations in the LPS (26), and the *lpsA* gene product can add either glucose or galactose in one of two alternative linkages to the same position in the LPS molecule (6).

As part of our ongoing studies of the LPS structure and genetics of a set of 26 NTHi strains from OM patients (3),

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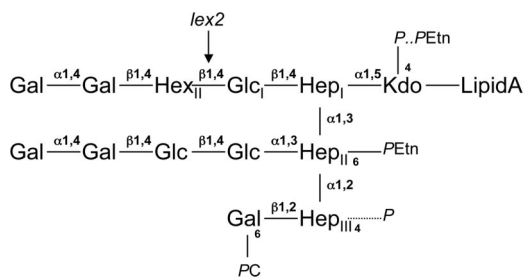


FIG. 1. Schematic representation of the structure of *H. influenzae* LPS, showing the relative position in biosynthesis that is influenced by the *lex2* locus. Hex_{II} indicates the position of the sugar added by Lex2B (linkage indicated by an arrow) to Glc. The example shown is the structure for a serotype b strain, RM7004 (29), where HexII is a glucose. The following molecules are present in the structure: Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; Hex, hexose (Glc or Gal); Glc, D-glucose; Gal, D-galactose; PEtn, phosphoethanolamine; P, phosphate; PC, phosphocholine.

detailed analysis of the LPS from NTHi strains 432 and 1124 (40; unpublished data) has shown that the second hexose in the OS extension from HepI is a galactose rather than the glucose described previously at this position (14). We wanted to determine whether this sugar specificity was determined by *lex2B* alone, in a way analogous to that seen for the *lpsA* gene, or whether an alternative gene is involved. In the present study, we further characterize the *lex2* locus of *H. influenzae* and show that the *lex2B* gene exhibits sequence polymorphism between strains that direct the attachment of either a β-glucose or a β-galactose as the second hexose in the OS extension from the proximal heptose of *H. influenzae* LPS. Lex2B is a glycosyltransferase that contributes to LPS structural heterogeneity through both its phase-variable expression and allelic polymorphisms that direct addition of alternative sugars.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The 26 NTHi strains used in this study are a subset of the strains which were isolated from children with OM by the Finnish Otitis Media Study group (3). *H. influenzae* strain RM118 (Rd; strain KW20) (19), serotype b strain RM153 (Eagan) (2), and NTHi strain 2019 (11, 31) have been described previously. *H. influenzae* strains were grown in brain heart infusion (BHI) broth supplemented with 10 μg ml⁻¹ hemin, 2 μg ml⁻¹ NAD, or agar (1%, wt/vol) at 37°C. When required, kanamycin was added at 10 μg ml⁻¹.

Escherichia coli strain DH5α (15) was used for DNA cloning and propagation of plasmid DNA and was cultured in Luria broth or on agar plates at 37°C. When necessary, isopropyl-β-D-thiogalactopyranoside (IPTG) (40 μg ml⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg ml⁻¹), ampicillin (100 μg ml⁻¹), or kanamycin (50 μg ml⁻¹) was added to the medium.

DNA procedures. Restriction enzymes and DNA-modifying enzymes were purchased from Roche or New England Biolabs and were used according to the manufacturers' instructions. Plasmid DNA was prepared from *E. coli* strains by alkaline lysis (34). Chromosomal DNA was prepared from *H. influenzae* by a method described elsewhere (33).

For Southern blot analysis (34), DNA probes for hybridization were labeled using the GE Healthcare ECL system according to the manufacturer's instructions. The membranes were prehybridized at 42°C for 1 to 4 h in hybridization buffer and then hybridized in the same buffer with labeled DNA probe overnight at 42°C. Filters were subsequently washed twice in primary wash buffer (6 M urea, 0.4% sodium dodecyl sulfate [SDS], 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) for 20 min at 42°C and then once in 2× SSC for 5 min at 42°C before they were developed with detection reagents and hybridized bands were visualized by autoradiography using ECL film.

PCR amplification and DNA sequencing. The oligonucleotide primers used for PCR amplification and DNA sequencing are listed in Table 1 and were

designed using the *lex2* sequences available in the databases. PCR amplification was routinely carried out as described previously (18). Prior to DNA sequencing, the *lex2* genes were amplified by PCR using primers blex2for and lex2CHP1. The DNA sequences of the templates were determined using primers blex2for, lex2CHP1, oligo1, and oligo2, a BigDye DNA sequencing kit (PE Applied Biosystems), and an automated ABI 3730 sequencer. DNA sequences were collated and analyzed using the GCG sequence analysis program (7). Sequence homologies were determined using the GenBank DNA and protein sequence databases through the National Center for Biotechnology Information BLAST network server (1).

Cloning of *lex2* for allelic exchange and mutation. A PCR product containing the *lex2A* and *lex2B* genes and 500 bp of upstream and downstream flanking DNA sequence was amplified from chromosomal DNA of NTHi strains 432, 1008, 1124, 1247, 1292, and 2019 using primers lex2RegForA and lex2RegRevB2 (Table 1) and then cloned into plasmid pCR2.1TOPO (Invitrogen). pCR2.1TOPO carries an ampicillin resistance cassette as well as a kanamycin resistance (Kan^r) cassette, so the insert was subcloned into plasmid pT7Blue (Novagen) to enable a Kan^r cassette to be inserted into the cloned DNA to permit selection following subsequent transformation into *H. influenzae*. The *lex2* inserts were excised from pCR2.1TOPO by digestion with BamHI and NsiI, the pT7Blue plasmid was digested with BamHI and PstI, and then the two DNAs were ligated together before transformation into *E. coli*.

A Kan^r cassette, released from pUC4Kan by digestion with HincII, was inserted 110 bp upstream of the *lex2* locus in the plasmid clone from each strain at an SnaBI restriction site. Following ligation and transformation into *E. coli*, correct plasmid constructs were confirmed by restriction digestion and PCR amplification. Plasmid clones plex2Gal₄₃₂, plex2Glc₁₀₀₈, plex2Gal₁₁₂₄, plex2Glc₁₂₄₇, plex2Glc₁₂₉₂, and plex2Gal₂₀₁₉, representing the sequences from the target strains, were selected for subsequent transformation into *H. influenzae*.

To inactivate the *lex2B* gene, the Kan^r cassette from pUC4Kan was inserted into a unique StyI site located 209 bp downstream of the initiation codon of *lex2B* in plasmid pCR2.1lex2 (14). This plasmid construct was introduced into *E. coli*, and a correct plasmid clone (pDL1) was verified by restriction digestion and PCR amplification.

Transformation of *H. influenzae*. For allelic exchange, plasmid DNA containing the cloned *lex2* genes and flanking sequences was linearized by restriction digestion and then transformed into selected *H. influenzae* strains by the MIV method (16). Putative transformed colonies were regrown on BHI agar plates containing kanamycin. PCR amplification products obtained using primers lex2RegForA and lex2CHP1 were sequenced to confirm that correct and complete allelic exchange of the *lex2* locus had occurred in the target strain.

The *lex2* locus was inactivated in strains 432, 1008, 1124, 1247, 1292, and 2019 by transformation with linearized plasmid pDL1. Following transformation and regrowth on kanamycin-containing medium, correct mutant strains were confirmed by PCR amplification of extracted DNA with primers blex2for and lex2CHP1.

LPS phenotypic analysis. For gel fractionation of LPS, whole-cell lysates were prepared from *H. influenzae* strains grown overnight as described by Serino and Virgi (37). The lysates were fractionated by Tricine-SDS polyacrylamide gel electrophoresis (PAGE) (24), and LPS profiles were detected by staining with silver (Quicksilver; GE Healthcare).

For immunoblotting, *H. influenzae* strains grown overnight in sectors on BHI agar plates were incubated with the appropriate LPS-specific MAb as described previously (9). MAb 4C4 was provided by E. J. Hansen (University of Texas), and MAb LLA5 was provided by J. Richards (National Research Council, Canada).

LPS structural analysis. The LPS structures from NTHi transformant strains 1008lex2Gal₁₁₂₄ and 1124lex2Glc₁₀₀₈ were elucidated using a combination of mass spectrometry and nuclear magnetic resonance (NMR) techniques and a protocol outlined previously (40). Briefly, cells from 5-liter batch cultures (five

TABLE 1. Oligonucleotide primers used for *lex2* genes and the adjacent sequence in this study

Primer	Sequence
blex2for	5'-AAATGAATAGGTAGGGG
lex2CHP1	5'-GGATGGGCAACTTATGGCG
lex2RegForA	5'-GAAGCGGTGTGAGTTTAG
lex2RegRevB2	5'-CCGATGCAACCAGTAAGTGC
oligo1	5'-GCGGTTGAATGCAAAGGG
oligo2	5'-ATGGCTAGCATAACAGCC

	120	*	140	*	160	*	180
1008	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1124	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1247	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1003	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1231	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
Eagan	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1232	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
375	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1181	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1292	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1180	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1268	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1200	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
432	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1209	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1233	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
1207	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
176	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
667	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
162	: -----						
486	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
981	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
477	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
2019	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						
285	: SDKNTFEFFWLQPDRLKNKRKLLISNFGNLSIYQFSKGFAGATGYLLTPQAARKFLTQSKWEYLTVDVTMD						

FIG. 2. Alignment of a segment of the translated amino acid sequences encoded by the *lex2B* gene for 25 strains. Shading indicates polymorphisms between the sequences, in particular, at the position of amino acid 157, which can be either a threonine (T) or an alanine (A). Strain 162 has a predicted truncated open reading frame due to a 2-bp deletion.

1-liter lots) were harvested after overnight growth, and LPS was extracted from lyophilized bacteria using phenol-chloroform-light petroleum. Reduced core OS material was obtained after mild acid hydrolysis of LPS. The results of multiple-step tandem electrospray ionization-mass spectrometry ESI-MS (ESI-MSⁿ) experiments performed with permethylated and dephosphorylated OS samples were recorded in the positive ion mode with a Finnigan LCQ iontrap mass spectrometer (Finnigan-MAT, San Jose, CA) coupled to a 2690 Waters high-performance liquid chromatography system (Waters, Milford, MA) using a microbore C₁₈ column [Phenomenex LUNA 5 μm C18(2)] at 21°C. The mobile phase gradient used had constant concentrations of 1% acetic acid and 0.1 mM sodium acetate and was changed gradually over time so that it contained methanol (solvent A) and water (solvent B) at a flow rate of 0.08 ml min⁻¹. The gradient was as follows: 50% solvent A at 0 min, 100% solvent A at 50 min, 100% solvent A at 60 min, and 50% solvent A at 70 min.

NMR spectra were recorded for OS samples in deuterium oxide (D₂O) at 22°C and 25°C. Spectra were acquired with a JEOL 500-MHz spectrometer using standard pulse sequences as previously described (40). For interresidue correlation, two-dimensional nuclear Overhauser effect (NOE) spectroscopy experiments with a mixing time of 250 ms were used. Total correlation spectroscopy experiments were done using a mixing time of 180 ms.

Nucleotide sequence accession numbers. The GenBank accession numbers for sequences determined in this study are FJ376687 to FJ376710.

RESULTS

In *H. influenzae* type b strains, *lex2B* has been shown to encode the glycosyltransferase required to add the second glucose in the OS extension from HepI of the LPS (Fig. 1). In studies of the LPS structure of NTHi strains, a galactose has been identified at the same position in a minority of strains, including NTHi strains 1124 (40) and 2019 (11, 31). To test the hypothesis that the alternative additions of glucose and galactose were dependent on the *lex2* locus, we sequenced the *lex2A* and *lex2B* genes of 25 of the 29 *H. influenzae* strains examined. The locus was not amplified by PCR in four strains (NTHi strains 723, 1158, 1159, and Rd), and Southern blot analysis confirmed that the locus was not present in strains 1158, 1159, and Rd, although it was present in strain 723 (data not shown). The lack of PCR amplification of the *lex2* locus in strain 723

could have resulted from polymorphisms in the primer binding regions or from the locus being present in a different chromosomal location. The sequence of each gene was highly similar between strains (there was at least 97.9% DNA sequence identity between strains), and translated amino acid sequences are shown aligned in Fig. 2. The size of the combined *lex2A* and *lex2B* reading frames is approximately 1,060 bp. *lex2A* is the smaller gene and has a reading frame around 320 bp long that contains the tetranucleotide repeat tract, in which the number of 5'-GCAA repeats varies from 3 to 41 between strains (Table 2). The *lex2A* and *lex2B* reading frames are separated by a small 13-bp intergenic region. It has been reported that the glycosyltransferase encoded by *lex2B* is functional only if *lex2A* is expressed ("on") (14, 21); this occurs when there are 3+3n repeats in *lex2A*. Thus, in 9 of the 25 strains *lex2* expression would be predicted to be "on," and in the rest of the strains it would be predicted to be "off" (Table 2). The Lex2A sequence shares no significant homology to the sequences of other proteins in the databases. It is interesting that the *lex2A* reading frames of NTHi strains 162, 1003, and 1247 contained a 5-bp deletion that introduced a frameshift, likely resulting in an inactive Lex2A protein. The *lex2B* reading frame is approximately 740 bp long and encodes a glycosyltransferase (14). The *lex2B* gene of strain 162 contains a 2-bp deletion 200 bp into the reading frame, resulting in a frameshift. Based on the assumption that Lex2B adds a sugar in an equivalent position in the LPS in strains, we hypothesized from structural data available for the LPS of our 29 strains that Lex2B was able to add either a glucose or a galactose depending on its sequence.

When the translated sequences of *lex2B* were aligned, there were a total of 21 polymorphic sites. A correlation between the specific Lex2B sequence and the hexose (HexII in Fig. 1) that is known to occur at the second position in the OS extension from HepI in the LPS from the strain was observed (Table 2). The specificity of addition of either a glucose or a galactose

TABLE 2. Number of tetranucleotide repeats, expression status of *lex2A*, and predicted specificity of hexose addition in our test strains

Strain	No. of repeats in <i>lex2A</i>	Lex2A expression ^a	DNA sequence ^b	Amino acid in sequence ^c	Predicted hexose added ^d
162	25	Off ^e	GCT	A	(Glc)
176	23	Off	GCT	A	(Glc)
285	41	Off	ACT	T	(Gal)
375	19	Off	ACT	T	(Gal)
432	18	On	ACT	T	Gal
477	15	On	GCT	A	<u>Glc</u>
486	22	Off	GCT	A	(Glc)
667	25	Off	GCT	A	<u>Glc</u>
723	ND ^f	ND	Present		
981	12	On	GCT	A	Glc
1003	14	Off ^e	GCT	A	(Glc)
1008	9	On	GCT	A	Glc
1124	3	On	ACT	T	Gal
1158			Absent		
1159			Absent		
1180	18	On	GCT	A	Glc
1181	18	On	GCT	A	Glc
1200	17	Off	GCT	A	(Glc)
1207	25	Off	ACT	T	(Gal)
1209	26	Off	ACT	T	(Gal)
1231	20	Off	GCT	A	<u>Glc</u>
1232	20	Off	GCT	A	<u>Glc</u>
1233	26	Off	ACT	T	(Gal)
1247	15	(On) ^e	GCT	A	Glc
1268	18	On	GCT	A	(Glc)
1292	18	On	GCT	A	Glc
2019	19	Off	ACT	T	Gal
Rd			NP		
Eagan	20	Off	GCT	A	(Glc)

^a The locus is predicted to be expressed (“on”) for 3+3n repeats.
^b DNA sequence encoding amino acid 157 in the *lex2B* gene. Present, present as determined by Southern blotting; absent, absent as determined by Southern blotting; NP, not present in the genome sequence.
^c Amino acid at position 157 for each *lex2B* gene.
^d Bold type indicates that the predicted hexose agrees with the known LPS structure; parentheses indicate that the predicted hexose is not present due to a lack of *lex2A* expression or an alternative extension in the LPS; and underlining indicates that the LPS structure is unknown.
^e The *lex2A* sequence contains a 5-bp deletion.
^f ND, not determined.

correlated with a single amino acid change at position 157 in Lex2B (Fig. 2), a position in the glycosyltransferase sequence similar to that observed for a related characteristic in previous studies of LpsA (6). Eight of 25 strains contain a threonine (T) at position 157, corresponding with the predicted potential to express a galactose in the LPS at the HexII position (Table 2). Conversely, the other strains contain an alanine (A) at position 157, consistent with Lex2B being able to add a glucose.

Based on the Lex2B sequence data and our knowledge of the LPS structures of the strains, we chose six NTHi strains, strains 432, 1008, 1124, 1247, 1292, and 2019, for further study. Lex2B from strains 432, 1124, and 2019 is predicted to add a galactose, and LPS structural studies have confirmed that a galactose residue is indeed found as HexII in each of these strains (11, 40; unpublished data). For the other three strains, Lex2B is predicted to add a glucose, a finding that is also supported by LPS structural data available for each strain (E. K. H. Schweda, unpublished data).

To confirm our prediction of different functions for allelic variants of *lex2*, plasmid constructs containing the *lex2* locus

TABLE 3. Relative levels of MAb binding

Strain	Level of binding to ^a :	
	MAb 4C4	MAb LLA5
1008	++++	+
1008lex2Glc₁₀₀₈ ^b	++++	+
1008lex2Gal₁₁₂₄	+	+
1008lex2B	+	+
1124	++	+
1124lex2Gal₁₁₂₄	++	+
1124lex2Glc₁₀₀₈	++	+
1124lex2B	++	+
1247	++++	++
1247lex2Glc₁₂₄₇	++++	++
1247lex2Gal₄₃₂	++	++
1247lex2Gal₁₁₂₄	++	++
1247lex2Glc₁₀₀₈	++++	++
1247lex2B	++	++
2019	+++	++++
2019lex2Gal₂₀₁₉	++	++++
2019lex2Glc₁₂₄₇	++	++++
2019lex2Glc₁₂₉₂	++	++
2019lex2B	++	+++

^a Relative levels of MAb binding to strains compared to the corresponding parent and *lex2* mutant strains. +++++, strong binding; +++, intermediate binding; ++, weak binding; +, background.
^b Bold type indicates allelic exchange strains investigated in this study.

cloned from strains in which the locus normally directs addition of a glucose (strains 1008, 1247, and 1292) were used for transformation to exchange the *lex2* genes by allelic replacement in strains that naturally add a galactose (strains 432, 1124, and 2019). Reciprocal transformations were also carried out using the glucose-specific *lex2* plasmid clones and the target strains that naturally express a galactose in the LPS. As a control for the possibility that insertion of the Kan^r cassette into the intergenic region adjacent to *lex2* influenced LPS structure, *lex2* plasmid constructs were also transformed into the homologous strains. Transformed *H. influenzae* strains were designated as follows: recipient strain followed by *lex2* followed by the sugar added by the donor strain, with the donor strain indicated by a subscript. For example, if strain 1008 was transformed with the *lex2* locus from 1124 (a strain that normally adds a galactose as HexII [Fig. 1]), the resulting strain was designated 1008lex2Gal₁₁₂₄. The DNA sequence of the *lex2* locus in each transformed strain was determined to ascertain that the complete locus had been replaced by reciprocal recombination and that the number of tetranucleotide repeat units was compatible with Lex2 expression. Table 3 lists the transformed strains. In later analyses we focused on transformed strains made using plasmid constructs derived from strains 432, 1008, 1124, and 1292, and each transformed strain was shown by DNA sequencing to contain a *lex2* locus with an “on” number of repeats. The *lex2* locus was also inactivated in each of the six strains chosen for study (Table 3).

The LPS phenotypes of allelic exchange and mutant strains were first investigated by comparing the electrophoretic profiles to those of the corresponding wild-type strains following Tricine-SDS-PAGE (Fig. 3). For strain 1008, a strain in which Lex2B would normally be predicted to add a glucose as HexII (Fig. 1), the LPS profile of strain 1008lex2Glc₁₀₀₈ was the same as that of the parent strain (Fig. 3A). In contrast, the 1008

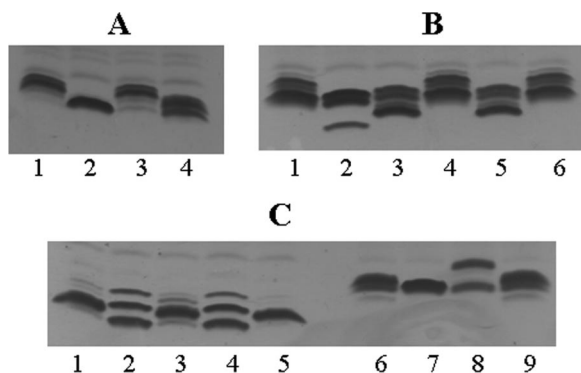


FIG. 3. Electrophoretic profiles of LPS from the NTHi parent and corresponding allelic exchange and mutant strains. (A) Strain 1008 and corresponding mutant and allelic exchange strains. Lane 1, 1008; lane 2, 1008*lex2B*; lane 3, 1008*lex2Glc*₁₀₀₈; lane 4, 1008*lex2Gal*₁₁₂₄. (B) Strain 1247 and corresponding mutant and allelic exchange strains. Lane 1, 1247; lane 2, 1247*lex2B*; lane 3, 1247*lex2Gal*₄₃₂; lane 4, 1247*lex2Glc*₁₀₀₈; lane 5, 1247*lex2Gal*₁₁₂₄; lane 6, 1247*lex2Glc*₁₂₄₇. (C) Strains 2019 and 1124 and corresponding mutant and allelic exchange strains. Lane 1, 2019; lane 2, 2019*lex2B*; lane 3, 2019*lex2Glc*₁₂₄₇; lane 4, 2019*lex2Glc*₁₂₉₂; lane 5, 2019*lex2Gal*₂₀₁₉; lane 6, 1124; lane 7, 1124*lex2B*; lane 8, 1124*lex2Glc*₁₀₀₈; lane 9, 1124*lex2Gal*₁₁₂₄.

lex2B mutant strain had a truncated LPS profile, consistent with the loss of one or two sugars from the major glycoforms (Fig. 3A). The LPS profile for the allelic exchange strain 1008*lex2Gal*₁₁₂₄, in which the *lex2* locus was replaced by the locus from strain 1124 and was expected to add a galactose, is intermediate between the *lex2B* mutant and parent patterns. This is consistent with inclusion of a galactose in LPS glycoforms at a position that might lead to shorter oligosaccharide chains from HepI. Strain 1247, another strain in which *lex2* naturally adds a glucose, displayed conservation of the LPS profile similar to that of the parent strain following allelic exchange in strains 1247*lex2Glc*₁₀₀₈ and 1247*lex2Glc*₁₂₄₇ (Fig. 3B), indicating that homologous and heterologous *Glc*-specific *lex2B* alleles encode the same function. When transformed with a galactose-specific allele, strains 1247*lex2Gal*₄₃₂ and 1247*lex2Gal*₁₁₂₄ have truncated LPS glycoforms (Fig. 3B). This indicates that independent *Gal*-specific *lex2B* genes have equivalent functions once they are inserted into the genome of strain 1247. The LPS from mutant strain 1247*lex2B* displayed the most truncated LPS profile, which is consistent with it having no hexose extension off *GlcI* in the major glycoforms (Fig. 3B).

Strain 1124, a strain in which *Lex2B* would normally be predicted to add a galactose, showed an LPS profile for the *lex2* mutant strain different from that of the parent strain, with the upper band of the two major bands absent (Fig. 3C). As expected, the allelic exchange involving the homologous gene (1124*lex2Gal*₁₁₂₄) resulted in an LPS profile that is highly similar to that of the parent strain. For strain 1124*lex2Glc*₁₀₀₈ expressing the different functional category of the *lex2* locus (i.e., glucose specific), there is an increase in the size of some LPS glycoforms compared to the parent strain, likely due to addition of a glucose as HexII and perhaps further extensions from this glucose. For 2019, another strain where *lex2* naturally adds a galactose, the situation is somewhat more complex. There is a clear difference between the LPS profile of the *lex2*

mutant strain and that of the parent, and both lower- and higher-molecular-weight glycoforms are present. Strain 2019*lex2Gal*₂₀₁₉ displayed an LPS profile equivalent to that of the parent strain, and the allelic exchange strain 2019*lex2Glc*₁₂₉₂ expressed an LPS pattern similar to that seen for the *lex2* mutant. This observation was explained after sequencing of the *lex2A* repeat tract in the transformed strain. A number of 5'-GCAA repeats inappropriate for expression of *lex2A* were present in the genome following transformation and culture; a *lex2* "off" phenotype was expected to be equivalent to that of a *lex2* mutant strain. For 2019*lex2Glc*₁₂₄₇, the LPS pattern was similar to that of the wild type, indicating that some *lex2*-dependent glycoform extension had occurred in this strain.

Immunoblotting analysis of this set of strains using MAbs 4C4 and LLA5 revealed some differences in binding between isogenic strains. MAb 4C4 interacts with a digalactoside epitope when it is present at some positions in the LPS of *H. influenzae* (14), particularly when it is located at the terminus of a globotetraose extension from either HepI or HepII (Fig. 1). A globotetraose extension is found in *GlcI* of the LPS of NTHi strain 1008 (E. K. H. Schweda, unpublished). Consistent with this, the wild-type strain reacted with MAb 4C4, while the *lex2* mutant strain and the allelic exchange strain 1008*lex2Gal*₁₁₂₄ did not react with MAb 4C4, confirming that the corresponding epitopes had been altered in the LPS of these strains. A similar observation was made for NTHi strain 1247; the wild type (in which *lex2* incorporates a *Glc*) reacted with MAb 4C4, yet two independent allelic exchange strains, 1247*lex2Gal*₄₃₂ and 1247*lex2Gal*₁₁₂₄, did not react with this MAb. With MAb LLA5, an antibody that reacts with the high-molecular-weight extensions that can be attached to *GlcI* (Fig. 1) in some strains (20), only wild-type strain 2019 had a significant level of reactivity (Table 3). This reactivity was not greatly diminished in the *lex2* mutant strain but was significantly reduced in the allelic exchange strain 2019*lex2Glc*₁₂₉₂. This is assumed to reflect an alteration of the balance of alternative OS extensions that can be synthesized from HepI in this strain background, which exhibits a complex LPS expression pattern.

The structure of OS material from NTHi strains 1124*lex2Glc*₁₀₀₈ and 1008*lex2Gal*₁₁₂₄ was investigated in detail by using ESI-MSⁿ and NMR, and the resulting data were compared with the data for the corresponding parent strains. Detailed information on the structure of LPS glycoforms for wild-type strains 1124 (40) and 1008 (E. K. H. Schweda, unpublished) is available from previous studies. ESI-MSⁿ performed with dephosphorylated and permethylated OS is a powerful tool for providing sequence and branching information for the various isomeric glycoforms present in heterogeneous mixtures. Our focus was to profile OS extensions from HepI.

In the ESI-MS spectrum of 1124*lex2Glc*₁₀₀₈, six sodiated adduct ions ($[M+Na]^+$) were observed at m/z 1671.5, 1875.3, 2079.9, 2120.8, 2282.7, and 2488.2, corresponding to Hex3, Hex4, Hex5, Hex4·HexNAc, Hex6, and Hex7 glycoforms (Fig. 4A). In order to obtain sequence and branching information, these molecular ions were further fragmented in MS² and MS³ experiments. One Hex7 glycoform was observed by fragmenting the molecular ion at m/z 2488.2. The isomer was

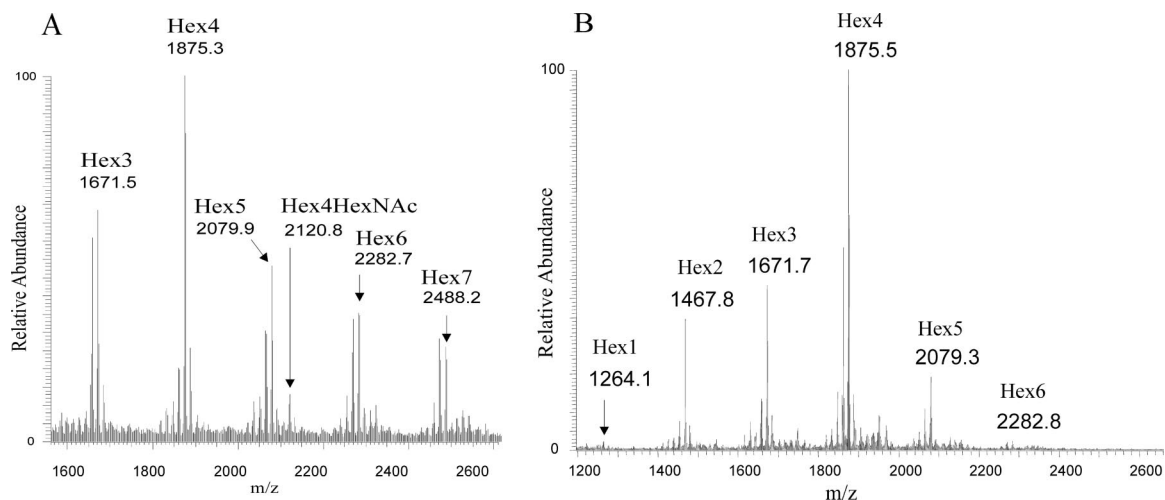


FIG. 4. High-performance liquid chromatography–ESI-MS full-scan spectra (positive mode) for dephosphorylated and permethylated OS of 1124lex2Glc₁₀₀₈ (A) and 1008lex2Gal₁₁₂₄ (B). The singly charged sodiated ions observed correspond to the following molecules: *m/z* 1264.1, Hex₁ · Hep₃ · reduced anhydro-2-keto-3-deoxyoctulosonic acid (AnKdo-ol); *m/z* 1467.8, Hex₂ · Hep₃ · AnKdo-ol; *m/z* 1671.7, Hex₃ · Hep₃ · AnKdo-ol; *m/z* 1875.5, Hex₄ · Hep₃ · AnKdo-ol; *m/z* 2079.3, Hex₅ · Hep₃ · AnKdo-ol; *m/z* 2120.8, HexNAc · Hex₄ · Hep₃ · AnKdo-ol; *m/z* 2282.8, Hex₆ · Hep₃ · AnKdo-ol; and *m/z* 2486.6, Hex₇ · Hep₃ · AnKdo-ol.

defined by the resulting fragment ions at *m/z* 2269.9, 2065.7, 1861.7, 1613.6, and 1365.9 corresponding to consecutive loss of residues from tHex-Hex-Hex-HepIII-HepII. The structure was confirmed in MS³ experiments with *m/z* 1613.6, which resulted in ions at *m/z* 1396.0, 1192.8, 987.3, and 785.4 due to loss of terminal tHex-Hex-Hex-Hex. By analogy, structures were determined for the remaining glycoforms (data not shown). It could be concluded that strain 1124lex2Glc₁₀₀₈ expresses LPS glycoforms similar to those of wild-type NTHi strain 1124 (40) except that they do not have HexNAc-Hex-Hex-Hex but rather have Hex-Hex-Hex-Hex extending from HepI.

In the ESI-MS spectrum of 1008lex2Gal₁₁₂₄, six sodiated adduct ions ([M+Na]⁺) were observed at *m/z* 1264.1, 1467.8, 1671.7, 1875.5, 2079.3, and 2282.8 corresponding to Hex1 to Hex6 glycoforms (Fig. 4B). One Hex6 glycoform was observed by fragmenting the molecular ion at *m/z* 2282.8. The isomer was defined by the resulting fragment ions at *m/z* 2065.6, 1861.2, 1657.6, 1409.9, and 1161.7 corresponding to consecutive loss of residues from tHex-Hex-Hex-HepIII-HepII. The structure was confirmed in MS³ experiments for *m/z* 1409.9, which resulted in ions at *m/z* 1192, 987.8, and 783.6 due to loss of terminal tHex-Hex-Hex. Similarly, structures were obtained for the remaining glycoforms (data not shown). It was concluded that 1008lex2Gal₁₁₂₄ expresses LPS glycoforms that are structurally similar to those of NTHi wild-type strain 1124 (40) but not to LPS glycoforms containing HexNAc. 1008lex2Gal₁₁₂₄ did not express any of the Hex7 glycoforms that NTHi wild-type strain 1008 displays (E. K. H. Schweda, unpublished data), nor does it have any tetrasaccharide units off HepI in its LPS.

The identity of the hexose linked to GlcI in OS was established by one-dimensional and two-dimensional NMR experiments as described previously (40). In the ¹H NMR spectra, the anomeric proton resonances from HepI, HepII, and HepIII residues were typically recognized between 5.0 and 6.0 ppm. As described previously, the anomeric protons of HepI

and HepIII have close chemical shifts occurring between 5.0 and 5.2 ppm, whereas the anomeric proton of HepII occurs further downfield between 5.5 and 5.9 ppm (35).

The anomeric proton of GlcI is easily identified by interresidue NOE connectivities between proton pairs GlcI H1 and HepI H4/H6 (35). The chemical shift of the anomeric proton of Glc linked to O-2 of HepIII (GlcII) is easily identified due to interresidue NOE connectivities between H1 GlcII H1 and HepIII H1/H2. The chemical shift of the anomeric proton of Gal/Glc (GlcIII) linked to O-4 of Glc I has previously been shown to resonate exceptionally downfield at δ 4.8 in glycoforms in which HepII does not have oligosaccharide extensions and HepIII is replaced by globotetraose (40; unpublished data). In glycoforms with truncated versions of globotetraose linked to HepIII, the anomeric proton of Gal linked to GlcI shifts more upfield and occurs in a region between δ 4.77 and 4.64 (40).

In the ¹H NMR spectrum of 1124lex2Glc₁₀₀₈, anomeric resonances corresponding to the triheptosyl moiety (HepI to HepIII) were identified at δ 5.04, 5.68, and 5.09, respectively. The typical resonances of a Gal residue linked to GlcI as observed by Yildirim and colleagues were absent (40). The anomeric proton of GlcI was assigned to δ 4.54 based on its interresidue NOE to H4/H6 of HepI (Fig. 5A). H-4 of GlcI was found at δ 3.70 (data not shown). The anomeric proton of GlcII was assigned to δ 4.69 based on its interresidue NOE to H1/H2 of HepIII (Fig. 5A). We concluded that the anomeric proton of GlcIII overlapped that of GlcII at δ 4.69 based on the occurrence of an interresidue NOE to H-4 of GlcI at δ 3.70. This was corroborated by a heteronuclear multiple-bond correlation experiment in which H-4 of GlcI showed connectivity to C-1 of GlcIII at δ 103.00 (data not shown). Thus, in strain 1124lex2Glc₁₀₀₈ a Glc residue replaced O-4 of GlcI instead of Gal, as has been described previously for the LPS of the parent strain (40).

In the ¹H NMR spectrum of 1008lex2Gal₁₁₂₄, anomeric res-

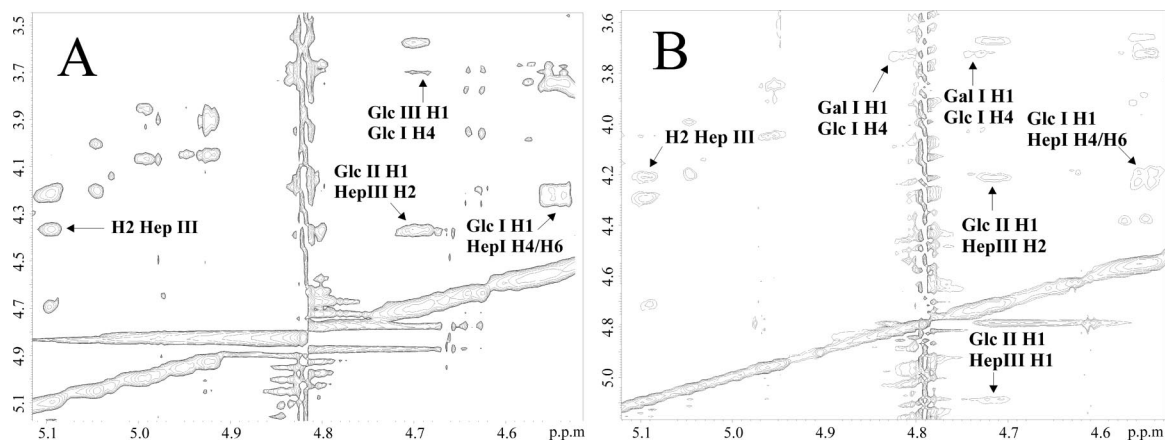


FIG. 5. Selected regions of two-dimensional gradient NOE spectroscopy spectra (mixing time, 250 ms) of OS material derived from strains 1124lex2Glc₁₀₀₈ (A) and 1008lex2Gal₁₁₂₄ (B). Cross peaks of significant interresidue NOE connectivities are labeled for GlcI, GlcII, GalI, and GlcIII.

onances corresponding to the triheptosyl moiety (HepI to HepIII) were identified at δ 5.05, 5.62, and 5.09, respectively. Interestingly, two anomeric protons of Gal residues observed at δ 4.82 and 4.74 were absent in the corresponding spectra of the wild-type strain (unpublished data). The anomeric proton of GlcI was assigned to δ 4.55 based on its interresidue NOE to H4/H6 of HepI (Fig. 5B). H-4 of GlcI was observed at δ 3.72 (data not shown). The anomeric proton of GlcII was assigned to δ 4.71 based on its interresidue NOE to H1/H2 of HepIII (Fig. 5B). The anomeric protons at δ 4.82 and 4.74 both showed interresidue NOE to H-4 of GlcI (Fig. 5B). Thus, Gal replaced O-4 of GlcI in the LPS of strain 1008lex2Glc₁₁₂₄, unlike the Glc found at the same position in the parent strain. The occurrence of two anomeric signals of this Gal residue is in agreement with the observation of longer and shorter extensions from HepIII.

Some biological significance for the differential function of the *lex2* genes was sought for these allelic exchange strains through the bactericidal assay; changes in LPS glycoforms often correlate to altered susceptibility of the expressing strain to serum killing. For strain 1247, the serum resistance of the allelic exchange strains 1247lex2Glc₁₂₄₇ and 1247lex2Glc₁₀₀₈, each with a glucose included as HexII, was very similar to that of the parent strain (data not shown). For the *lex2* mutant and the galactose-specific exchange strains 1247lex2Gal₁₁₂₄ and 1247lex2Gal₄₃₂, serum resistance was somewhat reduced compared to that of the parent strain (data not shown), a finding consistent with truncation of the LPS glycoforms observed upon SDS-PAGE. For strain 1124, the homologous exchange strain again showed resistance to killing equivalent to that of the parent strain, and the *lex2* mutant derivative showed some reduction in serum resistance (data not shown). The allelic exchange strain 1124lex2Glc₁₀₀₈, with a Gal swapped for a Glc, was less resistant to killing; this correlates with an apparent increased glycoform size for LPS compared to the wild type (Fig. 3C). For NTHi strains 1008 and 2019 there was little apparent change in serum resistance within each set of isogenic strains tested (data not shown).

DISCUSSION

A characteristic of the LPS of *H. influenzae* is the intrastrain heterogeneity of structure generated through phase variation by frequent and reversible “on”-“off” switching of translation of LPS biosynthetic loci containing tetranucleotide repeat tracts. The combinatorial effect of having multiple phase-variable genes in one strain provides a potential for extensive heterogeneity of glycoform structure. We have now shown that one of these loci, *lex2*, is the source of further heterogeneity of LPS structure through allelic sequence variation. Lex2B is the first glycosyltransferase in *H. influenzae* whose expression is phase variable and that is able to add alternative sugars at the same position in the *H. influenzae* LPS molecule.

Lex2B is a member of family 25 of the glycosyltransferase A superfamily of glycosyltransferases that transfer an activated monosaccharide residue (from UDP-Glc or UDP-Gal) to an acceptor molecule, which in this case is LPS. This is the same glycosyltransferase family that contains another *H. influenzae* LPS glycosyltransferase, LpsA, which has been extensively studied in our laboratory and shown to add either a glucose or a galactose in a β 1-2 or β 1-3 linkage in LPS biosynthesis (6). The glycosyltransferase A superfamily 25 glycosyltransferases possess a DXD motif, found at positions 99 to 101 in the Lex2B sequence (data not shown), a conserved amino acid motif that binds Mn^{2+} and is part of the catalytic domain for the donor sugar. Lex2B is predicted to use an inverting catalytic mechanism in which the α -linked donor sugar is transferred to the LPS to form a β -linked product. GlcI, the sugar residue which acts as the acceptor for Lex2B, is universally present in all *H. influenzae* LPS structures elucidated to date. It is therefore not unsurprising that the *lex2* locus is found in a majority of NTHi strains tested, although this is not the only means by which sugars can be added to GlcI. GlcI can also be replaced by either an LD-Hep residue (28) or a DD-Hep residue (27) or by addition of two alternative four-sugar units (5), making the OS extensions from HepI the most heterogeneous OS extensions in structure from the *H. influenzae* LPS inner core.

The specificity of Lex2B for adding a glucose or a galactose

is predicted to be at amino acid 157, where there is an absolute correlation between the presence of a T directing a galactose addition and the presence of an A directing a glucose addition. This is a location similar to that of the amino acid at position 151 that was shown by site-directed mutagenesis to direct specificity for LpsA, where again a T was required for galactose incorporation (6). Indeed, this specificity is likely conserved in family 25 glycosyltransferases across a range of bacterial and other species. Position 157 of Lex2B is the only variant residue in a region of the protein sequence that is absolutely conserved in all NTHi sequences investigated (Fig. 2).

Adding the second glucose in the extension from HepI of the LPS inner core backbone, as seen for Lex2B in serotype b strains in previous studies (14, 21) and NTHi strain 1008 in our study, facilitates further oligosaccharide extension that includes digalactoside-containing moieties (Gal- α -1 to 4-Gal- β -1 to 4-Glc- β -1 to 4-Glc- α) that bind MAbs 4C4 and 5G8 (13, 14, 22). The presence of these digalactoside moieties can have biological consequences, and several studies have shown that the digalactoside on *H. influenzae* LPS can enhance the ability of the organism to escape the bactericidal activity of serum in *in vitro* assays and facilitates intravascular survival in the infant rat model of *H. influenzae* infection (4, 10, 12, 19, 22, 23, 39). The importance to the bacterium of varying the expression of the Gal-Gal-Glc moiety added to GlcI can be inferred from the fact that addition of each of these three consecutive sugars is dependent upon the action of an independent phase variably expressed glycosyltransferase (12).

The mechanism by which the *lex2A* reading frame and the repeats in this reading frame affect the phase-variable expression of the Lex2B phenotype is not clear. A relationship between the numbers of repeat units in the *lex2A* tract and incorporation of the digalactoside (measured by MAb 5G8 binding) has previously been shown (21). The genes are co-transcribed (14), and it has been shown that in *Helicobacter pylori* expression of cotranscribed genes can co-phase vary through transcriptional-translational coupling (8). However, in the study of Griffin et al., translational fusions with a reporter gene indicated that each gene is translated independently, and a nonpolar mutation of *lex2A* resulted in an inactive Lex2B phenotype (14). In our study, strain 1247 contained a *lex2A* sequence with a 5-bp deletion, which implies that Lex2A was inactive, but this does not correspond with LPS structural data that indicate that Lex2B is active (i.e., the LPS contains a hexose attached to GlcI [unpublished]). Data base searches with the Lex2A sequence reveal no significant matches (data not shown), and thus the contribution of this sequence, which is unique to *H. influenzae* glycosyltransferases, to the function of the *lex2* locus remains unclear and requires further study.

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