Genes required for the synthesis of heptose-containing oligosaccharide outer core extensions in *Haemophilus influenzae* lipopolysaccharide

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Heptose-containing oligosaccharides (OSs) are found in the outer core of the lipopolysaccharide (LPS) of a subset of non-typable *Haemophilus influenzae* (NTHi) strains. Candidate genes for the addition of either L-glycero-D-manno-heptose (LD-Hep) or D-glycero-D-manno-heptose (DD-Hep) and subsequent hexose sugars to these OSs have been identified from the recently completed genome sequences available for NTHi strains. *losA1/losB1* and *losA2/losB2* are two sets of related genes in which *losA* has homology to genes encoding glycosyltransferases and *losB* to genes encoding heptosyltransferases. Each set of genes is variably present across NTHi strains and is located in a region of the genome with an alternative gene organization between strains that contributes to LPS heterogeneity. Dependent upon the strain background, the LPS phenotype, structure and serum resistance of strains mutated in these genes were altered when compared with the relevant parent strain. Our studies confirm that *losB1* and *losB2* usually encode DD-heptosyl- and LD-heptosyl transferases, respectively, and that *losA1* and *losA2* encode glycosyltransferases that play a role in OS extensions of NTHi LPS.

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INTRODUCTION

Haemophilus influenzae is a common commensal of the upper respiratory tract of the human host but is also an important cause of disease. The majority of strains are unencapsulated (non-typable) *H. influenzae* (NTHi) that can by contiguous spread cause infection, such as otitis media (OM) and lower respiratory tract infections, particularly in infants. Occasionally, strains can be invasive, causing bacteraemic infections such as meningitis and septicaemia. Lipopolysaccharide (LPS) is the major glycolipid on the bacterial cell surface, is essential for *H. influenzae* and can have a role in both commensal behaviour and the pathogenesis of *H. influenzae* infections. *H. influenzae* LPS comprises a membrane-anchoring lipid A portion linked by a single 2-keto-3-deoxyoctulosonic acid (Kdo) molecule to a heterogeneous oligosaccharide (OS) composed mainly of neutral heptose (Hep) and hexose (Hex) sugars (Zamze & Moxon, 1987). Each Hep of

a conserved trisaccharide inner core (HepI–HepIII) (Fig. 1a) can be a point for the addition of Hex sugars and further chain extension (Schweda *et al.*, 2007, 2008). The degree and pattern of saccharide extension and the inclusion of phosphate- and non-phosphate-containing substituents all contribute to the structural heterogeneity of *H. influenzae* LPS. The expression of several epitopes of *H. influenzae* LPS is switched on and off at high frequency, so-called phase variation (Patrick *et al.*, 1987; Weiser *et al.*, 1989), a translational control mechanism involving micro-satellites that is adaptive and advantageous for survival of bacteria confronted by the differing conditions, including the immune response, within the host.

Structural analyses of LPS have revealed that a fourth heptose (HepIV), either as a D-glycero-D-manno-heptose (DD-Hep) (Månsson *et al.*, 2003a) or L-glycero-D-manno-heptose (LD-Hep) (Månsson *et al.*, 2003b) enantiomer, can be included as part of the OS extension from HepI in a subset of NTHi strains (Fig. 1a). This HepIV residue can be substituted by further hexose extensions (Houliston *et al.*, 2007; Månsson *et al.*, 2003b), can be branched (Månsson *et al.*, 2003a) or can be substituted by phosphocholine

Abbreviations: DD-Hep, D-glycero-D-manno-heptose; Hep, heptose; LC-ESI-MSⁿ, liquid chromatography-electrospray ionization-multiple step tandem MS; LD-Hep, L-glycero-D-manno-heptose; NTHi, non-typable *H. influenzae*; OM, otitis media; OS, oligosaccharide.

Fig. 1. Schematic representation of HepIV in the LPS of *H. influenzae* and the organization of the encoding genes. (a) The conserved tri-heptose-containing inner core structure (HepI–HepIII) with the location of HepIV indicated by a box. R₁, R₂ and R₃ represent addition of H or sugar(s) dependent upon the strain. Hep, heptose; Glc, glucose; Kdo, 2-keto-3-deoxyoctulosonic acid; PEtn, phosphoethanolamine; P..PEtn, pyrophosphoethanolamine. (b) Organization of the *los* genes determined from genome sequences. (i) The *losA1* and *losB1* genes for strain R2846; (ii) the *losA2* and *losB2* genes for strains R2846 and R2866. The relative positions and orientations of the reading frames are represented by arrows drawn to scale, with the common gene names below and reading frame numbers derived from Artemis analysis of the complete NCBI genome sequence above. Flanking genes are represented by grey arrows. The corresponding chromosomal organization in strain Rd is shown for comparison in each case.

(PCho) (Fox *et al.*, 2008). The gene repertoire required for the synthesis of the *H. influenzae* LPS molecule has been identified in a number of key strains (Hood *et al.*, 1996a, 2001) following analyses based upon the whole-genome sequence of strain Rd (Fleischmann *et al.*, 1995). However, the genes responsible for the incorporation of HepIV and its substitution were not identified by these methods, as these LPS epitopes, and presumably their genetic determinants, are not found in strain Rd. Following the completion of further genome sequences for NTHi strains (Erwin *et al.*, 2005; Harrison *et al.*, 2005), a candidate gene for a novel heptosyltransferase (*losB*) was identified. *losB* is homologous to a gene characterized in the related species *Haemophilus ducreyi* (Tullius *et al.*, 2002) that directs the incorporation of a fourth heptose in the LPS of this species (Post *et al.*, 2007). The *losB* gene is found in a two-gene 'island' in a minority of *H. influenzae* strains (Erwin *et al.*, 2005) alongside *losA*, predicted to encode a phase-variable glycosyltransferase that can influence serum resistance of the bacteria (Erwin *et al.*, 2006b). Structural analysis of LPS from an NTHi *losB* mutant strain has confirmed that *losB* encodes a heptosyltransferase (Lundström *et al.*, 2008). We investigated the role of two homologous sets of *losA* and *losB* genes in the synthesis of heptose-containing OSs in the outer core of NTHi LPS.

METHODS

Bacterial strains and culture conditions. The *H. influenzae* parent strains used in this study are listed in Table 1. Our 25 NTHi strains (Cody *et al.*, 2003) and strain R2846 (Erwin *et al.*, 2006b) are isolates from OM. NTHi strain R2866 is an isolate from invasive disease (Erwin *et al.*, 2005) and NTHi strain 2019 is a clinical isolate that has been used in LPS structural studies (Phillips *et al.*, 1992). *H. influenzae* strain RM118 (Rd; strain KW20) is a type d-derived laboratory strain lacking a capsule, and strain RM153 (Eagan) is a serotype b isolate from invasive disease (Anderson *et al.*, 1972). Strains were grown at 37 °C in brain heart infusion (BHI) broth supplemented with haemin (10 µg ml⁻¹) and NAD (2 µg ml⁻¹) or on BHI agar (1% w/v) supplemented with 10% (v/v) Levinthal's reagent. For selection following transformation, kanamycin (10 µg ml⁻¹), streptomycin (50 µg ml⁻¹), erythromycin (20 µg ml⁻¹) or tetracycline (2 µg ml⁻¹) was added to the growth medium.

Escherichia coli strain DH5α, used for DNA cloning and plasmid propagation, was grown at 37 °C in Luria–Bertani (LB) broth supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or erythromycin (300 µg ml⁻¹), as required (Sambrook *et al.*, 1989).

PCR amplification and recombinant DNA methodology.

Restriction endonucleases and DNA-modifying enzymes were obtained from Roche or New England Biolabs and used according to the suppliers' instructions. Plasmid DNA preparation was performed as described elsewhere (Sambrook *et al.*, 1989). Chromosomal DNA was prepared from *Haemophilus* by the method described previously (Preston *et al.*, 1996).

Table 1. Results of PCR and LPS analyses of the fourth heptose of NTHi strains

Strain	HepIV in LPS	<i>losA1/losB1</i> region*	<i>losA2/losB2</i> region
162	—	—	—
176	DD-Hep	<i>losA1/losB1</i>	<i>losA2/losB2</i> †
285	—	<i>lic2B/lic2C</i>	—
375	—	—	—
432	—	<i>lic2B/lic2C</i>	—
477	DD-Hep	<i>losA1/losB1</i>	—
486	—	<i>lic2B/lic2C</i>	—
667	—	<i>lic2B/lic2C</i> §	—
723	—	—	—
981	DD-Hep	<i>losA1/losB1</i>	—
1008	—	<i>lic2B/lic2C</i>	—
1124	—	—	—
1158	DD-Hep	<i>lic2B/lic2C</i>	<i>losA2/losB2</i> †
1159	DD-Hep	<i>lic2B/lic2C</i>	<i>losA2/losB2</i> †
1180	—	<i>lic2B/lic2C</i>	—
1181	—	<i>lic2B/lic2C</i>	—
1200	—	<i>lic2B/lic2C</i>	—
1207	LD-Hep	—	<i>losA2/losB2</i> ‡
1209	LD-Hep	—	<i>losA2/losB2</i> ‡
1231	DD-Hep	<i>losA1/losB1</i>	<i>losA2/losB2</i> †
1232	DD-Hep	<i>losA1/losB1</i>	<i>losA2/losB2</i> †
1233	LD-Hep	—	<i>losA2/losB2</i> ‡
1247	—	—	—
1268	—	<i>lic2B/lic2C</i>	—
1292	—	<i>lic2B/lic2C</i>	—
R2846	DD-Hep	<i>losA1/losB1</i>	<i>losA2/losB2</i> †
R2866	LD-Hep	—	<i>losA2/losB2</i> ‡
2019	—	<i>lic2B/lic2C</i>	—
Eagan	—	<i>lic2B/lic2C</i>	—
Rd	—	—	—

*Alternative gene arrangements are indicated.

†‡Size of the *hgt1* gene produced with primer pair 2846A and 2846F (†0.93 kb, i.e. R2846-like; ‡1.53 kb, i.e. R2866-like).

§PCR product of a size smaller than that predicted for *lic2B/lic2C* due to the presence of a partial *lic2B* gene.

DNA fragments were amplified by PCR; conditions were 1 min periods of denaturation (94 °C), annealing (50 °C) and polymerization (72 °C) for 30 cycles. To clone the *losA1* and *losB1* genes, the region of DNA between the *infA* and *ksgA* genes was amplified using primers infrev (5'-CCACGTGACCGTTTCCAAC-3') and ksgArev (5'-GCATATCTTGAATCACATC-3') from genomic DNA isolated from strains R2846, 176 and 981. A 1 µl volume of the PCR-amplified product of 2.47 kbp was ligated with plasmid pT7Blue (Novagen) and transformed into *E. coli*. For R2846, the PCR product was initially cloned into the vector pCR2.1 (Invitrogen), then the insert, released on a *Bam*HI–*Xho*I fragment, was subcloned into compatible *Bam*HI–*Sal*I sites in pT7Blue. Recombinant plasmids were confirmed by restriction endonuclease digestion and PCR amplification, then the target genes were mutagenized by insertion of a kanamycin-resistance cassette (*kan*^R) (from pUC4Kan, GE Healthcare) or a tetracycline-resistance cassette (*tet*^R) [from pHVT1 (Danner & Pifer, 1982)]. The *losA1* gene was cut at a *Nsi*I site, then ligated with a *kan*^R released by digestion with *Pst*I, whilst the *losB1* gene was interrupted by ligating

kan^R, released with *Acc*I, into a unique *Bst*BI site within the reading frame. The *losB1* gene was also digested at the same *Bst*BI site, end-filled using Klenow and dNTPs, then disrupted by inserting a *tet*^R released by digestion with *Hind*III, then end-filled with Klenow, to give plasmid p2846A3.1BstBItet1.

The *losA2* and *losB2* genes were amplified using primers 2846C (5'-TGGTCGTGCAGAACGCTT-3') and 2846D (5'-AGTATGTGCG-TTCTGCC-3'), and the neighbouring genes *hgt1* and *L31* using primers 2846A (5'-TAAAAACATGGTGGGTGG-3') and 2846B (5'-AAGAATAATAGCACGCGC-3') from strains R2846 and 176. Aliquots (1 µl) of the PCR-amplified products of 1.75 and 1.31 kb, respectively, were ligated with plasmid pT7Blue (Novagen) and transformed into *E. coli*. Recombinant plasmids containing the *hgt1/L31* and *losA2/losB2* genes from NTHi strain R2846 and the *losA2/losB2* genes from NTHi 176 were confirmed by restriction endonuclease digestion and PCR amplification using the specific primers above, then were used to inactivate the target genes by insertional mutagenesis using a *kan*^R. Genes *hgt1* and *losA2* were cut at *Mfe*I sites within the respective reading frame, then ligated with a *kan*^R released by digestion with *Eco*RI, and *losA2* was interrupted by ligating the same *kan*^R into an *Eco*RI site within the reading frame. The gene *losB2* was digested at a unique *Nru*I site within the reading frame, end-filled using Klenow and dNTPs, and then disrupted by inserting a *kan*^R released by digestion with *Hinc*II.

The oligonucleotide primer pair infrev/ksgArev was used to screen for the presence of genes inserted between *infA* and *ksgA* in each of the NTHi strains by PCR amplification. To determine whether these genes are *lic2B* and *lic2C*, primers specific for each gene (*lic2B* 5'-GTC-TTTCGTTTCTAACTG-3'/*lic2BB* 5'-AAGTATGATCCTCAAATG-3' for *lic2B* and *lic2BC* 5'-CAATTTCACTAACTTGCC-3'/*lic2BA* 5'-CATTAGCGATGAGTTCC-3' for *lic2C*) were used. Correspondingly, to determine whether the *losA1* and *losB1* genes were present, the primer pairs R2846GTfor (5'-CAAGAAGAGCAATTATTTC-3')/R2846GTrev (5'-GGTTCAATACCATAGGTG-3') and R2846hepfor (5'-GGTGTCTATCTGCACCAAAC-3')/R2846heprev (5'-GATCTCA-TTAACATTATCG-3') were used, respectively.

Primer pairs *losA2A* (5'-GATATAATCGCTCAACGG-3')/*losA2B* (5'-TCCAGTGAGTTGTAACGC-3') and *losB2A* (5'-GTAAAGTTGG-GTCTAGG-3')/*losB2B* (5'-TATGCGGATAATCGGCTG-3') were used to amplify specifically the *losA2* and *losB2* sequences, respectively, and primers 2846G (5'-GGCAGAAACGCACATACT-3')/2846H (5'-GGGCGACCTATACATTAG-3') the two genes downstream of *losB2* from the NTHi strains. The oligonucleotide primer pairs 2846A/2846B and 2846C/2846D were used to screen for the presence of the *hgt1/L31* and *losA2/losB2* regions of DNA, respectively, in each of the *H. influenzae* strains.

Construction of mutant strains. A 1 µg sample of recombinant plasmid containing an interrupted target gene was linearized by restriction digestion, then used to transform *H. influenzae* strains by the M-IV medium method (Herriott *et al.*, 1970). All transformants were recultured on BHI/kanamycin or BHI/tetracycline, then confirmed as mutants by PCR amplification using the oligonucleotide primers described above and when appropriate by DNA sequencing.

Complementation of *losB1*. Plasmid pEJ18 contains both an *E. coli* and an *H. influenzae* origin of replication plus an erythromycin-resistance gene (*E. Robinson*, unpublished results). The cloned *losA1/losB1* region from R2846 was released from our recombinant plasmid by digestion with *Bam*HI and *Xho*I, then ligated into pEJ18 digested with *Nru*I and *Xho*I. An in-frame deletion of the *losA1* gene was generated by digestion with *Dra*III, blunting of the ends and religation to produce plasmid pEJ18Δ*losA1*/ *losB1*+. pEJ18Δ*losA1*/ *losB1* + was transformed into *E. coli* strain SM10λpir, which was then used as donor in conjugation with strain R2846*losA1strep*^R (strain

R2846/*losA1* transformed to streptomycin resistance using chromosomal DNA from RM154, a spontaneously resistant derivative of strain Eagan). Transconjugants were selected for on BHI plates containing streptomycin to select against the *E. coli* donor strain and erythromycin to select for the recipient *H. influenzae* harbouring the plasmid.

Analysis of LPS by electrophoresis. The patterns of LPS isolated from strains upon Tricine-SDS-PAGE (T-SDS-PAGE) were analysed as described previously (Hood *et al.*, 1996a).

Serum resistance assay. A sample of 5×10^2 bacteria cultured on plates was assayed for survival against the killing effect of normal human serum as described previously (Hood *et al.*, 1999). Experiments were carried out in triplicate.

Structural analysis of purified LPS. LPS purified from bacteria (Hood *et al.*, 1996b) was subject to structural elucidation following procedures described previously (Lundström *et al.*, 2008). Briefly, sugars were identified as their alditol acetates using authentic standards. Core OS fractions of R2486/*losA1* and R2486/*losA2* were obtained following mild acid hydrolysis of LPS and subsequent gel filtration chromatography. For 176/*losB1* and 176/*losB2*, we applied a micro-method involving mild acid hydrolysis of O-deacylated LPS, reduction and dephosphorylation of resulting OS material in one reaction vial prior to permethylation as outlined previously (Gulin *et al.*, 2003). Dephosphorylation was obtained after treatment with 48 % hydrogen fluoride. Methylation analysis was accomplished on dephosphorylated OS using methyl iodide in DMSO in the presence of lithium methylsulfinylmethanide. Liquid chromatography-electrospray ionization-multiple step tandem MS (LC-ESI-MSⁿ) experiments on permethylated OS samples were performed in the positive ion-mode $[M + Na]^+$ on a Finnigan LCQ ion trap mass spectrometer coupled to a Waters HPLC system.

RESULTS

Genes required for biosynthesis of heptose-containing OSs

OS extensions present in NTHi LPS can contain a fourth heptose, as either DD-Hep or LD-Hep, that can be further substituted, or branched, and thus the genetics underlying their biosynthesis is likely complex. In our search to identify genes required for the biosynthesis of heptose-containing OSs, we investigated two chromosomal loci found in the genome sequences of NTHi strains. One of these loci contains two genes reported in *H. influenzae*, *losA1* and *losB1* (formerly called *losA* and *losB*), that are homologues of LPS biosynthesis genes in the related species *H. ducreyi* (Erwin *et al.*, 2005). In *H. ducreyi* these genes, known as *lgbA* and *lgbB*, encode a galactosyltransferase and D-glycero-D-manno-heptosyltransferase, respectively (Post *et al.*, 2007; Tullius *et al.*, 2002). *losA1* and *losB1* are located between the *infA* and the *ksgA* genes, and if present are in a position in the *H. influenzae* genome equivalent to that of the LPS genes *lic2C* and *lic2B* (Erwin *et al.*, 2005; High *et al.*, 1996; Hood *et al.*, 2004a) (Fig. 1b), but share no homology to them. Thus, the *infA/ksgA* region represents a particular site of genetic diversity between strains (Erwin *et al.*, 2005). The *losA1* (AAY56464.1) and *losB1* (AAY56463.1) genes are present in the genome sequence of NTHi strain R2846

(Erwin *et al.*, 2005), but are not found in either NTHi strain R2866 or strain 86-028NP. At a second distant chromosomal locus, further related genes, *losA2* and *losB2*, were identified by homology searches in the genomes of strains R2846 and R2866, but not 86-028NP. The *losA2* and *losB2* genes in the R2846 genome sequence form part of a 10-gene island of approximately 8 kb in length that comprises mainly genes inferred to play roles in iron metabolism (Fig. 1b). This group of genes is located in a position equivalent to that between genes *HI0862* and *HI0861* in the strain Rd genome sequence; in Rd there is only a 190 bp gap between these reading frames (Fig. 1b). For strain R2846, the *losA1* and *losA2* genes are 63 % homologous and the transcribed products are 61 % identical. Similarly, the *losB1* and *losB2* gene products are 68 % similar and 58 % identical to each other, albeit that *LosB1* is predicted to be 14 aa shorter than *LosB2*. The *losA* and *losB* genes are contiguous at each of the two chromosomal sites (Fig. 1b); *losA2* and *losB2* reading frames are separated by only 2 bp. In strain R2846, the *losA2* reading frame is truncated, encoding a protein of only 44 aa, in contrast to the 225 aa protein encoded by *losA1*. Upstream of *losA2* in strain R2846 is a frameshifted ribosomal protein gene (*L31*) and upstream of this is a gene encoding a further novel glycosyltransferase (ZP_01784022), *hgt1* (haemophilus glycosyltransferase 1), which has no significant homology to other *H. influenzae* genes. Five genes in this island downstream of *losB2*, and separated by the *fpg* gene, are predicted to encode the components of an ABC transport system (nhi1564–nhi1568 in Fig. 1b) involved in the transport of divalent cation-containing compounds, and nhi1569 has homology to genes encoding autotransporter adhesins. We would predict from the juxtaposition of many of these genes that their transcription would be co-dependent. The *fpg* reading frame has high homology to part of *mutM*, a gene involved in DNA repair that is located at a separate chromosomal location (*HI0964* in the Rd genome sequence). Each gene product from this island, with the exception of nhi1569, was almost identical in sequence to equivalent contiguous genes identified in *Pasteurella multocida* PM70 (1 341 541–1 348 996 bp on the genome sequence at <http://cmr.jcvi.org>).

In strain R2866, although the organization of the *losA2/losB2* region of DNA is overall similar (Fig. 1b), the *hgt1* gene is 522 nt longer than that in R2846 and the predicted gene product (ZP_00156727) shares homology with characterized glycosyltransferases [43 % identity with the galactosyltransferase Lic2A from *H. influenzae* (High *et al.*, 1996)]. Also in R2866, the *losA2* gene encodes a predicted full-length and likely functional 222 aa protein of a length similar to the proteins produced in both *H. ducreyi* and *P. multocida*.

We investigated the distribution of the *losA1/losB1* and *losA2/losB2* chromosomal loci by PCR amplification in our set of 25 NTHi strains, each expressing LPS that had been structurally characterized (Schweda *et al.*, 2007), using combinations of internal and external primers specific for

each locus. Between *infA* and *ksgA*, five strains contained *losA1/losB1*, 11 contained *lic2B/lic2C*, eight had no genes present, and one contained a sequence smaller than predicted for *lic2B/lic2C* due to the presence of a partial *lic2B* gene. The distribution of these alternative gene arrangements amongst strains was similar to that found for sets of throat-, OM- and invasive disease-related NTHi isolates (Erwin *et al.*, 2005). For the second locus of interest, *losA2* and *losB2* genes were identified in eight of our 25 NTHi strains in a similar organization (Fig. 1b). Five of the eight NTHi strains contained the shorter *hgt1* sequence similar to that of strain R2846, and three NTHi strains had the longer R2866-like sequence (Table 1). By structural analysis, one or both of the *losA1/losB1* and *losA2/losB2* gene pairs were present in strains known to configure a fourth heptose, either an LD-Hep or a DD-Hep, in their LPS (Table 1). Only three of our 25 NTHi strains, 176, 1231 and 1232, contained both the *losA1/losB1* and *losA2/losB2* gene sets, similar to strain R2846. Although each strain that included LD-Hep as HepIV in its LPS contained at least the *losA2/losB2* genes, no complete correlation between the presence of either gene set and the nature of HepIV could be made.

Effect of mutation of *losA* and *losB* genes

To investigate the roles of these two chromosomal loci in LPS synthesis, we mutated relevant genes in *H. influenzae* strains expressing a HepIV in their LPS. Mutants were obtained with either the *losA1* or *losB1* genes knocked out in strain R2846 (DD-Hep) and 176 (DD-Hep) backgrounds, and the *losA2* or *losB2* genes knocked out in the R2846, 176, 1207 (LD-Hep) and 1158 (DD-Hep) strain backgrounds. No NTHi strain mutated in the predicted glycosyltransferase gene, *hgt1*, was obtained. The LPS profile for each of these mutant strains was compared with that for the relevant parent strain. The LPS was truncated for the two strains R2846 (DD-Hep) and 176 (DD-Hep) mutated in the *losB1* or *losA1* gene (Fig. 2a). Some

more minor changes in LPS profile were also observed for both *losA2* and *losB2* mutants, with the mutants in strain R2846 having LPS patterns skewed to the lower-molecular-mass end of the range seen in the parent strain (Fig. 2a). LPS from the double mutant strain R2846*losB1losB2* was truncated similarly to that isolated from the R2846*losB1* single mutant strain. No difference in LPS profile, when compared with the wild-type, was found for either the *losA2* mutant or the *losB2* mutant in strain 1158 (DD-Hep) (data not shown), and only some minor changes for mutants in strain 1207 (LD-Hep) (Fig. 2b). Although *losB1* mutations in strains expressing DD-Hep as HepIV showed the biggest change in LPS profile compared with the wild-type, indicating that LosB1 would be the DD-heptosyl-transferase, no complete correlation between LosB1 and LosB2 distribution and HepIV specificity in mutant strains was found.

For any given strain background tested, the LPS phenotypes of strains mutated in either the *losA* or the *losB* gene from the same locus were similar. This result strongly suggests co-dependent transcription of the *losA* and *losB* genes and a polar effect of mutation of *losA* on *losB* expression. We predict that LosA would add a hexose as part of any further extension from HepIV, with LosA1 predicted to add a glucose and LosA2 a galactose based on sequence similarities to other glycosyltransferases (Deadman *et al.*, 2006, 2009). We cannot confirm *losA* function in the relevant mutant strain as the HepIV-containing acceptor glycoform is absent. To establish the function of *losA1*, we constructed a derivative of strain R2846*losA1* in which *losB1* function was complemented *in trans* on a plasmid. For this strain R2846Δ*losA1/B1*+, the molecular mass of LPS glycoforms observed was increased when compared with R2846*losA1* (Fig. 2c).

HepIV-containing LPS glycoforms have previously been shown to include epitopes that influence serum resistance of NTHi strains (Erwin *et al.*, 2006b; Houliston *et al.*, 2007). For our strains, the killing effect of normal human

Fig. 2. Electrophoretic profiles of LPS isolated from strains mutated in LPS biosynthesis genes compared with the parent strain (wt). (a) *losA* and *losB* mutants for strains R2846 and 176; (b) *losA2* and *losB2* mutants for strain 1207; (c) strain R2846, the *losA1* mutant and the complemented *losA1* strain (Δ *losA1/B1* +).

serum was somewhat enhanced for each of the *losA1*, *losB1*, *losA2* and *losB2* mutants of both strain 176 (Fig. 3a) and strain R2846 (Fig. 3b) when compared with the respective parent strain. The double mutant R2846*losB1/losB2* was one of the most serum-sensitive strains in this NTHi background (Fig. 3b). For NTHi strain R2866, there was little difference in serum resistance between the *losA2* or *losB2* mutant strain and the parent strain (data not shown).

Structural confirmation of *losA* and *losB* function

To properly interpret the changes in LPS phenotype observed with *losA* and *losB* mutant strains, the structure of LPS was determined for isogenic strains in the NTHi 176 and R2846 backgrounds. Sequence and branching details of the various glycoforms in core OS material were obtained using LC-ESI-MSⁿ in the positive mode. Analyses of LPS from R2486*losA1* indicated the total loss of DD-Hep (HepIV), virtually the same result obtained previously for strain R2486*losB1* (Lundström *et al.*, 2008) (Tables 2 and 3). For the complemented *losA1* mutant, methylation analysis of LPS from R2846Δ*losA1*/B1+ revealed major amounts of terminal DD-Hep, complete loss of 4-substituted DD-Hep, and a decrease of terminal Glc when compared with the wild-type strain (Table 2). This supports *LosB1* function being restored in this *losA1* mutant background. In the LC-ESI-MSⁿ mass spectrum of dephosphorylated and permethylated OS from strain R2846Δ*losA1*/B1+ (positive mode, Fig. 4a), a major

sodiated singly charged adduct ion ($[M+Na]^1+$; m/z 1716) was detected, corresponding to Hex₂Hep₄AnKdo-ol (Hex2Hep glycoform). By fragmenting this ion (Fig. 4a) we confirmed that HepIV is predominantly a terminal DD-Hep, in contrast to the wild-type strain, in which glucose is terminal (Lundström *et al.*, 2008). For strain R2846*losA2*, compositional analyses of LPS indicated near identical ratios of sugars to those of strain R2846Δ*losA1*/B1+ (data not shown). In the LC-ESI-MS spectrum of dephosphorylated and permethylated OS from R2846*losA2*, major sodiated adduct ions ($[M+Na]^1+$) were observed at m/z 1920 and 2124 (Fig. 4b), corresponding to Hex3Hep and Hex4Hep glycoforms, respectively (Table 3). In these latter glycoforms, HepIV is DD-Hep, which is further extended, since 4-substituted DD-Hep was detected in methylation analysis (Table 2). Thus, structural data for R2846*losA2* LPS were similar to those from R2846*losB2* and the wild-type strain (Lundström *et al.*, 2008). These data are consistent with the hypothesis that *losB1* encodes addition of the DD-Hep that is incorporated as HepIV exclusively in the wild-type strain, and that *LosA1* functions as a glycosyltransferase, adding a glucose to this HepIV.

In a previous study, structural analysis of LPS from strain R2846*losB1* indicated that although DD-Hep was not expressed, minor quantities of LD-Hep were found in its place as HepIV (Lundström *et al.*, 2008). LPS from R2486*losB2* was very similar to that of the wild-type strain, and LPS from the R2846*losB1/losB2* mutant showed no

Fig. 3. Serum bactericidal assay of the relative killing of wild-type and mutant NTHi strains by normal human serum. The percentage survival of bacteria (relative to t_0) is plotted against doubling dilutions of human serum for NTHi strains 176 (a) and R2846 (b). The data shown are the mean from three repeat experiments; error bars, SD.

Table 2. Methylation analysis of dephosphorylated OS samples derived from 176 and R2846 mutant strains

Methylated sugar*	$t_{gm}^{\dagger}$	Linkage assignment	Relative detector response (%)					
			176 <i>losB1</i>	176 <i>losB2</i>	R2846 <i>losA1</i>	R2846 <i>losA2</i>	R2846 <i>ΔlosA1/B1</i> +	R2846wt‡
2,3,4,6-Me ₄ -Glc	1.00	D-Glcp-(1-	22	17	19	18	7	13
2,3,4,6-Me ₄ -Gal	1.06	D-Galp-(1-	25	14	200	16	12	6
2,3,6-Me ₃ -Gal	1.22	-4)-D-Galp-(1-	Trace	Trace	Trace	1	Trace	2
2,3,6-Me ₃ -Glc	1.23	-4)-D-Glcp-(1-	2	1	4	1	Trace	1
2,4,6-Me ₃ -Gal	1.27	-3)-D-Galp-(1-		Trace	6	Trace		4
2,3,4-Me ₃ -Glc	1.28	-6)-D-Glcp-(1-	3	18	4	14	19	16
2,3,4,6,7-Me ₅ -Hep	1.32	DD-Hep-(1-					14	
2,3,4-Me ₃ -Gal	1.36	-6)-D-Galp-(1-			2	1	Trace	Trace
2,3,4,6,7-Me ₅ -Hep	1.39	LD-Hep-(1-	Trace		5	Trace	2	Trace
2,3-Me ₂ -Glc	1.48	-4,6)-D-Glcp-(1-		2	1	4	4	3
2,3,6,7-Me ₄ -Hep	1.53	-4)-DD-Hep-(1-		18		16		17
2,3,6,7-Me ₄ -	1.58	-4)-LD-Hep-(1-						Trace
3,4,6,7-Me ₄ -Hep	1.59	-2)-LD-Hep-(1-	33	18	29	19	23	10
2,6,7-Me ₃ -Hep	1.69	-3,4)-LD-Hep-(1-	12	12	10	8	19	17
2,3,7-Me ₃ -Hep	1.71	-4,6)-LD-Hep-(1-	3		Trace			
2,3,4,6-Me ₃ -GalN	1.75	D-GalpNAcp-(1-		Trace	Trace	Trace	Trace	2
2,3,6-Me ₃ -GlcN	1.88	-4)-D-GlcNAcp-(1-		Trace	Trace	Trace	Trace	4

*2,3,4,6-Me₄-Glc represents 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol-1d₁, etc.

†Retention times (t_{gm}) are reported relative to 2,3,4,6-Me₄-Glc.

‡For comparison (from Lundström *et al.*, 2008).

HepIV. To broaden these findings, we investigated a *losB1* mutant of NTHi strain 176, which, as wild-type, expresses predominantly DD-Hep as HepIV (our unpublished results). Compositional analyses of LPS from strain 176*losB1* revealed no DD-Hep by GLC-MS analysis (data not shown). In the LC-ESI-MS spectrum of dephosphorylated and permethylated OS from 176*losB1*, the major sodiated adduct ion ($[M + Na]^+$) was observed at m/z 1468, corresponding to a Hex2 glycoform (Table 3). Minor ions at m/z 1716, 1920, 2124 and 2328 corresponded to Hex2Hep, Hex3Hep, Hex4Hep and Hex5Hep, respectively, indicating glycoforms that contain HepIV. Since LPS from 176*losB1* did not contain DD-Hep, the fourth heptose was concluded to be a non-core LD-Hep. MS² and MS³ experiments confirmed this heptose to be part of the outer core (Table 3). The 4,6-substituted LD-Hep observed in methylation analysis was concluded to be HepIV. Analyses of LPS from strain 176*losB2* gave virtually the same major glycoforms as the wild-type strain, with a major Hex3Hep glycoform in which HepIV is a 4-substituted DD-Hep (Table 2). In contrast to 176*losB1*, however, no traces of 4,6-substituted Hep could be detected (Table 2), indicating that it did not express non-core LD-Hep. These data are consistent with *LosB2* having an LD-heptosyltransferase function.

DISCUSSION

Genetic diversity across NTHi underlies the significant variation in surface structures within and between strains that facilitates adaptation in the human host. LPS structure

can influence both the carriage of the bacterium and the likelihood of disease following NTHi infection (Erwin *et al.*, 2006a; Schweda *et al.*, 2007).

The two regions of the chromosome containing *losA* and *losB* genes investigated in our study are examples of NTHi genome diversity that contribute to the substantial heterogeneity of LPS structure that is observed between strains. In our set of 25 NTHi strains, three alternative arrangements between *infA* and *ksgA* are: no genes present (32 %), or the *lic2B/lic2C* (48 %) or *losA1/losB1* (20 %) gene pair present. Association studies test links between the presence or absence of a particular DNA sequence in the genome and the ability of the relevant strains to cause disease (Erwin *et al.*, 2005; Harrison *et al.*, 2005). In an earlier study, either the *losA1/losB1* or *lic2B/lic2C* genes were found in a majority of throat and OM NTHi isolates, but were absent in 40 % of invasive isolates, although no definitive correlation with disease could be established for these genes (Erwin *et al.*, 2005). In another study, *lic2B* had a skewed distribution amongst NTHi strains, being associated with one half of OM isolates but only 14 % of strains isolated from the throat (Pettigrew *et al.*, 2002).

The *losA2* and *losB2* genes, present in 32 % of our 25 NTHi isolates, are part of a 10-gene island containing other genes with functions apparently directed mainly to divalent ion acquisition whose overall organization is conserved between strains. This island is located only three reading frames distant from the intergenic region (between *H10864* and *H10865* in the strain Rd genome sequence) where the

Table 3. Glycoform composition elucidated by LC-ESI-MSⁿ (positive mode) of permethylated and dephosphorylated OS samples obtained from 176 and R2846 mutant strains

The glycoforms present and their relative abundance are indicated on the left of the table for each strain. All glycoforms contain Hep₃AnKdo-ol. The right-hand side of the table details which glucose residues, denoted by the letters k, l, m, n, o, p, q, r and s in the structure above the table, are present. Numbers greater than 1 indicate that multiple glucose residues of similar type are added to the same starting position. The relative isomer abundance for each glycoform is indicated on the extreme right of the table (A–E).

Glycoform	Relative glycoform abundance* (%)					Structure									Relative isomer abundance†				
	176losB1	176losB2	2846losA2‡	2846losA1	R2846ΔlosA1/B1 +	k	l	m	n	o	p	q	r	s	A	B	C	D	E
	A	B	C	D	E														
Hex	3	1		4	6	0	0	0	0	1	0	0	0	0	H	L		H	H
						0	0	0	0	0	0	0	1	0	L	H		L	
Hex ₂	82	1	Trace	86	25	0	0	0	0	1	0	0	1	0	H	H	H	H	H
Hex ₃	5	1		Trace		0	0	0	1	1	0	0	1	0	H	H		H	
Hex ₄	1			Trace		0	0	0	2	1	0	0	1	0	H			H	
Hex ₁ Hep				Trace		0	0	0	0	1	1	0	0	0				H	
Hex ₂ Hep	1	2	2	8	69	0	0	0	0	1	1	0	1	0	M	L	L	H	H
						0	0	0	0	1	1	1	0	0	M	M	M		
Hex ₃ Hep	2	89	86	Trace		0	0	0	0	1	1	1	1	0	M	H	H	H	
						0	0	0	0	1	1	1	0	1	M				
Hex ₄ Hep	5	3	12			0	0	0	1	1	1	1	1	0			H		
						0	0	0	0	1	1	1	1	1	H				
						0	0	0	0	1	1	2	1	0		M			
						0	0	0	1	1	1	1	1	0		M			
Hex ₅ Hep	1	1				0	0	0	0	1	1	2	1	1	H				
						0	0	0	0	1	1	3	1	0		H			
Hex ₃ HexNAC ₁				Trace		0	1	1	1	1	0	0	0	0				H	
Hex ₄ HexNAC ₁				2		0	1	1	1	1	0	0	1	0				H	
Hex ₅ HepHexNAC ₁		1	Trace			0	1	1	1	1	1	1	1	0		H	H		
Hex ₅ HepHexNAC ₂		1	Trace			1	1	1	1	1	1	1	1	0		H	H		

*Relative abundance (percentage for each glycoform calculated from the detected molecular ions in the MS¹ spectrum).
†Relative abundance for structural isomers of each glycoform calculated from the intensity of the product ions in the MS² spectrum: H (high, >80%), M (medium, 80–30%), L (low, 30–2%).
‡Data virtually identical to those for R2846 wild-type and R2846losB2 (Lundström *et al.*, 2008).

lic3B gene, encoding an LPS-specific sialyltransferase, is variably present amongst NTHi strains (Fox *et al.*, 2006), and this is separated by a further gene from the chromosomal location where the *hmg* locus (between *HI0865* and *HI0875* in the strain Rd genome sequence), encoding the incorporation of a four-sugar unit into the LPS, is present in about 60 % of NTHi strains (Hood *et al.*, 2004b). Both *lic3B* and the *hmg* locus can enhance LPS

sialylation, a crucial factor in the pathogenesis of OM in an experimental model of NTHi infection (Bouchet *et al.*, 2003). The *losA2/losB2*, *lic3B* and *hmg* loci were distributed such that any combination of up to two of the three regions was found in a given strain background, but no strain contained all three (data not shown). The reason why the *losA/losB* genes are located in regions of the genome with variable insertion of genes is not clear. A

Fig. 4. LC-ESI-MS full-scan spectra of the derivatized LPS (permethylated OS) from R2846 mutant strains. (a) R2846 Δ losA1/B1+, (b) R2846/losA2. The MS/MS spectra of the boxed ions and the corresponding glycoform structures are presented in (i) and (ii). The spectrum for R2846/losA2 in (b) is virtually identical to spectra for the R2486 parent and R2486/losB2 strains described previously (Lundström *et al.*, 2008).

syntenic series of genes highly homologous (>98 %) to the *losA2/losB2* island is present in *P. multocida*, consistent with a recent exchange of this region of DNA between genera.

Based upon structural analysis of LPS from the isogenic mutants in the strain 176 and R2846 backgrounds, *losB1* encodes a DD-heptosyltransferase and *losB2* an LD-heptosyltransferase. Both proteins are active in these strains, as a *losB1* mutation switches incorporation from the normal DD-Hep to an LD-Hep, the former being incorporated exclusively in the wild-type (Lundström *et al.*, 2008). When considering the distribution of *los* genes across NTHi, each strain in which HepIV is the LD-Hep enantiomer contains the *losB2* gene, and with the exception of isolates 1158 and 1159 (DD-Hep), all strains expressing DD-Hep as HepIV contain the *losB1* gene. DNA sequence analysis of *losB2* from strain NTHi 1158 revealed a sequence highly homologous to *losB2* from strains R2846 and 1207, differing uniquely at only two amino acids (data not shown). However, comparison of these sequence polymorphisms with the amino acid sequences of heptosyltransferases from other species (<http://www.cazy.org>) where the specificity for DD- or LD-Hep addition is known revealed that these changes might direct 1158 *LosB2* function in a manner closer to that of *LosB1* in other NTHi strains. One of the amino acids is located in the putative active site of the protein, and work is under way to confirm that these sequence polymorphisms direct the specificity of the heptose addition. In *H. ducreyi*, the *losB* homologue, *lbgB*, encodes a DD-heptosyltransferase

required to incorporate a heptose equivalent to HepIV in *H. influenzae*, and it and the adjacent *losA* homologue, *lbgA*, are flanked by genes encoding L31 ribosomal protein and exonuclease III (Post *et al.*, 2007). This organization is similar to that of the *losB2* gene in NTHi, but *LbgB* has a function similar to that of *LosB1*. The juxtaposition of the *losA* and *losB* genes makes it unsurprising that mutation of *losA* has an apparent polar effect on *losB* expression, resulting in an LPS phenotype in which no HepIV is incorporated. Through complementation of *losB1* function in a R2846/losA1 mutant strain we showed that HepIV was now included in the LPS but with no further OS elongation. This is consistent with *LosA1* being a glucosyltransferase required to add the next hexose onto HepIV. By analogy and based on sequence differences compared with *LosA1*, we conclude that *LosA2* acts as a galactosyltransferase for HepIV substitution, in the respective NTHi strains.

In strain R2846, the *losA2* and *hgt1* genes are truncated due to frameshifts and are likely non-functional. In an earlier study of *losA1* in NTHi, tandem copies of the octanucleotide 5'-CGAGCATA-3' controlled variable expression of the gene and the serum-resistant phenotype of the bacteria (Erwin *et al.*, 2006b). This repeat tract is not present in *losA2*. When *losA1* was inactivated in strain R2846 in the study of Erwin *et al.* (2006b), a truncation of the LPS was found, similar to the results in our study, but no corresponding change in LPS phenotype was observed when the number of octanucleotide repeats changed from one permissive for translation of the gene to one not, and

vice versa. This discrepancy likely reflects the co-dependence of *losA1* and *losB1* transcriptional (but not translational) expression indicated in our study.

H. influenzae has several strategies to enhance LPS structural variation within and between strains. These include phase-variable expression of LPS biosynthesis genes, sequence polymorphisms directing alternative LPS phenotypes, and differences in the number and nature of LPS genes between strains. This list can now be refined to include mutually exclusive LPS gene sets found at the same genomic location. The alternative gene organizations at the *infA/ksgA* site in the genome direct either OS elongation from the middle heptose (HepII) (*lic2B/lic2C*) of the LPS inner core or inclusion and substitution of HepIV in OS extensions from HepI (*losA1/losB1*), LPS patterns that must be mutually exclusive and that can influence the disease-causing properties of the molecule (Erwin *et al.*, 2005; Hood *et al.*, 2004a).

In conclusion, we have identified two sets of genes that add and substitute a fourth heptose in OS extensions in some NTHi LPS. These genes underlie a further example of the extreme genetic and phenotypic diversity of LPS between strains that exists to increase the fitness of the bacteria during interactions with the host.

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