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Digalactoside Expression in the Lipopolysaccharide of *Haemophilus influenzae* and Its Role in Intravascular Survival

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Digalactoside (galα-1-4 galβ) structures of the lipopolysaccharide (LPS) of *Haemophilus influenzae* are implicated in virulence. A confounding factor is that tetranucleotide repeats within the *lic2A*, *lgtC*, and *lex2* genes mediate phase-variable expression of the digalactosides. By deleting these repeats, we constructed recombinant strains of RM153 constitutively expressing either one or two LPS digalactosides. Expression of two digalactosides, rather than one, was associated with increased virulence of *H. influenzae* in vivo.

Lipopolysaccharide (LPS) is one of the major virulence determinants of the human pathogen *Haemophilus influenzae*. The number of hexoses and phosphate groups replacing the triheptose (HepI to HepIII) backbone (Fig. 1) is variable within any strain owing to high-frequency translational switching (phase variation) of LPS genes containing repeat tracts. Phase-variable LPS genes include *lic2A* and *lgtC*, which are involved in the assembly of galα-1-4 galβ into the oligosaccharide extensions from the conserved triheptose backbone (7, 10), and *lex2*, which is involved in completion of the HepI-attached diglucoside acceptor for this digalactoside (Fig. 1) (4). The investigation of the association between digalactoside expression on *H. influenzae* LPS and virulence has relied on the monoclonal antibody (MAb) 4C4 for detecting the expression of the digalactoside (1, 2, 12, 13). The findings from these studies have been difficult to interpret because of the confounding factor of phase variation and the different numbers and locations of the digalactoside on oligosaccharide extensions in different strains (7, 17, 18).

Recently, it has been shown that MAb 4C4 binds digalactosides that are part of the extensions from both HepI and HepII of the triheptose LPS backbone (Fig. 1) (4, 5). A type b clinical strain, RM153, used in previous virulence studies (9, 16), typically generates LPS molecules containing only four hexose sugars (17), depending on whether *lic2A*, *lgtC*, or *lex2* is out of frame (4, 7, 10). This is in contrast to the related strain RM7004, which has up to nine hexose sugars in its LPS due to these three loci being predominantly in frame (Fig. 1). Knowing the LPS structure, the genes required for digalactoside assembly, and the fact that tetranucleotide repeats mediate phase variation, we constructed recombinant strains of RM153 in which variable expression of digalactosides was eliminated by removing the repeat tracts located within each of these genes. Briefly, two isogenic strains were constructed by transformation (6) using appropriate chromosomal DNA or plasmid constructs in which the repeats had been deleted for *lic2A* (8), *lex2* (5), or *lgtC*. All three genes were constitutively expressed in the first strain, RM153*lic2A*+t*lgtC*+*lex2*+k, to facilitate expression of two digalactosides, while in the second strain, RM153*lic2A*+t*lgtC*+*lex2*−k, *lex2* was mutated such that only a single digalactoside in the extension from HepII was expressed (Fig. 1).

The strains were constructed as follows. An in-frame deletion of the repeat tract of *lic2A* in RM153 was generated by transformation with chromosomal DNA of a derivative of RM7004 in which the 5′-CAAT-3′ repeats had been deleted (8). Our construct differed from that of High and coworkers (8) only in that the *kanR* antibiotic resistance cassette was replaced with *tetR*. Into this strain (designated RM153*lic2A*+t), we introduced an in-frame deletion of the repeats in *lex2A* by transformation with pB*lex2*Δ5′-GCAA-3′k (4) and selection for kanamycin resistance. The resultant strain, RM153*lic2A*+*lex2*+k, was then transformed with pU*lgtC*Δ5′-GACA-3′*lacZ*, in which *lacZ* is fused to the 5′ end of *lgtC* (Fig. 2C). The disrupted *lgtC* gene of a *lacZ*-positive transformant of RM153*lic2A*+*lex2*+k was rescued by transformation with pU*lgtC*Δ5′-GACA-3′, which carries an in-frame deletion of the repeat tract of *lgtC* (Fig. 2B). Briefly, pU*lgtC*Δ5′-GACA-3′ and pU*lgtC*Δ5′-GACA-3′*lacZ* were created as follows. A primer (LGTC3268R) was designed to include BglII and XhoI sites followed by sequence complementary to sequence upstream of and including the initiation codon of *lgtC* (Fig. 2A). Another primer, LGTCRPS1 (5′-TCGAGATCTACGGACT GTCAGTCAGACAATG-3′), was designed to include a BglII site followed by sequence immediately downstream of the repeat tract (Fig. 2A). Plasmid pBSHI, incorporating the region encompassing *lgtC* of RM153 (Fig. 2A), was used as a source of DNA. The region upstream of *lgtC* was amplified using primers LGTC3268R (5′-TGACTGACAGTCCGTCCGTCA GATCTCGAGACGCGTTCATGAAATTATCTCTGATT-3′) and 6024J (5′-TCGTAAGGAATAAGCGTG-3′), and the downstream region was amplified using primers LGTCRPS1 and T7 (Fig. 2A) (23). These fragments were cloned separately and then fused using the BglII site and other appropriate cloning sites in the vector plasmids to generate pUClgtCΔ5′-

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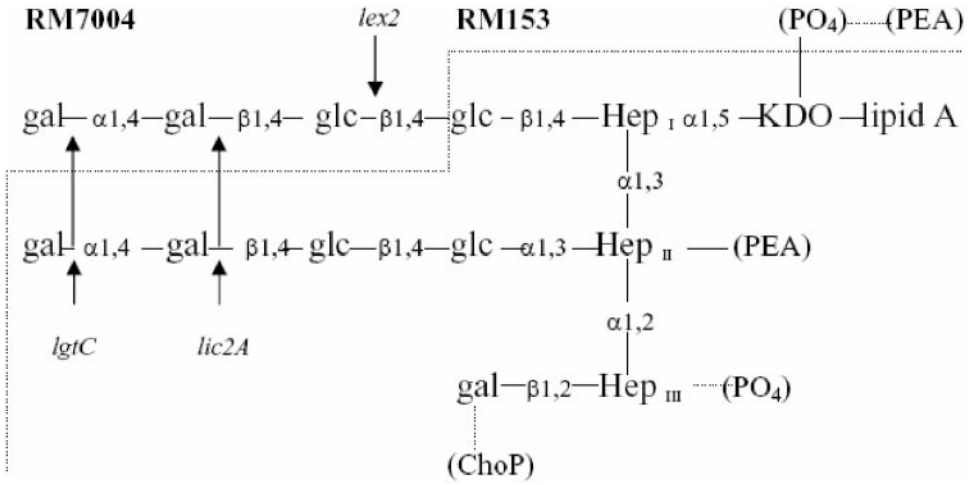

FIG. 1. Schematic representation of the structure of the fully extended LPS glycoforms of *H. influenzae* type b strains RM153 (17) and RM7004 (18). The LPS of strain RM7004 is similar in structure to that of RM153 except for an additional extension from the glucose attached to the first heptose due to *lex2A* being in frame in this strain, as opposed to out of frame in RM153 (4). Represented in the LPS structure are the following: Hep, LD-heptose; Glc, glucose; Gal, galactose; P, phosphate; ChoP, phosphorylcholine; PEA, phosphoethanolamine. A dotted line indicates the substituents that are variably present. The places of action of *lex2*, *lgtC*, and *lic2A* are indicated. The proximal-to-distal heptoses are numbered I to III accordingly.

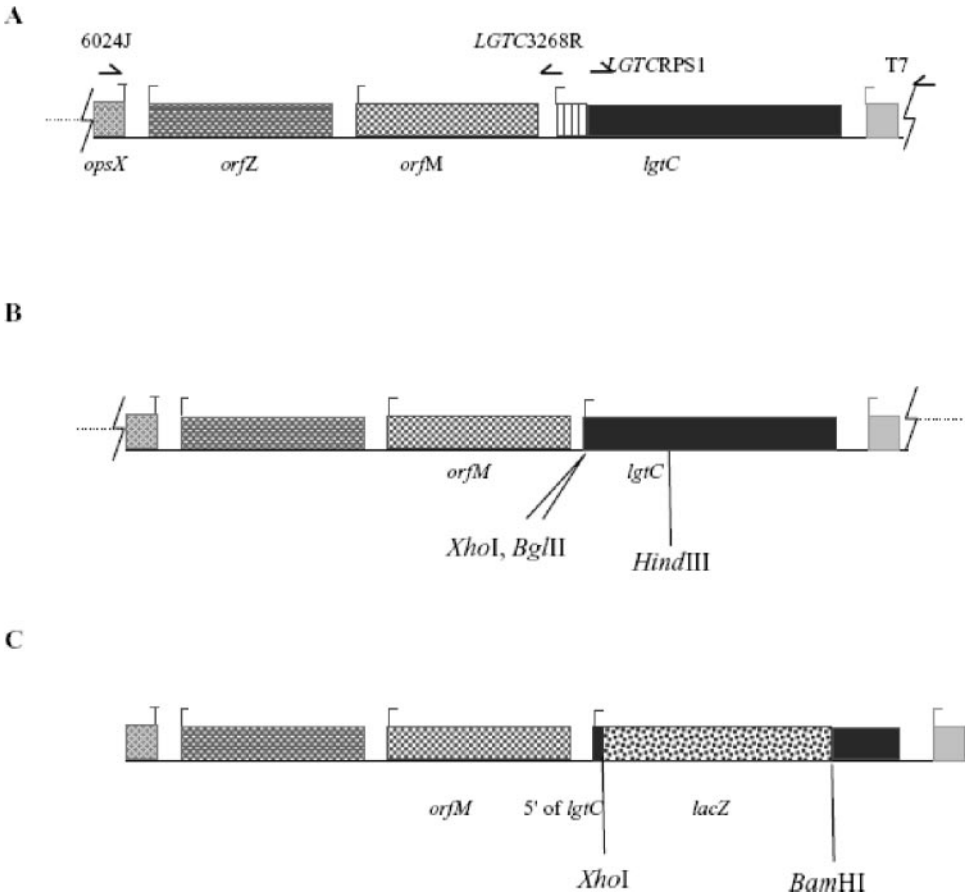

FIG. 2. Schematic representation of plasmids pBSH1 (A), pUC*lgtC*Δ5′-GACA-3′ (B), and pUC*lgtC*Δ5′-GACA-3′*lacZ* (C). The primers and their orientations and the restriction sites used to generate these constructs are shown. The open reading frames are represented by open boxes, and initiation codons are represented by ⌈. The repeats of *lgtC* are shown as a vertically striped box.

TABLE 1. Numbers of CFU of strains RM153*lic2A*+t*lgtC*+*lex2*+k and RM153*lic2A*+t*lgtC*+*lex2*−k per ml of blood extracted from the tail vein of rats

Rat	No. of cells/ml of strain:		Competition ratio[b]
	RM153*lic2A*+ t*lgtC*+*lex2*+k	RM153*lic2A*+ t*lgtC*+*lex2*−k	
1	4.00×10^5	3.20×10^{5a}	1.25
2	7.67×10^7	8.20×10^{6a}	9.35
3	2.54×10^7	1.48×10^{6a}	17.16
4	9.15×10^7	1.23×10^{7a}	7.44
5	1.60×10^5	4.00×10^{4a}	4.00
6	1.33×10^7	2.04×10^{6a}	6.52
7	4.53×10^6	1.08×10^{5a}	41.94
8	1.24×10^6	4.00×10^{4a}	31.00
9	5.00×10^6	5.60×10^{5a}	8.93
10	1.27×10^7	7.60×10^{5a}	16.71
11	1.12×10^6	1.20×10^{5a}	9.33
12	1.12×10^6	4×10^{4a}	28.00
13	6.14×10^4	1.25×10^{5a}	0.49
14	9.60×10^5	4.80×10^{5a}	2.00
15	2.40×10^5	6.12×10^{4a}	3.92
16	1.60×10^{4a}	1.35×10^5	0.12
17	8.40×10^{5a}	6.00×10^5	1.40
18	3.28×10^{6a}	3.20×10^6	1.03
19	4.00×10^{5a}	2.00×10^5	2.00
20	3.64×10^{4a}	1.03×10^5	0.35
21	2.26×10^{5a}	4.44×10^4	5.09
22	4.22×10^{5a}	1.06×10^6	0.40
23	2.00×10^{5a}	1.60×10^5	1.25
24	1.24×10^{6a}	6.40×10^5	1.94
25	3.27×10^{5a}	6.40×10^4	5.11
26	2.64×10^{6a}	4.64×10^6	0.57
27	4×10^{4a}	7.60×10^5	0.05
28	1.42×10^{7a}	5.35×10^7	0.27

[a] Strains carrying the streptomycin resistance gene.
[b] Competition ratio, value obtained by dividing the number of CFU of strain RM153*lic2A*+t*lgtC*+*lex2*+k by that for strain RM153*lic2A*+t*lgtC*+*lex2*−k.

TABLE 2. Negative-ion electrospray-mass spectrometry analysis and proposed composition for the major components of O-deacylated LPS of strain RM153*lic2A*+t*lgtC*+*lex2*+k

Mol wt	Composition[a]	Relative abundance
2,601	Lipid A, Kdo-P, 3Hep, PEA, 4Hex	0.20
2,724	Lipid A, Kdo-PPEA, 3Hep, PEA, 4Hex	0.15
2,763	Lipid A, Kdo-P, 3Hep, PEA, 5Hex	1.00
2,886	Lipid A, Kdo-PPEA, 3Hep, PEA, 5Hex	0.80
2,925	Lipid A, Kdo-P, 3Hep, PEA, 6Hex	0.40
3,048	Lipid A, Kdo-PPEA, 3Hep, PEA, 6Hex	0.40
3,087	Lipid A, Kdo-P, 3Hep, PEA, 7Hex	0.15
3,210	Lipid A, Kdo-PPEA, 3Hep, PEA, 7Hex	0.10
3,249	Lipid A, Kdo-P, 3Hep, PEA, 8Hex	0.07
3,372	Lipid A, Kdo-PPEA, 3Hep, PEA, 8Hex	0.08
3,411	Lipid A, Kdo-P, 3Hep, PEA, 9Hex	0.03
3,534	Lipid A, Kdo-PPEA, 3Hep, PEA, 9Hex	0.02

[a] Kdo-P, 2-keto-3-deoxyoctulosonic acid replaced by monophosphate; Hep, *LD*-heptose; PEA, phosphoethanolamine; Hex, hexose; Kdo-PPEA, 2-keto-3-deoxyoctulosonic acid replaced by pyrophosphoethanolamine.

GACA-3′ (Fig. 2B). Plasmid pUC*lgtC*Δ5′-GACA-3′*lacZ* was created by replacing the XhoI-HindIII fragment of pUClgtCΔ5′-GACA-3′ with a XhoI-BamHI fragment of pGΔZMCS (3), incorporating *lacZ* without an initiation codon. Transformants constitutively expressing all three loci were selected by their ability to react with MAb 4C4 and designated RM153*lic2A*+t*lgtC*+*lex2*+k.

To obtain organisms expressing one digalactoside only, *lex2* was disrupted in a strain in which *lic2A* and *lgtC* expression was constitutive. This strain was constructed as follows. RM153*lic2A*+t was transformed with a construct carrying *lgtC* disrupted by *kanR* (10), and then *lgtC* expression was restored by transformation with pUC*lgtC*Δ5′-GACA-3′ (Fig. 2B). Transformants were selected for their restored ability to react with MAb 4C4. Finally, *lex2* was disrupted by transformation with pDL2 (4), and transformants were selected by growth on kanamycin. The resultant strain was designated RM153*lic2A*+t*lgtC*+*lex2*−k. To confirm that the loci lacked repeats and were maintained constitutively in frame in these strains, the repeat region was amplified by PCR and sequenced using appropriate primers. Note that a plus in a strain designation indicates that the relevant gene lacks repeats and is therefore constitutively expressed. A minus in a strain designation indicates a disrupted gene that is not expressed. The designations "t" and "k" indicate that selection for these genes was dependent upon tetracycline or kanamycin antibiotic resistance cassettes, respectively.

The magnitude of bacteremia for organisms expressing two digalactosides, compared to that for organisms expressing one digalactoside, was investigated in infant rats to assess the role of galα-1-4 galβ expression in intravascular survival in an in vivo model.

Prior to mixed infection of infant rats, isogenic streptomycin-resistant (Str^r) mutants were obtained for RM153*lic2A*+t*lgtC*+*lex2*+k and RM153*lic2A*+t*lgtC*+*lex2*−k. Use of these mutants permitted discrimination between these strains following challenge of the rats with a mixed inoculum by plating blood cultures from infected animals onto medium with or without streptomycin. The strains were transformed with chromosomal DNA of a spontaneous Str^r clone of RM153. The resultant Str^r isogenic strains showed no alteration in their LPS compared to that of their progenitors and maintained the deletions in the repeat tracts. Each of the four recombinant strains showed no difference in growth rate (data not shown).

Twenty-eight 5-day-old Sprague-Dawley rats were each given mixed infections by the intraperitoneal route (9): 15 rats were inoculated with approximately 150 CFU of RM153*lic2A*+t*lgtC*+*lex2*+k and 150 CFU of Str^r RM153*lic2A*+t*lgtC*+*lex2*−k, while 13 received 150 CFU of each strain in which the antibiotic resistance marker was switched so that the former strain carrying the Str^r marker was now streptomycin sensitive. Forty-eight hours after inoculation, significantly higher numbers of bacteria expressing two digalactosides than of the single-digalactoside-expressing strain were recovered from tail vein blood from infant rats, as determined by the nonparametric Mann-Whitney U test (P value, 0.0287) (Table 1). The paired data in Table 1 were also used to derive a competition ratio for bacteria expressing two digalactosides versus one digalactoside (ratio determined by dividing the number of CFU of strain RM153*lic2A*+t*lgtC*+*lex2*+k by that of strain RM153*lic2A*+t*lgtC*+*lex2*−k). The average ratio was higher when the single-digalactoside-expressing strain was streptomycin resistant, although this antibiotic resistance mutation could be associated with a small fitness deficit. However, as the average ratio was greater than 1 in both groups of animals, this

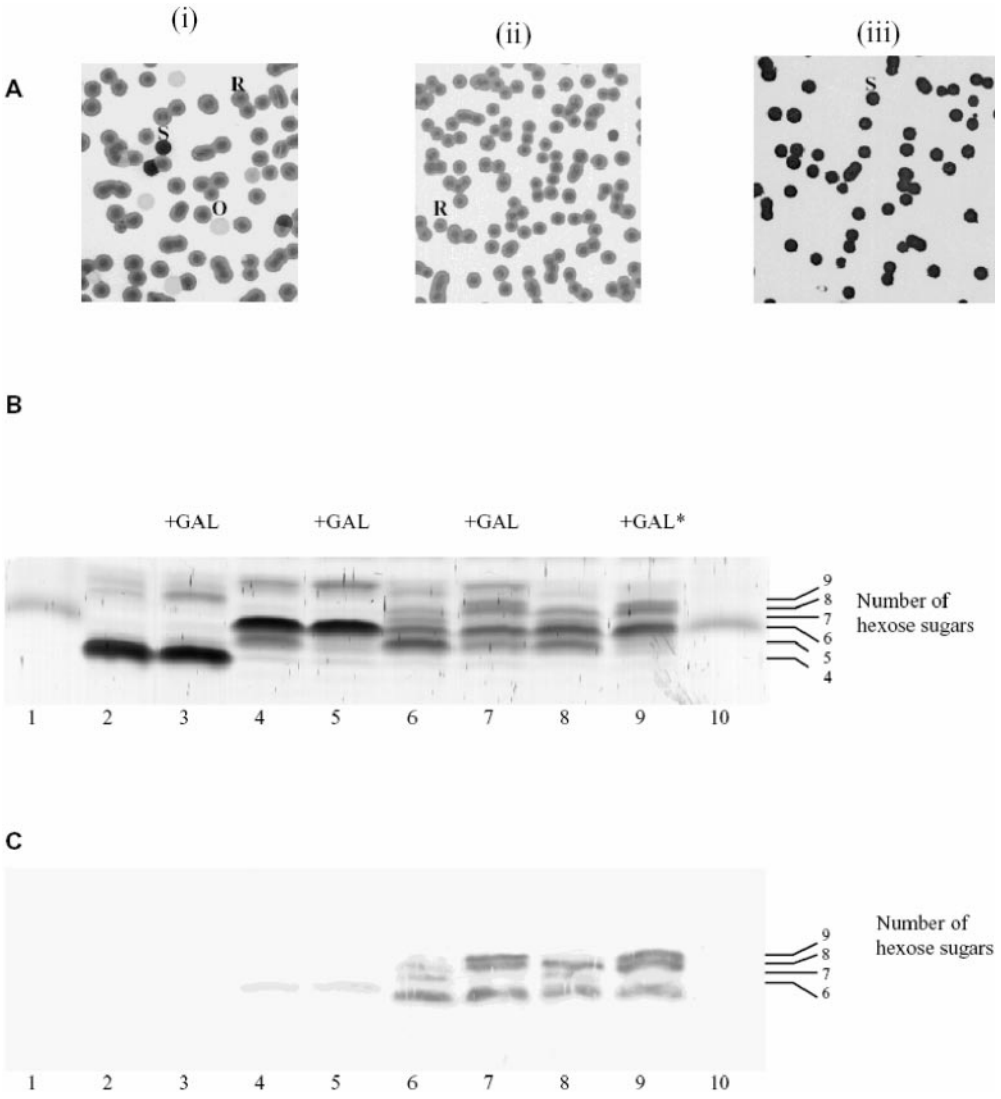

FIG. 3. Analysis of the LPS of strains RM153, RM153*lic2A*+t*lgtC*+*lex2*−k and RM153*lic2A*+t*lgtC*+*lex2*+k by immunoblotting and electrophoresis. (A) Colonies of strains (i) RM153, (ii) RM153*lic2A*+t*lgtC*+*lex2*−k, and (iii) RM153*lic2A*+t*lgtC*+*lex2*+k were tranferred to nitrocellulose membranes and incubated with MAb 4C4 (21). Intermediate reacting (R), strong (S,) and negative or off (O) MAb 4C4 phenotypes are indicated. (B and C) PAGE analysis of bacterial lysates (22) visualized by silver staining (20) (B) and by transfer to nitrocellulose for incubation with MAb 4C4 (24) (C) from *H. influenzae* strains grown in the absence and presence (+GAL) of galactose. Lanes 1 and 10, standard protein markers; lanes 2 and 3, RM153; lanes 4 and 5, RM153*lic2A*+t*lgtC*+*lex2*−k; lanes 6 and 7, RM153*lic2A*+t*lgtC*+*lex2*+k; lanes 8 and 9, RM7004. The number of hexose sugars predicted to be present in each LPS glycoform (band) represented is indicated.

result provides further evidence that increased digalactoside expression leads to enhanced virulence.

In order to substantiate the data from the animal studies, we investigated the LPS expression patterns of the two isogenic test strains used to infect rats. These strains had been cultured in 1% galactose, which was previously shown to encourage the incorporation of galactose into the LPS (16).

First, the reactivity of colonies of the test strains was investigated using MAb 4C4 (21). RM153*lic2A*+t*lgtC*+*lex2*−k showed the reactive (R) phenotype only, suggesting the expression of a single digalactoside, while RM153*lic2A*+t*lgtC*+*lex2*+k demonstrated the strongly reacting (S) phenotype only, indicative of the expression of two digalactosides (Fig. 3A) as documented for RM7004 (4).

Second, duplicate sodium dodecyl sulfate-polyacrylamide gels were run with whole-cell lysates (14, 22). One gel was stained with silver (20), while the other was blotted onto a membrane and reacted with MAb 4C4 (24). The predominant band of LPS from RM153*lic2A*+t*lgtC*+*lex2*−k showed migration equivalent to that expected for glycoforms comprising six hexose sugars and showed staining, albeit weak, with MAb 4C4, thus confirming the presence of a single digalactoside (Fig. 3). Strain RM153*lic2A*+t*lgtC*+*lex2*+k showed additional bands; each incremental band size is considered to represent an additional hexose (9). A band corresponding to the presence of nine hexose sugars and reactive with MAb 4C4 was detected for RM153*lic2A*+t*lgtC*+*lex2*+k, as for RM7004, which typically contains each of the three loci in frame and

expresses two digalactoside-containing oligosaccharides (Fig. 3) (18).

Finally, the presence of nine hexose sugars in RM153*lic2A*+ t*lgtC*+*lex2*+k was confirmed by electrospray-ionization mass spectrometry (Table 2) (15). Compositional sugar analysis (15) indicated a 5:4 ratio of galactose to glucose, analogous to the fully extended glycoform of RM7004.

In conclusion, variants capable of expressing two rather than one digalactoside were more virulent in vivo. These data provide strong indications of the importance of the galα-1-4 galβ digalactoside structure of LPS in virulence, but some caveats must be considered in interpreting these results. First, the relevance of findings from an infant rat model for humans is of course open to question, especially since host cells in the rat express digalactosides in which the galactose linkage is α1-3β (19). In contrast, humans express an α1-4β digalactoside identical to that found on the LPS of *H. influenzae*. Another important potentially confounding factor is the extent of LPS sialylation. We cannot exclude the possibility that the differences in virulence observed could be attributed, at least in part, to differences in sialylated LPS glycoforms (11), or indeed other unrecognized but relevant and subtle differences in LPS structure that are independent of digalactoside expression.

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