Carbohydrate Research 346 (2011) 1262–1266

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

The role of *lic2B* in lipopolysaccharide biosynthesis in *Haemophilus influenzae* strain Eagan

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ARTICLE INFO

Article history:
Received 7 February 2011
Received in revised form 6 April 2011
Accepted 7 April 2011
Available online 13 April 2011

Keywords:
Haemophilus influenzae
Lipopolysaccharide
lic2B
Mass spectrometry
Glucosyltransferase

ABSTRACT

Lipopolysaccharide (LPS) biosynthesis in *Haemophilus influenzae* involves genes from the *lic2* locus that are required for chain extension from the middle heptose (HepII) of the conserved triheptosyl inner-core moiety. Lic2C initiates the process by attaching the first glucose to HepII, but the gene encoding for the enzyme adding the next β-D-Glc*p*- is uncharacterized. Lic2B is the candidate glucosyltransferase; however, in previous investigations, mutation of *lic2B* resulted in no hexose extension from HepII, likely due to a polar effect on the *lic2C* gene.

In this study we complemented a *lic2B* knock-out mutant of *H. influenzae* strain Eagan with a functional *lic2C* gene and investigated its LPS by mass spectrometry and 2D NMR spectroscopy. Lic2B was found to encode a glucosyltransferase responsible for the linkage of β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→ extending from O-3 of the central heptose of the triheptosyl inner-core moiety, L-α-D-Hep*p*-(1→2)-[*P*Etn→6]-L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*-(1→5)-[*PP*Etn→4]-α-Kdo-(2→6)-lipid A.

Haemophilus influenzae is a commensal bacterium colonizing the human nasopharynx and upper respiratory tract that can also be a pathogen. It exists in encapsulated (type a–f) and unencapsulated (non-typeable) forms. One of its major virulence determinants is the outer membrane glycolipid lipopolysaccharide (LPS). LPS from *H. influenzae* consists of a conserved glucose-substituted triheptosyl inner-core moiety L-α-D-Hep*p*-(1→2)-[*P*Etn→6]-L-α-D-Hep*p*-(1→3)-[β-D-Glc*p*-(1→4)]-L-α-D-Hep*p* linked to lipid A via Kdo 4-phosphate. The outer core has been found to mimic human glycosylation patterns, and the pattern of oligosaccharide extensions is extremely variable within and between strains.[1] Further heterogeneity is introduced by a variety of non-carbohydrate substituents, for example, acetates (Ac), phosphate (*P*) and phosphoethanolamine (*P*Etn) that are added in non-stoichiometric amounts.[1]

The LPS structure of *H. influenzae* type b strain Eagan has been established and is shown in Figure 1.[2–4] Extension from the middle heptose includes a terminal P^k-blood group antigen, α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→, linked to α-D-Glc*p* attached to the middle heptose (HepII).[2]

Detailed investigations of the function of the numerous genes required for LPS biosynthesis in *H. influenzae* have been carried out in several typeable and non-typeable strains, amongst them strain Eagan. The *lic2* locus consists of four genes (*lic2A*, *ksgA*, *lic2C*, *lic2B*) and is required for chain extension from HepII.[5] Lic2C initiates the process by attaching the first glucose to HepII.[5] The enzyme adding the next β-D-Glc*p* is uncharacterized but the P^k antigen is assembled by Lic2A attaching β-D-Gal*p* to β-D-Glc,[5,6] and LgtC completing the terminal digalactosyl unit by adding α-D-Gal*p*-.[6] Lic2B is a candidate transferase potentially responsible for the attachment of β-D-Glc*p* to O-4 of α-D-Glc*p* from HepII. However, in previous investigations, mutation of the *lic2B* gene was thought to inactivate Lic2C activity through a polar effect on the downstream encoding gene, thus allowing no hexose extension from HepII.

In this study we investigated the role of Lic2B in *H. influenzae* strain Eagan*lic2B*lic2C+, a *lic2B* mutant of strain Eagan complemented with a separate functional *lic2C* gene maintained on a plasmid.

Abbreviations: Ac, acetate; *An*Kdo-ol, reduced anhydro Kdo; CE, capillary electrophoresis; Gal, galactose; Glc, glucose; GlcNAc, 2-acetamido-2-deoxy-glucose; Hep, L-*glycero*-D-*manno*-heptose; Hex, hexose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LipidA-OH, O-deacylated lipid A; LPS, lipopolysaccharide; LPS-OH, O-deacylated lipopolysaccharide; MS^n, multiple step tandem mass spectrometry; OS, oligosaccharide; *P*Etn, phosphoethanolamine; *PP*Etn, pyrophosphoethanolamine; tHep, terminal heptose; tHex, terminal hexose.

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doi:10.1016/j.carres.2011.04.016

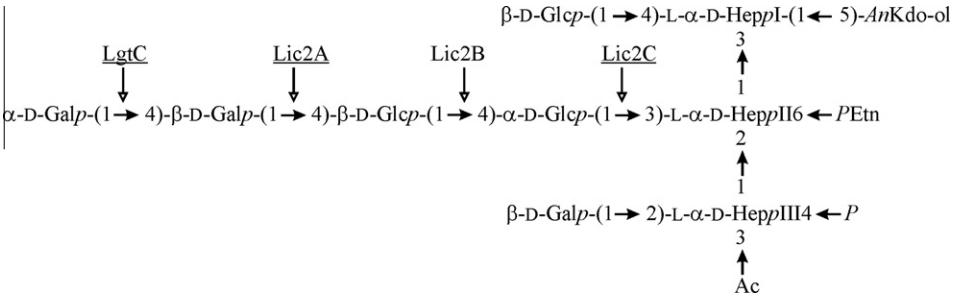

Figure 1. The established structure of the fully extended oligosaccharide from *H. influenzae* strain Eagan.[2] The underlined genes are those characterized as adding the respective hexose linkage indicated by the arrow. The proposed function of the *lic2B* gene product is indicated.

The mutant bacteria were grown in liquid culture and the LPS was extracted by a modified phenol/chloroform/light petroleum method[7] and purified by ultracentrifugation. Structural analysis was accomplished on O-deacylated LPS (LPS-OH) and on reduced core oligosaccharide (OS) material.

Compositional sugar analysis of LPS-OH confirmed the presence of D-glucose (Glc), D-galactose (Gal), 2-amino-2-deoxyglucose (GlcN) and L-*glycero*-D-*manno*-heptose (Hep) in a ratio of 31:19:17:33 as identified by GLC–MS of their corresponding alditol acetate derivates. Compositional sugar analysis of OS material showed Glc, Gal, and Hep in the ratio 40:21:39. The absence of GlcN confirmed this sugar as being part of lipid A.

The linkage positions of the saccharide residues were determined by methylation analysis on dephosphorylated OS, which revealed terminal (t)-Glc, t-Gal, 2-substituted Hep, 3,4-substituted Hep, and 2,3-substituted Hep in a ratio of 27:11:13:28:21.

The glycoform composition of LPS-OH was determined by capillary electrophoresis–electrospray ionization mass spectrometry (CE–ESIMS) in the negative-ion mode. The ESIMS spectrum revealed abundant molecular peaks corresponding to doubly and triply deprotonated ions (Table 1). Major glycoforms containing three hexoses were detected at *m*/*z* 812.0/1218.3 and 853.1/1279.7 corresponding to $Hex_3{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$ and $Hex_3{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn_2{\cdot}LipidA\text{-}OH$, respectively. Minor ions at *m*/*z* 703.9/1056.4 and 758.1/1137.4 indicated glycoforms with the respective compositions of $Hex_1{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$ and $Hex_2{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$, respectively.

The compositions of OS were deduced from ESIMS experiments in the positive-ion mode (Table 1). The major ions *m*/*z* 705.6/1407.3 and 727.1/1449.3 corresponded to Hex3 glycoforms with respective compositions $Ac_{0\text{-}1}{\cdot}Hex_3{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$. A molecular ion at *m*/*z* 1125.5 corresponded to a glycoform with the composition $Ac_1{\cdot}Hex_1{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$. Other minor ions at *m*/*z* 1245.4 and 1287.4 corresponded to the doubly and singly charged ions of the glycoforms with respective compositions $Ac_{0\text{-}1}{\cdot}Hex_2{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$.

In accordance with the suggested mutant the highest glycoform observed was the Hex3 glycoform.

In order to establish the sequence and branching details of the various glycoforms dephosphorylated and permethylated OS material was analyzed by liquid chromatography–multiple step ESIMS (LC–$ESIMS^n$) in the positive-ion mode. The mass spectrum (Fig. 2A) showed sodiated singly charged adduct ions ($[M+Na]^{1+}$) corresponding to the glycoforms $Hex_{1\text{-}3}{\cdot}Hep_3{\cdot}AnKdo\text{-}ol$ (*m*/*z* 1263.8, 1467.9, 1671.9).

The MS^2 spectrum of the ion at *m*/*z* 1671.9 ($Hex_3{\cdot}Hep_3{\cdot}AnKdo\text{-}ol$, Fig. 2B) revealed fragment ions originating at *m*/*z* 1453.9 and 1205.4 due to the subsequent loss of a tHex-HepIII unit. The sodiated fragment ion corresponding to tHex-HepI-Kdo at *m*/*z* 754.3, and its sodiated counter ion at *m*/*z* 941.3 indicated a Hex3 glycoform with one hexose residue substituting each heptose residue. This was confirmed by further fragmenting the ions *m*/*z* 1205.4 and 941.3. The MS^3 spectrum of *m*/*z* 1205.4 (Fig. 2C) revealed the ion at *m*/*z* 753.3 due to loss of Hex-HepII. The MS^3 spectrum of *m*/*z* 941.3 (Fig. 2D) revealed, inter alia, the ion at *m*/*z* 489.3 corresponding to tHex-HepIII.

The major glycoform structures in OS from strain Eagan*lic2-B*lic2C+ were elucidated by detailed ^1H, ^{13}C, and ^{31}P NMR analyses. ^1H and ^{13}C NMR resonances were assigned using gradient chemical shift correlation techniques (COSY, TOCSY, and HMQC experiments). The chemical shift data are given in Table 2 and confirm the carbohydrate backbone as shown in Fig. 1 with the exception of the elongation from α-D-Glc*p* linked to HepII. Subspectra corresponding to the individual glycosyl residues were identified on the basis of spin–connectivity pathways delineated in the ^1H chemical shift correlation maps, the chemical shift values, and the vicinal coupling constants. The monosaccharide sequences of the major glycoforms were confirmed from transglycosidic NOE connectivities between anomeric and aglyconic protons on adjacent residues

Table 1
Negative ion CE–ESIMS data from O-deacylated LPS and ESIMS data (positive-ion mode) for oligosaccharide from *H. influenzae* Eagan*lic2B*lic2C+ with proposed composition[a]

Observed ions *m*/*z*				Molecular mass (Da)		Relative abundance	Proposed composition
$(M-3H)^{3-}$	$(M-2H)^{2-}$	$(M+2H)^{2+}$	$(M+H)^{1+}$	Observed	Calculated	%	
703.9	1056.4			2114.8	2114.8	5	$Hex_1{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$
758.1	1137.4			2277.1	2276.9	6	$Hex_2{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$
812.0	1218.3			2438.8	2439.1	76	$Hex_3{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn{\cdot}LipidA\text{-}OH$
853.1	1279.7			2561.9	2562.1	13	$Hex_3{\cdot}Hep_3{\cdot}Kdo{\cdot}P{\cdot}PEtn_2{\cdot}LipidA\text{-}OH$
			1125.5	1126.5	1125.9	4	$Ac{\cdot}Hex_1{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$
			1245.4	1246.4	1246.0	6	$Hex_2{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$
			1287.4	1288.4	1288.1	5	$Ac{\cdot}Hex_2{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$
		705.6	1407.3	1408.3	1408.2	39	$Hex_3{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$
		727.1	1449.3	1450.3	1450.2	46	$Ac{\cdot}Hex_3{\cdot}Hep_3{\cdot}PEtn{\cdot}AnKdo\text{-}ol$

[a] Calculations were accomplished using the following masses: LipidA-OH: 953.02; Kdo: 220.18; *An*Kdo-ol: 222.20; Hep: 192.17; Hex: 162.14; *P*Etn: 123.05; *P*: 79.98; Ac: 42.02.