Biosynthesis of Cryptic Lipopolysaccharide Glycoforms in *Haemophilus influenzae* Involves a Mechanism Similar to That Required for O-Antigen Synthesis

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It is generally thought that mucosal bacterial pathogens of the genera *Haemophilus*, *Neisseria*, and *Moraxella* elaborate lipopolysaccharide (LPS) that is fundamentally different from that of enteric organisms that express O-specific polysaccharide side chains. *Haemophilus influenzae* elaborates short-chain LPS that has a role in the pathogenesis of *H. influenzae* infections. We show that the synthesis of LPS in this organism can no longer be as clearly distinguished from that in other gram-negative bacteria that express an O antigen. We provide evidence that a region of the *H. influenzae* genome, the *hmg* locus, is involved in the synthesis of glycoforms in which tetrasaccharide units are added en bloc, not stepwise, to the normal core glycoforms, similar to the biosynthesis of an O-antigen.

Haemophilus influenzae is an important cause of human disease worldwide. Lipopolysaccharide (LPS) is the major glycolipid of the bacterial cell wall and in *H. influenzae* is a target for host immune responses and influences both the commensal and pathogenic behavior of the organism (11). *H. influenzae* LPS comprises a membrane-anchoring lipid A moiety (endotoxin) linked to oligosaccharide chains that extend from the bacterial cell surface (Fig. 1). LPSs from a number of different strains have been analyzed and shown to be composed of a common L-glycero-D-manno-heptose-containing inner-core trisaccharide unit attached to the lipid A moiety via a phosphorylated 2-keto-3-deoxyoctulosonic acid residue (6, 12, 19–25, 27–29). Each of the heptose (Hep) residues can provide a point for the addition of a hexose (Hex) residue, which in turn can lead to oligosaccharide chain extensions. The nature of the oligosaccharide chains and the degree to which they contain noncarbohydrate substituents show intra- and interstrain variation that can have profound effects upon the virulence of the organism. Several of the surface-exposed epitopes of *H. influenzae* LPS are subject to high-frequency phase variation (11, 14, 34), an adaptive mechanism that is advantageous for survival of bacteria confronted by the differing microenvironments and immune responses of the host. It is perceived that *H. influenzae* LPS, as well as short-chain LPSs from other species, is fundamentally different from other gram-negative LPSs that contain an O antigen. This distinction has led many investigators to designate the glycolipid of *H. influenzae* as a lipooligosaccharide (8).

In recent studies, it was found that cryptic LPS glycoforms that are more complex than those previously characterized were apparent under altered culture conditions (5, 10, 16, 31). Specifically, when *H. influenzae* strains were grown on solidified brain heart infusion (BHI) medium enriched with sialic acid, we detected units of four sugars that were incorporated into LPS (Fig. 1). We propose that these tetrasaccharides, one of which includes sialic acid, are synthesized and incorporated into LPS via an isoprenoid lipid carrier; this mechanism is the principle for O-antigen synthesis in other bacteria. The genetic basis for the biosynthesis of these units has been investigated and is described here.

MATERIALS AND METHODS

Bacterial strains and media. The *H. influenzae* strains used in this study were the serotype b disease isolate RM153 (Eagan) (13) and the serotype d-derived strain RM118 (Rd), the strain for which the complete genome sequence has been obtained. Nontypeable strains were obtained from Juhani Eskola as part of a Finnish Otitis Media Cohort study and are mainly isolates obtained from the middle ear. The 25 representative nontypeable *H. influenzae* isolates used in this study were generally diverse based on their position within a phylogenetic tree that had been used to select them (4). *H. influenzae* strains were routinely cultured on solidified brain heart infusion medium supplemented with 10% Levinthal's reagent and, when appropriate, Neu5Ac (25 µg ml⁻¹). For selection following transformation, kanamycin (10 µg ml⁻¹) was included in the growth medium.

Investigation of the genetic basis for cryptic LPS glycoform synthesis. More than 40 LPS-related genes have been identified and investigated in our previous analysis of the *H. influenzae* genome sequence (9, 13) and other studies (unpublished data). Mutations in these genes were established in strains RM118 and RM153. Plasmid constructs containing cloned LPS biosynthesis genes (Table 1) disrupted by insertion of antibiotic resistance cassettes were transformed into these strains by the method of Herriott et al. (7). Mutant strains were confirmed by PCR with locus specific primers as described previously (9, 12, 13). PCR amplification was carried out in buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8), 0.01% (wt/vol) gelatin, and 2.5 mM MgCl₂. Thirty cycles of PCR were performed, with each cycle consisting of 1-min periods of denaturation at 94°C,

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FIG. 1. Structures of LPSs from *H. influenzae* strains used in this study. Each heptose of the inner-core trisaccharide can provide the point for oligosaccharide extensions. Represented in the panels are the published fully extended LPS structures for strains RM118 (28) and RM153 (23, 30) and the structures determined for strain RM118 following growth in the presence of sialic acid (5, 10). Sugars to the left of the vertical dashed lines in the lower panel are those added when sialic acid is present; Neu5Ac competes with the addition of the α -D-Galp to the lactose extended from the distal heptose residue (10). Represented in the LPS structure: Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; Glc, D-glucose; Gal, D-galactose; GalNAc, N-acetylgalactosamine; PEtn, phosphoethanolamine; P, phosphate; PCho, phosphocholine; LipA, lipid A. Acetate and glycine can substitute at various positions on the inner and outer cores.

annealing at 50°C, and extension at 72°C. PCR amplification was similarly used to investigate the presence and organization of the *hmg* locus in different strains by using primers designed against the Rd genome sequence and the DNA sequence obtained from nontypeable *H. influenzae* strain 176. For PCR amplification of *rffG*, oligonucleotide primers glnA1 (5'-TTTTGTCCAGAGCTG AAT) and pepB2 (5'-CGACCTTCGCGCATCAGTATT) were used. DNA sequences were determined and analyzed as previously described (4).

Analysis of lipopolysaccharide. Isolated LPS was analyzed by Tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and silver staining (Quicksilver; Amersham Pharmacia). Immunological analysis of LPS with monoclonal antibodies (MAbs) was performed as described previously (13). MAb LLA5 was produced by immunization of mice with killed whole cells of strain RM118 in which the *lic1* and *lpsA* genes were inactivated (M. Gidney, personal communication).

LPS structural analysis. Purified LPS was prepared from bacteria grown on BHI plates supplemented with Neu5Ac and was analyzed by electrospray mass spectrometry (ES-MS) following O deacylation or delipidation of the LPS sample as described previously (5, 9, 23, 28).

RESULTS

Growth of *H. influenzae* in the presence of exogenous sialic acid results in the synthesis of cryptic LPS glycoforms. The *H. influenzae* strains for which there is the most detailed knowledge of LPS structure and the genes required for its synthesis are the serotype b strain RM153 (Eagan) (13, 23, 30) and the serotype d-derived strain RM118 (Rd) (9, 28). This structural detail had been obtained exclusively for LPS isolated from bacteria cultured in liquid. Following growth of these two independent *H. influenzae* strains on solid medium containing

sialic acid, there was a substantial increase in higher-molecular-weight glycoforms, identifiable by sodium dodecyl sulfate-PAGE, compared to growth in liquid or with no added sialic acid (Fig. 2A). Notable was the incremental increase in the molecular size of the largest LPS glycoform as determined by gel electrophoresis. These higher-molecular-weight glycoforms were estimated to be equivalent to the addition of a three- to four-sugar unit to the lower-molecular-weight bands, based upon our experience. Routinely, this increase in size was more prominent for LPS derived from strain RM153 than for that from strain RM118. Treatment with neuraminidase indicated that these glycoforms were sialylated (Fig. 2A and F).

Mass spectrometry has established that a sialic acid-containing tetrasaccharide unit was added to four-hexose-containing glycoforms of strain RM118 following growth on BHI plates with added sialic acid (5). In the present study, we identified a similar tetrasaccharide unit in the LPS from strain RM153 by MS (Fig. 3; Table 1). The MS data are consistent with the tetrasaccharide unit consisting of α -Neu5Ac-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 4)- β -D-GlcpNAc-(1 $\rightarrow$ 3)- β -D-Galp- (SiaT in Table 1), the same structure identified in strain RM118 extended from the β -D-Glcp- attached to the first Hep of the inner core (Fig. 1 and 3; Table 1) (5). The MS spectrum for strain RM153 is shown in Fig. 4. Recent studies of cryptic glycoforms in LPS derived from nontypeable *H. influenzae* strains identified a related structure, (PEtn $\rightarrow$ 6)- α -D-GalpNAc-(1 $\rightarrow$ 6)- β -D-Galp-

TABLE 1. Negative-ion ES-MS data and proposed compositions of major LPS glycoforms of *H. influenzae* strains RM118 and RM153 and selected RM153 mutants^a

Strain	Glycoform	[M - 3H] ³⁻	[M - 2H] ²⁻	<i>M_r</i>	Proposed composition ^b			Reference
					R1	R2	R3	
RM118	4 Hexose ^b	962.0		2889.0	PCho	H	Globotriose	5
	4 Hexose + SiaT ^b	1179.7		3542.1	H	H	Globotriose	5
	4 Hexose + SiaT ^b	1235.0		3708.0	PCho	H	Globotriose	5
	3 Hexose ^c	523.5	785.5	1573.5	PCho	H	Lactose	This study
	4 Hexose ^c	645.1	968.1	1938.1	PCho	H	Globotriose	This study
	3 Hexose ^c + GaT	807.7	1212.0	2426.1	PCho	H	Lactose	This study
RM153 ^b	4 Hexose	905.5		2719.5	H	Cellobiose	Gal	This study
	4 Hexose + SiaT ^d	1180.5		3543.3	H	Cellobiose	Gal	This study
RM153 <i>wbaP</i> ^b	4 Hexose	907.5	1361.5	2725.3	H	Cellobiose	Gal	This study
RM153 <i>orfE</i> ^b	4 Hexose	905.5		2719.5	H	Cellobiose	Gal	This study
RM153 <i>wecA</i> ^b	4 Hexose	907.5	1361.5	2725.3	H	Cellobiose	Gal	This study
	4 Hexose + SiaT ^d	1180.5		3543.3	H	Cellobiose	Gal	This study

^a Unit residue (incremental mass): Gal, D-galactopyranose (162.15); PEtn, phosphoethanolamine (123.05), PPEtn, phyrophosphoethanolamine (203.05), PCho, phosphocholine (165.05); KDO, 3-deoxy, 2-keto octulosonic acid (220.16), lactose, β-D-Galp-(1→4)-β-D-Glcp- (324.30); globotriose, α-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-GlcpNac-(486.45); cellobiose, β-D-Glcp-(1→4)-β-D-Glcp- (324.30); SiaT, α-Neu5Ac-(2→3)-β-D-Galp-(1→4)-β-D-GlcpNac-(1→3)-β-D-Galp-(818.54); GaT, PEtn→6)-α-D-GalpNac-(1→6)-β-D-Galp-(1→4)-β-D-GlcpNac-(1→3)-β-D-Galp-(854.75); *anhydro*-KDO (222.20).

^b ES-MS of O-deacylated LPS samples indicated ions from both the KDO-PPEtn and KDO-P series of glycoforms (see Fig. 4). Only data for the KDO-PPEtn series of glycoforms are given. R1, R2, and R3 are substitutions as illustrated in Fig. 3.

^c Hexose* refers to core oligosaccharide with *anhydro*-KDO at reducing end, prepared by mild acid hydrolysis (28).

^d Quadruply charged ions ([M - 4H]⁴⁻) were observed at *m/z* 884.5 for 4 Hexose + SiaT glycoforms.

(1→4)-β-D-GlcpNac-(1→3)-β-D-Galp-, which likewise appears to be synthesized only as a complete tetrasaccharide unit (Gidney, personal communication). A MAAb, LLA5, which specifically recognizes a terminal epitope of this oligosaccharide showed that both strains RM118 and RM153 produce small amounts of glycoforms with this alternative (PEtn→6)-α-D-GalpNac-containing tetrasaccharide unit, in addition to the more prevalent sialylated form described above (Gidney, personal communication). Further mass spectral analysis confirmed the presence of the (PEtn→6)-α-D-GalpNac-containing glycoform in the LPS from strain RM118 (GaT in Table 1). Both the sialic acid and (PEtn→6)-α-D-GalpNac tetrasaccharide unit-containing glycoforms evidently migrate to equivalent positions upon electrophoresis. The nonsialylated glycoform can be visualized as the faint upper band remaining after neuraminidase treatment of RM153 wild-type LPS (Fig. 2F).

Tetrasaccharide units of cryptic LPS glycoforms are synthesized en bloc and require genes located in a distinct chromosomal locus (*hmg*). The genetic basis for the synthesis of these tetrasaccharide units was investigated by establishing mutations in LPS-related genes of known or unknown function in strains RM118 and RM153 (Table 2). The identification, cloning, and mutagenesis of these genes have been described previously (9, 13). Putative LPS biosynthesis genes comprised over 40 candidates, of which around 20 have been assigned functions in the synthesis of the LPS oligosaccharides of strains RM153 (13) and RM118 (9). Although our screen focused upon genes of unknown function, several LPS genes of characterized function were included to exclude them from having

a direct role in the synthesis of the tetrasaccharide units and to further confirm the point of attachment in the LPS of the strains under study. The LPSs from the RM118 and RM153 mutants grown on solid medium in the presence of sialic acid were isolated and analyzed by PAGE for comparison with the wild type (Fig. 2B to E). In each case where a higher-molecular-weight glycoform was identified, neuraminidase treatment showed it to contain sialic acid; examples are shown in Fig. 2F. In no case did mutation in a gene required for the biosynthesis of the sialic acid-containing cryptic glycoforms cause merely a truncation of the tetrasaccharide unit, a finding consistent with the hypothesis that the unit was added en bloc. Most of the genes involved cluster in a single chromosomal locus, hereafter referred to as the *hmg* (for high-molecular-weight glycoform) locus. Seven of the 10 contiguous open reading frames (ORFs) (Fig. 5) showed homology to gene sequences in the public database that are associated with O-antigen or polysaccharide synthesis in other gram-negative organisms (13, 17, 33). The genes of the *hmg* locus were atypical in that previous studies had shown that most *H. influenzae* LPS biosynthetic genes were not clustered but were scattered throughout the genome (13). Flanking the *hmg* locus are the genes encoding glutamine synthetase (*glnA*) and peptidase B (*pepB*). A number of the genes in the *hmg* locus share homology with glycosyltransferases required to link sugars, and others have homology to gene products involved in sugar unit transport or O-antigen unit addition. HI0866 has homology to *wzz* in *Actinobacillus*, a gene functioning as an O-antigen chain length determinant in other organisms. HI0867 is a close homologue of another *H. influ-*

FIG. 2. Profiles of LPSs isolated from strains RM153 and RM118 and their respective mutants, after gel electrophoresis and staining with silver. Panel A shows the profile for strain RM118 after growth in broth or on plates with added sialic acid, before (–) and after (+) neuraminidase treatment. Panels B and C correspond to strain RM153, and panels D and E correspond to strain RM118, all grown on plates with added sialic acid. The gene mutated in each strain is given below the relevant profile (9, 13); wt represents wild type. Panel F shows the LPS profiles before (–) and after (+) neuraminidase treatment of strain RM153 and representative mutants. Shown by an arrow on each panel is the position of the glycoforms expressing the sialic acid-containing tetrasaccharide unit. The other prominent lower-molecular-weight bands correspond to the LPS glycoforms previously characterized for each strain.

enzae LPS-related gene, the first ORF of the *lsg* locus (*lsgA*) (1), and has homology to transporters for capsule repeat units in other organisms. HI0867 might have some role as a flippase in the transport of the tetrasaccharide units across the cytoplasmic membrane. HI0868 and HI0869 have homology to number of glycosyltransferases predominantly involved with O-antigen synthesis or capsule polysaccharide biogenesis in other bacteria. HI0870 and HI0870.1 share no significant homology with any other database sequence. HI0871 has significant homology with *lst* of *Haemophilus ducreyi*, a gene encoding an LPS sialyltransferase in this organism, and has been previously termed *orfY* (13) and *siaA* (16). HI0872 has significant homology with the *wbaP* (formerly *rjbP*) genes, which are crucially required to initiate O-antigen unit formation in other gram-negative bacteria. HI0873 has significant homology with the *rffG* and *rmlB* (formerly *rjbB*) genes, which are required for biosynthesis of the sugar rhamnose in other organisms. HI0874 has low homology with *waaL* which encodes a ligase for the addition of O-type sugar units to the growing LPS molecule. Mutation of the ORFs HI0867, HI0870, and HI0873 had no apparent effect upon LPS biosynthesis in the two primary strains investigated, and an interruption of HI0866 only partially reduced the amount of LPS glycoforms containing the tetrasaccharide unit in strain RM118. The average base composition (G+C content) of the ORFs of the *hmg* locus is just under 30%, which is significantly different from the *H. influenzae* strain Rd genome average of 38.2%.

The observations made from gel electrophoresis were confirmed by structural analysis of LPSs isolated from several representative mutant strains derived from RM153. MS analysis of O-deacylated LPS indicated that while the wild-type strain clearly contained significant levels of glycoforms containing the tetrasaccharide units, these glycoforms were absent from strains in which the *wbaP* or *orfE* gene had been mutated (Table 1; Fig. 4). No strain analyzed showed any evidence for inclusion of intermediates in the biosynthesis of these tetrasaccharide units into the LPS. Only the prominent lower-molecular-weight bands evident on the acrylamide gels (Fig. 2) could be detected in the LPSs of these mutant strains.

A preliminary analysis of mutant strains by using MAb LLA5 indicated that a similar pattern of genes, but not includ-

ing *siaA*, was required for expression of the (PEtn→6)-α-D-GalpNAc-containing unit (data not shown).

In each case where a mutant strain has been made for a gene in the *hmg* locus, the relevant reading frame has been disrupted by insertion of a kanamycin resistance cassette. Due to the close proximity of the contiguous reading frames within the locus, some polar effects of mutation on downstream genes could not be ruled out in all cases. Preliminary analysis of transcripts from the *hmg* locus by reverse transcription-PCR has confirmed the expression of each of the reading frames (data not shown). Some low level of cotranscription could be detected for the 3' portion of, but not the full-length, HI0871 with HI0872. The regulation of expression of the *hmg* locus is evidently somewhat complex, and further investigation will be required to establish the full pattern of genes expressed under relevant growth conditions.

Cryptic glycoforms are synthesized in a majority of, but not all, *H. influenzae* strains. The organization of the *hmg* locus was investigated by PCR and Southern analysis in a range of nontypeable *H. influenzae* clinical isolates from otitis media and strains RM118 and RM153. A striking finding was that the entire *hmg* locus was absent in 40% of the strains tested. There was an exact correlation between the presence of the *hmg* locus and the capacity to make these cryptic glycoforms, based on gel electrophoresis and immunoblotting. All strains expressing these glycoforms had the *hmg* locus. Sixteen of 17 strains possessing the locus reacted with MAb LLA5, and in 11 of these 17 strains, the inclusion of apparently similar-sized units in the glycoforms was visualized by gel electrophoresis. Some of the remaining six strains produce relatively larger LPS glycoforms, which may include other oligosaccharide extensions from the inner core that compete with attachment of the tetrasaccharide unit.

PCR analysis indicated some difference in the central portion of the locus between strain RM118 and all other strains tested (data not shown). A representative nontypeable *H. influenzae* strain was selected, which contains the *hmg* locus and which expresses higher-molecular-weight LPS glycoforms containing the tetrasaccharide units that are lost when representative genes of the *hmg* locus are mutated (Fig. 5B). DNA sequence analysis of the central 5.4-kb portion of the *hmg* locus from nontypeable *H. influenzae* strain 176 (4), deposited as EMBL AJ579378, showed 80% overall sequence similarity to the equivalent section of the strain Rd genome sequence. Some regions within this sequence indicated more significant sequence divergence. The putative sialyltransferase gene, *siaA*, was 64% homologous to HI0871 (*orfY*), and the 1.3 kb of upstream sequence was only 42% homologous to that in strain Rd. The DNA sequence found in strain 176 was much more related to that reported for *siaA* and the surrounding DNA for serotype b strain A2 (16). The 2.1 kb of DNA sequence upstream of HI0871 indicated some different gene organization when the nontypeable *H. influenzae* and A2 sequences were compared with that of strain Rd. HI0869 is truncated in strain Rd, and the longer gene sequence in other strains includes the region designated HI0870 in the strain Rd genome sequence (Fig. 5). There are two putative small ORFs between HI0869 and *siaA* in the nontypeable *H. influenzae* sequence which differ from HI0870.1 given in the Rd genome sequence. PCR analysis using Rd- and nontypeable *H. influenzae*-specific prim-

FIG. 4. ES-MS spectra for the O-deacylated LPSs obtained from strains RM153 (panel A), RM153 *wecA* (panel B), RM153 *wbaP* (panel D), and RM153 *orfE* (panel E) grown on plates supplemented with Neu5Ac. Inset panel C is a precursor-ion MS spectrum from strain RM153 for *m/z* 290, indicative of sialylated glycoforms. The regions of the spectra corresponding to the ions from the lower-molecular-weight glycoforms (4Hex) and the higher-molecular-weight sialylated glycoforms (4Hex + SiaT) are indicated. The spectra are somewhat heterogeneous due to the presence of salt (sodium and potassium) and water adducts; only the nonadducted ions are labeled in the spectra.

ers designed against the respective sequence indicated that the organization and sequence of genes in the central portion of the *hmg* locus in strain Rd were the exception when compared to all other strains tested (data not shown).

In strains that are unable to synthesize cryptic glycoforms, *hmg* is absent and is replaced by DNA with homology to *rffG*. In strains lacking the *hmg* locus, PCR amplification using *glnA*-

and *pepB*-specific primers showed that the genes were consistently separated by only approximately 1.1 kb of DNA. Sequence analysis of this DNA from nontypeable *H. influenzae* strains 375 and 486 indicated that it contained a homologue of the *rffG* gene. The sequence was only 66% homologous to HI0873 in the strain Rd genome sequence and to *rmlB* in nontypeable *H. influenzae* strain 176. In strains 375 and 486,

FIG. 4—Continued.

rffG contains several frameshifts (data not shown), likely indicating that the gene is nonfunctional. PCR analysis indicated that related sequences of comparable length are found in all other nontypeable *H. influenzae* strains tested that lack the *hmg* locus (data not shown).

Transfer of the *hmg* locus into nontypeable *H. influenzae* strain 375 enables the bacteria to elaborate cryptic LPS glycoforms. To investigate whether the genes within the *hmg* locus

are sufficient to permit synthesis of LPS glycoforms containing the tetrasaccharide units, we isolated DNA containing the locus from strain RM118 and transferred the entire locus into nontypeable *H. influenzae* strain 375 by transformation. Strain 375hmg+ was shown now to contain the complete *hmg* locus, with it having replaced the *rffG* locus present in the parent strain (data not shown). LPS from strain 375hmg+ now contained apparent high-molecular-weight cryptic glycoforms

TABLE 2. Genes mutated to investigate the biosynthesis of the cryptic LPS glycoforms in *H. influenzae*

Gene ^a	Function	Reference	Effect of mutation on cryptic glycoforms of strain ^b :	
			RM118	RM153
<i>cld</i> (HI0866)		13	(+)	—
<i>lsgI</i> (HI0867)		13	—	—
<i>kfiC</i> (HI0868)		13	+	+
<i>orfE</i> (HI0869)		13	+	+
<i>orfO</i> (HI0870)		13	—	—
<i>orfY</i> (HI0871)		13	(+) ^c	(+)
<i>wbaP</i> (HI0872)		13	+	+
<i>rmlB</i> (HI0873)		13	—	—
<i>cap5j</i> (HI0874)			+	+
<i>lsgA</i> (HI1700)		1	+	(+) ^c
<i>lsgB</i> (HI1699)		1	(+) ^c	+
<i>lsgC</i> (HI1698)		1	(+) ^c	ND ^d
<i>lsgD</i> (HI1697)		1	—	—
<i>lsgE</i> (HI1696)		1	—	—
<i>lsgF</i> (HI1695)		1	—	—
<i>orfM</i> (HI0260)		13	—	—
<i>mrsA</i> (HI1337)		13	—	—
<i>wecA</i> (HI1716)		13	(+) ^c	(+)
<i>lgtF</i> (HI0653)	β-Glucosyltransferase	9	+ ^c	+ ^c
<i>lgtC</i> (HI0258)	α-Galactosyltransferase	13	— ^c	—
<i>lpsA</i> (HI0765)	β-Glycosyltransferase	9,13	— ^c	— ^c
<i>lex2B</i>	β-Glucosyltransferase	14	ND	—
<i>pgmB</i> (HI0740)	Phosphoglucomutase	13	+ ^a	+ ^c
<i>lic2C</i>			ND	— ^c
<i>siaB</i> (HI1279)	CMP-Neu5Ac synthetase	12	+	+
<i>lic1</i> (HI1537-40)	Addition of phosphocholine	34	—	—
<i>lic2A</i> (HI0550)	β-Galactosyltransferase	9	—	—
<i>kdka</i> (HI0260.1)	KDO kinase	13,36	— ^c	—

^a The genes in the lower part of the table are those which have a characterized function in *H. influenzae* LPS synthesis.

^b LPS profiles for a majority of mutant strains are represented in Fig. 2. +, (+), and —, loss of, reduction in intensity of, and no effect on the cryptic LPS glycoforms observed compared to wild type.

^c LPS profile indicates that mutation has other effects upon core LPS glycoforms.

^d ND, not determined.

(Fig. 5) and reacted with MAb LLA5, unlike that of the parent strain.

The synthesis of cryptic glycoforms is influenced by genes other than those located in the *hmg* locus. Based on our PAGE analysis, additional genes, located outside *hmg*, influence expression of the glycoforms containing the sialylated tetrasaccharide unit in some strains (Table 2; Fig. 2). A number of LPS biosynthesis genes of characterized function were included in our study. The *lgtF*, *lic2C*, and *lpsA* genes encode the glycosyltransferases responsible for adding the first hexose unit to the first, second, and third heptoses, respectively, of the inner core backbone of *H. influenzae* LPS (reference 9 and unpublished data). Mutation of *lgtF*, but not *lic2C* (*lic2orf3*), and *lpsA* in both strains RM118 and RM153 resulted in loss of the tetrasaccharide unit, consistent with the structural conclusions for strains RM118 and RM153 that the tetrasaccharide units are located in the oligosaccharide extension from the first heptose (5). Mutation in two other genes, *lgtC* and *lic2A* (9, 13), encoding galactosyltransferases required for multiple other steps in *H. influenzae* LPS synthesis, did not affect inclusion of the tetrasaccharide units.

The *lsgA* and *lsgB* genes in strain RM153 and the *lsgB* and *lsgC* genes in strain RM118 each influence the cryptic LPS glycoforms when mutated. Sequences from the *lsg* locus have previously been shown to direct the synthesis of chimeric LPS molecules in *Escherichia coli* (26), although the precise function of most individual genes has not been determined. The second ORF in the locus, *lsgB*, has been shown to have some role in LPS sialylation in strain A2 (1, 16). We hypothesize that the cryptic glycoforms are deficient unless they are sialylated or capped by (PEtn→6)-α-D-GalpNAc- and that LsgB is a candidate sialyltransferase for this purpose. The requirement for sialylation was confirmed for the *siaB* mutant (12), which is unable to add sialic acid to macromolecules. The effects observed after mutation of *lsgA*, a close homologue of HI0867, in strain RM153 and of *lsgC* in strain RM118 were upon the entire LPS profile, and the precise nature of these remains unexplained. A high-molecular-weight LPS band that is sensitive to neuraminidase remains in the RM153 *lsgA* mutant strain (Fig. 2F). Mutation by the combined deletion of *lsgAC* or *lsgDF* in other strains had little apparent effect upon synthesis of the sialic acid-containing tetrasaccharide unit (data not shown). Mutation of *rfe*, a homologue of the gene now renamed *wecA*, reduced the amount of tetrasaccharide units observed, or altered the LPS profile, in both strains. *wecA* encodes a polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferase, which is required for an alternative O-antigen initiation reaction whereby GlcNAc or GalNAc is added as the initial sugar to the isoprenoid lipid carrier. Neuraminidase treatment of LPS isolated from the RM153 *wecA* mutant indicated that there was only a reduction in the amount of glycoforms containing tetrasaccharide units and that these glycoforms remain sialylated (Fig. 2). MS analysis of O-deacylated LPS derived from strain RM153 *wecA* confirmed that LPS glycoforms containing the sialylated tetrasaccharide units were still present in significant amounts (Table 1; Fig. 4). Mutation of the phosphoglucomutase gene (*pgmB*) significantly reduced the amount of tetrasaccharide units incorporated, likely due to the effect on the availability of nucleoside-activated glucose, a precursor for LPS synthesis, and the severe truncation of the respective LPS (13). Mutation of *lic1*, a locus required for the incorporation of phosphocholine into *H. influenzae* LPS (35), did not influence incorporation of the tetrasaccharide unit. In strain RM118, the glucose attached to the first heptose is heavily substituted with phosphocholine (28).

The data accumulated in our analysis are consistent with the *hmg* locus being the primary genetic determinant for incorporation of a tetrasaccharide unit(s) into the LPSs of a majority of *H. influenzae* strains by a mechanism related to O-antigen synthesis in other bacteria.

DISCUSSION

We propose that the sialyl- and (PEtn→6)-α-D-GalpNAc-containing tetrasaccharide units described here represent a novel mechanism for elaborating related structures in the LPS that are relevant to the commensal and pathogenic behavior of *H. influenzae*. This mechanism introduces the equivalent of a single O-antigen unit into the LPS and is contrary to the long-held belief that there exists some fundamental difference between the LPSs of certain gram-negative bacteria, typified by

FIG. 5. The genes of the *hmg* locus in *H. influenzae* strains. Panel A, representation of the locus in strain Rd. Arrows represent the relative location and the direction of transcription of each of the reading frames given in The Institute for Genomic Research database (<http://www.tigr.org>), with the appropriate designation (HI number) given above. The relative gene organization of the central portion of the *hmg* sequence for nontypeable *H. influenzae* strain 176 is given below for comparison, with the relevant gene designation. *glnA* and *pepB* are the flanking genes. For Rd, regions of DNA outside recognized reading frames that show some homology to O-antigen genes from other organisms are shown as dotted lines. The small square downstream of HI0870 is a tract of a repeated pentanucleotide (5'-TTATC). Panel B shows the LPS profiles for nontypeable *H. influenzae* strain 176 and the respective *wbaP* mutant. wt, wild type. Panel C shows the higher-molecular-weight glycoforms in the LPS of nontypeable *H. influenzae* strain 375 transformed with the *hmg* locus, compared to the wild type, after gel electrophoresis and staining with silver. -, represents before treatment with neuraminidase; +, after treatment with neuraminidase.

members of the enterobacteria, and the LPS of *H. influenzae*. *E. coli* and *Salmonella* synthesize an O antigen, typically a repeated two- to six-sugar unit, which is added to the core at a late stage in LPS biogenesis. The units of the O antigen are synthesized with, and added to the LPS core via, an isoprenoid lipid carrier intermediate, making the process fundamentally distinct from our understanding of core biosynthesis, where sugars are added sequentially to the growing oligosaccharide attached to lipid A. It is well documented that the LPS of *H. influenzae* is comprised of only lipid A and oligosaccharide core components. The lack of O polysaccharide (O-PS) in the LPS of *H. influenzae* has led many workers to rename the molecule lipooligosaccharide.

It was evident from our analysis of LPS sialylation in *H. influenzae* that the method used to culture the bacteria was crucial to the level of sialylation observed but also that it could induce LPS glycoforms which could not be explained in terms of the knowledge of LPS structure at that time. The structure of the sialyl-containing tetrasaccharide unit (5) is identical to that of the lacto-*N*-neotetraose (lNnt) unit characterized for the LPS of the pathogenic neisseriae. In *Neisseria* spp., the lNnt is synthesized by the sequential addition of sugar units to the growing α -chain of the LPS molecule (15, 32). However, the striking feature of the synthesis of the structure in *H. influenzae*

is that the structure appears to be synthesized then added only as a complete unit, similar to the synthesis of many O-antigen repeat units. The details of the complex process of O-antigen synthesis have mainly been obtained from investigations of the enterobacteria, largely with *Salmonella* and *E. coli*. Individual O units are synthesized in the cytoplasm, where sugar monomers are transferred sequentially to an undecaprenol-phosphate lipid carrier. One of two initiation reactions catalyzed by WbaP (galactose) or WecA (GlcNAc or GalNAc) is known to be required for the initial attachment of the relevant sugar to the lipid carrier involved in the biosynthesis process. A particularly striking homology is seen for the *H. influenzae wbaP* gene compared with the same gene from other organisms. WbaP is the initiator of O-antigen synthesis in many other bacteria, adding a galactose residue to the isoprenoid lipid carrier to which the remainder of the sugars of the O unit are condensed. This role would agree with our observation that the initial sugar added as the proximal residue of the cryptic tetrasaccharide units is indeed a galactose. Assembly of the O-PS occurs at the periplasmic face of the cytoplasmic membrane. Transfer and ligation of the O-PS to the lipid A-core terminates the polymerization process. Thus, lipid-linked O-antigen units must be transported (flipped) across the membrane. The components required for the transmembrane export define the

two main pathways for O-antigen assembly, the Wzx (flipase)/Wzy (polymerase)- and the Wzm/Wzt (ABC transporter)-dependent pathways (reviewed in reference 17). HI0867 has some homology to polysaccharide unit transporters (flipases) present in other bacteria, indicating that the likely transport mechanism resembles the Wzx/Wzy system typically observed in *E. coli* and *S. enterica* heteropolymer O antigens. It is common for Wzx proteins to show little homology between bacteria (17). The lack of phenotype associated with mutation of HI0867 in the test strains in this study might be due to the presence of the close homologue *lsgA* at a separate genomic location within the *lsg* gene cluster. This duplication may also explain some changes in the LPS observed when *lsgA* was mutated and underlies the complexity associated with LPS synthesis in this bacterium. It has been reported that WbaP itself could be implicated in the transport process (33). HI0866 also has low homology to the chain length determinant (Wzz), which interacts with the polymerase (Wzy) to control the chain length of the O antigen formed, although the sequence is truncated in the Rd genome sequence. There is no evidence for homologues of the components (Wzm/Wzt) required for the ABC transporter or of the processive transferases required for the synthase-dependent pathways of O-antigen assembly described for some organisms. Mutation of *wecA* (formerly called *rfe*), an alternative initiator of O-unit synthesis, had some effect upon the LPS profiles of strains RM118 and RM153 but did not prevent tetrasaccharide unit incorporation. The evidence suggests that in *H. influenzae* there exists a modified or incomplete O-antigen biogenesis system; this might explain the relative inefficiency with which the relevant tetrasaccharide units are incorporated into the LPS during in vitro growth. The alternative tetrasaccharide structures found in *H. influenzae* LPS are likely variants of the same, or closely related, biosynthetic process, with perhaps only the final step(s), including capping with sialic acid or α -D-GalpNAc-(PEtn), being unique to each structure. The distribution of these two structures varies between strains after in vitro growth. The precise contribution of individual genes to biosynthesis of the tetrasaccharide units remains to be elucidated; such analysis is complicated by the "all-or-nothing" en bloc addition of these moieties. Evidence for related sialyl- and α -D-GalpNAc-(PEtn)-containing tetrasaccharide units in *H. influenzae* has been inferred from recent analysis of LPS from a serotype b strain, A2 (16). It is also possible that other genes outside the *hmg* locus, such as those in the *lsg* locus, may play some role in elaboration of these and related LPS glycoforms in some strains. A more detailed analysis of these genes will be required to understand their role in synthesis of the cryptic glycoforms.

The incorporation of a single unit by an O-antigen-type mechanism has been described previously, particularly for the pathogenic bacterium *Bordetella pertussis* (2). In this organism, the addition of a single trisaccharide unit to form so-called A-form LPS is dependent upon the function of a *wbaP* homologue.

Upon investigation of the *hmg* locus across a number of strains, we found that the genes either were all present together or were completely absent. This distribution of genes suggested that the entire *hmg* region is gained, or lost, en bloc. A majority of strains tested maintained the *hmg* region of

DNA. The aberrant base composition of the *hmg* locus suggests that this region of DNA has been gained by horizontal transfer from another bacterium. Transfer of the *hmg* locus into a strain that does not naturally contain it provided evidence that gain of this DNA is all that is required to direct LPS biosynthesis to include the tetrasaccharide units. The transfer of O-antigen genes between organisms is a known mechanism for O-antigen variation of bacterial pathogens (18).

Twenty-four of the 25 nontypeable *H. influenzae* strains investigated in our study are known to express sialylated glycoforms (3). The sialylated INnt side chain represents one of the frequent sialylated glycoform types, but other sialylation sites, such as a lactose from the distal heptose, are found in some strains (12, 16, 20).

It is clear that the biosynthesis of LPS in *H. influenzae* is more complex than has previously been predicted. There is evidence to suggest that the expression of the tetrasaccharide units in *H. influenzae* LPS has biological relevance. It is known that a single sialylated INnt side chain elaborated through this mechanism contributes to the resistance of the organism to the killing effects of normal human serum (10). The relative contributions of the sialyl- and (PEtn $\rightarrow$ 6)- α -D-GalpNAc-containing tetrasaccharide units to the virulence of strains expressing these structures is under investigation. It is tempting to speculate upon the chance that any strain of this naturally transformable organism could gain, or indeed have, those extra gene functions that are necessary to synthesize a fully extended O antigen.

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