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Note

Structural investigation of lipopolysaccharides from nontypeable *Haemophilus influenzae*: investigation of inner-core phosphoethanolamine addition in NTHi strain 981

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Abstract—LPS of NTHi comprises a conserved tri-L-*glycero*-D-*manno*-heptosyl 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*-(1→5)-α-Kdo*p*) in which addition of *P*Etn to the central heptose (HepII) in strain Rd is controlled by the gene *lpt6*. It was recently shown that NTHi strain 981 contains an additional *P*Etn linked to O-3 of the terminal heptose of the inner-core moiety (HepIII). In order to establish whether *lpt6* is also involved in adding *P*Etn to HepIII, *lpt6* in strain 981 was inactivated. The structure of the LPS of the resulting mutant strain 98l*lpt6* was investigated by MS and NMR techniques by which it was confirmed that the *lpt6* gene product is responsible for addition of *P*Etn to O-6 of HepII in strain 981. However, it is not responsible for adding *P*Etn to O-3 of HepIII since the 981*lpt6* mutant still had full substitution with *P*Etn at HepIII.

Keywords: *Haemophilus*; Phosphoethanolamine; Phosphocholine; Lipopolysaccharide; *P*Etn transferase

Haemophilus influenzae is a Gram-negative pathogenic bacterium that routinely colonises the human upper respiratory tract and which can be found both in encapsulated (types a–f) and unencapsulated (nontypeable) forms. While the incidence of disease caused by *H. influenzae* type b (invasive diseases, including meningitis and septicaemia) has greatly reduced in recent years due to the development of conjugate vaccines, there exists no vaccine against nontypeable *H. influenzae* (NTHi). NTHi strains are a common cause of otitis media and respiratory tract infections and their lipopolysaccharide (LPS) molecules have been shown to be important for colonisation, bacterial persistence and survival in the circulatory system. Structures of *H. influenzae* LPS have been extensively investigated. A structural model consisting of a conserved tri-L-*glycero*-D-*manno*-heptosyl 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*-(1→5)-α-Kdo*p*), in which each of the three heptose residues can provide a point for elongation by oligosaccharide chains or for attachment of noncarbohydrate substituents, is now well established.[1–18] *H. influenzae* LPS can undergo phase variation between defined oligosaccharide structures, leading to an extensive repertoire of glycoforms within and between strains. The addition of phosphate-containing substituents, including monophosphate (*P*), phosphoethanolamine (*P*Etn), pyrophosphoethanolamine (*PP*Etn) and phosphocholine (*P*Cho), as well as glycine and *O*-acetyl substituents contributes to the structural variability of these molecules. The genes involved in LPS biosynthesis have been investigated extensively in the type b strain Eagan and in the genome reference strain Rd.[19,20]

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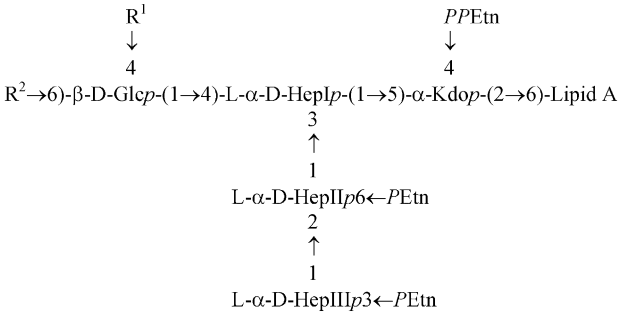
Gene functions have been identified that are responsible for most of the steps in the biosynthesis of the OS portion of their LPS molecules. *P*Cho addition has been shown to be directed by the products of the *lic1* locus. Recently, the gene *lpt6* was shown to be involved in the addition of *P*Etn to the central heptose (HepII) of the inner-core of *H. influenzae*.[21]

Our recent studies have focussed on the structural diversity of LPS expression and the genetic basis for that diversity in a representative set consisting of 25 NTHi clinical isolates obtained from otitis media patients.[10,12–14,18,22,23] *H. influenzae* nontypeable strain 981 is one of these strains and the structure of its LPS was characterised previously (Structure 1).[22]



Structure 1 R^1 = β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1 or truncated versions thereof;

R^2 = *P*Cho or β-D-Gal*p*-(1→4)-D-α-D-Hep*p*-(1

It was found to have four phosphorylation sites: (i) *PP*Etn at O-4 of Kdo; (ii) *P*Cho at O-6 of the glucose residue linked to the proximal heptose of the inner-core (HepI) in a subpopulation of glycoforms; (iii) *P*Etn at O-6 of HepII and (iv) an additional *P*Etn unit at O-3 of the distal heptose of the inner-core (HepIII). To date *P*Etn substituting HepIII has been only observed in a small subset of NTHi strains,[18,24] however the gene(s) responsible for its addition has not been identified. NTHi strain 981 was thus suitable for further studies to investigate biosynthetic pathways of LPS phosphorylation.

In this study, we addressed the question of whether *lpt6* is bifunctional and involved in adding *P*Etn to HepIII or not. Thus, *lpt6* in strain 981 was inactivated and the resulting mutant strain 98l*lpt6* was investigated by mainly MS and NMR techniques. In our search for the gene involved in addition of *P*Etn to HepIII, we investigated another mutant in which the gene HI1064 was inactivated. HI1064 had been identified through the low homology of its translated product to Lpt3, the transferase required to add *P*Etn to the distal heptose in the inner core of *Neisseria meningitidis* LPS.[25]

The strains were grown in liquid culture and the LPS was isolated by phenol–chloroform–light petroleum extraction. Compositional sugar analysis of the LPS samples identified glucose (Glc), galactose (Gal), 2-amino-2-deoxy-glucose (GlcN), D-*glycero*-D-*manno*-heptose (D,D-Hep) and L-*glycero*-D-*manno*-heptose (L,D-Hep) as the constituent sugars by GLC–MS analysis of their corresponding alditol acetate derivatives. The relative ratios for 981*lpt6* were 32:51:6:3:7 and for 981HI1064 27:30:10:10:23.

O-Deacylation of LPS by treatment with anhydrous hydrazine under mild conditions afforded water-soluble material (LPS-OH) which was subjected to ESI-MS analyses. Similar to observations from the wild-type strain, the ESI-MS spectrum of LPS-OH from 981*lpt6* (negative mode, Table 1) showed ions corresponding to two subpopulations of glycoforms, that is, glycoforms having three heptoses and mainly *P*Cho (Hep3-glycoforms), and glycoforms with compositions comprising four heptoses but lacking *P*Cho (Hep4-glycoforms). Thus, major quadruply charged ions were observed at *m/z* 650.1/680.9 and 697.3/728.2 corresponding to glycoforms with the respective compositions *P*Cho·Hex_3·Hep_3·*P*Etn_{1-2}·P_1·Kdo·Lipid A-OH and Hex_4·Hep_4·*P*Etn_{1-2}·P_1·Kdo·Lipid A-OH. Since, no ions corresponding to glycoforms with three *P*Etn were detected (see Structure 1 for comparison), it was indicated that the *lpt6* mutation resulted in removal of one of the substituents. In analogy with the investigation on the NTHi 981, wild-type (wt) strain[22] sequence and branching details of the various glycoforms present in 981*lpt6* were determined using ESI-MS^n on dephosphorylated and permethylated oligosaccharide material obtained after mild acid hydrolysis of LPS. The results were in principle identical to those obtained for NTHi 981wt (data not shown) showing Hep3 and Hep4 glycoforms with chain extensions only from HepI and a branching GlcI residue (Structure 1).

The ESI-MS spectrum of 981HI1064 revealed major ions corresponding to only Hep4-glycoforms. Major

Table 1. Negative ion ESI-MS data and proposed compositions for LPS-OH of NTHi strains 981*lpt6* and 981HI1064

Observed ions (*m/z*)		Molecular mass (Da)		Relative abundance (%)		Proposed composition
$(M-4H)^{4-}$	$(M-3H)^{3-}$	Observed	Calculated	*lpt6*	1064	
609.1	812.0	2440.4	2439.2	2	—	$Hex_3{\cdot}Hep_3{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
639.6	853.3	2562.7	2562.2	2	—	$Hex_3{\cdot}Hep_3{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
650.1	867.2	2604.5	2604.3	15	—	$PCho{\cdot}Hex_3{\cdot}Hep_3{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
680.9	908.3	2727.8	2727.3	19	—	$PCho{\cdot}Hex_3{\cdot}Hep_3{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
690.3	920.7	2765.2	2766.4	3	—	$PCho{\cdot}Hex_4{\cdot}Hep_3{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
721.8	962.4	2890.7	2889.5	3	—	$PCho{\cdot}Hex_4{\cdot}Hep_3{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
741.5	989.0	2970.0	2969.6	1	—	$PCho{\cdot}HexNAc_1{\cdot}Hex_4{\cdot}Hep_3{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
772.3	1029.9	3093.0	3092.7	2	—	$PCho{\cdot}HexNAc_1{\cdot}Hex_4{\cdot}Hep_3{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
616.3	822.2	2469.4	2469.2	7	—	$Hex_2{\cdot}Hep_4{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
647.2	863.0	2592.4	2592.2	4	—	$Hex_2{\cdot}Hep_4{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
697.3	930.3	2793.6	2793.5	19	—	$Hex_4{\cdot}Hep_4{\cdot}PEtn_1{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
728.2	971.3	2916.9	2916.5	22	48	$Hex_4{\cdot}Hep_4{\cdot}PEtn_2{\cdot}P_1{\cdot}$Kdo·Lipid A-OH
758.7	1012.2	3039.2	3039.6	1	52	$Hex_4{\cdot}Hep_4{\cdot}PEtn_3{\cdot}P_1{\cdot}$Kdo·Lipid A-OH

Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Hex, 162.14; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; *P*, 79.98; *P*Etn, 123.05; *P*Cho, 165.13 and Lipid A-OH, 953.02. Relative abundance was estimated from the area of molecular ion peak relative to the total area (expressed as percentage). Peaks representing less than 3% of the base peak are not included in the table.

quadruply charged ions at *m/z* 728.2/758.7 corresponded to species with the respective compositions $Hex_4{\cdot}Hep_4{\cdot}PEtn_{2-3}{\cdot}P_1$Kdo·Lipid A-OH. The number of *P*Etn substituents was not changed compared to the wild-type strain but interestingly, glycoforms containing *P*Cho were not detected.

Methylation analysis of LPS derived samples revealed the presence of terminal Gal, 4-substituted Gal, 4-substituted Glc, 4-substituted-D,D-Hep and 3,4-disubstituted Hep as the major sugar components (Table 2), confirming the basic structures identified in NTHi 981.

LPS-OH samples of both strains and oligosaccharide material (OS) derived from 981HI1064 after mild acid hydrolysis were investigated by ^1H–^1H chemical shift correlation experiments (DQF-COSY, TOCSY and NOESY) and heteronuclear ^1H–^{13}C and ^1H–^{31}P NMR correlation studies in the ^1H detected mode (HSQC and HMQC, respectively) as described earlier. The NMR data of 981*lpt6* indicated an identical carbohydrate backbone structure and similar glycoform patterns of those reported for NTHi 981 wt (data not shown). However, chemical shift data obtained for the inner-core region in the Hep3- and Hep4-glycoforms of 981*lpt6* showed considerable differences and are presented in Table 3.

The ^1H NMR spectrum of LPS-OH from strain 981*lpt6* is shown in Figure 1B. In the anomeric region >4.8 ppm, the resonance of the α-linked GlcN in the lipid A was observed at δ 5.46. The remaining signals corresponded to heptose and galactose residues, which were identified in the 2D COSY, TOCSY and NOESY spectra. Two anomeric signals corresponding to HepII residues were identified at δ 5.77 and 5.63. The signal at δ 5.77 was assigned to HepII of the Hep4-glycoforms, whereas the signal at δ 4.63 corresponded to the Hep3-glycoforms based on observations made on the wild-type strain in which H-1 of HepII in the Hep4 glycoforms was more downfield shifted.[22] NOESY correlations between anomeric resonances of HepII and HepIII[26] confirmed the presence of two distinct HepIII residues at δ 5.19 (Hep3-glycoforms) and 5.13 (Hep4-glycoforms). A signal at δ 5.14 was

Table 2. Linkage analysis data for LPS-OH from *H. influenzae* NTHi strain 981*lpt6* and dephosphorylated OS derived from *H. influenzae* NTHi strain 981HI1064

Methylated sugar[a]	T_{gm}[b]	Relative detector response (%)		Linkage assignment
		981*lpt6*	981HI1064	
2,3,4,6-Me_4-Gal	1.00	31	23	D-Gal*p*-(1→
2,3,6-Me_3-Gal	1.17	13	1	→4)-D-Gal*p*-(1→
2,3,6-Me_3-Glc	1.18	24	13	→4)-D-Glc*p*-(1→
2,4,6-Me_3-Gal	1.21	3	2	→3)-D-Gal*p*-(1→
2,3,4,6,7-Me_5-Hep	1.34	—	4	L,D-Hep*p*-(1→
2,3-Me_2-Glc	1.44	4	14	4,6)-D-Glc*p*-(1→
2,3,6,7-Me_4-Hep	1.48	13	14	→4)-D,D-Hep*p*-(1→
3,4,6,7-Me_4-Hep	1.54	4	8	→2)-L,D-Hep*p*-(1→
2,4,6,7-Me_4-Hep	1.59	1	—	→3)-L,D-Hep*p*-(1→
2,6,7-Me_4-GlcN	1.65	1	20	→3,4)-L,D-Hep*p*-(1→

[a] 2,3,4,6-Me_4-Glc represents 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol-1-d_1, etc.
[b] Retention times (T_{gm}) are reported relative to 2,3,4,6-Me_4-Gal.

Table 3. ^{1}H and ^{13}C NMR chemical shifts corresponding to the tri-L-*glycero*-D-*manno*-heptosyl inner-core moiety (L-α-D-HepIII*p*-(1→2)-L-α-D-HepII*p*-(1→3)-[β-D-Glc*p*-(1→4)]-L-α-D-Hep*p*-(1→) present in the Hep3- and Hep4-glycoforms of LPS-OH from 981*lpt6*

Residue	Glycose unit	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6_A/C-6	H-6_B	H-7_A/C-7	H-7_B
HepI	→3,4)-L-α-D-Hep*p*-(1→	5.14	4.11	4.03	4.22[a]	—[b]	4.05[a]		—	—
		100.4	70.8	72.5	74.3		69.1			
HepII	→2)-L-α-D-Hep*p*-(1→	5.77[c]	4.26	3.92	3.91	—	—		—	—
		99.2	80.1	69.1	66.8	—	—		—	
		5.63[d]	4.26	3.92	3.91					
		99.7	80.1	69.1	66.8					
HepIII	L-α-D-Hep*p*-(1→	5.19[d]	4.23	4.31	3.89	—	—		—	—
	3	102.5	70.5	76.8	65.8	—	—		—	
	↑	5.13[c]	4.22	4.31	—					
	*P*Etn	102.5	70.5	76.8						
GlcI	→4)-β-D-Glc*p*-(1→	4.56[d]	3.51	3.57	3.70	3.69	4.29	4.29		
	6									
	↑	103.6	73.8	75.3	78.6	74.0	64.1			
	*P*Cho									
	→4,6)-β-D-Glc*p*-(1→	4.54[c]	3.57	—	3.66	3.65	3.89	4.14		
		103.6	73.8	—	77.7	73.4	66.8			
	→6)-β-D-Glc*p*-(1→	4.49[c]	3.54	—	3.57	3.57	3.79	4.11		
		103.6	73.8	—	—	74.2	—			
*P*EtnII		4.16	3.27							
		62.5	40.8							
*P*Cho		4.38	3.69							
		60.2	66.7							

Data was recorded in D_2O at 30 °C. Signals corresponding to *P*Cho methyl protons and carbons occurred at 3.23/54.5 ppm.
[a] H-4/H-6 of HepI were identified by NOE from GlcI.
[b] (—) Not found owing to the complexity of the spectrum.
[c] Corresponds to the residue in the Hep4-glycoform.
[d] Corresponds to the residue in the Hep3-glycoform.

assigned to H-1 of HepI. Based on chemical shift data, signals at δ 4.94 and 4.91 were assigned to correspond to terminal and 3-substituted α-D-Gal residues. Signals corresponding to the D,D-heptose residue HepIV were identified at δ 5.02 and 4.91. In the region from δ 4.65 to 4.43, subspectra of β-linked hexose residues were observed. *Inter alia*, H-1 of the β-linked GlcN in the lipid A was observed at δ 4.60. Furthermore, subspectra corresponding to GlcI in the Hep3- and Hep4 glycoforms were identified at δ 4.56 and 4.49. In agreement with previous reports,[1–18] interresidue NOE connectivities between GlcI H-1 and HepI H-4/H-6 were observed confirming the unambiguous assignment of chemical shifts of GlcI. Notably, we were not able to assign the resonances of H-6/C-6 of HepII in 981*lpt6*. In other investigations, H-6/C-6 of this residue were easily distinguishable and downfield shifted to $\delta \sim 4.6/75$ due to O-6 substitution with *P*Etn.[1–18] Figure 2C shows the HSQC spectrum of LPS-OH from 981 wt in which the cross peak at δ 4.56/74.6 corresponds to H-6/C-6 of HepII. In the ^{1}H–^{31}P NMR spectrum a cross peak at δ −1.3/4.56 is observed (Fig. 3C). The absence of these cross peaks in the corresponding spectra of 981*lpt6* (Figs. 2B and 3B) indicated that the phosphorylation pattern of HepII had changed.

In the ^{1}H NMR spectrum, the signal for the methyl protons of *P*Cho was observed at δ 3.23 and characteristic spin-systems for ethylene protons from this residue and from the two *P*Etn residues indicated by ESI-MS were found at δ 4.38/3.69 (*P*Cho), 4.25/3.35 (*PP*Etn) and 4.16/3.27 (*P*Etn). In the ^{1}H–^{31}P NMR spectrum, a cross peak from δ 4.25 to −12.6 corroborated the presence of *PP*Etn which is linked to Kdo.[10] Furthermore, in that spectrum (Fig. 3B) correlations between the signal at δ −1.7 and the signals from H-6 protons of GlcI (δ 4.29) and the methylene protons of *P*Cho (δ 4.38) confirmed the *P*Cho substituent to be located at O-6 of this residue in the Hep3-glycoform of the mutant strain. Correlations between the ^{31}P NMR signal at δ −1.8 and H-3 of HepIII (δ 4.31) and the methylene proton pair of *P*Etn (δ 4.16) confirmed *P*Etn to be linked to HepIII.

The ^{1}H NMR spectrum of LPS-OH from strain 981HI1064 is shown Figure 1A. The resonance of the α-linked GlcN in the lipid A resonated at δ 5.46. In agreement with ESI-MS data, no intense signal corresponding to *P*Cho at δ 3.23 was observed. The signal corresponding to one anomeric proton of HepII was observed at δ 5.94. The remaining anomeric signals corresponded to heptose and galactose residues, which were identified in the 2D COSY, TOCSY and NOESY spectra. In the ^{1}H–^{31}P NMR spectrum, a cross peak from δ 4.25 to −12.6 corresponded to *PP*Etn at O-4 of Kdo.[10] In that spectrum, phosphorylation at O-6 of HepII was indicated by the cross peak at δ −1.3/4.58 (Fig. 3A). NMR spectra of OS derived from 981HI1064 were virtually identical to those reported for the Hep4 glycoforms observed in 981 wt.[22] Thus, signals for H-6/C-6

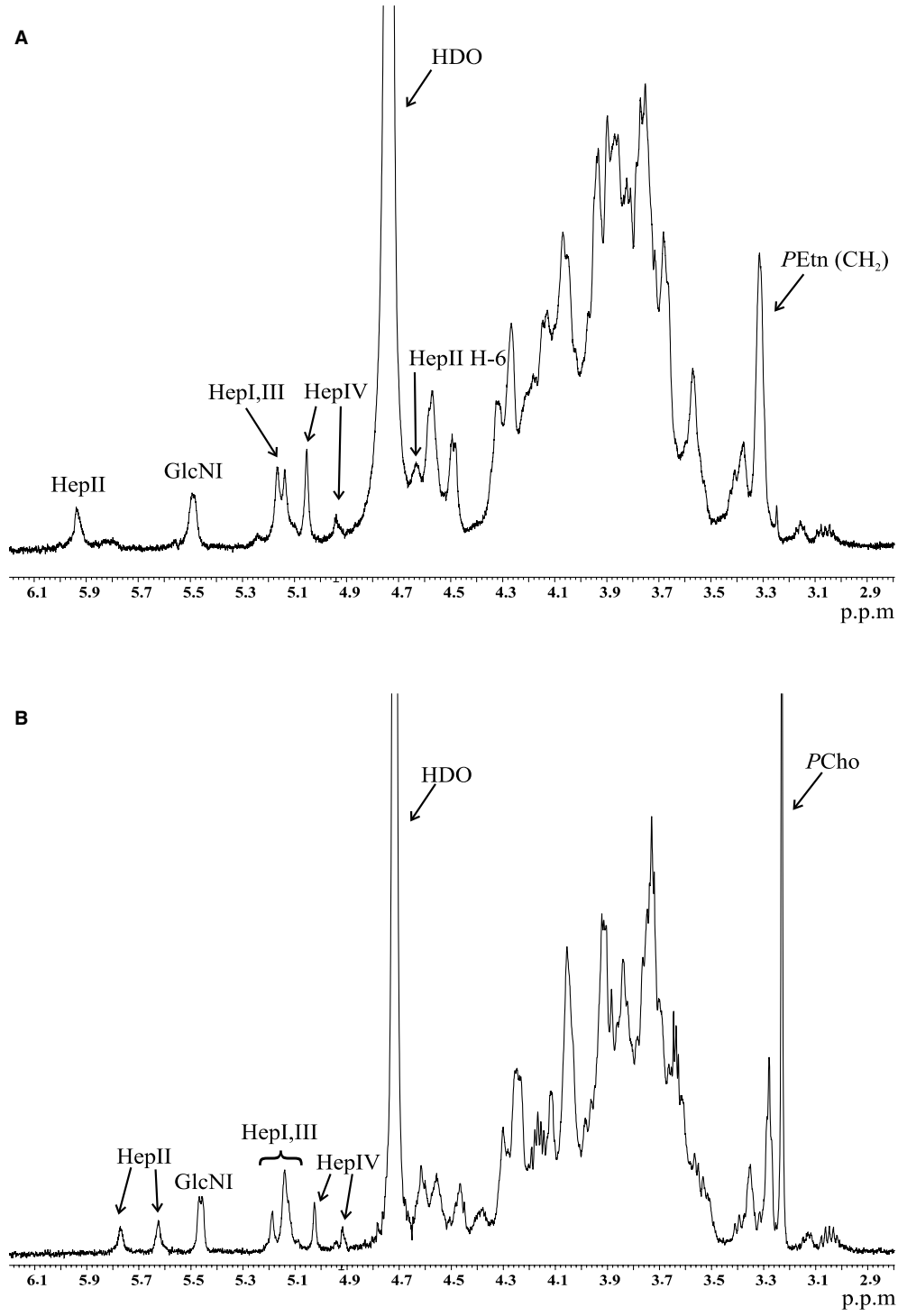


Figure 1. 500 MHz ^{1}H NMR spectra of *O*-deacylated LPS derived from nontypeable *H. influenzae* mutant strains 981HI1064 (A) and 981*lpt6* (B) and in D_2O at 30 °C.

of HepII were detected at δ 4.57/75.4 (Fig. 2A) and those for *P*EtnI and *P*EtnII methylene resonances were observed at δ 4.15/62.5, 3.29/40.5 and 4.17/62.5, 3.29/40.5, respectively. It was concluded that the substitution of the inner-core region with *P*Etn had not changed in this mutant strain compared to the wt strain. However, mutation in HI1064 had eliminated Hep3-glycoforms and only Hep4-glycoforms were identified. Notably, traces of a *P*Cho crosspeak at δ 3.23/54.5 and 3.69/66.7 were observed (Fig. 2A), indicating a minor population of Hep3-glycoforms in the mutant. The genetic locus responsible for adding *P*Cho is *lic1* and for NTHi strain 981 we have proposed that *lic1* competes with the still unknown gene adding the D,D-Hep residue to

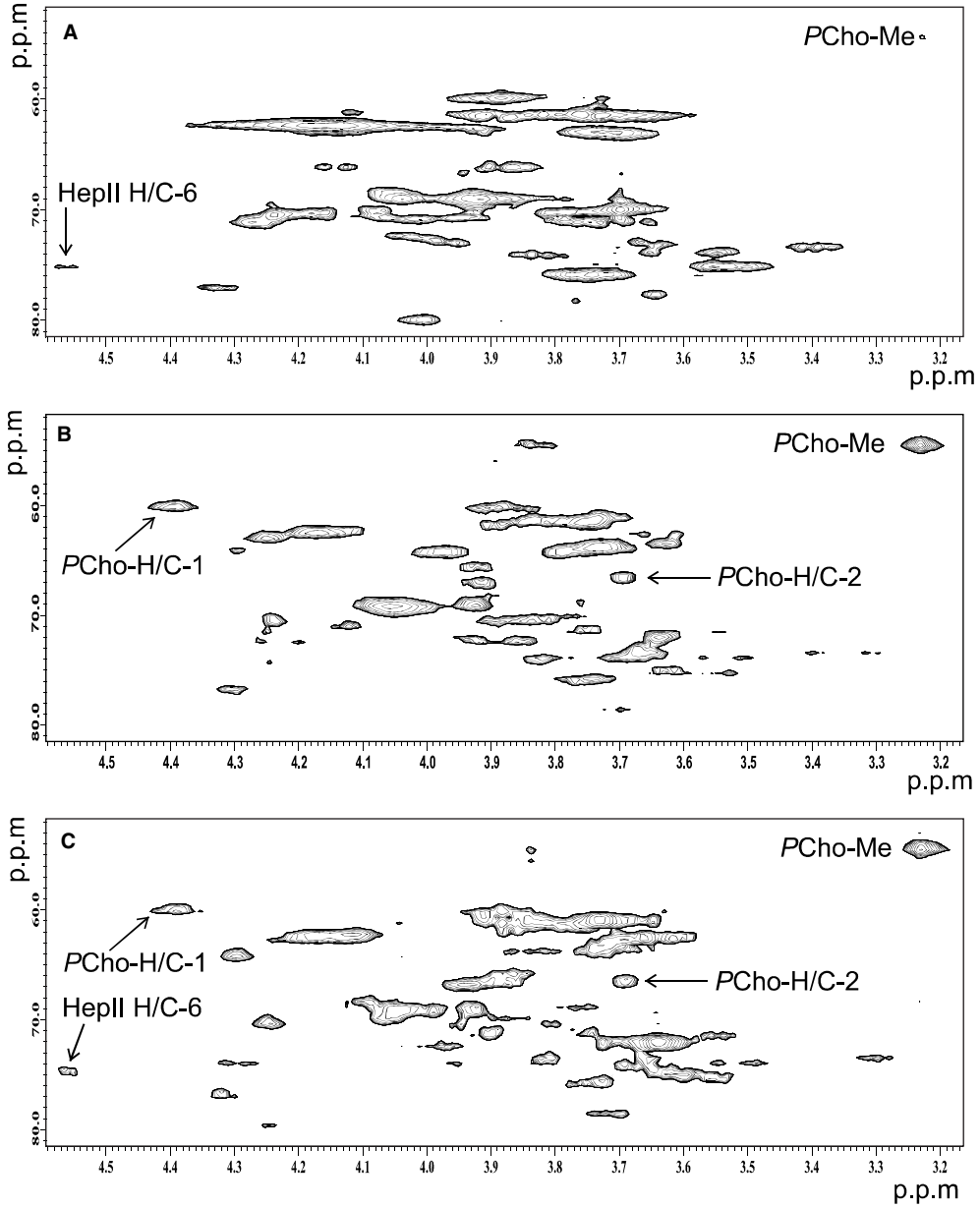


Figure 2. Parts of the 500 MHz heteronuclear ^{1}H–^{13}C HSQC spectra of (A) OS derived from 981HI1064, (B) LPS-OH derived from 981*lpt6* and (C) LPS-OH derived from 981 wt. Assignments are labelled.

O-6 of GlcI.[22] The resemblance in phenotype of the HI1064 mutant to a *lic1* mutant was investigated further. It was found that phase variation of the *lic1A* gene, such that it was no longer translated in the HI1064 mutant strain, resulted in a lack of *P*Cho incorporation that could be reversed in colonies at low frequency upon regrowth and selection with an antibody that specifically recognises *P*Cho (data not shown).

In conclusion, this study showed that *lpt6* has the same function in NTHi strain 981 as strain Rd, the strain for which the complete genome sequence has been determined. However, it is not responsible for adding *P*Etn to O-3 of HepIII since the 981*lpt6* mutant still had full substitution with *P*Etn at HepIII and none at HepII. The function of HI1064 is still unknown, however it does not seem to be involved in pathways of LPS oligosaccharide phosphorylation.

1. Experimental

1.1. Construction of mutant strains, bacterial cultivation and preparation of LPS

Strain NTHi 981 has been described before.[22,27] Strain 981 was mutated by transformation[28] with linearised

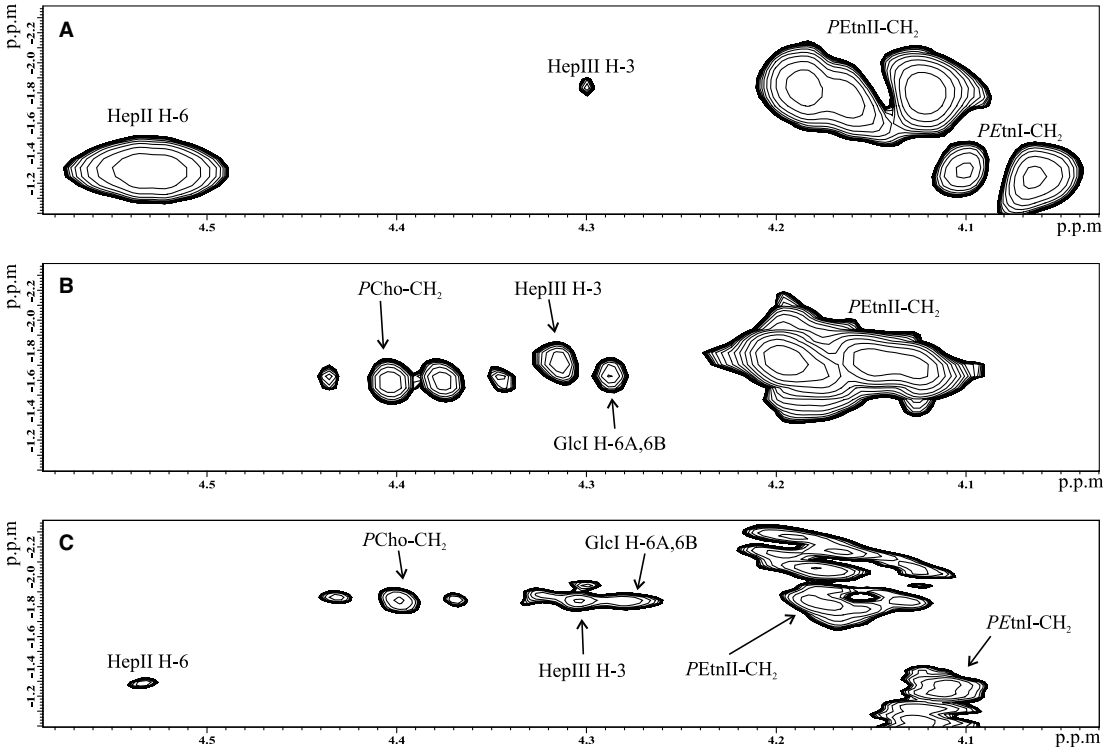


Figure 3. Parts of the 500 MHz heteronuclear ^{1}H–^{31}P HMQC spectra of (A) OS derived from 981HI1064, (B) LPS-OH derived from 981*lpt6* and (C) LPS-OH derived from 981 wt. The experiments were run with regard to an average coupling constant of 8 Hz. Assignments are labelled.

plasmid containing the *H. influenzae lpt6* gene interrupted by insertion of a kanamycin resistance cassette,[21] to give strain 981*lpt6*. Strain 981HI1064 was obtained following transformation of NTHi 981 with plasmid comprising the HI1064 gene cloned into plasmid pT7Blue (Novagen), then interrupted by insertion of a kanamycin resistance cassette obtained from plasmid pUC4kan into the HI0164 reading frame. Mutant strains were confirmed by PCR and Southern analyses as described before.[19] Bacteria were grown in brain–heart infusion broth supplemented with haemin (10 μg mL^{-1}) and NAD (2 μg mL^{-1}). LPS was extracted by the phenol/chloroform/light petroleum method, as described previously.[2]

1.2. Preparation of OS material

1.2.1. *O*-Deacylation of LPS. *O*-Deacylation of LPS was achieved with anhydrous hydrazine as described previously.[29]

1.2.2. Mild acid hydrolysis of LPS. Reduced core OS material was obtained after mild acid hydrolysis (1% aqueous acetic acid, pH 3.1, 100 °C, 2 h) as previously described.[22]

1.2.3. Dephosphorylation. Dephosphorylated OS material was obtained after treatment with 48% hydrogen fluoride as described previously.[12]

1.3. Mass spectrometry and NMR spectroscopy

GLC–MS, ESI-MS and ESI-MS^n were performed as described previously.[10,22,23]

NMR spectra were obtained at 30 °C on a JEOL JNM-ECP500 spectrometer using the previously described experiments.[18]

1.4. Analytical methods

Sugars were identified as their alditol acetates as previously described.[30] Methylation analysis was performed as described earlier.[12] The relative proportions of the various alditol acetates and partially methylated alditol acetates obtained in sugar- and methylation analyses correspond to the detector response of the GLC–MS.

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