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Elucidation of the Monoclonal Antibody 5G8-Reactive, Virulence-Associated Lipopolysaccharide Epitope of *Haemophilus influenzae* and Its Role in Bacterial Resistance to Complement-Mediated Killing

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The phase-variable locus *lex2* is required for expression of a *Haemophilus influenzae* lipopolysaccharide (LPS) epitope of previously unknown structure. This epitope, which is reactive with monoclonal antibody (MAb) 5G8, has been associated with virulence of type b strains. When strain RM118 (from the same source as strain Rd), in which the *lex2* locus and MAb 5G8 reactivity are absent, was transformed with *lex2* DNA, transformants that were reactive with MAb 5G8 were obtained. Surprisingly, the 5G8 reactivity of these transformants was phase variable, although the *lex2* locus lacked tetrameric repeats and was constitutively expressed. This phase variation was shown to be the result of phase-variable expression of phosphorylcholine (PCho) such that MAb 5G8 reacted only in the absence of PCho. Structural analysis showed that, compared to RM118, the *lex2* transformant had acquired a tetrasaccharide, Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β1,4, linked to the proximal heptose (HepI). A terminal GalNAc was detected in a minority of glycoforms. LPS derived from a mutant of RM7004, a virulent type b strain which naturally expresses *lex2* and has LPS containing the same tetrasaccharide linked to HepI as the sole oligosaccharide extension from the inner core, confirmed that GalNAc is not a part of the MAb 5G8-reactive epitope. Thus, MAb 5G8 specifically binds to the structure Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β attached via a 1,4 linkage to HepI of *H. influenzae* LPS, and we show that the ability to synthesize this novel tetrasaccharide was associated with enhanced bacterial resistance to complement-mediated killing.

Haemophilus influenzae is a human-specific, gram-negative coccobacillus. It is usually a commensal present in the upper respiratory tract but has the propensity to cause disease. Encapsulated strains, mainly *H. influenzae* type b (Hib) strains, cause meningitis and septicemia in unvaccinated children (30), and nonencapsulated strains typically cause acute lower respiratory tract infections and otitis media.

A major component of the *H. influenzae* cell wall influencing both commensal and virulence behavior is the lipopolysaccharide (LPS) molecule (5, 14, 17). The structure of *H. influenzae* LPS includes a conserved triheptose (HepI to HepIII) inner core linked via a single 2-keto-3-deoxy-octulosonic acid (Kdo) molecule to lipid A, from which there are oligosaccharide extensions (outer core), mainly hexose sugars, that vary from one strain to another (see Fig. 1). *H. influenzae* LPS lacks the repetitive side chains (often referred to as O antigens) typical of some pathogenic gram-negative bacteria but displays great complexity in its outer core sugars and other, nonsugar substituents such as phosphorylcholine (PCho) (20). Several of these sugars and nonsugar substituents of *H. influenzae* LPS are phase variable, a finding initially demonstrated by colony immunoblotting using monoclonal antibodies (MAbs) specific for *H. influenzae* LPS (16, 17). Of these MAbs, most Hib disease isolates were reactive with MAb 4C4 and some also reacted with MAb 5G8 (7). Spontaneous acquisition of reactivity to MAb 5G8 by a relatively avirulent, serum-sensitive Hib strain was associated with resistance to killing by infant rat serum and enhanced virulence in an infant rat model of infection (16). Similar observations were reported for other Hib strains (17) and for strains already expressing the MAb 4C4-reactive epitope (2, 3, 16, 17). Subsequent studies have showed that MAb 4C4 binds to an epitope incorporating a terminal digalactoside (Gal-α1,4-Gal-β-) extending from the second heptose (HepII) of the LPS of strain RM153 (32). Phase variation of this digalactoside is mediated by translational switching of two galactosyl transferase-encoding genes, *lic2A* (9) and *lgtC* (12), that contain tetranucleotide repeats within the 5′ ends of their reading frames. While the structure of the MAb 5G8-reactive epitope is unknown, its expression is associated with the expression of the MAb 4C4-reactive epitope and with the expression of the phase-variable locus *lex2* (15). This locus comprises two reading frames: *lex2A*, which contains tetranucleotide repeats, and *lex2B.* Both genes are necessary for the addition of a second glucose to the glucose attached to HepI of *H. influenzae* LPS (6).

We have now fully characterized the MAb 5G8-reactive epitope in two strains for which the detailed structure of the LPS is available, RM7004 and RM118 (Fig. 1) (21, 23). We used genetics to modify RM118, a strain which does not naturally react with MAb 5G8, so that it expresses *lex2*, synthesizes

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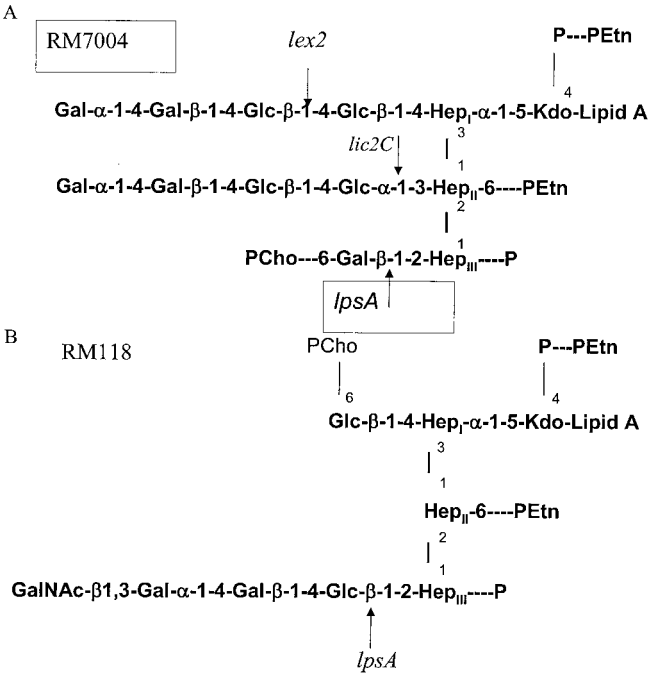

FIG. 1. Schematic representation of the structure of the fully extended LPS glycoforms of *H. influenzae* strains RM7004 (A) and RM118 (B). (A) A globotriose (Gal-α1,4-Gal-β1,4-Glc-β1,4-) is linked to the glucose attached to both HepI and HepII of the LPS of strain RM7004, and a single galactose, partially replaced with PCho, is linked to HepIII (21). (B) A single glucose, replaced with PCho, is linked to HepI, and a globotetraose unit extends from HepIII of the LPS of strain RM118 (23). Represented in the LPS structures are the following molecules: heptose (Hep), glucose (Glc), galactose (Gal), phosphate (P), PCho, and phosphoethanolamine (PEtn). Dashed lines indicate the substituents that are variably present. The sites of action of key loci in LPS synthesis are indicated by arrows.

tetrasaccharide Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β1,4, and displays phase-variable MAb 5G8 reactivity as well as enhanced bacterial resistance to complement-mediated killing.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. influenzae* type d-derived strain RM118 is from the same source as strain Rd used for the genome sequencing project (4), and the type b strain RM7004 is a disease isolate (31). *H. influenzae* strains were grown at 37°C in brain heart infusion medium supplemented with hemin (10 μg ml^{-1}) and NAD (2 μg ml^{-1}) in liquid medium or Levinthals supplement (10% [vol/vol]) (1) on solid medium. When appropriate, kanamycin (10 μg ml^{-1}) or tetracycline (4 μg ml^{-1}) was included in the medium.

Escherichia coli strain DH5α was used to propagate cloned plasmids and was grown at 37°C in Luria-Bertani broth (26) supplemented with ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}), and tetracycline (12 μg ml^{-1}) as appropriate.

Recombinant DNA methodology and nucleotide sequence analysis. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim and used according to the manufacturer's instructions.

Plasmid DNA was prepared from *E. coli* strains by the alkaline-lysis method (26). Chromosomal DNA was prepared from *Haemophilus* by the method described previously (22). Oligonucleotide primers were purchased from Sigma-Genosys (Cambridge, United Kingdom). PCR amplification was performed using *Taq* polymerase (Promega) in 50-μl reaction volumes in a Perkin-Elmer DNA thermal cycler. The Qiaex II gel extraction kit (Invitrogen) was used to purify restriction-digested DNA fragments prior to their ligation to generate plasmid constructs.

Preparation of plasmid constructs containing modified LPS genes. The plasmid construct p11$_2$ incorporating the *lpsA* gene disrupted by a Kan^r gene was previously described (10). Plasmid clone p11$_3$ was generated by restricting p11$_2$ with EcoRI to remove the Kan^r gene and replace it with the Tet^r gene (derived from Tn*10*).

To delete genes HI0753 and HI0754 from *H. influenzae* strain RM118, primers LEX2PR1 (5′-TATCCCAAGCTTGATTCAGTTTGGTTTGCAGGA-3′) and lex2CHP1 (5′-GGAATGGGCAACTTATGGCG-3′) were used to amplify by PCR a 2,759-bp fragment encompassing these genes from strain RM118 chromosomal DNA. The product obtained was cloned into plasmid pCR2.1, generating clone pCR2.1HI0753-4. The Kan^r gene in the plasmid replicon was inactivated by restricting with NcoI, which cuts the plasmid uniquely in this gene, treating with Klenow enzyme and deoxynucleotide triphosphates to fill in the recessed ends, and religating the plasmid. The resultant plasmid, pCR2.1HI0753-4N, was digested with SspI to remove a region spanning from 79 bp downstream of the potential initiation codon of HI0753 to 330 bp upstream of the potential stop codon of HI0754. The HincII fragment of pUC4k (Pharmacia) incorporating the Kan^r gene was ligated in place of this region to give plasmid pCR2.1HI0753-4Nk.

A PCR-based method was used to generate an in-frame deletion of the repeat tract of *lex2A* in plasmid pB7004E*lex2* (6). Primers BgA (5′-GCAAGATCTTC AAGACTATCTAAAATTTC-3′) and BgB (5′-GGAAGATCTGAGTGACTT AAATAGTACA-3′) were designed to anneal immediately adjacent to, but on opposite sides of, the repeat tract of *lex2A* and in reverse orientations, and each has a unique BglII restriction enzyme site near its 5′ end. These primers were each used pairwise with an appropriate plasmid-specific primer to generate products from pB7004E*lex2*. A PCR amplification product incorporating the 5′ end of *lex2A* and the 3′ end of the upstream gene *purL* was obtained by PCR amplification with primer BgA and the plasmid-specific T7 universal primer (28). This product was ligated into pCR2.1 (Invitrogen) to generate clone pCR2.1BgA. A PCR amplification product incorporating a sequence 3′ of the repeats of *lex2A* and *lex2B* and the 3′ end of gene HI0755 was obtained using primer BgB and the plasmid-specific M13 reverse universal primer (28) and was ligated into pCR2.1 to generate clone pCR2.1BgB. The cloned fragments released by digestion of pCR2.1BgA with SnaBI-BglII and pCR2.1BgB with BglII-StyI were ligated into SnaBI-StyI-digested pB7004E*lex2* (6), replacing the original *lex2* and adjacent sequence. Correct clones were designated pB*lex2*Δ5′-GCAA-3′.

For selection following transformation, an antibiotic resistance gene was inserted into the construct downstream of *lex2B* by using a restriction enzyme site created by PCR. Primers BaA (5′-CGCGGATCCACTGCTTTGTCTACTATA TC-3′) and BaB (5′-CGCGGATCCTGTTAATGTTTATTTTTAAATATTAG-3′), each incorporating a BamHI site near its 5′ end, were designed to anneal on opposite strands and in reverse orientations from the nucleotide 27 bp downstream of the stop codon of *lex2B*. A PCR product incorporating most of *lex2B* and the first 27 bp of the downstream intergenic sequence was generated using primers BaA and P1 (5′-GCGGTTGAATGCAAAGGG-3′) and was ligated into pCR2.1 to generate clone pCR2.1BaA. The region containing the remaining intergenic sequence downstream of *lex2B* and most of the downstream gene, HI0755, was amplified by PCR using primers BaB and P2 (5′-ATGCCACGTG AAATTTCCG-3′) and chromosomal DNA of strain RM7004 as a template. The product obtained was ligated into pCR2.1 to generate clone pCR2.1BaB. The StyI-BamHI fragment of clone pCR2.1BaA, the Kan^r gene of pUC4k (Pharmacia) released by digestion with BamHI, and the BamHI-XhoI fragment of clone pCR2.1BaB were ligated into the plasmid pB*lex2*Δ5′-GCAA-3′ digested with StyI and XhoI. The XhoI site is located in the polylinker of pB*lex2*Δ5′-GCAA-3′ downstream of *lex2B*. The clone was designated pB*lex2*Δ5′-GCAA-3′k. This construct was sequenced using primer lex2seq (6) to verify the sequence of the DNA where the repeats were deleted. Plasmid pB*lex2*Δ5′-GCAA-3′t was obtained from pB*lex2*Δ5′-GCAA-3′k by replacement of the Kan^r gene with the Tet^r gene.

Transformation of *H. influenzae*. *H. influenzae* strains were transformed with the appropriate linearized plasmid (8), and the recombinant strains were verified by PCR amplification and Southern analysis (26).

Plasmid constructs pB*lex2*Δ5′-GCAA-3′k, pB*lex2*Δ5′-GCAA-3′t, and pCR2.1HI0753-4N were used to transform strain RM118 to give strains RM118*lex2*+k, RM118*lex2*+t, and RM118HI0753-4kan, respectively. Plasmid p11$_2$ was used to transform strain RM118*lex2*+t to generate strain RM118*lex2*+t*lpsA*-k. Plasmid p11$_3$ was used to transform a mutant strain of RM7004 disrupted in *lic2C* (RM7004*lic2C*-k) to generate strain RM7004*lic2C*-k*lpsA*-t.

Analysis of LPS by colony immunoblotting. A freshly grown single colony of *H. influenzae* was serially diluted in phosphate-buffered saline, and an appropriate dilution was plated to obtain approximately 500 CFU per plate. After overnight growth, the colonies were transferred onto a nitrocellulose filter, dried, and then subjected to reactions with the appropriate MAbs as previously described (25).

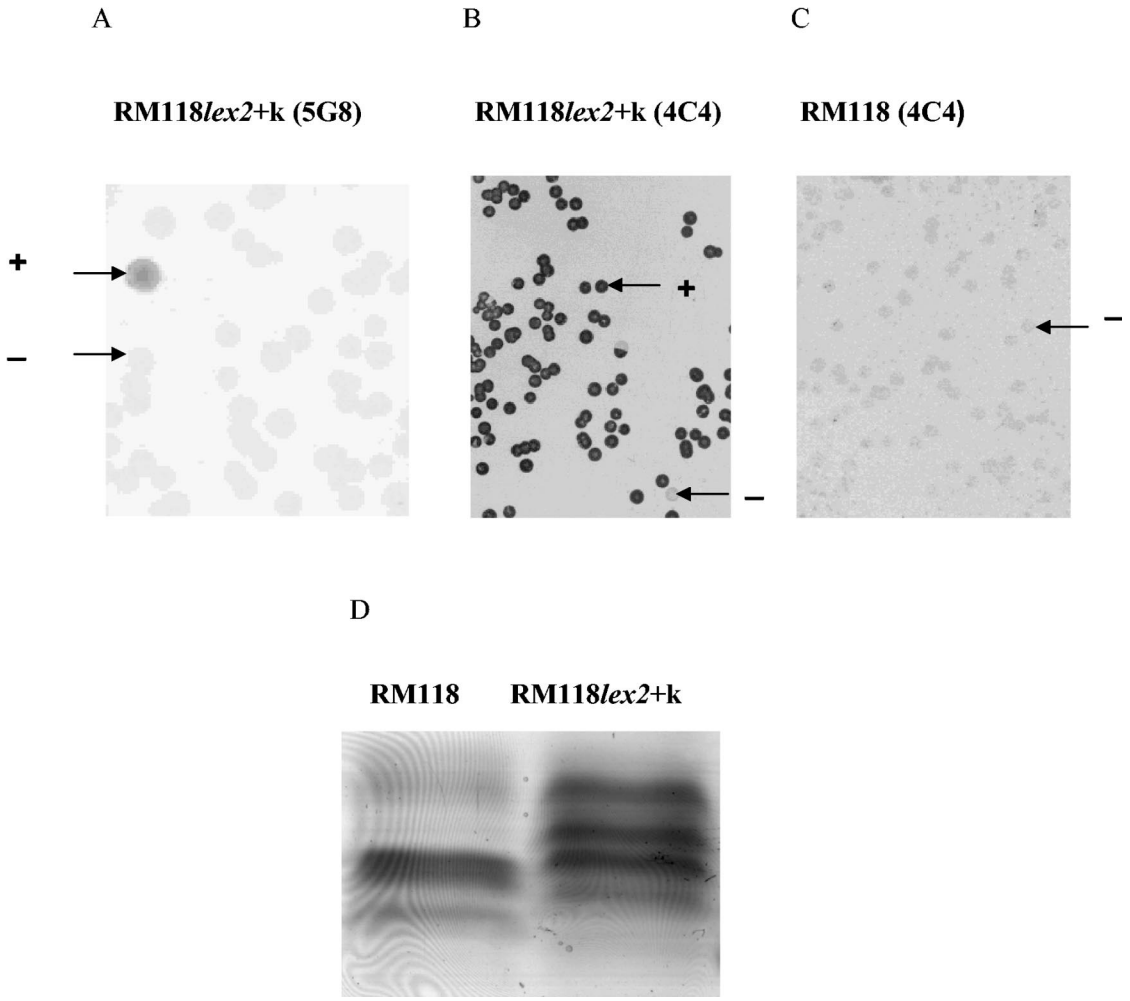

FIG. 2. Analysis of LPS molecules of *H. influenzae* strains RM118*lex2*+k and RM118 by colony immunoblotting with the indicated MAbs (A to C) and by gel fractionation (D). The levels of MAb binding by individual colonies are shown by arrows and indicated by + or −, representing reactivity and no reactivity, respectively. The faint upper bands seen in the profile of RM118 LPS correspond to four sugar units that can be added in the absence of *lex2* (13).

Gel fractionation of LPS and Western immunoblotting. Whole-cell lysates of *H. influenzae* strains were prepared as previously described (27) and then fractionated by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (18). LPS was visualized by staining with silver (Quicksilver; Amersham) (24) or was electrophoretically transferred onto a nitrocellulose membrane for Western analysis (29, 34).

LPS structural analyses. Structural analyses of LPS using mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques were carried out as described previously (19).

Serum resistance assay. Bacteria were assayed for survival against the killing effect of normal human serum as described previously (11).

RESULTS

Engineering an RM118 strain expressing *lex2* with acquired reactivity to MAb 5G8. Plasmid pB*lex2*Δ5′-GCAA-3′k, incorporating *lex2A* and *lex2B*, was constructed with the tetranucleotide repeat tract of *lex2A* removed to leave a non-phase-variable, constitutively translated gene. Any confounding effect of *lex2* phase variation upon analysis of function after transfer into strain RM118 was therefore negated.

A minority of colonies of the recombinant strain RM118*lex2*+k reacted with 5G8, unlike the parent strain, which does not react with this MAb (33). The remaining colonies showed no reactivity (Fig. 2A). Several colonies showed a sectored phenotype, indicative of phase-variable expression of the respective LPS epitope. We tested the reactivity of this strain with MAb 4C4, since 5G8 reactivity is associated with 4C4 reactivity (7) and the latter is associated with the expression of a digalactoside in *H. influenzae* LPS (32). Unlike those of the parent strain (Fig. 2C), most colonies of RM118*lex2*+k reacted (+ phenotype), while a minority of colonies demonstrated either strong (++ phenotype) or no (− phenotype) reactivity (Fig. 2B). When colonies of the − phenotype were isolated and the cells were dispersed and replated, a minority of the resultant colonies again displayed the + and ++ phenotypes (data not shown). Thus, transformation of strain RM118 with the *lex2* locus resulted in the phase-variable expression of an LPS epitope, which is presumed to include a digalactoside.

In the genome sequence of strain Rd (equivalent to RM118), in place of the *lex2* locus are two genes, HI0753 and HI0754, of unknown function (6). Reciprocal recombination in strain RM118*lex2*+k had replaced these two genes by the in-frame

TABLE 1. Negative ion electrospray-MS data and proposed compositions of O-deacylated LPS molecules from strains of RM118 transformed with *lex2*[a]

Strain	Result for:					Proposed composition of LPS
	$[M-3H]^{3-}$	$[M-2H]^{2-}$	Observed molecular ion	Calculated molecular ion	Relative intensity	
RM118*lex2*+k	866.4	1,300.4	2,602.9	2,603.2	0.2	PCho, 3Hex, 3Hep, PEtn, Kdo-P, lipid A
	907.4	1,362.6	2,725.9	2,726.3	0.2	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	920.7	1,386.1	2,765.9	2,766.3	0.4	PCho, 4Hex, 3Hep, PEtn, Kdo-P, lipid A
	961.8	1,443.4	2,888.8	2,889.3	0.5	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	974.8	1,462.5	2,927.1	2,928.4	0.5	PCho, 5Hex, 3Hep, PEtn, Kdo-P, lipid A
	1,015.9	1,524.5	3,050.8	3,051.4	0.8	PCho, 5Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	1,028.8		3,089.8	3,093.5	0.3	PCho, 6Hex, 3Hep, PEtn, Kdo-P, lipid A
	1,042.7		3,131.5	3,131.6	0.4	PCho, 5Hex, HexNAc, 3Hep, PEtn, Kdo-P, lipid A
	1,069.8	1,605.7	3,213.4	3,213.6	0.4	PCho, 6Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	1,083.6	1,625.9	3,253.9	3,254.7	1.0	PCho, 5Hex, HexNAc, 3Hep, 2PEtn, Kdo-P, lipid A
	1,096.7		3,293.5	3,293.7	0.3	PCho, 6Hex, HexNAc, 3Hep, PEtn, Kdo-P, lipid A
	1,137.5	1,707.0	3,415.6	3,416.7	0.5	PCho, 6Hex, HexNAc, 3Hep, 2PEtn, Kdo-P, lipid A
	1,150.7		3,455.9	3,455.9	0.3	PCho, 7Hex, HexNAc, 3Hep, PEtn, Kdo-P, lipid A
	1,191.8		3,577.8	3,578.9	0.3	PCho, 7Hex, HexNAc, 3Hep, 2PEtn, Kdo-P, lipid A
	1,218.8	1,827.6	3,659.4	3,659.1	0.3	PCho, 7Hex, 2HexNAc, 3Hep, PEtn, Kdo-P, lipid A
RM118*lex2*+t*lpsA*-*k*		1,056.2	2,114.4	2,113.9	0.1	1Hex, 3Hep, PEtn, Kdo-P, lipid A
		1,056.2	2,114.4	2,113.9	0.1	1Hex, 3Hep, PEtn, Kdo-P, lipid A
		1,118.0	2,237.4	2,236.9	0.1	1Hex, 3Hep, 2PEtn, Kdo-P, lipid A
		1,139.0	2,279.4	2,279.9	0.2	PCho, 1Hex, 3Hep, PEtn, Kdo-P, lipid A
		1,219.8	2,441.5	2,442.1	0.2	PCho, 2Hex, 3Hep, PEtn, Kdo-P, lipid A
		1,281.5	2,565.0	2,565.1	0.2	PCho, 2Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	866.9	1,300.9	2,603.9	2,603.2	0.6	PCho, 3Hex, 3Hep, PEtn, Kdo-P, lipid A
	907.8	1,362.7	2,727.5	2,726.3	0.4	PCho, 3Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	921.0	1,381.8	2,765.6	2,766.3	1.0	PCho, 4Hex, 3Hep, PEtn, Kdo-P, lipid A
	961.9	1,443.7	2,889.0	2,889.3	0.9	PCho, 4Hex, 3Hep, 2PEtn, Kdo-P, lipid A
	988.7	1,483.8	2,969.5	2,969.5	0.5	PCho, HexNAc, 4Hex, 3Hep, PEtn, Kdo-P, lipid A
	1,029.6	1,544.9	3,091.9	3,092.5	0.9	PCho, HexNAc, 4Hex, 3Hep, 2PEtn, Kdo-P, lipid A

[a]Average mass units were used for calculation of molecular weight based on proposed composition as follows: lipid A, 952.00; Hex, 162.15; HexNAc, 203.19; Hep, 192.17; Kdo-P, 300.16; phosphoethanolamine (PEtn), 123.05; and PCho, 165.05.

lex2 locus. To ensure that the altered LPS phenotype of this strain was due to the gain of *lex2* rather than the loss of genes HI0753 and HI0754, these two genes were mutated in strain RM118 by transformation with construct pCR2.1HI0753-4Nk. Strain RM118HI0753-4k showed no alteration in the PAGE fractionation pattern of LPS or in reactivity with MAbs 4C4 and 5G8 compared with the parent strain (data not shown). Thus, disruption of HI0753 and HI0754 had no effect upon LPS biosynthesis.

Characterization of the LPS of the *lex2* transformant strain RM118*lex2*+k. PAGE fractionation of the LPS isolated from strain RM118*lex2*+k showed two or three additional lower-mobility bands compared with the profile of the parent strain (Fig. 2D). Thus, expression of *lex2* in RM118 resulted in the addition of sugars to the LPS of this strain.

MS analysis of purified O-deacylated LPS of strain RM118 *lex2*+k indicated that three additional hexose and two additional HexNAc sugars were present in this strain compared to the parent strain (Table 1). The presence of the first HexNAc was detected subsequent to the incorporation of five hexoses, and the presence of the second HexNAc was observed after the incorporation of seven hexoses into the LPS of this strain. For the latter glycoform, sugar composition analysis suggested a glucose-to-galactose ratio of almost 1 to 1. This analysis also indicated that both HexNAc residues were *N*-acetylgalactosamine (GalNAc) (Table 1). These data are consistent with our prediction that the expression of *lex2* in strain RM118 would result in the addition of a further single glucose and a digalactoside to the glucose (GlcI) attached to HepI of the LPS of this strain, as reported for strain RM7004 (6). In strain RM118, GalNAc is the final residue added to the globotetraose unit at the third heptose after assembly of four hexoses. Detection of the addition of GalNAc in glycoforms comprising five or more hexose sugars in strain RM118*lex2*+k indicates that the addition of the glucose by *lex2* to GlcI occurs prior to the addition of the GalNAc of the globotetraose unit from HepIII.

Localization of the MAb 5G8 epitope in strain RM118*lex2*+t*lpsA*-k allows expression of only the oligosaccharide extension from HepI. In the LPS of strain RM118, there is no oligosaccharide extension from HepII (Fig. 1) (23). To confirm that the additional sugars in the LPS of strain RM118*lex2*+k were added to the oligosaccharide extending from HepI, and not to that extending from HepIII, *lpsA*, which is required for the addition of the first glucose to HepIII of strain RM118 (Fig. 1), was disrupted. Mutation of *lpsA* to give strain RM118*lex2*+t*lpsA*-k prevents any oligosaccharide extension from HepIII in this strain background (11).

Upon colony immunoblotting of strain RM118*lex2*+t*lpsA*-k with MAb 5G8, a minority of colonies reacted (+ phenotype) while the remainder showed no reactivity with this MAb (data not shown). This pattern of reactivity was similar to that of RM118*lex2*+k and confirms that the gain of reactivity with MAb 5G8 is associated with the assembly of an oligosaccharide chain extending from HepI. Upon colony immunoblotting of strain RM118*lex2*+t*lpsA*-k with MAb 4C4, a majority of colonies demonstrated reactivity (+ phenotype) while a minority

TABLE 2. ^{1}H NMR chemical shifts for the O-deacylated LPS from strain RM118*lex2*+t*lpsA*-k

Residue	Proton resonance					Nuclear Overhauser effect
	H-1	H-2	H-3	H-4	H-5	
HepI	5.15	4.13	4.07	ND	ND	4.31 Kdo, H-5
HepII	5.78	4.26	ND[a]	ND	ND	4.07 HepI H-3
HepIII	5.17	4.06	ND	ND	ND	4.26 HepII H-2 5.78 HepII H-1
α-GlcN	5.40	3.77	ND	ND	ND	
β-Glc (GlcI)	4.56	3.61	≈3.70	≈3.70	≈3.70	4.26 HepI H-4 4.03 HepI H-6
β-Glc (GlcII)	4.66	3.64	≈3.71	≈3.71	≈3.71	3.70 GlcI H-4
β-Gal (GalI)	4.53	3.58	3.74	4.04	3.79	3.71 GlcII H-4
t-α-Gal (GalII)	4.94	3.83	3.90	4.02	ND	4.04 GalI H-4
-3-α-Gal (GalII)	4.91	3.88	3.95	ND	ND	4.04 GalI H-4
β-GalNac	4.62	3.94	3.75	ND	ND	3.95 GalII H-3
β-GlcN	4.62	3.90	3.80	ND	ND	ND

[a] ND, not determined.

demonstrated no reactivity (− phenotype) or strong (++ phenotype) reactivity. Several of the MAb 4C4-reactive colonies showed sectoring, indicating that the oligosaccharide extending from HepI included a digalactoside and that this was likely expressed in a phase-variable manner (data not shown).

Electrospray ionization-MS analysis of the O-deacylated LPS of strain RM118*lex2*+t*lpsA*-k indicated a range of glycoforms containing between two and four hexose sugars (Table 1). Most glycoforms contained a PCho residue, and HexNAc was detected in the highest-molecular-weight glycoform. Sugar composition analysis of this glycoform suggested that the glucose-to-galactose ratio was almost 1 to 1 and that the HexNAc was GalNAc. ^{1}H NMR analysis of the O-deacylated LPS of strain RM118*lex2*+t*lpsA*-k confirmed that the expression of *lex2* resulted in the addition of a second glucose to HepI of the LPS, to which a digalactoside and finally a GalNAc residue were added (Table 2). This analysis confirmed the absence of sugar extensions from HepII and HepIII and the prediction that the arrangement of and linkages between the sugars of the oligosaccharide extending from HepI of strain RM118*lex2*+t*lpsA*-k (Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β1,4-) were indistinguishable from those of strain RM7004, except for the addition of a terminal GalNAc.

Definition of the MAb 5G8-reactive epitope in strain RM118*lex2*+t*lpsA*-k by sequential immunoblotting. To further characterize the epitope recognized by MAb 5G8 in strain RM118*lex2*+t*lpsA*-k, we conducted sequential colony immunoblotting using MAbs 5G8, 4C4, and TEPC-15. The latter antibody binds PCho, which is located as a substituent of the glucose attached directly to HepI (Fig. 1). Colonies of strain RM118*lex2*+t*lpsA*-k were grown on a plate and immunoblotted with MAb 4C4, and the colonies was then regrown and immunoblotted with MAb 5G8. The process was repeated for immunoblotting with MAb TEPC-15. This approach allowed superimposition of MAb binding patterns for individual colonies. For MAb 5G8, as before, a minority of colonies demonstrated the + phenotype while the majority of colonies were of the − phenotype (Fig. 3A). Each of these MAb 5G8 + phenotype variants corresponded exclusively to each of the colonies that demonstrated the MAb 4C4 ++ phenotype (Fig. 3B). It can be concluded that expression of the MAb 5G8 + phenotype and that of the MAb 4C4 ++ phenotype are coincident in the LPS of strain RM118*lex2*+t*lpsA*-k. The third in the series of immunoblots using MAb TEPC-15 explained this behavior. For MAb TEPC-15, most of the colonies reacted (+ phenotype) while a minority of variants demonstrated no reactivity (− phenotype) (Fig. 3C). Each of these MAb TEPC-15-nonreactive variants corresponded to each of the MAb 5G8 + phenotype-4C4 ++ phenotype variants. From these results, we conclude that 5G8 specifically reacts with the tetrasaccharide Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β1,4- extending from HepI and requires nonexpression of PCho in that oligosaccharide and that the reactivity with MAb 4C4 is diminished (++ to +) but not abolished by the presence of PCho.

Genetic modification of strain RM7004 to expose the 5G8-reactive epitope. Since MAb 5G8 was raised against a Hib strain (7), we wished to confirm our findings with Hib strain RM7004, which naturally synthesizes LPS containing an oligosaccharide that we would predict to contain the MAb 5G8 epitope. In addition, we set out to determine whether the

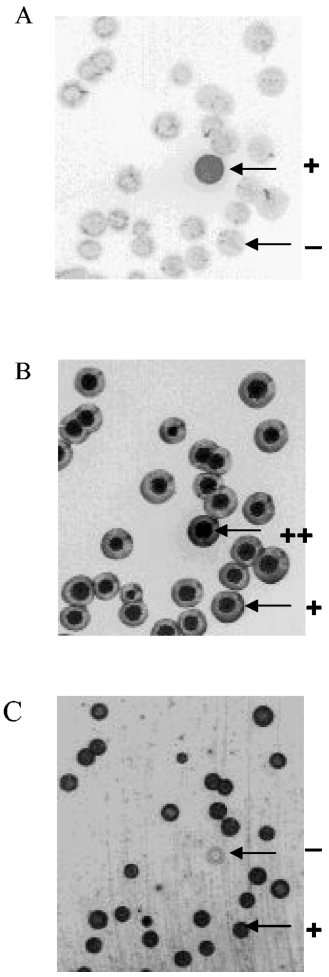

FIG. 3. Sequential immunoblotting of colonies of strain RM118 *lex2*+t*lpsA*-k5G8+ with MAbs 5G8 (A), 4C4 (B), and TEPC-15 (C). The levels of MAb binding by individual colonies are shown by arrows and indicated by +, ++, and − for reactivity, strong reactivity, and no reactivity, respectively.

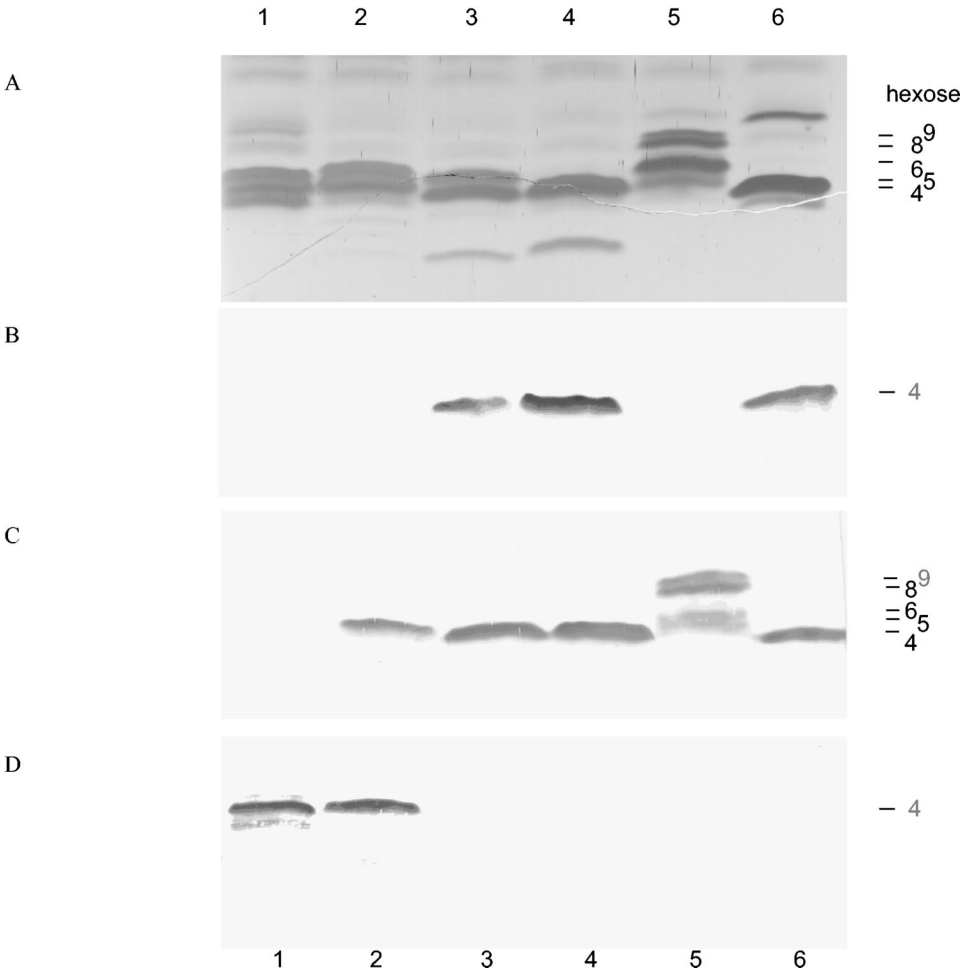

FIG. 4. Fractionation and Western analysis of LPS molecules isolated from *H. influenzae* strains. Panel A shows the fractionation pattern after tricine-sodium dodecyl sulfate-PAGE; results of Western analysis with MAbs 5G8 (B), 4C4 (C), and TEPC-15 (D) are shown below. Strains are RM118 (lane 1), RM118*lex2*+t*lpsA*-k5G8+ (lane 2), RM118*lex2*+t*lps*A-k (lane 3), RM7004*lpsA*-t*lic2orf3*-k (lane 4), RM7004 (lane 5), and DL42 (lane 6) The estimated numbers of hexose sugars included in each glycoform are indicated by the scale on the right-hand side.

terminal GalNAc played any part in this epitope, as strain RM7004 does not incorporate GalNAc into its LPS (21).

The genes *lic2C* and *lpsA*, which are known to be involved in the addition of the first hexose to HepII and HepIII, respectively, in this strain (Fig. 1A) (11), were inactivated to generate strain RM7004*lpsA*-t*lic2C*-k. We anticipated that the outer core LPS of this strain would consist of a tetrasaccharide extending from HepI only, nearly equivalent in composition to the LPS from strain RM118*lex2*+t*lpsA*-k but without the terminal GalNAc residue and without PCho attached to the first glucose. Unlike the parent strain RM7004, which did not react with 5G8, the majority of colonies of strain RM7004*lpsA*-t*lic2C*-k reacted strongly with this MAb (data not shown). Both single mutants, RM7004*lpsA*-t and RM7004*lic2C*-k, reacted with MAb 5G8, although less intensely than the double mutant strain (data not shown). Upon sequential immunoblotting of the double mutant strain with MAb 4C4, each of the MAb 5G8 + phenotype colonies reacted with MAb 4C4 and, conversely, each of the MAb 5G8 − phenotype colonies showed no reactivity with MAb 4C4 (data not shown). None of the colonies reacted strongly with MAb TEPC-15 (data not shown).

These findings confirmed our prediction that the LPS from strain RM7004*lpsA*-t*lic2C*-k, which contains a natural tetrasaccharide extending from HepI resembling that from strain RM118*lex2*+t*lpsA*-k but incorporates no terminal GalNAc, was indeed 5G8 reactive.

Comparison of the MAb 5G8-reactive LPS glycoforms in transformant strains of RM118 and RM7004 by Western analyses. Strain RM118*lex2*+t*lpsA*-k was enriched for MAb 5G8-reactive cells by culturing a single MAb 5G8 + variant colony designated RM118*lex2*+t*lpsA*-k5G8+. Lysates were prepared and then fractionated from this strain and nonenriched cells of the following strains: RM118*lex2*+t*lpsA*-k, RM118, RM7004, RM7004*lpsA*-t*lic2C*-k, and DL42, a 5G8 + control strain (15) (Fig. 4A).

LPS bands of equivalent mobilities from strains RM118*lex2*+t*lpsA*-k5G8+ and RM7004*lpsA*-t*lic2C*-k, as well as one from strain DL42, reacted with MAb 5G8 upon immunoblotting. The nonenriched strain RM118*lex2*+t*lpsA*-k did not react (Fig. 4B). The lower-mobility band of strain RM118*lex2*+t*lpsA*-k5G8+ LPS is presumed to correspond to the GalNAc- and PCho-depleted LPS glycoform and did not react with MAb

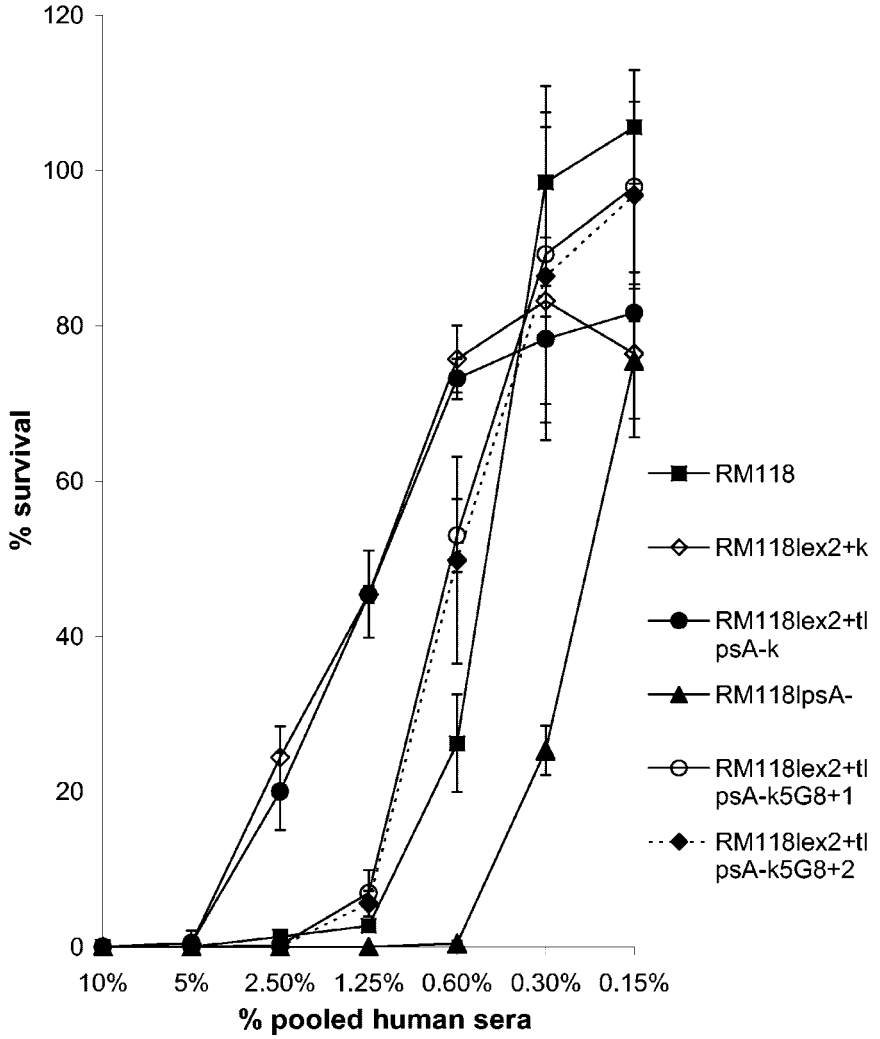

FIG. 5. Resistance of *H. influenzae* strains to the killing effect of normal human serum. Representative results for strain RM118 and derived mutants are shown. Organisms (5×10^2) were added to doubling dilutions of serum. Error bars from the averaging of the data from three experiments are displayed.

5G8. Thus, it appears that 5G8 binds to the equivalent glycoforms of strains RM118*lex2*+t*lpsA*-k and RM7004*lpsA*-t*lic2C*-k. In a parallel experiment using MAb 4C4, LPS bands of equivalent mobilities from strains RM118*lex2*+t*lpsA*-k, RM7004*lpsA*-t*lic2C*-k, and DL42 that reacted with MAb 5G8 reacted with MAb 4C4 also (Fig. 4C). This further supports our finding that MAb 5G8 binds the oligosaccharide extending from HepI that comprises a diglucoside and a terminal digalactoside. The presence of the GalNAc likely inhibits the binding of MAbs to the tetrasaccharide. TEPC-15 bound to LPS glycoforms from strains RM118 and RM118*lex2*+t*lpsA*-k, both of which contain an abundance of PCho (Table 1; Fig. 4D) (23). Western analysis showed that the LPS of strain RM118*lex2*+t*lpsA*-k was recognized by MAb TEPC-15 and not by MAb 5G8, confirming that the presence of PCho prevented 5G8 from binding to the LPS. Furthermore, the reduced binding of MAb 4C4 to the LPS of strain RM118*lex2*+t*lpsA*-k compared to that to the LPS of strain RM118*lex2*+t*lpsA*-k5G8+ supports our earlier observation that the presence of PCho reduces, but does not prevent, MAb 4C4 binding in RM118*lex2*+t*lpsA*-k. Unlike strain RM118, PCho is predominantly not expressed on the LPS of strain RM7004 (21) and when expressed substitutes for the single galactose residue extending from HepIII. Details of the structure of the LPS of strain DL42 are not known.

Strain RM118*lex2*+t*lpsA*-k displays altered serum resistance. To investigate the biological relevance of the MAb 5G8-reactive epitope in *H. influenzae* LPS, we studied the relative levels of resistance of strain RM118 and its transformants to the killing effect of pooled normal human sera. Typically, the percent serum concentration required to kill 60% of wild-type RM118 organisms was approximately 0.8%. With the *lpsA* mutant, organisms were killed to the same level by approximately 0.2% serum. This relates to the loss of the digalactoside-incorporating oligosaccharide from the LPS of the RM118 *lpsA* mutant strain (Fig. 5), confirming previous findings (11). However, in the *lpsA* mutant expressing *lex2* (RM118*lex2*+t*lpsA*-k), the level of serum resistance was greatly increased to above that found for the wild type, such that approximately 1.5% serum was required to kill 60% of organisms of this strain (Fig.

5). This level was diminished in two independent MAb 5G8 + phenotype variants of this strain, although the level of resistance was greater than that of strain RM118. Strain RM118*lex2*+k showed a level of resistance to serum killing similar to that of RM118*lex2*+t*lpsA*-k. These findings enabled us to draw the following conclusions.

The digalactoside extending from HepIII confers protection against complement-mediated killing, as seen by the significant reduction in resistance of the *lpsA* mutant. The oligosaccharide incorporating the digalactoside from HepI protects organisms of strain RM118*lex2*+t*lpsA*-k from complement-mediated killing, as seen by the large increase in the survival rate of this strain compared to that of the wild type. In RM118*lex2*+t*lpsA*-k, despite the loss of the HepIII-linked digalactoside and therefore the subsequent loss of protection conferred by this epitope, the expression of the digalactoside-containing oligosaccharide from HepI overcompensates, allowing significantly greater resistance to killing of this strain than to that of the wild-type strain. This protection is mediated by both digalactoside and PCho since MAb 5G8 + phenotype variants of this strain, lacking PCho, showed some diminution in survival.

DISCUSSION

Of 126 Hib strains investigated by Gulig and coworkers, 74 strains (59%) reacted with MAb 5G8, suggesting that the expression of the LPS determinant recognized by this MAb is relatively conserved in these clinical strains (7). Moreover, this frequency is likely to be an underestimate since in the absence of in vivo selection during in vitro passage, Hib strains are reported to switch off the expression of their virulence-associated LPS epitopes that react with MAbs 5G8 and 4C4 (17, 33). The association made between strains that are reactive with MAb 5G8 and strains that are highly virulent and serum resistant (16, 17) has generated much interest in elucidating the structure of this LPS epitope.

The initial finding that MAb 5G8 reactivity was associated with expression of the *lex2* locus was key to our investigation (15). The reactivity of colonies of Hib strains with 5G8 was shown to be dependent upon a number of in-frame tetranucleotide repeats located within the *lex2A* gene (15). We previously showed that the *lex2* locus is required for the addition of a second glucose to HepI of *H. influenzae* LPS to complete the acceptor for a digalactoside (6). We set out to characterize the structure of the MAb 5G8-reactive epitope in a strain devoid of capsule, to prevent interference of capsule with our serum resistance study, and expressing genetically characterized, minimally complex LPS. This strain, RM118, when transformed with the *lex2* locus acquired reactivity to MAb 5G8. To localize the 5G8-reactive epitope within the LPS molecule, which we predicted would extend from HepI, we further disrupted the *lpsA* gene to prevent oligosaccharide extension from HepIII in the *lex2* transformant strain. LPS from this double mutant strain retained MAb 5G8 reactivity and contained a single tetrasaccharide capped by GalNAc extending from HepI of the inner core. This tetrasaccharide was nearly identical to that extending from HepI of the LPS of strain RM7004 except for the presence of PCho, which is encoded by the phase-variable locus *lic1* (34). We showed by disrupting *lic2C* and *lpsA* in strain RM7004 (Fig. 1) that this mutant strain has only a tetrasaccharide extending from HepI which lacks GalNAc and PCho and that these two moieties are not part of the MAb 5G8-reactive epitope. Thus, we have compelling evidence that MAb 5G8 binds the tetrasaccharide Gal-α1,4-Gal-β1,4-Glc-β1,4-Glc-β1,4 that extends from HepI of the LPS of *H. influenzae*. This is the first complete genetic and structural elucidation of an epitope binding an LPS-specific MAb in *H. influenzae.*

Furthermore, we provide the first definitive evidence that MAb 4C4 binds the digalactoside extending from HepI of *H. influenzae* LPS in two strains, RM118*lex2*+t*lpsA*k and RM7004*lic2C*-k*lpsA*-t, that extend sugars from HepI only. Prior to this study, it was certain only that MAb 4C4 bound the digalactoside extending from the second heptose in the LPS molecules of Hib strains (32). Since MAb 4C4 does not bind to the digalactoside-containing globotetraose that extends from HepIII of the LPS of strain RM118, we speculate that the recognition of the digalactoside by MAb 4C4 is influenced by the location of the epitope and the structural conformation of the complete LPS molecule. It may be that the diglucoside acceptor synthesized prior to the digalactoside is essential for MAb 4C4 binding. This acceptor is available as an extension from both HepI and HepII in strain RM7004 (21), whereas HepIII is replaced by only a single glucose in strain RM118 (23).

The two MAbs 4C4 and 5G8 differ in their degrees of specificity of binding. MAb 4C4 recognizes the digalactoside-containing tetrasaccharide regardless of whether it extends from HepI or HepII, whereas MAb 5G8 binds exclusively the digalactoside-containing tetrasaccharide extending from HepI. In the presence of PCho linked to the glucose attached to HepI, MAb 4C4 binding is reduced, whereas MAb 5G8 binding is abolished.

In all Hib strains with characterized LPS molecules, there is no PCho substituting for the glucose attached to HepI. Rather, PCho is attached to the hexose extending from HepIII, where it would not appear to influence MAb 5G8 binding, as seen in our RM7004-derived strains.

It is clear from this study that the MAb 5G8 epitope confers significant resistance to serum killing, and this finding allows us to understand the association made previously between MAb 5G8 reactivity and virulence in type b strains. Since the LPS epitope in question incorporates a digalactoside and this epitope is reported to mimic human antigens found on a variety of cell types, including erythrocytes, protection occurs through presentation of a "self antigen" (32). The digalactoside extending from the third heptose also appeared to confer serum resistance, as seen by the large decrease in resistance of the *lpsA* mutant to serum.

Strain RM118*lex2*+k can synthesize oligosaccharides from both HepI and HepIII, along with PCho, and therefore may have been expected to be the most resistant strain. However, this strain did not survive as well as RM118*lex2*+t*lpsA*-k. In strain RM118*lex2*+k, only a minority of glycoforms contain fully extended oligosaccharides incorporating the digalactoside (Table 1). In contrast, in strain RM118*lex2*+t*lpsA*-k, the majority of glycoforms incorporate the digalactoside (Table 1).

PCho contributed to the serum resistance of RM118*lex2*+

tlpsA-k, as seen by some reduction in resistance in the PCho-negative variants.

In summary, the digalactosides included in oligosaccharide extensions from both HepI and HepIII in *H. influenzae* LPS confer resistance to killing by human serum and PCho plays a role in this protection.

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REFERENCES

1. **Alexander, H. E.** 1965. The *Haemophilus* group. Pitman Medical Publishing, London, United Kingdom.
2. **Cope, L. D., R. Yogev, J. Mertsola, J. C. Argyle, G. H. McCracken, Jr., and E. J. Hansen.** 1990. Effect of mutations in lipooligosaccharide biosynthesis genes on virulence of *Haemophilus influenzae* type b. Infect. Immun. **58:** 2343–2351.
3. **Cope, L. D., R. Yogev, J. Mertsola, J. L. Latimer, M. S. Hanson, G. H. McCracken, Jr., and E. J. Hansen.** 1991. Molecular cloning of a gene involved in lipooligosaccharide biosynthesis and virulence expression by *Haemophilus influenzae* type b. Mol. Microbiol. **5:**1113–1124.
4. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
5. **Flesher, A. R., and R. A. Insel.** 1978. Characterization of lipopolysaccharide of *Haemophilus influenzae*. J. Infect. Dis. **138:**719–730.
6. **Griffin, R., A. D. Cox, K. Makepeace, J. C. Richards, E. R. Moxon, and D. W. Hood.** 2003. The role of *lex2* in lipopolysaccharide biosynthesis in *Haemophilus influenzae* strains RM7004 and RM153. Microbiology **149:**3165–3175.
7. **Gulig, P. A., C. F. Frisch, and E. J. Hansen.** 1983. A set of two monoclonal antibodies specific for the cell surface-exposed 39K major outer membrane protein of *Haemophilus influenzae* type b defines all strains of this pathogen. Infect. Immun. **42:**516–524.
8. **Herriott, R. M., E. Y. Meyer, M. Vogt, and M. Modan.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101:**517–524.
9. **High, N. J., M. E. Deadman, and E. R. Moxon.** 1993. The role of a repetitive DNA motif (5′-CAAT-3′) in the variable expression of the *Haemophilus influenzae* lipopolysaccharide epitope alpha Gal(1–4)beta Gal. Mol. Microbiol. **9:**1275–1282.
10. **Hood, D. W., M. E. Deadman, T. Allen, H. Masoud, A. Martin, J. R. Brisson, R. Fleischmann, J. C. Venter, J. C. Richards, and E. R. Moxon.** 1996. Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. Mol. Microbiol. **22:**951–965.
11. **Hood, D. W., M. E. Deadman, A. D. Cox, K. Makepeace, A. Martin, J. C. Richards, and E. R. Moxon.** 2004. Three genes, lgtF, *lic2C* and *lpsA*, have a primary role in determining the pattern of oligosaccharide extension from the inner core of *Haemophilus influenzae* LPS. Microbiology **150:**2089–2097.
12. **Hood, D. W., M. E. Deadman, M. P. Jennings, M. Bisercic, R. D. Fleischmann, J. C. Venter, and E. R. Moxon.** 1996. DNA repeats identify novel virulence genes in *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **93:** 11121–11125.
13. **Hood, D. W., G. Randle, A. D. Cox, K. Makepeace, J. Li, E. K. H. Schweda, J. C. Richards, and E. R. Moxon.** 2004. Biosynthesis of cryptic lipopolysaccharide glycoforms in *Haemophilus influenzae* involves a mechanism similar to that required for O-antigen synthesis. J. Bacteriol. **186:**7429–7439.
14. **Hood, D. W., and E. R. Moxon.** 2002. Gene variation and gene regulation in bacterial pathogenesis, p. 19–40. *In* D. A. Hodgson and C. M. Thomas (ed.), Society for General Microbiology Symposium, vol. 61. Switches, signals, regulons and cascades: control of bacterial gene expression. Cambridge University Press, Cambridge, United Kingdom.
15. **Jarosik, G. P., and E. J. Hansen.** 1994. Identification of a new locus involved in expression of *Haemophilus influenzae* type b lipooligosaccharide. Infect. Immun. **62:**4861–4867.
16. **Kimura, A., and E. J. Hansen.** 1986. Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. Infect. Immun. **51:**69–79.
17. **Kimura, A., C. C. Patrick, E. E. Miller, L. D. Cope, Jr., G. H. McCracken, and E. J. Hansen.** 1987. *Haemophilus influenzae* type b lipooligosaccharide: stability of expression and association with virulence. Infect. Immun. **55:** 1979–1986.
18. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilising tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis. J. Immunol. Methods **126:**109–117.
19. **Lysenko, E., J. C. Richards, A. D. Cox, A. Stewart, A. Martin, M. Kapoor, and J. N. Weiser.** 2000. The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. Mol. Microbiol. **35:**234–245.
20. **Månsson, M., D. W. Hood, E. R. Moxon, and E. K. H. Schweda.** 2003. Structural diversity in lipopolysaccharide expression in nontypeable *Haemophilus influenzae*. Identification of L-*glycero*-D-*manno*-heptose in the outer-core region in three clinical isolates. Eur. J. Biochem. **270:**610–624.
21. **Masoud, H., A. Martin, P. Thibault, E. R. Moxon, and J. C. Richards.** 2003. Structure of extended lipopolysaccharide glycoforms containing two globotriose units in the *Haemophilus influenzae* serotype b strain, RM7004. Biochemistry **42:**4463–4475.
22. **Preston, A., D. Maskell, A. Johnson, and E. R. Moxon.** 1996. Altered lipopolysaccharide characteristic of the I69 phenotype in *Haemophilus influenzae* results from mutations in a novel gene, *isn*. J. Bacteriol. **178:**396–402.
23. **Risberg, A., G. Alvelius, and E. K. Schweda.** 1999. Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* strain RM.118-26. Eur. J. Biochem. **265:**1067–1074.
24. **Roche, R. J., N. J. High, and E. R. Moxon.** 1994. Phase variation of *Haemophilus influenzae* lipopolysaccharide: characterisation of lipopolysaccharide from individual colonies. FEMS Microbiol. Lett. **120:**279–284.
25. **Roche, R. J., and E. R. Moxon.** 1995. Phenotypic variation in *Haemophilus influenzae*: the interrelationship of colony opacity, capsule and lipopolysaccharide. Microb. Pathog. **18:**129–140.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Serino, L., and M. Virji.** 2000. Phosphorylcholine decoration of lipopolysaccharide differentiates commensal *Neisseriae* from pathogenic strains: identification of *licA*-type genes in commensal *Neisseriae*. Mol. Microbiol. **35:** 1550–1559.
28. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. **16:**7583–7600.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology **24:**145–149.
30. **Turk, D. C., and J. R. May.** 1967. *Haemophilus influenzae*, its clinical importance. The English Universities Press, London, United Kingdom.
31. **van Alphen, L., T. Riemens, J. Poolman, C. Hopman, and H. C. Zanen.** 1983. Homogeneity of cell envelope protein subtypes, lipopolysaccharide serotypes, and biotypes among *Haemophilus influenzae* type b from patients with meningitis in The Netherlands. J. Infect. Dis. **148:**75–81.
32. **Virji, M., J. N. Weiser, A. A. Lindberg, and E. R. Moxon.** 1990. Antigenic similarities in lipopolysaccharides of *Haemophilus* and *Neisseria* and expression of a digalactoside structure also present on human cells. Microb. Pathog. **9:**441–450.
33. **Weiser, J. N., A. A. Lindberg, E. J. Manning, E. J. Hansen, and E. R. Moxon.** 1989. Identification of a chromosomal locus for expression of lipopolysaccharide epitopes in *Haemophilus influenzae*. Infect. Immun. **57:**3045–3052.
34. **Weiser, J. N., M. Shchepetov, and S. T. Chong.** 1997. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of *Haemophilus influenzae*. Infect. Immun. **65:**943–950.

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