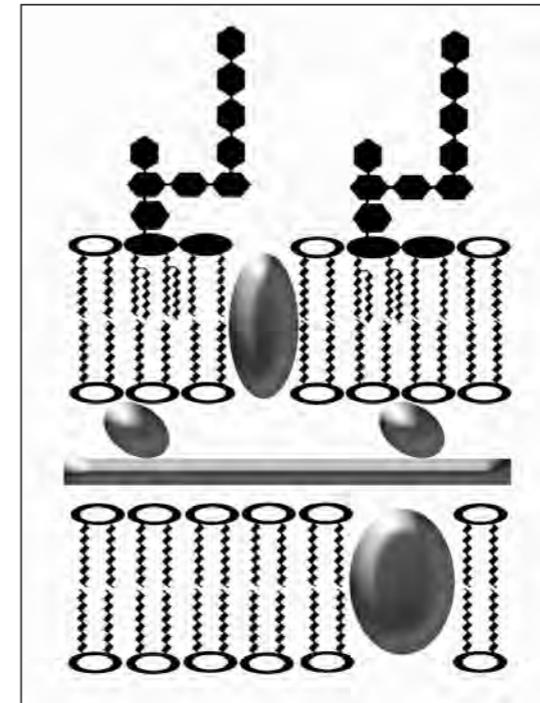


Thesis for doctoral degree (Ph.D.)
2008

Detailed structural studies towards the understanding of lipopolysaccharide glycan expression in non-typeable *Haemophilus influenzae*



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From Clinical Research Center
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Abstract

Haemophilus influenzae (Hi) is a host-adapted Gram-negative bacterium that regularly colonizes the respiratory tract of humans. Hi is an important cause of disease worldwide and exists in encapsulated and unencapsulated (non-typeable, NT) forms. Lipopolysaccharide (LPS) is a characteristic surface component of the bacteria and has been shown to be an important virulence factor. A great variety of both inter- and intra-strain LPS glycoform structures have been detected and structurally elucidated as well as the genes that are involved in LPS biosynthesis. The knowledge of LPS biosynthetic genes and their related structures has facilitated *in vivo* studies of LPS in virulence. An ultimate goal is to use this knowledge in the developments of LPS-based vaccines.

In this thesis, LPS from three NTHi strains taken from patients with otitis media has been structurally elucidated. The inter-strain differences between the closely related strains 1268 and 1200 compared to the sequenced strain R2846 were very apparent. All three strains indicated great intra-strain heterogeneity regarding both glucose extensions and non-carbohydrate substituents. Furthermore, the strains showed structural outer-core features that had previously not been detected in other Hi strains. In addition to the structural elucidation of LPS from the wild-type strains, the biosynthesis of the outer-core LPS region was investigated using combined genetics and structural studies. Two heptosyltransferase gene candidates, *losB1* and *losB2* were shown to direct the expression of outer-core heptose in strain R2846. Furthermore, LPS from several *lpsA* mutant strains were structurally elucidated in order to identify which part of the gene sequence of *lpsA* is responsible for directing the addition of glucose and galactose to the distal inner-core heptose via alternative linkages. LPS was also analyzed to compare changes in glycoforms between *in vivo* and *in vitro* grown bacteria and also importantly, in order to study the expression patterns of LPS during different stages of chinchilla middle-ear infection. It was found that as disease progressed LPS glycoforms became more truncated and less complex. Furthermore, glycoforms containing sialic acid were absent after 9 days post-infection.

In order to obtain a complete detailed structural LPS analysis several different methods and techniques were used. Briefly, LPS was isolated by extraction from lyophilized bacteria. LPS was then either subjected to *O*-deacylation to remove ester linked fatty acids of lipid A or subjected to mild acid hydrolysis in order to release the entire lipid A moiety. The three products, LPS-OH (obtained by *O*-deacylation) and OS and lipid A (obtained by mild hydrolysis) were further chemically degraded and derivatized or analysed directly by different mass spectrometric (MS) and nuclear magnetic resonance (NMR) techniques.

List of Publications

This thesis is based on the following papers which will be referred to by their Roman numerals I-IV.

- I. **Specific amino acids of the glycosyltransferase LpsA direct the addition of glucose or galactose to the terminal inner core heptose of *Haemophilus influenzae* lipopolysaccharide via alternative linkages.**
Deadman ME, Lundström SL, Schweda EK, Moxon ER, Hood DW.
Journal of Biological Chemistry, 281(40): 29455-29467, (2006)
- II. **Novel globoside-like oligosaccharide expression patterns in nontypeable *Haemophilus influenzae* lipopolysaccharide.**
Lundström SL, Twelkmeyer B, Sagemark MK, Li J, Richards JC, Hood DW, Moxon ER, Schweda EK.
European Journal of Biochemistry, 274(18): 4886-4903, (2007)
- III. **Structural analysis of the lipopolysaccharide from non-typeable *Haemophilus influenzae* strain R2846**
Lundström SL, Li J, Deadman ME, Hood DW, Moxon ER, Schweda EK.
Submitted to Biochemistry in December 2007.
- IV. **Application of CE-ESI-MS and LC-ESI-MSⁿ to profile glycoform expression during *Haemophilus influenzae* pathogenesis in the chinchilla model of experimental otitis media**
Lundström SL*, Li J*, Månsson M, Figueira M, Leroy M, Goldstein R, Hood DW, Moxon ER, Richards JC, Schweda EK.
Submitted to Infection and Immunity in December 2007.
* SLL and JL contributed equally to this work.

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List of abbreviations

1D	One-dimensional
2D	Two-dimensional
Ac	Acetate
CE	Capillary electrophoresis
CID	Collision induced dissociation
COSY	Correlation spectroscopy
CPS	Capsular protein
CRP	C-reactive protein
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization
Gal	Galactose
GalNAc	2-acetamido-2-deoxygalactose
GaT	[(PEtn→6)αDGalpNAc(1→6)βDGalp(1→4)βDGlcNAc(1→3)βDGalp(1→4)βDGlc(1→)]
GC	Gas chromatography
Glc	Glucose
GlcNAc	2-acetamido-2-deoxyglucose
Gly	Glycine
GPC	Gel-permeation chromatography
Hep	Heptose
DD-Hep	D- <i>glycero</i> -D- <i>manno</i> -heptose
LD-Hep	L- <i>glycero</i> -D- <i>manno</i> -heptose
Hex	Hexose
HexNAc	N-acetylhexoseamine
Hi	<i>Haemophilus influenzae</i>
Hib	<i>Haemophilus influenzae</i> type b
HMBC	Heteronuclear multiple-bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPAEC-PAD	High-performance anion-exchange chromatography pulsed amperometric detection
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
Kdo	3-deoxy-D- <i>manno</i> -oct-2- <i>ulsonic</i> -acid
AnKdo-ol	Reduced anhydro Kdo
lipid A-OH	O-deacylated lipid A
LPS	Lipopolysaccharide
LPS-OH	O-deacylated LPS
MA	Methylation analysis
MS	Mass spectrometry
MEF	Middle ear fluid
MS/MS	Tandem mass spectrometry
MS ⁿ	Multiple step tandem mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NDP	Nucleotide diphosphate
Neu5Ac	N-acetylneuraminic acid
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser enhancement

NOESY	NOE spectroscopy
NTHi	Non-typeable <i>Haemophilus influenzae</i>
OS	Oligosaccharide
PAF	Platelet activating factor
rPAF	Platelet activating factor receptor
P	Phosphate
PCho	Phosphocholine
PCP	Phenol:chloroform:light petroleum
PCR	polymerase chain reaction
PEMAA	Partially ethylated and methylated alditol acetate
PerMA	Permethylation analysis
PEtn	Phosphoethanolamine
PMAA	Partially methylated alditol acetate
PPETn	Pyrophosphoethanolamine
PS	Polysaccharide
QIT	Quadrupole ion trap
SA	Sugar analysis
sBHI	Brain heart infusion broth supplemented
SiaT	Sialyllacto- <i>N</i> -neotetraose
SIM	Selective ion monitoring
SRM	Selective reaction monitoring
TIC	Total ion chromatogram
TFA	Trifluoroacetic acid
TLR4	Toll like receptor 4
TOCSY	Total correlation spectroscopy
TQ	Triple quadrupole
uCA	Unsupplemented chocolate agar

1 Introduction

In nature, carbohydrates are important biomolecules forming both simple and complex structures either alone or covalently linked to proteins and lipids. Early studies of carbohydrates were often focused on plant glycan structures such as cellulose, starch and pectins, largely because of their wide range of industrial applications. Since then carbohydrates have been discovered to play key roles in various biological events and glycobiology has emerged as an interesting but also challenging research area. Carbohydrates have a potential information content that is several orders of magnitude higher than any other macromolecule due to the broad range of monomers (>100) of which they are composed and the different ways in which these monomers are joined. In a sugar residue one or more of several different hydroxyl groups can be glycosylated, thus allowing the formation of branched structures. Furthermore, the glycosidic linkage can lead to one of two different stereoisomers, the α - or β -glycoside. The feature of carbohydrates to include branched structures is unique among biomolecules and can provide even short oligosaccharide chains with a large numbers of isomers.

Lipopolysaccharide (LPS) is an important constituent of the outer membrane of Gram-negative bacteria. It has been shown that LPS participates in many physiological processes and plays a key role in the pathogenesis and manifestation of Gram-negative infection. In this thesis, LPS from the outer membrane of a number of Gram-negative *Haemophilus influenzae* (Hi) strains and mutant derivatives have been structurally elucidated. This knowledge will facilitate the understanding of the role taken by LPS in virulence. The ultimate goal for our group and our collaborators is to relate genetics, structure and host interaction behaviour of non-typeable (NT)Hi LPS to facilitate the development of a vaccine.

1. 1 The Gram-negative membrane

Bacteria are classified as Gram-positive and Gram-negative depending on the structure of their outer membranes. Gram-positive bacteria have a relatively thick cell wall consisting of a cytoplasmic membrane that includes a thick layer of protective peptide polysaccharide conjugates, peptidoglycan. In contrast, Gram-negative bacteria have a much more defined cell wall structure that consists of a sheet of peptidoglycan that is situated between the cytoplasmic inner- and outer-phospholipid bilayer membranes (**Figure 1**). In addition to specific membrane proteins, the outer membrane is covered with lipopolysaccharides (LPS). LPS is often referred to as endotoxin and exists in two forms, rough (R) and smooth (S). Both forms contain lipid A which anchors the core oligosaccharide units to the membrane. In addition, the S-form contains O-antigen polysaccharide (O-specific chain), that consist of repeating oligosaccharide units (1, 2). Notably, LPS from the bacterium investigated in this thesis (Hi) does not express these O-specific chains (3).

Various Gram-positive and Gram-negative bacteria can produce capsular polysaccharides (CPS). Hi can produce six (a-f) capsular serotypes (3). The extracellular polysaccharide capsule is loosely bound to the outer membrane and contains repeating units of oligosaccharides (OS). These OS are often acidic due to the presence of uronic, ulsonic and/or phosphate groups.

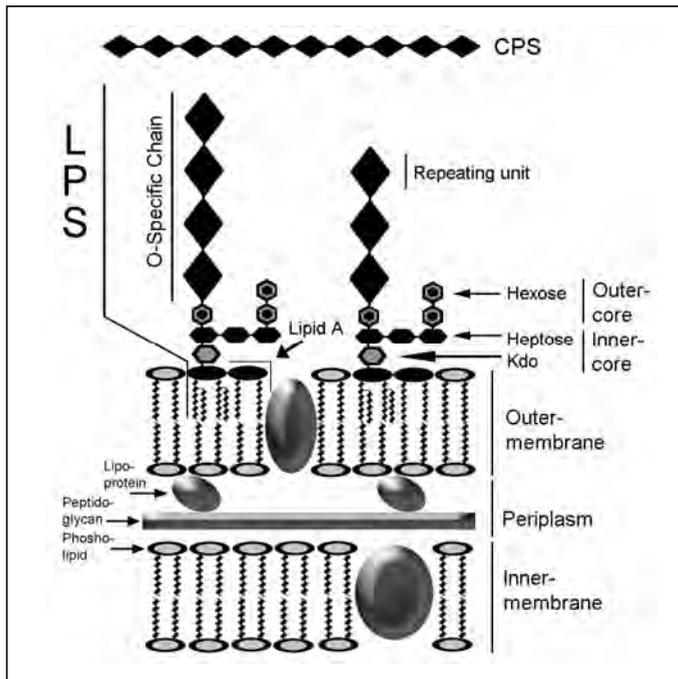


Figure 1. Schematic representation of the cell wall of Gram-negative bacteria.

2 *Haemophilus influenzae*

Haemophilus influenzae (Hi) is a host-adapted Gram-negative bacterium that regularly colonizes the respiratory tract of humans (3-11). It exists either as a commensal or as a pathogen within the host. The rod shaped bacterium is non-spore forming, non-motile and in microscopic appearance relatively small (1 x 0.3 µm). The generic name *Haemophilus*, means “blood-loving” and refers to its required growth factors haemin and/or NAD that are both present in blood. The species name *influenzae* originates from the erroneous belief that it was responsible for epidemic influenza at the time of its original description in 1892.

Hi isolates can be divided into encapsulated (typeable) and non-encapsulated (non-typeable) forms. The encapsulated strains are further subdivided into six serotypes, a-f, according to structural differences of the capsule. Compared to typeable Hi, non-typeable (NT)Hi strains have been found to contain a greater genetical diversity (12). A species level-ribotype dendrogram visualizing the genetic diversity of capsular- and NTHi-strains is shown in **Figure 2** (13). Before the introduction of a vaccine based on Hi type b capsule (Hib), this form was the main source of Hi disease and was causing infection of the central nervous system (meningitis), the respiratory tract (epiglottitis, pneumonia and empyema), the synovial joints (septic arthritis) and soft tissues (cellulitis), particularly in infants (5, 7, 8). In contrast to Hib, the other encapsulated types are rarely pathogenic. However, they can cause the same types of disease. NTHi strains are a common origin of otitis media (middle ear infection) and can also cause sinusitis, conjunctivitis (eye infection) as well as respiratory tract infections in patients with chronic bronchitis and cystic fibrosis (5, 6).

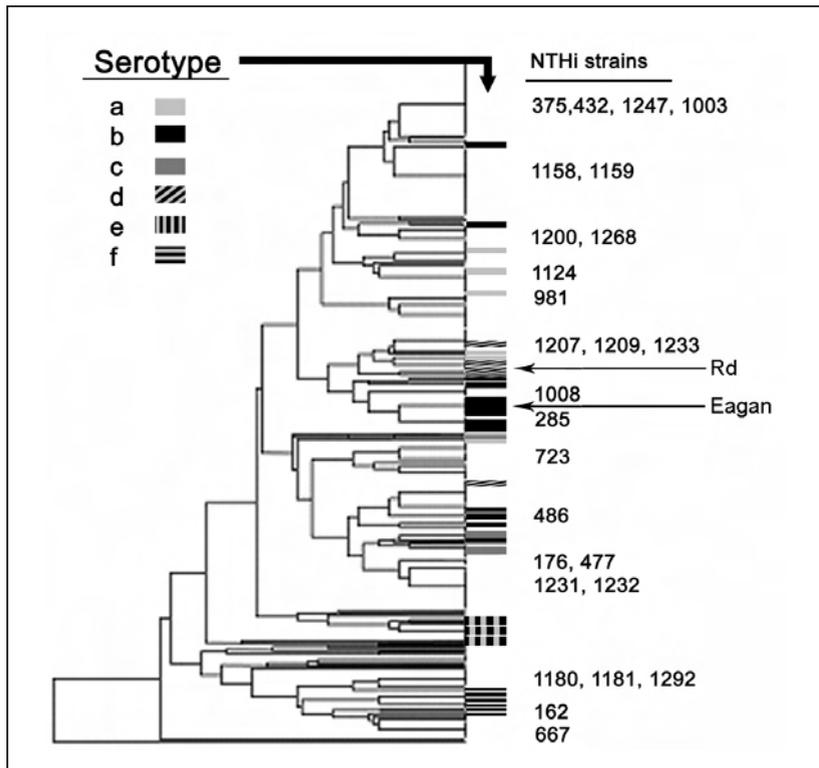


Figure 2. Species-level ribotyping dendrogram based on more than 400 Hi strains. Notable is the genetic diversity of the NTHi strains when compared to the capsular strains (13). Strain Rd and strain Eagan are also indicated.

The rate of NTHi colonization increases from infancy (20%, first 12 months), to early childhood (>50%, 5–6 year olds) and remains high in adults (11). Although NTHi can be treated with β -lactam antibiotics, such as ampicillin, resistance is becoming increasingly common (5, 9). In contrast to Hib which can become invasive and spreads via the blood stream, non-typeable strains normally cause disease by local invasion of mucosal surfaces (6, 7, 10, 11). The potential of Hi to cause disease highly depends upon its surface expressed carbohydrate antigens, capsular polysaccharide (CPS) or lipopolysaccharide (LPS) (14, 15).

2.1 Hi LPS structure

The membrane anchoring lipid A moiety of Hi LPS is linked to a 3-deoxy-D-manno-oct-2-ulsonic acid (Kdo) 4-phosphate residue which in turn is linked to a L-glycero-D-manno-heptose (Hep) trisaccharide unit (16). This molecular structure (**Figure 3**) forms the invariant inner-core of Hi LPS. The inner-core also consists of a β -D-glucose (GlcI) linked to the proximal heptose (HepI) in 4-position and a phosphoethanolamine (PEtn) linked in 6-position to the middle heptose (HepII).

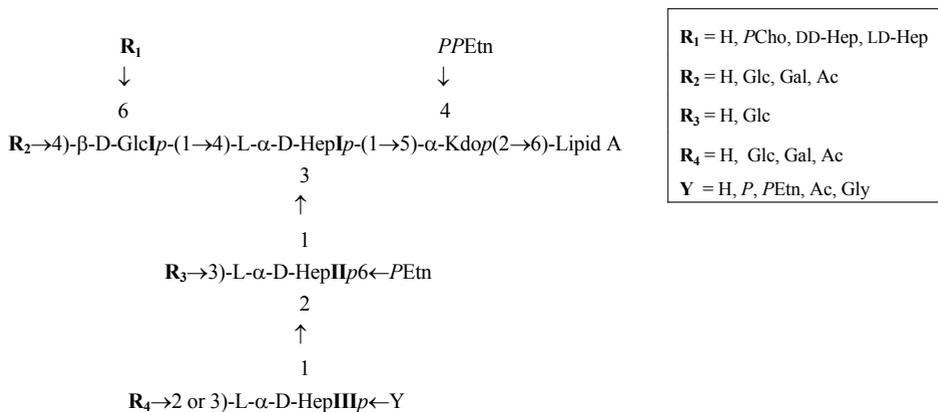


Figure 3. The invariant inner-core structure of Hi LPS.

The GlcI, HepII and the distal heptose (HepIII) each provide a point at which hexoses can be added and extended into oligosaccharide (OS) chains that will form the outer-core of the LPS molecule. The outer-core is very heterogeneous both between and within Hi strains. Substitution with non-carbohydrate substituents such as phosphate (*P*), phosphoethanolamine (*PE*tn), phosphocholine (*P*Cho), acetate (Ac) and glycine (Gly) are also frequently observed and contribute to the heterogeneity.

2.1.1 Outer-core

A diverse range of oligosaccharide structures have been identified as extensions from the triheptosyl inner-core unit (**Figure 4**) (16).

In every strain investigated to date, the proximal heptose (HepI) is substituted by β -D-Glc at O-4. This glucose (GlcI) can be further elongated with hexoses from two positions, O-4 and O-6. OS extensions from O-6 have been found only to be extended by heptoses (L- α -D-Hep or D- α -D-Hep) that additionally can be substituted with hexoses (17-21, III). Importantly, [α -Neu5Ac-(2 \rightarrow 8)- α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)] has been reported linked to LD-Hep from this position (20). In contrast, O-4 substitution from GlcI has only been observed with hexoses. Globotetraose [β -D-GalpNAc-(1 \rightarrow 3)- α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)], globoside [α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp] and lactose [β -D-Galp-(1 \rightarrow 4)- β -D-Glcp] have all been observed extending from HepI or GlcI (18, 22, 23). In addition, sialyllactose [α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp(1 \rightarrow)] and its disialylated counterpart have been detected extending from HepI (22). It has also been shown that Hi can express sialyllacto-*N*-neotetraose [α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)] and the related structure [(*PE*tn \rightarrow 6)- α -D-GalpNAc-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow), (SiaT and GaT) from HepI (24, 25, III).

The middle heptose (HepII) of the triheptosyl inner-core moiety has been found substituted at O-3 by α -D-Glc (23, 26-28, II). This glucose has been detected terminal, further extended at O-4 by globotriose [α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)] or as part of an extending globotetraose-like unit [β -D-GalpNAc-(1 \rightarrow 3)- α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow)] and/or as truncated versions thereof (II). In addition sialyllactose has been identified extending from HepII (II).

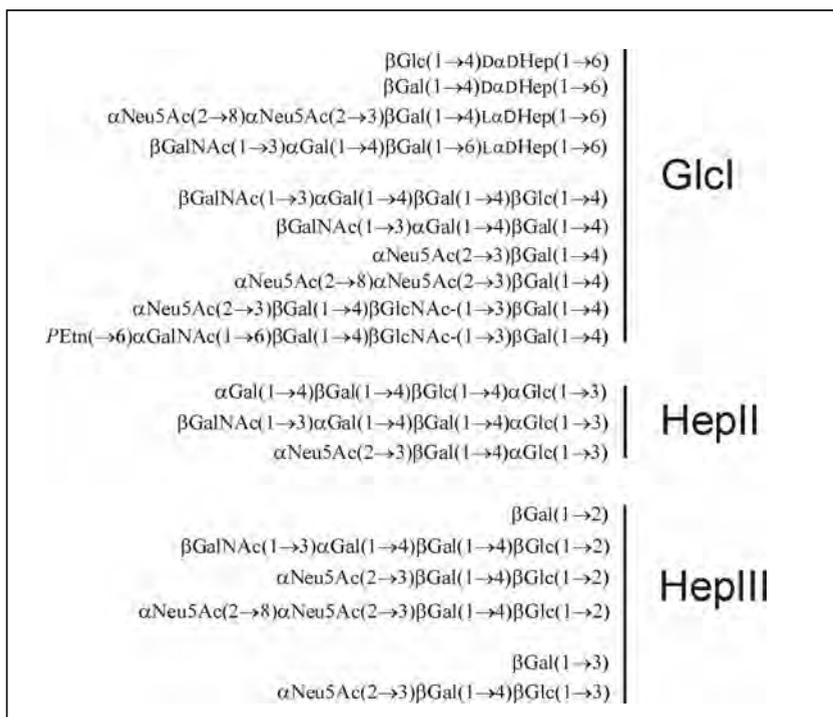


Figure 4. OS chains extending from GlcI, HepII and HepIII of the LPS inner-core unit. Truncated versions are possible. All sugars are pyranosidic and the hexoses have D-configuration.

The distal heptose (HepIII) can be substituted either at O-2 or O-3 position, by β -D-Gal or β -D-Glc, depending on the strain (19, 20, 22, 23, 26, 28-35, II, III). Whereas β -D-Gal has been found only as a terminal sugar, strains containing β -D-Glc at this position will show further hexose extensions at O-4 from the glucose. Globotetraose, disialyllactose and their truncated counterparts have each been found extending from HepIII (19, 20, 22, 26, 31-35).

2.1.2 Non-carbohydrate substituents

Both the inner- and outer-core glycosyl residues can be substituted by non-carbohydrate substituents. These units will contribute to the heterogeneity considerably since they can be located at several different positions in the same LPS molecule, often in non-stoichiometric abundances (16). In every strain investigated to date, Kdo is substituted at O-4 by pyrophosphoethanolamine (PPEtn) and HepII is substituted at O-6 by PEtn. The inner-core unit has also been observed substituted with P and PEtn at HepIII, but only in a limited number of strains (18, 20, 22, 27, 34, III).

PCho substitution is a common feature observed in Hi LPS. A majority of strains carry PCho at O-6 of GlcI (16). Other variants are known with substitution on O-6 of either β -D-Gal or β -D-Glc linked to HepIII, or on O-6 of α -D-Glc at HepII (26, 29, 36, 37, II, III). Furthermore, the external heptose linked to GlcI has been observed with PCho (20, 21, II). Notably, some strains can elaborate glycoforms that carry two PCho substitutions (20, 21, 29, III).

In addition to the phosphorylated substituents described above, *O*-acylating glycine (Gly) and acetates (Ac) are frequently observed in strains positioned at both outer- and inner-core residues (16). Gly is often linked to HepIII although it has also been detected linked to HepII, HepI and to Kdo in certain strains (38). Acetate has been detected linked to O-2 or O-3 positions of HepIII and also to the hexose linked to HepIII via O-6 or O-4 linkages (26, 29, 35, 39). Similarly, O-2 of HepI, as well as O-3, O-4 or O-6 of GlcI can be acetylated (19, 34, 36). In addition, the GaT epitope [(PEtn→6)- α -D-GalpNAc-(1→6)- β -D-Galp-(1→4)- β -D-GlcpNAc-(1→3)- β -D-Galp-(1→4)- β -D-Glcp-(1→)] was shown acetylated at the GlcNAc unit in Paper III. Notably, addition of *O*-acetyl groups does not seem to be equal between potential acceptor sites and certain positions seem to be more acetylated than others (16). Also, the presence of Gly and Ac groups may have been under reported. This is due to the methods used to isolate and prepare the LPS which can result in the removal of ester linked substituents.

2.1.3 Host mimicry

A characteristic feature of Hi LPS (**Table 1**) is that many of the OS-chains in the outer-core region mimic human biostructures (40-44). The observed molecular mimicry allows the bacterium to camouflage itself from the human defense system and thereby most likely contributes to the adherence to and invasion of human cells as well as evasion of the host immune response.

Table 1. Terminal Hi LPS structures that mimic human glycosphingolipids. *P*Cho substituted LPS mimics the platelet-activating factor (PAF), (a phospholipid) (Section 2.3.1) (42).

Host-mimicking structure	Trivial name
[Cho <i>P</i> →]	Phosphocholine
[β Gal-(1→4)- β Glc-(1→)]	Lactose
[α Neu5Ac-(2→3)- β Gal-(1→4)- β Glc-(1→)]	Sialyllactose
[α Gal-(1→4)- β Gal <i>p</i> -(1→)]	Digalactoside / Galabiose
[α Gal-(1→4)- β Gal-(1→4)- β Glc-(1→)]	Globotriose / Globoside
[β GalNAc-(1→3)- α Gal-(1→4)- β Gal-(1→4)- β Glc-(1→)]	Globotetraose
[α Neu5Ac-(2→3)- β Gal-(1→4)- β GlcNAc-(1→3)- β Gal-(1→4)- β Glc-(1→)]	Sialyllacto- <i>N</i> -neotetraose

2.1.4 Lipid A

The conserved lipid A unit comprises a β -2-amino-2-deoxy-D-glucopyranose-(1→6)- α -2-amino-2-deoxy-D-glucopyranose that is phosphorylated at C-1 of the reducing- and at C-4 of the non-reducing sugar (45, 46). The C-2/C-2' and C-3/C-3' positions are substituted by amide- and ester-linked 3-hydroxytetradecanoic acid chains, respectively (**Figure 5**). In addition the fatty acid chains on C-3' and C-2' are further esterified by tetradecanoic acid chains. Even though lipid A is considered conserved, some heterogeneity has been detected in this unit, mainly due to shorter length in the fatty acid chains. Two NTHi strains have also been observed to express lipid A molecules that are *O*-acetylated in the 3-hydroxytetradecanoic acid group attached to C-2 or C-3 (46). The biological role of acetylation in lipid A is not known, but it has been evidenced that the number, positions and lengths of fatty acid chains have a role in the toxicity and biological activity in Gram-negative bacteria (47).

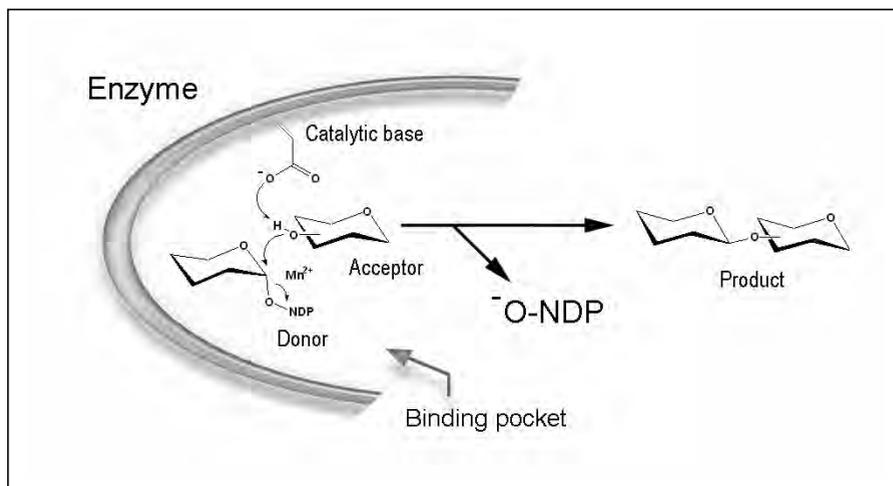


Figure 6. Reaction mechanisms for an inverting nucleotide diphosphate (NDP)-sugar glycosyltransferase. The reaction involves a single displacement in which an activated sugar donor is attacked by the acceptor leading to inversion of the anomeric configuration.

2.2.2 Biosynthesis: inner- and outer-core

The complete genome sequence of Hi strain Rd (originally type d) has been available since 1995 when it was published in *Science* by Fleischmann *et al.* as the first fully sequenced genome of an organism (52). This has facilitated a comprehensive study of LPS biosynthetic genes in the homologous strain RM118 (Rd') and in the type b strains Eagan (RM153) and RM7004 (53, 54). Recently the complete genome sequences of other Hi strains have also been elucidated (55, 56). In order to confirm LPS gene functions, mutant strains are made with a disruption in the genes of interest (21, 22, 26, 57, I, III). Structural elucidations of the mutant LPS, often with mass spectrometric and NMR techniques, will determine structural changes between the mutants and wild-type(s).

The inter- and intra-strain LPS diversity observed in Hi has been shown to be due to a set of biosynthetic genes that direct the enzymatic addition of substituents (16, 58). Variations of LPS between strains generally result from differences in the genetic blueprint available in each strain. The absence of genes and allelic variations in the gene sequence will contribute to variations in the substitution pattern. The observed diversity within a single strain can also be explained by phase-variable expression of particular genes. Phase-variation is a genetic mechanism in which the gene is switched on and off at high frequency resulting in reversible loss or gain of sugars and other units. Intra-strain variation of LPS also results from competition during biosynthesis where a sugar residue can function as the acceptor for two or more transferases (33).

The genes involved in assembly of the inner-core moiety are invariably present and carry out the same functions in strains Rd, Eagan and RM7004. These genes have also been found to have homologous counterparts in 25 NTHi strains, representative of the genetic diversity of the species (16, 59, 60). Additionally, the genes required for initiation of OS extensions from the triheptosyl unit have been identified as homologous for a number of strains (16).

The sequential addition of HepI, HepII and HepIII to Kdo is directed by *opsX*, *rfaF* and *orfH*, respectively. The gene *lgtF* directs GlcI to HepI, *lic2C* directs α -D-Glcp to HepII and *lpsA* directs β -D-Glcp or β -D-Galp to HepIII, (**Figure 7**). The *lpsA* gene is invariably present in all examined strains while *lic2C* has been found in only about half of the strains. Each Hi strain uniquely produces only one out of the four possible combinations for hexose extension from HepIII (β -D-Glcp-(1 \rightarrow 2), β -D-Glcp-(1 \rightarrow 3), β -D-Galp-(1 \rightarrow 2) or Galp-(1 \rightarrow 3)) and it has been shown that a specific allelic variant of the LpsA enzyme is responsible for the observed differences in the LPS (*I*). Remarkably, only one single key amino acid (at position 151) in LpsA determines if a glucose or galactose will be added. In addition it has been concluded that the 3' end of the *lpsA* gene directs the anomeric linkage of the added hexose.

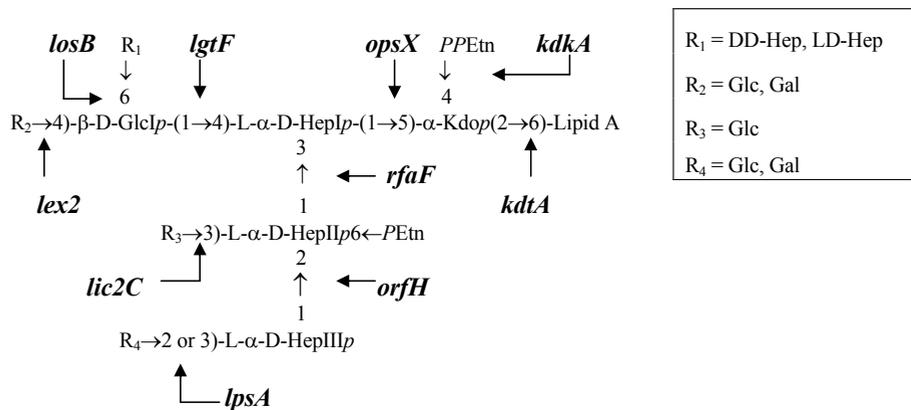


Figure 7. Genes involved in biosynthesis of inner-core LPS and the genes directing addition of R_{1-4} to the molecule.

LPS phase-variation of core sugars and also non-sugar modifications, is mediated by polymerase slippage in multiple tandem tetranucleotide repeats in seven characterized chromosomal loci; *lic1*, *lic2*, *lic3A*, *lic3B*, *lgtC*, *lex2A* and *oafA* (**Figure 8**) (54, 57, 61-63).

Notably, all these genes control expression of structural elements that have been shown important in the virulence behavior of the bacterium (*16*). The phase-variable genes (*lex2A*, *lic2A*, *lgtC* and *lic3A* / *lic3B*) that are involved in extensions of globoside and/or sialyllactose and disialylated lactose are shown in **Figure 9** (*16*, *22*, *23*, *53*, *63-65*).

Notably, the *lex2* loci is both phase-variable and exists in two allelic variants (Hood *et al.*, unpublished). Similar to the LpsA transferase (*I*), the difference between the additions of a Glc- or Gal-unit lies in the variation of one amino acid in Lex2B. Also, the sialyltransferase gene *lic3B* has been found to be bifunctional having the capacity to synthesize sialyllactose and disialyllactose extensions from HepIII (*65*). Preliminary data would suggest that the *lic3* locus also is involved in the biosynthesis of sialyllactose from HepI and HepII (*22*, *II*). Recently, Lic3B has been shown to catalyze the extension of a disialosyl unit to O-3 on a β -D-Galp-(1 \rightarrow 4)-L- α -D-HepIII unit linked to GlcI (*20*).

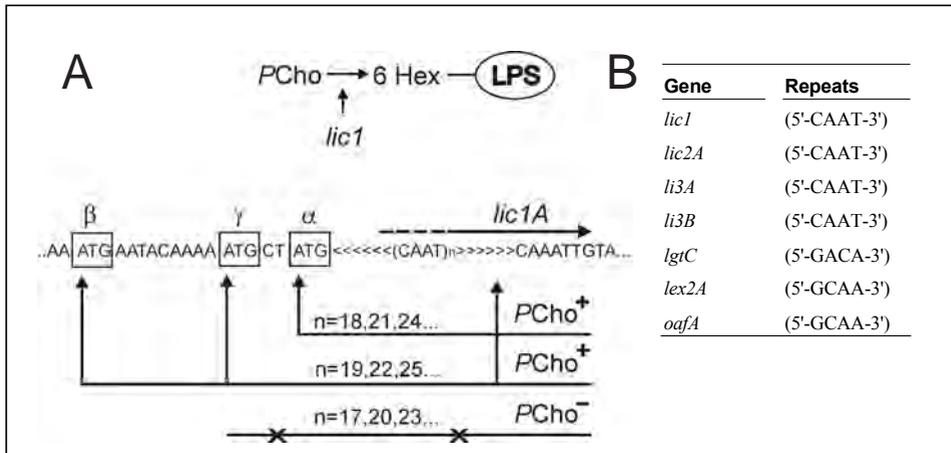


Figure 8. (A) Phase-variable expression of *PCho*, directed by the *lic1* locus (*lic1A-D*). Depending on the number of tandem repeats of 5'-CAAT-3', one of three initiation codons (α , β , γ) is either positioned in or out of phase with the remainder of the *lic1A* open reading frame (58). (B) Tetranucleotide repeats of phase-variable genes.

Lic1 and *oafA* mediate the addition of *PCho* and *O*-acetyl groups, respectively. The *lic1* locus comprises four genes (*lic1A* to *lic1D*) and of those *lic1D* is responsible for substituting *PCho* to the LPS molecule (21, 66, 67). Allelic variants of *lic1D* will direct *PCho* to different targets in the LPS molecule. Structural analysis of LPS from strains containing variant *lic1D* sequences has confirmed the role of the gene in the different positioning of *PCho* in LPS between strains (21, 67).

Comparative structural analyses of LPS from wild-type and *oafA* mutant strains have indicated that *oafA* directs Ac to HepIII (57). However, the mutants were still able to express minor amounts of *O*-acetyl groups at other sites in the LPS, indicating the presence of other yet unidentified *O*-acetylases.

Another gene that has been confirmed to be involved in LPS biosynthesis is *lgtD* which adds a terminal β -D-GalNAc(1 \rightarrow 3) residue to globotetraose (59). Furthermore, the gene *lpt6* has recently been shown to add *PEtn* to HepII (68). In addition to the phase variable sialyltransferase genes *lic3A* and *lic3B*, two other genes, *lsgB* (*orf2*) and *siaA* (*orfY*) have been implicated in the sialylation process of lacto-*N*-neotetraose (25, 69).

The addition of the tetrasaccharide units, α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4) and *PEtn* \rightarrow 6)- α -D-GalpNAc-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4) linked to GlcI is mediated by the *hmg* locus which includes *siaA*. The tetrasaccharide unit is added *en bloc*, by a biosynthetic mechanism similar to that seen for *O*-antigen biosynthesis (25), in contrast to the biosynthesis of all other known inner- and outer-core LPS units whereby sugars are added stepwise (59).

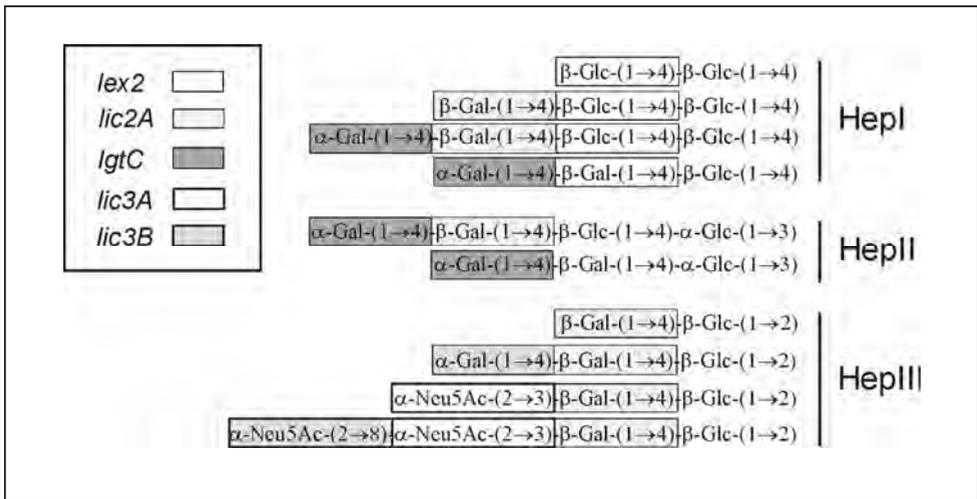


Figure 9. OS extensions from the inner-core unit that are added by the phase-variable genes *lex2*, *lic2A*, *lgtC*, *lic3A* and *lic3B*, respectively.

Recently, the genes *losB1* and *losB2* have been identified to express external DD- and LD-heptose linked to O-6 at GlcI (*III*). The *losB* genes are adjacent to genes, *losA1* and *losA2* (*losA1* to *losB1* and *losA2* to *losB2*). The *losA* genes are encoding glycosyltransferases predicted to direct the substitution of hexoses to the non-core heptose. The presence of the gene pair *losA1* and *losB1* precludes the presence of genes *lic2C* and *lic2B* being present (60). As mentioned above *lic2C* is responsible for the addition of the first hexose to HepII. *Lic2B* is a candidate for encoding the second hexose that can extend from the Hex linked to HepII.

2.2.3 Biosynthesis: Lipid A

Biosynthesis of lipid A (also known as the Raetz pathway), consists of nine enzymatic steps that are conserved among Gram-negative bacteria (70). However, lipid A can differ between species regarding the number of Kdo residues and chain length of fatty acids. This is due to variations in the enzymatic specificity. Briefly, uridine diphosphate (UDP)-GlcNAc is acylated in O-3 by LpxA. LpxC and LpxD then catalyze the deacylation of the amide linkage at O-2 and the addition of a second fatty acyl chain in an amide linkage to form UDP-2,3-diacylglucose amine (**A**). LpxH then cleaves the pyrophosphate bond of UDP yielding UMP and 2,3 diacylglucoseamine 1-phosphate (**B**). In the next step a disaccharide synthase (LpxB) catalyzes the characteristic β -1'-6 linkage by condensing **A** and **B**, with **B** serving as the acceptor molecule. Up until the formation of the lipid A disaccharide backbone, the sub-cellular localization of the biosynthesis is cytosolic. The latter steps of the pathway (catalyzed by LpxK, WaaA (KdtA), KdkA, LpxL (HtrB) and LpxM (MsbB)) are membrane-bound. Phosphorylation of the 4'-hydroxyl group is mediated by LpxK. In *Hi*, WaaA then catalyzes the addition of one Kdo sugar which is phosphorylated at O-4 by KdkA (71-73). The synthesis of lipid A is completed by addition of tetradecanoyl residues (14:0) to the 3-hydroxytetradecanoic acid (14:0(3-OH)) residues on 2' and 3' of the distal glucoseamine (catalyzed by LpxL and LpxM).

2.3 Colonization and Infection of NTHi

NTHi is part of the commensal microflora of the nasopharynx in most healthy individuals. Infections occur when bacterial cells migrate to the upper and lower respiratory tracts or if the balance of the normal flora is disturbed (10, 11, 74, 75). Thus, organisms spread contiguously to the middle ear, the sinuses or the lungs where they stimulate an inflammatory response producing symptomatic infection. Unlike Hib, NTHi strains rarely survive in the bloodstream to cause widespread infections and diseases such as meningitis.

In the initial step the pathogen adhere to the viscous fluid (mucus) that covers and protects the epithelium cells in the respiratory tract and also damages cilia on the cells (ciliostasis). The adherence to mucus is mediated by outer membrane proteins (OMP-2 and OMP-5) that will bind to mucin receptors (76). Ciliostasis occurs when LPS molecules are released from bacteria causing toxic effects mediated by lipid A (11, 47). In addition, a specific protein (protein D) has been shown to induce ciliary damage (77).

In the next step the bacteria adhere preferably to the surface of damaged epithelial cells and to non-ciliated cells. A number of Hi surface structures influence the process of adherence. One such determinant is pili that are hair like attachments found on the bacterial surface (11). Not all NTHi contain the *hif* locus that encodes the pili, however several other alternative adhesins including the autotransporter proteins Hap, HMW1/HMW2 and Hia/Hsf are used by NTHi to promote adherence (75, 78, 79). Furthermore, PCho coated LPS (Section 2.3.1) is important during this process (44). Notably, *hif*, *hmw* and *lic1* all are phase-variable which indicates the importance for the bacterium to adapt and persist under varying conditions, including the settings of the immune response (11).

When the bacteria have established themselves on the mucosal surface they are challenged to persist. This is achieved by the evasion of the hosts (innate and adaptive) immune defenses by several different mechanisms. For example the bacteria produce IgA1-protease which cleaves the most predominant immunoglobulin present in the nasopharynx (11, 80). Formation of microcolonies is likely important in conferring resistance to natural bacteriostatic compounds such as lactoferrin, lysozyme and peroxidases (11). Microcolony formation may also block access of antibodies to individual organisms, thereby impeding antibody dependent killing and phagocytosis. Several studies also suggest that NTHi can enter and survive within epithelial and non-epithelial cells where the bacteria are protected from host killing (11, 81, 82). This may even result in resistance to antibiotic treatment. In addition to phase-variation, several Hi biomolecules, among those OMP-2, OMP-5, IgA1 protease and pili, also undergo antigenic drift, which will facilitate avoidance of the immune response (11). This is achieved by an irreversible process that involves substitution of amino acids in immuno-dominant regions of the proteins.

2.3.1 The role of LPS in virulence

Several structural motifs detected in LPS have shown to be important virulence factors of NTHi. Due to the increased knowledge of different structural features in various strains and of the genes expressing them, this knowledge can be used in *in vivo* animal models in which the virulence behaviors between wild-type and mutant strains are compared.

Three host mimicking structural elements have been identified as having special biological significance, digalactoside [α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)], *P*Cho and sialic acid (Neu5Ac) (**Figure 10**) (16). Each unit helps camouflage the bacterium against host defense systems, giving the bacterium an advantage during adhesion and invasion of the host. Strikingly, they are all expressed by phase-variable genes.

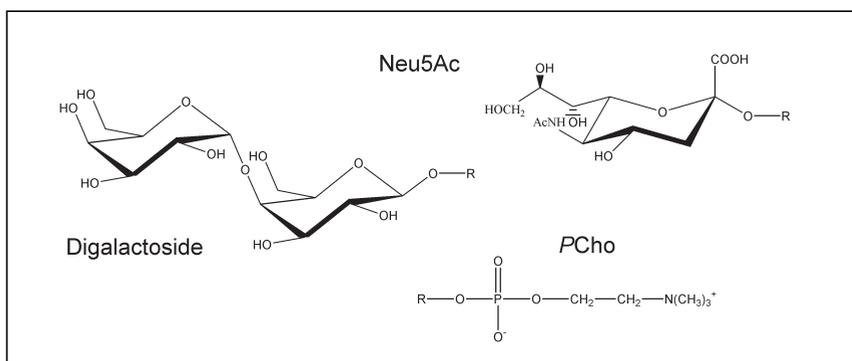


Figure 10. Structures of digalactoside, *P*Cho and Neu5Ac.

Digalactoside structural units have been shown to resist killing by naturally acquired antibody and complement present in human serum (83). This is believed to be due to molecular mimicry where the antigen prevents antibody stimulation and subsequent serum bactericidal activity. Furthermore, the expression of two digalactosides at once have indicated increased virulence by using a model of Hi infection *in vivo*, in which recombinant isogenic strains expressing either one or two digalactoside extensions were compared (84). Since digalactoside epitopes are expressed by phase-variable genes the presence of this epitope can vary. This can be advantageous in the alternative host compartments where the bacteria have different requirements (16).

*P*Cho substitution has been indicated to contribute to the ability of NTHi to colonize and persist within the human respiratory tract, at least partly by mediating bacterial adherence and invasion of the host epithelia (83). This is thought to be provided by a mechanism in which the NTHi bacilli adhere to bronchial epithelia cells through interaction between *P*Cho and the platelet activating factor receptor (rPAF) on the host cells (44). This interaction is suggested to initiate anti-inflammatory pathways. Since this would limit the inflammatory response it may be essential for NTHi in order to be a successful commensal. Bacterial mimicry of host membranes based on expression of *P*Cho also contributes to resistance of Hi to at least one human antimicrobial peptide (LL-37/hCap18). This peptide is found in the same host environment as Hi in concentrations that may be bactericidal (85). Expression of *P*Cho also renders the organism more susceptible to bactericidal activity of human serum (67). *P*Cho is the target of an acute phase reactant in serum, C-reactive protein (CRP), which mediates killing through activation of complement when bound to the organism. It has been shown that allelic variants of *P*Cho substitution show different sensitivity to CRP-mediated serum bactericidal activity regardless of the genetic background. Interestingly, transformant strains in which the *lic1* locus of strain Rd and strain Eagan were exchanged and compared to the wild type strains, indicated that CRP binds *P*Cho

more efficiently when substituted to a hexose on HepIII than glycoforms where *PCho* is substituted to GlcI.

Sialic acid (Neu5Ac) is an important virulence factor which facilitates the bacterium to evade the innate immune response of the host (16). It is believed that the mechanism by which sialylation of LPS contributes to virulence is a key to understanding the pathogenesis of NTHi otitis media and may also be relevant to other diseases caused by the pathogen.

However, the biological role of sialic acid incorporation is complex and many aspects are not yet well understood. Sialic acid has been suggested to camouflage LPS epitopes that are targets for the host immune system and to provide the pathogen access to host receptor systems (86). It has been shown that sialic acid coated Hi LPS helps the bacteria to resist the killing effect of normal human serum but has little effect on their attachment to, or invasion of, cultured human cells or neutrophils (33). Sialic acid has also been demonstrated to be an essential virulence factor in experimental otitis media by comparing isogenic proficient and deficient strains through a well described chinchilla otitis media model (87). Similarly, it has been demonstrated that sialic acid is important in the pathogenesis of otitis media in the gerbil middle ear model (88). In addition, recent studies have indicated that sialic acid has the capacity to hinder bacterial clearance in the middle ear and that sialylated LPS glycoforms are important in formation of biofilms (88-90). Incorporation of Neu5Ac in the LPS is dependent on an environmental supply of the sugar and also by the capacity of the bacterium for its uptake (91, 92). Notably, sialic acid has been shown to be present in all NTHi strains we have investigated to date (93, III).

In Paper IV the structural changes of LPS expression over time (2, 5 and 9 days after inoculation) were studied using a method developed for *in vivo* grown bacteria in the chinchilla middle ear. In this study sialylated glycoforms were required for early disease but a trend towards more truncated glycoforms was observed during the course of the infection and importantly sialylated glycoforms were completely absent at day 9.

The biological impact of *O*-acetylation has recently been shown (57). By using mutant strains containing inactivated *oafA* genes and by comparing them to wild-type strains, an increased killing effect of normal human serum was shown for strains where *O*-acetyl groups had been removed. Notably *oafA* is phase-variable similar to other virulence factors of Hi.

2.3.2 Endotoxin response in the host

When bacteria multiply, but also when they die and lyse, LPS (endotoxin) is set free from the bacterial surface. As mentioned above, the lipid A portion of the molecule has been shown to be endotoxically active and it has been evidenced that the number, positions and lengths of fatty acid chains have a role in the toxicity and biological activity of the bacterium (47). Data suggest that full endotoxic activity is observed when the molecule contains two glucoseamine residues, two phosphoryl groups and six fatty acids (saturated and in part 3-hydroxylated) including 3-acyloxyacyl groups with a defined chain length and in a distinct location.

Endotoxins give rise to both innate and adaptive immune responses (1, 94, 95). In contrast to the toxic effect of LPS in high doses, low doses of LPS are thought to be beneficial for the host, e.g. by causing immunostimulation and enhancing resistance to infections and malignancy. The primary target immune defense cells of NTHi LPS are

tissue macrophages which constitutively express the receptors CD14 as well as TLR4. LPS binding protein (LBP) catalyzes the transfer of released LPS to the membrane bound CD14. This in turn activates the TLR4 signaling pathway in conjunction with the coreceptor protein MD2. A cascade effect is mediated which will secrete proteins (cytokines), lipid mediators and reduced oxygen species (O-radicals) that will further give rise to multiple immune responses (**Figure 11**). A moderate infection (low) will result in mild fever and immune defense killing of bacteria. If large amounts of endotoxins are released (high) the infection can become severe since the high amounts of free cytokines, lipids and radicals will cause life-threatening shock, sepsis and tissue death.

2.3.3 Vaccine development

Since the introduction of a neoglycoconjugate vaccine for Hi type b (Hib) a dramatic reduction of meningitis in children has been observed world wide (7, 96-98). The vaccine is based on the type b capsule (polyribosylribitol phosphate, PRP) conjugated to a carrier protein.

Since polysaccharide antigens are poor immunogens due to their T-lymphocyte independent (TI) nature, linking the saccharide unit to a protein (e.g. toxin) leads the immune system to recognize the polysaccharide as if it were a T-lymphocyte dependent (TD) antigen. TD antigens induce an immune response that is long lasting due to formation of memory B and T lymphocytes.

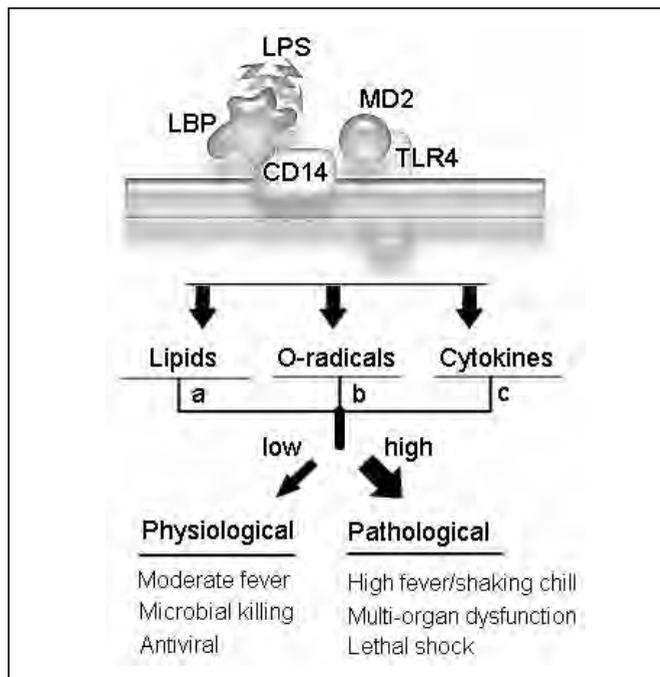


Figure 11. Macrophage-mediated activation of innate immunity by LPS. LBP catalyzes the transfer of LPS to CD14 on the phagocyte surface that in turn initiates the release of a wide spectrum of mediators via TLR4*MD2 (1). (a) PAF, prostaglandin, thromboxane e.g. (b) O_2^- , NO e.g. (c) interleukins, tumor necrosis factor e.g.

A vaccine that is directed to surface-exposed LPS, would be a possible candidate to control NTHi infections. However, in addition to the poor immunogenic characteristic of LPS, other problems regarding the heterogeneity of OS expression and the homology between carbohydrate structures on the bacterial surface and the host cell membranes makes development of NTHi vaccines a challenging task.

3 Structural elucidation of LPS

In order to elucidate the primary and secondary structure of LPS in Hi a number of chemical methods and analytical techniques are used. The identities of each monosaccharide, as well as their ring sizes, linkage positions and absolute- and anomeric-configurations have to be determined. In addition, the sequence and branching patterns of the oligosaccharide chains and the positioning of non-carbohydrate substituents are investigated. Since Hi LPS is very heterogeneous the primary and secondary structures of several different glycoforms are elucidated within one strain. No method alone can give full information of the LPS structures so a combination of techniques is necessary for a complete structural elucidation.

Briefly, LPS is isolated by extraction from lyophilized bacteria. In order to increase the solubility and to simplify the elucidation of the structure of the molecule, LPS can either be subjected to *O*-deacylation which will remove the ester-linked fatty acids of lipid A or be subjected to mild acid hydrolysis which will release the entire lipid A moiety. The three products, LPS-OH (obtained by *O*-deacylation) and OS and lipid A (obtained by mild hydrolysis) can then be chemically degraded and derivatized or analysed directly by different mass spectrometric (MS) and nuclear magnetic resonance (NMR) techniques. An overview of the methods used for structural elucidation of LPS is given in **Figure 12**.

3.1 Bacterial cultivation and LPS extraction

Strains are grown at 37 °C in brain heart infusion broth supplemented (sBHI) with haemin, 10 ug·mL⁻¹ and nicotinamide adenine dinucleotide (NAD) 2 ug·mL⁻¹. When appropriate (mutant strains) kanamycin, 10 ug·mL⁻¹ is also added. Mutant strains are constructed by transformation with plasmid constructs and confirmed by PCR amplification and Southern analyses (53, 99). After growth to late logarithmic phase, routinely in 5 lots of 1 L of sBHI, bacteria are killed and lyophilized. LPS is extracted by using the phenol:chloroform:light petroleum (PCP)-method (100). This procedure is known to give high yield and purity for non-encapsulated LPS. First, PCP (2:5:8) is added to lyophilized bacteria, the mixture is stirred (24 h, 21 °C) and centrifuged. The supernatant is then collected and the extraction step is repeated with fresh PCP (24 h, 21 °C). Chloroform and light petroleum are removed from the pooled supernatants by rotary evaporation. Acetone and diethylether are added to the phenol phase to a final ratio of acetone:ether:phenol, 5:1:1. The LPS is precipitated, centrifuged and washed with acetone (x 3) to remove phenol. The pellet is then dissolved in water and ultra-centrifuged (75 000 g, 4 °C, 16 h) in order to remove RNA and other impurities. It is proposed that pyrophosphoethanolamine (PPEtn) substituted to O-4 of Kdo can loose PEtn during the extraction due to the acidic properties of phenol (27).

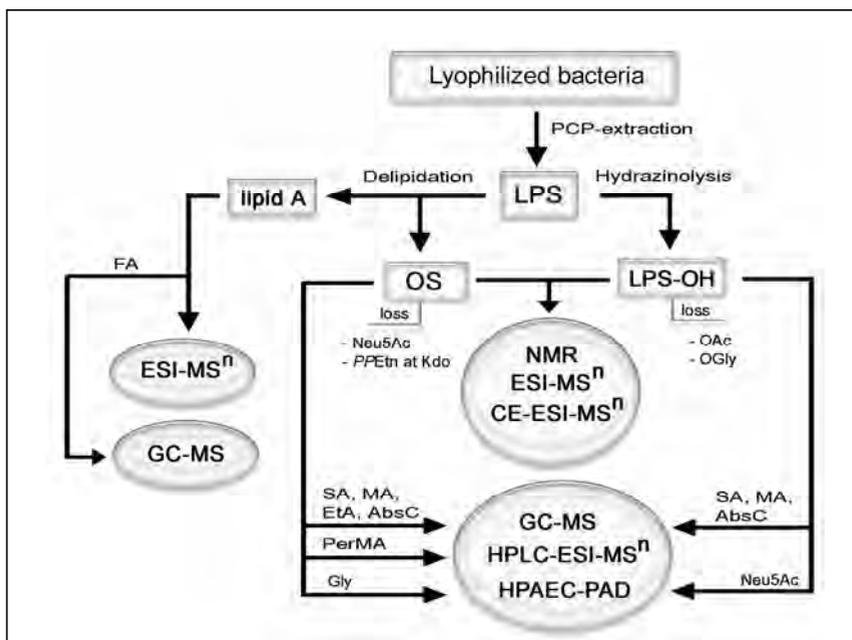


Figure 12. Summary of the methods used in order to obtain a detailed structural LPS elucidation. Fatty acid analysis (FA), analysis of PEMA (EtA) and absolute configuration analysis (AbsC). Gly and Neu5Ac are quantified using HPAEC-PAD.

In Paper IV samples were taken directly (2 to 10 days after inoculation) from the middle ear fluid (MEF) of chinchilla (87, 101). MEF samples were analyzed directly or after passage on solid chocolate agar (uCA) and liquid sBHI media (incubated overnight at 37 °C). All samples were frozen in 2% phenol and subjected to micro LPS extraction as follows: phenol was removed by low-speed centrifugation and washing with water (102). The bacterial cell-wall was disrupted with proteinase K followed by successive treatments of DNase and RNase to enhance release of free LPS.

3.2 Preparation of oligosaccharides and lipid A

3.2.1 O-deacylation of LPS with hydrazine

O-deacylation with anhydrous hydrazine (hydrazinolysis) under mild conditions is accomplished in order to remove ester linked fatty acids from the lipid A moiety (Figure 13) (103). Unfortunately, other ester linked substituents such as glycine and acetyl groups will also be eliminated by this treatment. However, in contrast to delipidated oligosaccharide (Section 3.2.3), O-deacylated LPS still includes intact sialic acid residues. O-deacylated LPS (LPS-OH), is obtained after treating the LPS with anhydrous hydrazine (40 °C, 1 h). The reaction mixture is cooled (0 °C) and surplus hydrazine destroyed by addition of cold acetone. The precipitated LPS-OH is washed twice with cold acetone by centrifugation.

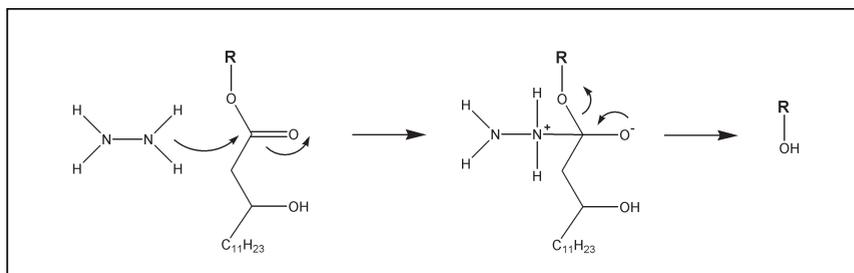


Figure 13. *O*-deacylation of ester linked fatty acid on the lipid A moiety using hydrazine.

3.2.2 Hydrolysis and neuraminidase treatment of LPS-OH

Sialic acid is detected and quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after hydrolysis (0.1 M HCl, 80 °C, 1 h) or neuraminidase treatment of LPS-OH (93).

3.2.3 Mild acid hydrolysis of LPS

Reduced core oligosaccharide (OS) material and free lipid A are obtained by mild acid hydrolysis (delipidation) of LPS in 1% acetic acid (pH 3.1, 100 °C, 2 h). The insoluble lipid A part is separated from the OS by centrifugation and after lyophilization of the supernatant, OS is purified by gel-permeation chromatography (GPC) (Bio-Gel: P-4, 800-4000 Da and G-15 \leq 1500 Da) using a refractive index detector.

The mild hydrolysis is achieved in the presence of borane-*N*-methylmorpholine complex for simultaneous reduction of the several anhydro-forms of Kdo (*An*Kdo-ol) that are formed by β -elimination of phosphate or pyrophosphoethanolamine from C-4 position (**Figure 14**) (104-106). Delipidation also results in the loss of Neu5Ac residues in OS due to hydrolysis of the ketosidic linkage.

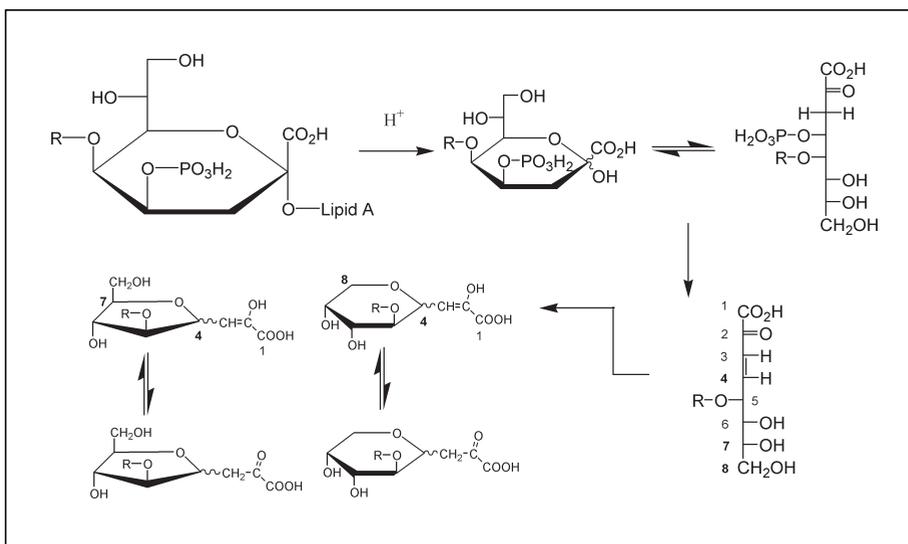


Figure 14. The formation of *An*Kdo-ol forms upon mild acid hydrolysis.

Furthermore, it can result in partial loss of fatty acids and also of the phosphate (*P*) group at C-1 in lipid A. Generally, ketoses, deoxy sugars and furanosides are acid labile but compared to hydrazinolysis, the substitution of glycine and acetate groups remains intact.

An alternative method for obtaining OS is by performing the mild hydrolysis in one step and then performing the reduction step separately (NaBH_4 , 1M NH_3 , 20 °C, 16 h). This method was used in Paper IV to prepare dephosphorylated and permethylated OS from LPS-OH trace samples.

3.2.4 Dephosphorylation and deacylation of OS

Dephosphorylation (48% HF, 4 °C, 48 h) can be achieved to remove *P*, *PEt*n and *PCho*. It is very important to keep a low temperature during the reaction and throughout the evaporation of HF or other glycosidic bonds may also be cleaved.

Deacylation of OS under mild conditions (1% NH_3 , 21 °C, 16 h) is performed to remove ester-linked substituents such as acetates and glycine in order to decrease the heterogeneity of the sample. In addition, base treatment of OS under harsher conditions (0.1 M NaOH, 21 °C, 30 min) is performed prior to HPAEC-PAD (38).

3.2.5 Compositional and linkage position analysis of oligosaccharides

To determine the identity and relative proportion of monosaccharide residues, LPS, LPS-OH and OS samples are subjected to sugar analysis (SA) (**Figure 15**) (107). The glycosidic linkages are cleaved by acid hydrolysis (**B**) (2 M TFA, 120 °C, 2 h) and the liberated sugars are then reduced (**C1**) (NaBH_4 , 1 M NH_3 , 21 °C, 16 h) to their corresponding alditols to prevent mutarotation. Finally, the alditols are acetylated (**D**) (Ac_2O /Pyridine, 1:1, v/v, 110 °C, 20 min) to increase the molecules volatility before analysis by gas chromatography - mass spectrometry (GC-MS).

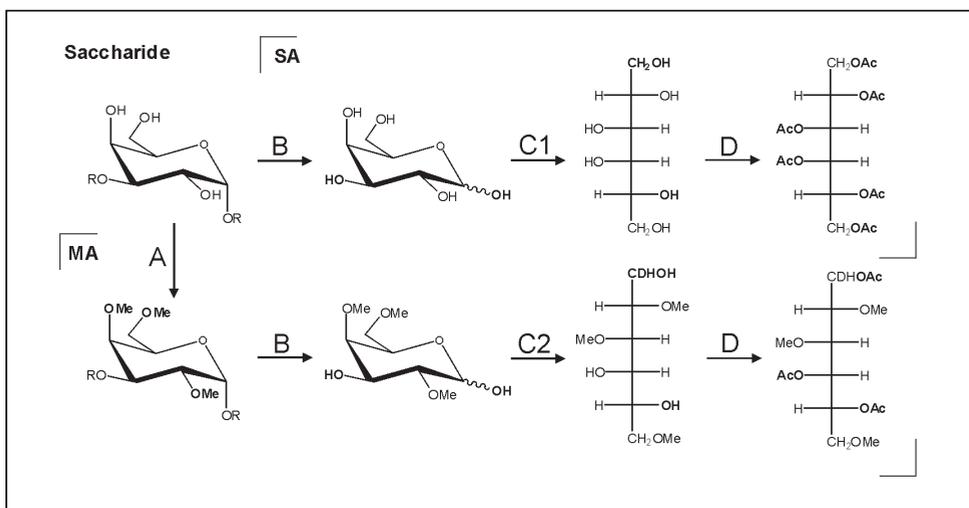


Figure 15. Principles for sugar analysis (SA), methylation analysis (MA) and permethylation analysis (PerMA). In PerMA only step A is performed.

Neu5Ac and Kdo are not detected by this procedure since they are degraded during the hydrolysis. Also, phosphorylated sugars will not be detected and *N*-acetylhexoseamine (HexNAc) residues will not be detected in stoichiometric amounts.

In order to determine the saccharides linkage positions LPS-OH and OS samples are subjected to methylation analysis (MA) (**Figure 15**) (108, 109). MA includes a methylation step (**A**) prior to SA in which free hydroxyl groups and other base-labile substituents will be methylated.

The sample is incubated with dimethyl sulphoxide (DMSO) (21 °C, 24-48 h) and then methylation is performed with methyl iodide in the presence of lithium methylsulphinylmethanide (BuLi, 40 °C, 1 h; MeI, 0 °C, 10 min, 21 °C, 16 h) (**Figure 16**). The methylated compounds are then recovered on a SepPak C18 cartridge and further derivatized by performing the same steps as in the sugar analysis (described above). The end product is referred to as partially methylated alditol acetates (PMAA) and analyzed by GC-MS. In order to easily identify the former position of the sugars carbonyl group the reduction step (**Figure 15 C2**) is performed with NaBD₄. The most critical step in MA is the formation of alkoxide ions from the hydroxyl groups. This relies on the material to be completely dissolved in DMSO. This can be a problem, in particular for LPS-OH which is therefore preacetylated (Ac₂O, 4-dimethylaminopyridine, 21 °C, 4-5 h) for increased solubility.

Phosphorylated sugars are not detected by MA. In order to determine the position of *P*-substituents, the former phosphate positions can be ethylated (**C'**) (DMSO 21 °C, 16 h; BuLi, 40 °C, 1 h; EtI, 0 °C, 10 min, 21 °C, 16 h) prior to methylation (**A'**) and dephosphorylation (**B'**) (**Figure 17**). The sample is then subjected to SA and the resulting partially ethylated and methylated alditol acetates (PEMAA) are analyzed by GC-MS. A similar method using perdeutero iodomethane instead of iodomethane has previously been used in order to structurally elucidate *P* substituents in LPS (27). Since the phosphate in *PE*tn and presumably *P*Cho can be subjected to both hydrolysis and migration in strong alkaline conditions (between positions 3/4, 6/7) the information obtained by the PEMAA is only indicative and should be used as a complement to other methods such as NMR and ESI-MSⁿ analyses (110).

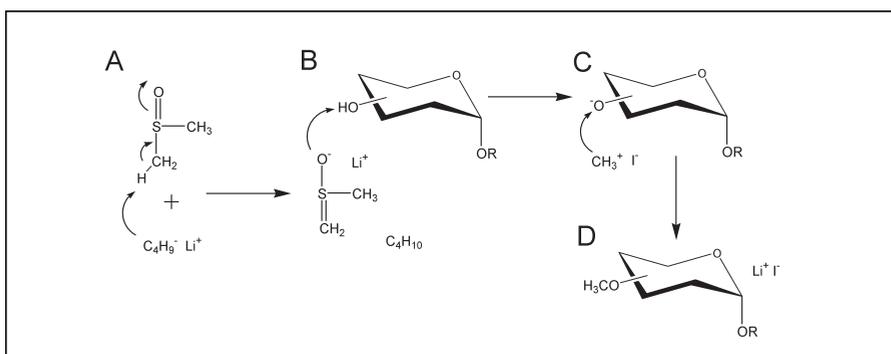


Figure 16. Reaction mechanism for the first step in MA. The sample is incubated with DMSO and then methylation is performed with methyl iodide in the presence of lithium methylsulphinylmethanide (formed between DMSO and BuLi).

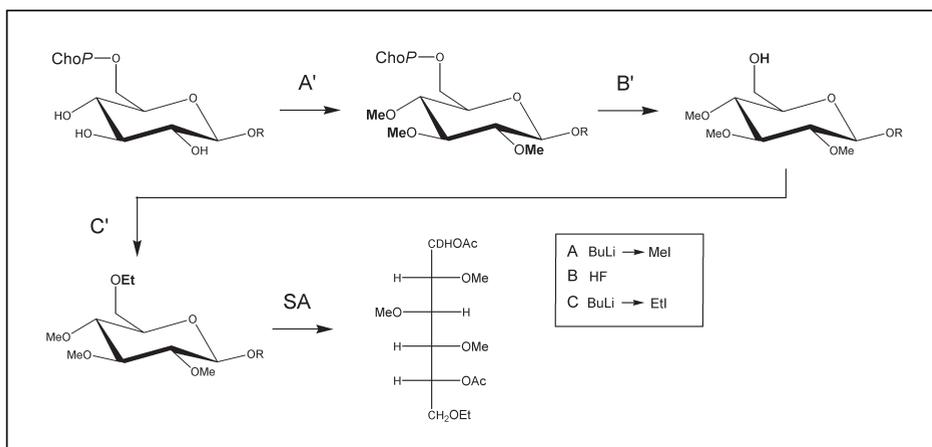


Figure 17. Principle for obtaining ethylated and methylated alditol acetates (PEMAA). The saccharide units are methylated (A'), dephosphorylated (B'), ethylated (C') and subjected to SA before GC-MS analysis.

To distinguish the absolute configurations (i.e. D or L) of a sugar, it is essential to convert them from enantiomers to diastereomers (*III*). This is achieved by Fischer glycosidation with a chiral alcohol, generally (+)-2-butanol. Briefly, LPS-OH or OS are hydrolyzed (2 M TFA, 120 °C, 2 h). If the sample contains *N*-acetylhexoseamine sugars, an extra re-*N*-acetylation step is then necessary (Ac₂O, 21 °C, 4 h). Thereafter, the glycoses are butanolysed ((+)-2-butanol, acetyl chloride, 85 °C, 8 h) and acetylated (Ac₂O/pyridine, 1:1, v/v, 110 °C, 20 min). Finally, the glycosides obtained are analyzed with GC-MS.

3.2.6 Preparation of oligosaccharide for sequence analysis

For determination of LPS sequence and branching patterns of hexoses and heptoses, OS samples are dephosphorylated and permethylated (**Figure 15 step A**). The OS is then subjected to high performance liquid chromatography - electrospray - multiple step tandem mass spectrometry (HPLC-ESI-MSⁿ) analyses (PerMA analysis).

3.2.7 Preparation of lipid A

The lipid A obtained after delipidation is purified by partition using chloroform:methanol:water (2:1:1) (46). After centrifugation the lower chloroform phase is reserved and the sample is subjected to ESI-MSⁿ. The fatty acids are identified as their corresponding fatty acid methyl esters (FAMES) by GC-MS. Briefly lipid A is treated with acid (HCl, 100 °C, 4 h) and the liberated fatty acids are then subjected to methanolysis (MeOH, acetyl chloride, 80 °C, 16 h).

3.3 Mass spectrometry analyses

Mass spectrometry (MS) is a useful and sensitive tool to detect and identify oligosaccharides and their modified derivatives. Generally the mass spectrometer is coupled to a chromatograph to facilitate separation and quantification of the compounds. In addition the detection will increase.

3.3.1 ESI-MS

ESI-MS is used to determine the compositions of sugars and non-carbohydrate substituents in LPS-OH and OS (112, 113). The distribution of molecular ions can be observed as doubly, triply and/or quadruply charged species that are interpreted according to $[m/z = (M-nH)^n]$ or $[m/z = (M+nH)^{n+}]$.

In order to improve the ionization of the molecules, a running solvent of acetonitrile/water or methanol/water with added NH_3 (negative mode) or HOAc (positive mode) is often used. To unambiguously determine signals originating from glycoforms containing Gly and Ac substituents, deacylated OS is also analyzed and compared to OS prior deacylation. Additionally, the decreased heterogeneity in the sample can reveal glycoforms that were not observed in the original OS (**Figure 18**).

3.3.2 ESI-MSⁿ and HPLC-ESI-MSⁿ

To gain further structural information the molecular ions are fragmented using tandem ESI-MS (ESI-MS/MS or ESI-MSⁿ). This is obtained by using a triple quadrupole (TQ) (ESI-MS/MS) or an ion trap (IT) (ESI-MSⁿ) and employing collision induced dissociation (CID). Both capillary electrophoresis (CE)-ESI-TQ-MS/MS and ESI-quadrupole ion trap-(QIT)-MSⁿ are used to determine the arrangements of non-carbohydrate substituents of LPS-OH and OS samples (112, 113).

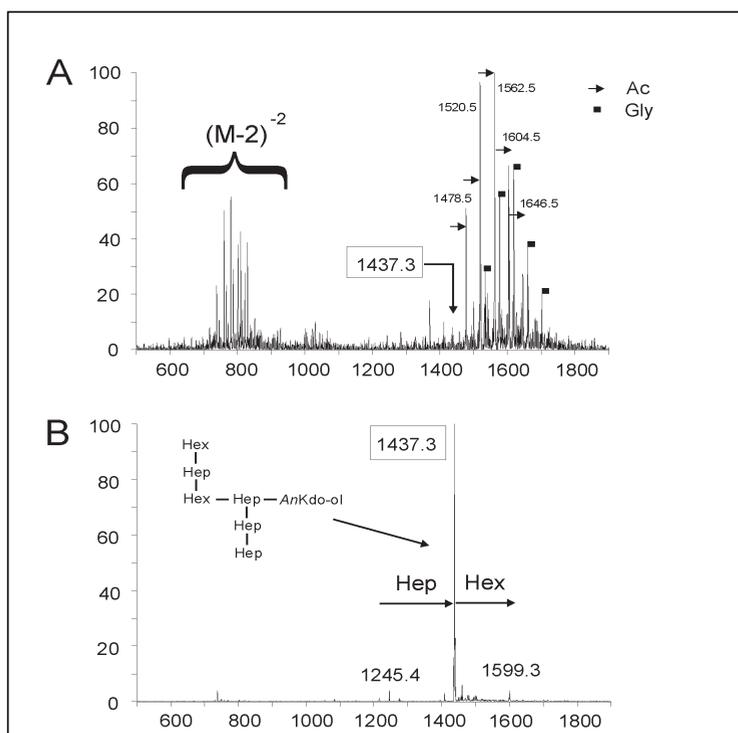


Figure 18. (A) ESI-MS spectrum of OS from the *lpsA* mutant of strain R2846. The sample is heavily acetylated and also glycosylated. (B) The same OS sample after O-deacylation indicates one major glycoform (Hex₂-Hep₄-AnKdo-ol).

ESI-TQ-MS/MS is an important tool to detect sialic acid in LPS-OH in which its presence is confirmed in precursor ion monitoring experiments by scanning for the loss of m/z 290 (Neu5Ac, negative mode) or m/z 274 (Neu5Ac-H₂O, positive mode) following CE-ESI-MS/MS (87, 112).

In order to gain more information, MS³ experiments can be obtained from a TQ analyzer, by using a method referred to as front end CID (38). In this method a high potential (180 V) between the orifice plate and the skimmer creates fragments of the sample before it enters Q1.

Compared to the TQ analyzer, a quadrupole ion trap (QIT) analyzer can not provide precursor ion scan experiments. However, MS²⁻⁵ experiments performed on a QIT analyzer are adequate for a complete structural elucidation of oligosaccharides and lipids regarding sequence- and branching-information (19, 46, 114-117).

Sequence analyses on dephosphorylated and permethylated OS samples are performed using ESI-QIT-MSⁿ via direct injection or coupled to a HPLC. The compounds are analyzed as pseudo molecular ions [M+Na]⁺ using a solvent containing 1 mM NaOAc in methanol:water or acetonitrile:water. When the HPLC is used the gradient is changed gradually towards an increasing hydrophobic condition to elute the compounds. In addition to the mass spectra, the HPLC will deliver a total ion chromatogram (TIC) with peaks of the separated glycoforms. The molecular ions of interest are then further fragmented in selective reaction monitoring (SRM) experiments using the QIT potential to create MSⁿ. Selective ion monitoring (SIM) is sometimes performed to detect trace amounts of material. In contrast to SRM, SIM will not give any sequence information since the selected ion is never fragmented.

Since dephosphorylation and permethylation in combination with added sodium acetate increase the MS response by several magnitudes, additional glycoforms are generally observed in the full scan MS spectrum that are not easily detected in underivatized samples (19, 114). Furthermore, MS²⁻⁵ on the molecular ions often reveals several isomeric compounds of the same composition. Fragmentation occurs by cleavage of the glycosidic bond with oxygen retention on the reducing end fragment (114-116). The sodium ion is then either ionizing the non reducing or the reducing end fragment, producing B_i or Y_i ions, respectively (Figure 19).

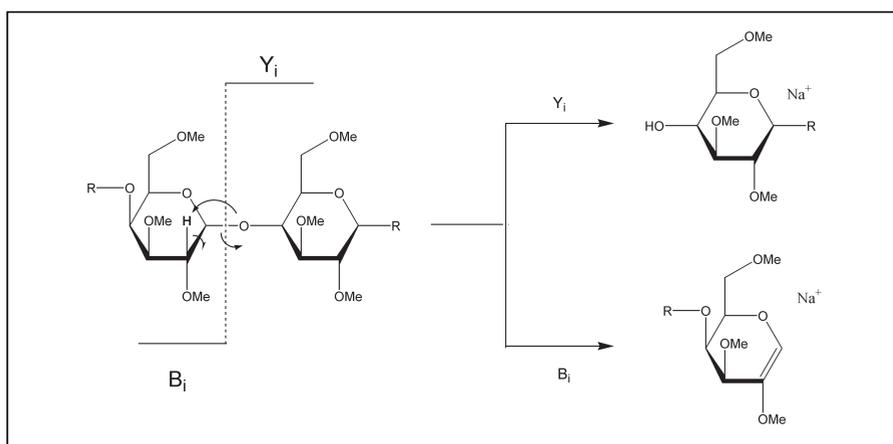


Figure 19. Fragmentation mechanism for the formation of B_i and Y_i ions.

When HexNAc residues are present, glycosidic cleavage at the reducing side of these residues is highly favored. The methyl tagging of the glycoses makes it easy to distinguish terminal and substituted units and it also allows the distinction between fragment ions generated by cleavage of a single glycosidic linkage and inner fragments resulting from the cleavage of two glycosidic linkages (**Figure 20**).

Linkage positions in permethylated oligosaccharides have previously been determined by interpretation of inner-ring cleavages (114, 115). However, such experiments have not been used here since the fragments were weak or completely absent. In ESI-MSⁿ experiments performed on lipid A, fragments originating from inner-ring cleavages of the disaccharide unit are generally detected though (see below).

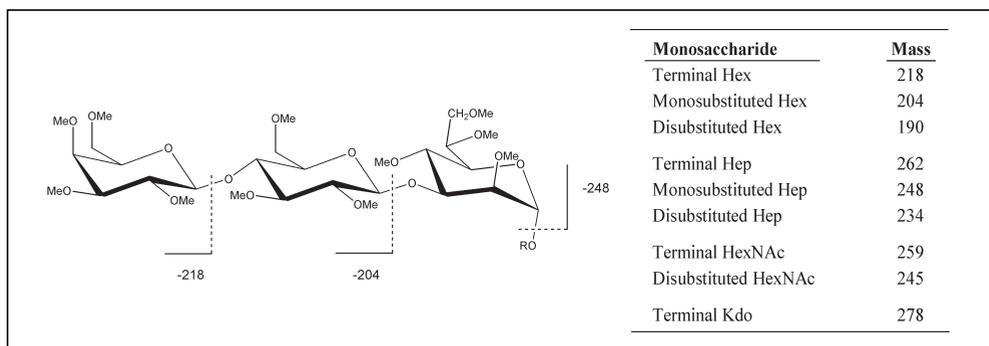


Figure 20. Differences in mass between terminal-, mono- and di- substituted permethylated saccharides.

ESI-QIT-MSⁿ run in negative mode (chloroform:methanol, 1:1, v/v, 10 μ l/min) is used to elucidate lipid A (46, 117). Since lipid A is conserved the analysis is mainly obtained to confirm the structure. Diphosphorylated lipid A is identified in the full scan spectrum as a singly charged negative ion at m/z 1825. Since lipid A can be degraded in the mild acid hydrolysis of LPS some variability in the molecule is usually observed and detected as singly charged ions at lower m/z values. The phosphate on C-4' of the non-reducing sugar is easily ionized which will enhance ionization of fragments at the non-reducing end of the molecule. Ions are mainly formed either due to ester-cleavage resulting in the loss of fatty acids and/or inner-ring cleavage which are diagnostic for the 1 \rightarrow 6 linkage of the disaccharide unit of lipid A.

3.3.3 GC-MS

Derivatized monosaccharides and fatty acids obtained for composition and linkage assignments are readily analyzed by a gas chromatograph (GC) coupled to an electron impact (EI) mass spectrometer (46, 118). Each compound's identity is given from retention times in the GC chromatogram and from the characteristic mass spectra obtained from the MS.

Briefly, the alditol acetates and (+)-2-butyl-acetylated glycosides obtained in SA and absolute configuration analyses are identified by their retention times in the GC chromatogram. In order to identify the substitution patterns of the sugar units, the mass spectra of their corresponding PMAA and PEMAA derivatives are interpreted.

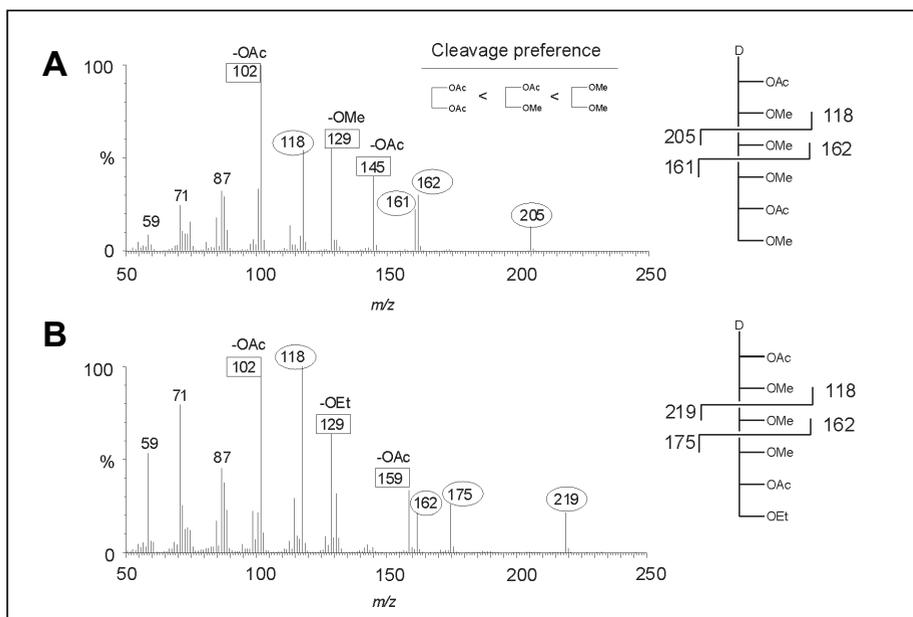


Figure 21. Mass spectra of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-1-*d*₁ (A) and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-6-*O*-ethyl-D-galactitol-1-*d*₁ (B). Primary fragments are indicated in circles and secondary fragments that are originating from the loss of OAc (-60 Da), OMe (-32 Da) and OEt (-46 Da) are indicated in squares.

The fragmentation patterns from characteristic primary and secondary cleavages of the alditols (**Figure 21**) are dependent upon the positioning of methyl, acetate or ethyl groups. Sugar identities will be determined from the GC chromatogram.

Fatty acids are identified by the retention times in the chromatogram and by characteristic fragmentation patterns in their respective mass spectra.

3.4 NMR spectrometry analyses

NMR is a powerful tool to structurally elucidate OS or LPS-OH glycoforms (113, 119). A combination of different NMR experiments will provide extensive structural information of the investigated sample. In most cases, each sugars identity and anomeric configuration is obtained, as well as OS sequence and linkage and branching patterns. In addition, the positions of non-carbohydrate substituents can be identified. In this thesis ¹H, ¹³C and ³¹P NMR experiments were used for detailed structural information of OS and deacylated OS samples. Chemical shifts were reported in p.p.m. referenced to internal sodium 3-trimethylsilylpropanoate-*d*₄ (δ_{H} 0.00), external acetone (δ_{C} 30.1) and external phosphoric acid (δ_{P} 0.00) standards. The OS sample is lyophilized in D₂O in several steps before analysis which will reduce the intensity of the HDO signal. Since this signal is temperature dependent, changing the temperature can help to visualize overlapped signals in the spectrum. Generally a temperature of around 20–25 °C (HDO, δ_{H} 4.8) is sufficient for OS analysis.

NMR is a non-destructive method so no sample is lost during an experiment; however compared to for example mass spectrometric techniques, NMR has a much lower

sensitivity. Generally spectra obtained using more scans and at high resolution will give the best results since the detection limit will increase and overlapping signals may be separated.

3.4.1 1D ^1H NMR

The heterogeneity of the sample and the quantities of characteristic structural units are observed in 1D ^1H NMR experiments (**Figure 22**). 1D ^1H experiments will also give valuable information of the glycosides anomeric conformation from vicinal coupling constants between H-1 and H-2 ($^3J_{\text{H1,H2}}$), (**Table 2**) (119).

The signals originating from the OS glycoforms are distributed in a region from about δ 2 to 6 (17-20, 22-24, 26-32, 34-37, 39, 106). The anomeric protons resonate at approximately δ 4.3-6.0. This downfield displacement is due to electron withdrawing properties of the ring oxygen and the oxygen in the glycosidic linkage. Anomeric signals from α -L-glycero-D-manno-heptoses and α -D-glycero-D-manno-heptoses from the inner- and outer-core will resonate at δ 5.0-5.9. Their α -manno configuration is indicated by the $^3J_{\text{H1,H2}}$ value around 2 Hz (**Table 2**). The anomeric signal of HepII is shifted downfield (~ 0.5 p.p.m.) compared to the other heptoses. Furthermore, the HepI anomer is split up into several less intense signals (δ 5.0-5.2), due to the heterogeneity of *AnKdo-ol*.

Table 2. Characteristic 3J -coupling patterns for Glc, Gal and Man.

Glc:	J_α (Hz) -3.6, 9.5, 9.5, 9.5
	J_β (Hz) -7.8, 9.5, 9.5, 9.5
	The coupling pattern is similar for GlcNAc
Gal:	J_α (Hz) -3.8, 10, 3.8, 1
	J_β (Hz) -8, 10, 3.8, 1
	The coupling pattern is similar for GalNAc
Man:	J_α (Hz) -1.8, 3.8, 10.0, 9.8
	J_β (Hz) -1.5, 3.8, 10.0, 9.8

The anomeric signals originating from α -Glc/GlcNAc and α -Gal/GalNAc residues are usually observed in the region δ 4.9-5.5 with the $^3J_{\text{H1,H2}}$ values of approximately 4 Hz indicating their α -gluco/galacto configuration (**Table 2**). In contrast, anomers of β -Glc/GlcNAc and β -Gal/GalNAc residues are generally detected at δ 4.3-4.7 having $^3J_{\text{H1,H2}}$ values about 8 Hz (**Table 2**).

Protons on ring carbons substituted with phosphate and acetate substituents can also be detected in the anomeric region. An important example is H-6 of HepII that occurs at approximately δ 4.6 since the sugar is linked in O-6 to PEtn. However, most ring protons resonate in the region δ 3.2–4.3 with subsequent overlaps that makes it impossible to assign the proton shifts of the glycoses in this area.

PCho is also easily detected in the 1D ^1H NMR spectrum by a characteristic singlet originating from the methylprotons (δ 3.23). In addition, the H-1 ethylene protons of PCho are resonating at δ \sim 4.35. The presence of PEtn can also be confirmed from an intense signal at 3.29, originating from the ethylene protons (H-2).

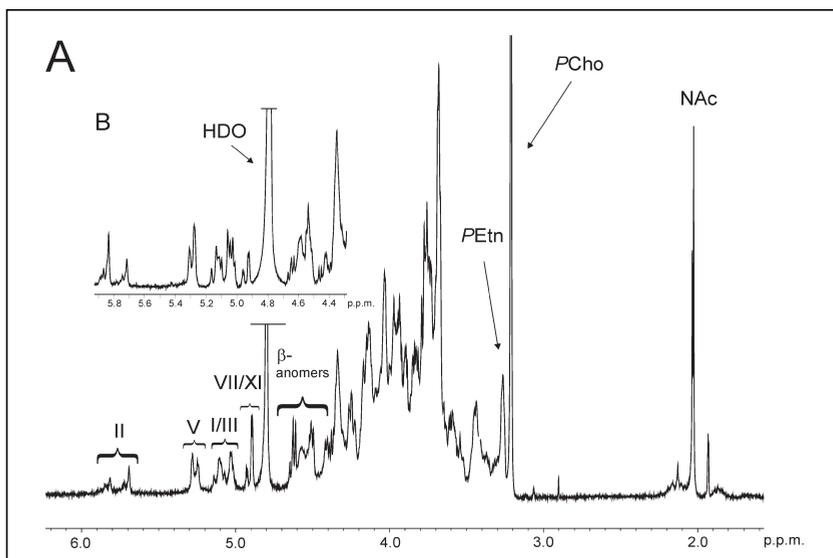


Figure 22. 1D NMR spectrum of strains 1268 (A) and 1200 (B). I, II and III correspond to H-1 of the inner-core heptoses. V indicates H-1 of α -glucose and VII and XI indicate H-1 of α -galactose.

The methyl groups of *O*-acetyl and *N*-acetyl substituents are detected at $\delta \sim 2.1$ and ~ 1.9 , respectively. In addition pairs of deoxyprotons of reduced *AnKdo*-ol are identified in the region $\delta 1.9$ -2.2. The 1D-spectra of strain 1268 and 1200 are shown in **Figure 22**.

3.4.2 2D homonuclear ^1H - ^1H - correlated NMR

^1H - ^1H - correlated spectroscopy (COSY) will give off-diagonal cross peaks between vicinal connected protons. In principle it is possible to obtain sequential resonance assignments stepwise around the ring starting from the well resolved anomeric protons. However, for complex saccharide molecules it is not possible to assign the entire spin system for each individual sugar using this method (119, 120). Since the ring protons are overlapping and/or can have small $^3J_{\text{H,H}}$ constants, only H-2 resonances and in some cases H-3 and H-4 of the glucose residues are determined. Some conclusions regarding the sugar identity can be drawn from the spectrum since the chemical shift of H-2 differs somewhat between different glycoses. Cross peaks between the deoxy protons originating from *anKdo*-ol are also easily detected from a COSY spectra.

^1H - ^1H - total correlated spectroscopy (TOCSY) correlations are obtained not only from a given ring proton to its neighbor but to other spins within the ring (i.e. within the same coupling network) (119, 120). This is achieved during a short period of time when the spins are locked, referred to as spin lock time or mixing time. Provided that a longer spin lock time (180 ms) is used and that the coupling constants are large, the whole spin-system of a sugar can be visualized as cross peaks from H-1. The relationship between the $^3J_{\text{H,H}}$ coupling constant and dihedral angle is visualized by the Karplus curve (**Figure 23**) (121).

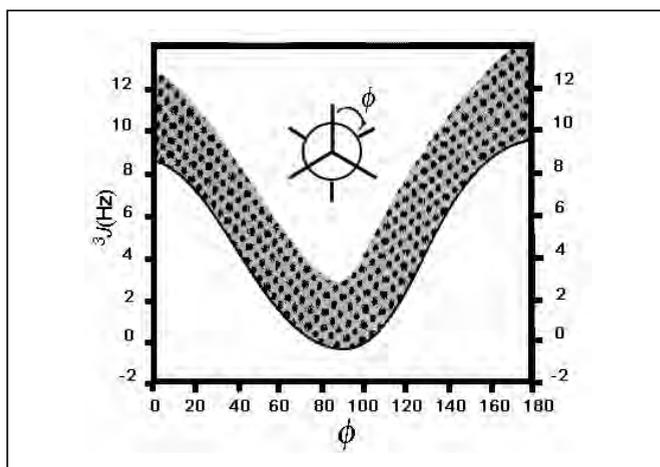


Figure 23. The Karplus curve, visualizing the relationship between 3J coupling and the dihedral angle. The coupling is the smallest when the torsion angle is close to 90° and largest at angles of 0 and 180° .

The small coupling constants between H-4 and H-5 of sugars in *galacto*- configuration (~ 1 Hz) and between H-1 and H-2 of sugars in *manno*- configuration (~ 1 -2 Hz) will prevent the net magnetism being transferred further (**Table 2**). Due to this phenomenon most of the ring protons originating from the heptoses in the OS (H-3 to H-7) as well as H-5 and H-6 in Gal and GalNAc residues are difficult to assign. However, the differences observed in spin couplings between the glycosides are also a benefit in order to determine the different sugar identities. By regulating the spin lock time to a shorter time period (150 ms) the intensities of the couplings from H-1 to H-3 and H-4 are increased which can sometimes be relevant for a complete proton assignment.

The TOCSY experiments in this thesis were performed in either absolute or phase sensitive mode. The phase sensitive spectra have higher resolution but the experiments are more time consuming. Several TOCSY spectra are shown in Paper I, Paper II and Paper III.

In nuclear Overhauser enhancement spectroscopy (NOESY) cross peaks are observed between protons that are close in space (3-5 Å) (*113*, *119*, *120*). The magnetization is transferred through dipolar couplings between spins during the mixing time (200/250 ms). A long mixing time can result in spin diffusion which results in cross peaks between protons that are further apart. NOESY experiments performed on OS will give both inter- and intra-residue NOE connections. These connections are of great importance for the structural elucidation. Intra-residue NOE connectivities will confirm that each sugar residue is present in the pyranosyl ring form as well as the anomeric configurations. Inter-residue NOE connectivities between anomeric and aglyconic protons on adjacent residues will provide sequence information (**Figure 24**). NOESY spectra are shown in Paper I and Paper II, respectively.

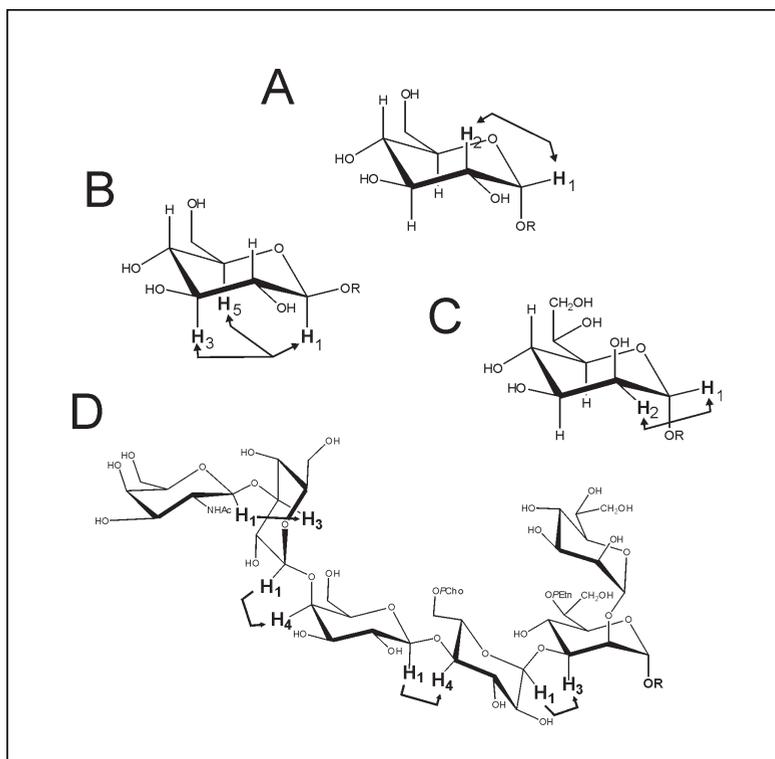


Figure 24. Intra- and inter-residue NOE connectivities between the anomeric proton and ring protons of various saccharides. (A) Intra-residue NOE connection of α -glucose. α -galactose will give the same connection. (B) Intra-residue NOE connections of β -glucose. β -galactose will give the same connections. (C) Intra-residue NOE connection of a heptose in α -manno-configuration. (D) Inter-residue NOE connections of a globotetraose-like unit extending from HepII.

3.4.3 2D heteronuclear ^1H - ^{13}C - and ^1H - ^{31}P - correlated NMR

For a complete structural elucidation of OS, carbon chemical shifts are assigned from heteronuclear multiple quantum coherence (HMQC) or heteronuclear single quantum coherence (HSQC) experiments between ^1H and ^{13}C nuclei (18, 19, 22, 23, 26-29, 31, 34-37, 39, 119, 120). To facilitate the assignment of carbon shifts, the proton shifts are normally assigned beforehand in the homonuclear proton experiments (described above). Anomeric carbon shifts are easily assigned at approximately δ 95-105 as well as carbonyl carbons that are found around δ 165-180. C-1 carbons from residues in β -configuration generally have higher chemical shifts than those originating from residues with α -configuration. Signals from ring carbons are found at δ 65-80. Substituted carbons are found down field compared to their unsubstituted counterparts. In contrast, substituted and unsubstituted hydroxymethyl carbons resonate at δ 60-66. C-2 of GlcNAc and GalNAc will resonate in the region δ 52-56 and the nitrogen bearing methylene carbon in *PEtn* and *Gly* will resonate at δ ~ 41. Furthermore, signals from the methyl carbons of *PCho*, *O*-acetyl and *N*-acetyl groups are found at δ ~55, ~21 and ~23, respectively. Using nondecoupled HSQC experiments the anomeric

$^1J_{C1,H1}$ couplings for pyranosidic α -anomers (170-175 Hz) are easily distinguished from those measured for β -anomers (160-167 Hz).

In order to elucidate the linkage positions of phosphate containing substituents in OS, 1H - ^{31}P HMQC experiments are performed where 3J scalar couplings between proton and phosphorus atoms give rise to cross peaks (18, 19, 22, 26-28, 31, 34-36). In addition, couplings to the ethylene protons of *PEtn* and *PCho* are detected. A 1H - ^{31}P HMQC spectrum is shown in Section 4.3.2.

Heteronuclear multiple bond coherence (HMBC) experiments are achieved to obtain $^3J_{C,H}$ couplings between ^{13}C and 1H nuclei (119). This experiment is useful in order to elucidate the oligosaccharide sequence patterns and sometimes to determine C-3 and C-5 shifts. However, in this thesis NOESY experiments have been used for sequence elucidation.

3.5 Other analytical techniques used

High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) is used in order to identify and also estimate the quantity of deliberated sialic acid and glycine from LPS-OH and OS, respectively (38, 93). HPAEC separates acidic compounds using a column containing pellicular anion exchange resin beads and an eluent at high pH. The PAD will then detect the compounds by measuring the electrical current generated by oxidation which will give excellent signal to noise ratios of very small quantities (picomoles).

Gel-permeation chromatography (GPC) is performed to fractionize and purify OS after delipidation. Since the method separates particles depending on size with larger molecules eluting faster, the leading OS fractions will contain glycoforms of higher molecular mass. The compounds are detected with a differential refractometer that measures the refractive index of the eluted solvent over time.

4 Structural analyses of LPS from Hi and NTHi strains

4.1 Paper I

Specific amino acids of the glycosyltransferase LpsA direct the addition of glucose and galactose to the terminal inner core heptose of *Haemophilus influenzae* lipopolysaccharide via alternative linkages.

4.1.1 Background

The Hi glycosyltransferase LpsA has been identified to be responsible for adding a Hex to the distal heptose (HepIII) of the inner-core of LPS in either β 1-2 or β 1-3 linkages (Section 2.1.1). In this paper we investigated the *lpsA* gene and its function in 28 Hi strains using various mutant and transformant strains and by elucidating their LPS structure. The 28 selected wild-type strains were representative of the genetic diversity of a collection of more than 400 Hi strains and included both capsulated and capsule-deficient (non-typeable) isolates collected over a 35 year period from diverse geographic regions.

4.1.2 Results

It was confirmed that the *lpsA* gene sequence was present in each of the 28 strains in the study. By comparing the gene sequence to matching glycosyltransferases in the same family, it was concluded that translation is initiated with an ATG (Met) initiation codon. The open reading frame would then include 256 amino acids.

Strikingly, the *lpsA* sequences of the selected wild-type strains were observed to be represented by two families. One major group of 22 strains (“Rd-like”) had limited polymorphism between sequences and 94-99% identity to strain Rd. The other six strains (“486-like”) resembled the *lpsA* gene of NTHi strain 486 and showed 95% identity to this strain. When the nucleotide sequences of strain Rd and 486 were compared, two regions (bp 161-210, 336-747) were detected that showed less homology (65%) than the rest of the *lpsA* gene sequence. To investigate whether or not the specific sequence of the *lpsA* gene was sufficient to direct the addition of either a Glc or Gal to HepIII via a β 1-2 or β 1-3 linkage, variant strains were constructed in which the allelic *lpsA* gene sequences were exchanged.

Cloned *lpsA* genes from strains Rd (β Glc-(1 \rightarrow 2), Eagan (β Gal-(1 \rightarrow 2)), 486 (β Glc-(1 \rightarrow 3)), and 176 (β Gal-(1 \rightarrow 2)) were used as donor DNAs to transform the wild-type strains and thereby obtain the reciprocal linkages through allelic exchange.

LPS analyses on core OS using methylation analyses and NMR experiments were performed in order to identify the hexose sugar and linkage to HepIII. In particular the inter-residue NOE connectivities between the Hex and HepIII were of importance for the structural elucidation since the β Hex-(1 \rightarrow 2) and β Hex-(1 \rightarrow 3) linkages to HepIII differ significantly. In order to unambiguously determine that only changes in the substitution pattern on HepIII was observed, *O*-deacylated and core oligosaccharide of each strain were analyzed using ESI-MS. Furthermore OS samples were sequence analyzed using ESI-MSⁿ. Sugars were also identified as their alditol acetates using authentic standards.

The structural analysis of LPS from strain Rd containing an *lpsA* gene from strain 486 (RdlpsA486) indicated that the transformant contained β -Glc O-3 substituted to HepIII in contrast to the β -Glc O-2 substituted to HepIII in the wild-type strain. Similarly, the transformants RdlpsA176 and 486lpsAEa indicated that the inserted *lpsA* gene expressed the structural allelic variant of strain 176 and of strain Eagan, respectively. In contrast, the major glycoform of 486lpsARd expressed terminal HepIII. Sequence analysis on the mutant indicated trace amounts of elongated HepIII but due to the low abundance, the identity of this hexose and its linkage position was not determined. The results are summarized in **Figure 25A**.

It was also observed that the presence of threonine at position 151 in the amino acid sequence is associated with the addition of Gal, regardless of the linkage position. Glc is added when cysteine, alanine or methionine is present at the position.

Using site-directed mutagenesis, the cloned *lpsA* gene from strain Rd and 176 were mutated such that Thr¹⁵¹ was changed to Cys¹⁵¹ in *lpsA* of strain 176 (T₁₅₁C176) and conversely Cys¹⁵¹ was changed to Thr¹⁵¹ in *lpsA* of strain Rd (C₁₅₁TRd). The altered *lpsA* genes were then transformed into strain Rd and Eagan by reciprocal exchange. The LPS from the strains were structurally investigated using the same methods as described above.

Experiments on strains RdlpsAC₁₅₁TRd and EaganC₁₅₁TRd both gave evidence that HepIII was substituted by β -D-Galp at the O-2 position. Similarly RdT₁₅₁C176 and

EaganT₁₅₁C176 contained LPS where HepIII was substituted by β-D-Glcp at the O-2 position. From these results (**Figure 25B**) it was concluded that the specificity of one amino acid (at position 151 in LpsA) is responsible for directing the addition of either a Glc or Gal to HepIII in Hi LPS.

In contrast to the sequence specificity observed in *lpsA* for the addition of either a Glc or a Gal, the sequence difference between β1-2 and β1-3 linkage related LpsA enzymes is much greater. To investigate whether the 5' or the 3' block of divergent sequence or both direct the specificity of linkage, chimeric genes were constructed. Plasmids containing chimeric *lpsA* genes, comprising the 5' portion of one and the 3' portion of a gene from a second strain were constructed to give plasmids p5'Rd/3'486, p5'486/3'Rd and p5'486/3'C₁₅₁TRd. Allelic transformants of strains Rd, Eagan and 486 were made and their respective LPSs were structurally analyzed using the same procedures as described above. In all transformants the 3' portion of the gene was directing the specific linkage of the Hex on HepIII (**Figure 25C**). It was therefore concluded that this portion is primarily responsible for determining the linkage position.

Notably, sequence analysis data obtained by LC-MSⁿ on mutant strains RdlpsA176, RdlpsAC₁₅₁TRd and Rd5'486/3'C₁₅₁TRd (i.e. directing βGal-(1→2 to HepIII) indicated that the mutant strains could express minor quantities of a disaccharide unit linked to HepIII (**Figure 26**). Due to the low abundance of these epitopes the hexose identities could not be elucidated.

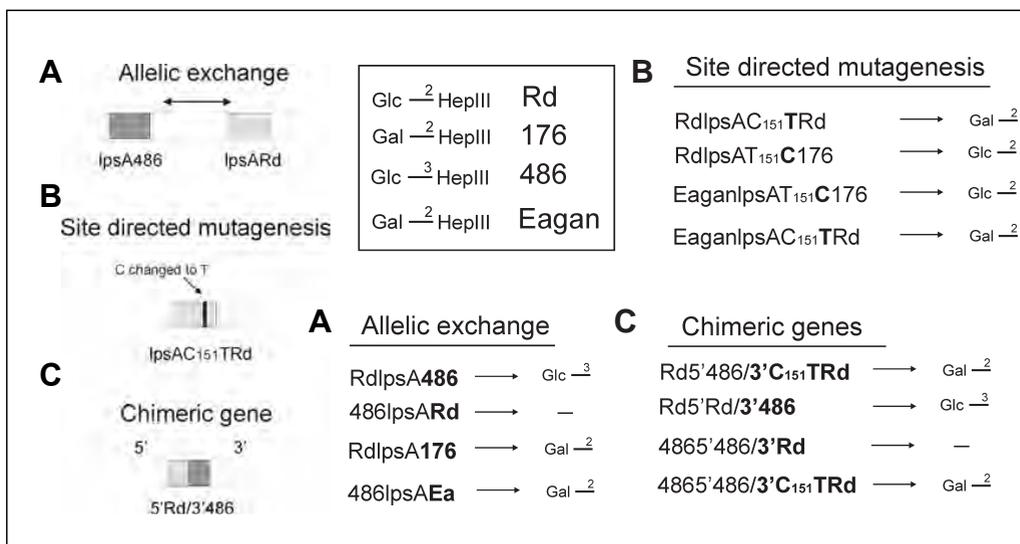


Figure 25. Summary of results. (A) Allelic exchange (B) Site directed mutagenesis (C) Chimeric genes. The hexose- and linkage-identity to HepIII of the wild-type strains are marked out within the square.

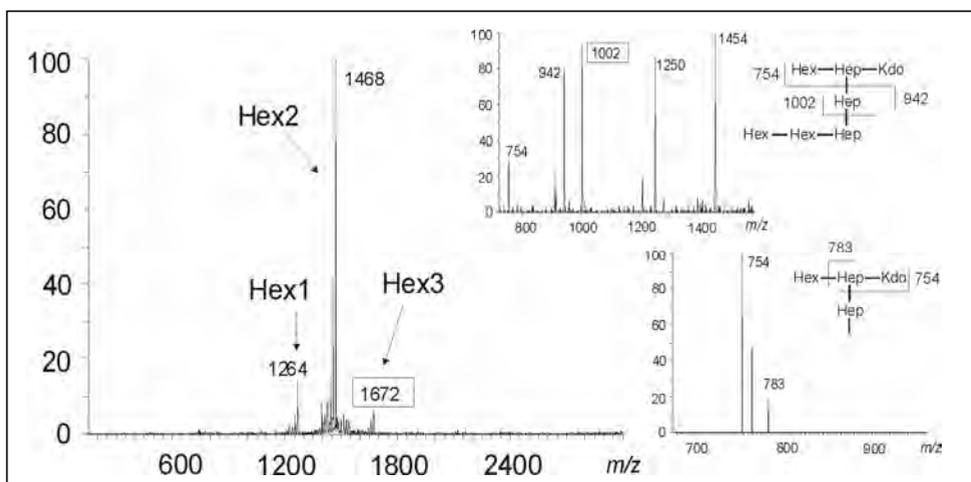


Figure 26. Sequence analysis on mutant strain RdIpsA176. (A) Full scan spectrum indicating a minor Hex3 glycoform. (B) MS² on this ion revealing fragments indicating an isomer containing a tHex-Hex- unit on HepIII. (C) MS³ on the ion at m/z 1002 confirmed this.

4.2 Paper II

Novel globoside-like oligosaccharide expression patterns in nontypeable *Haemophilus influenzae* lipopolysaccharide.

4.2.1 Background

Paper II is a structural elucidation of two closely related non-typeable Hi strains, 1268 and 1200. The strains are clinical isolates originating from the Finnish Otitis Media Study Group. Genetically, they have the same ribotype and by multilocus sequence typing have been shown to have identical nucleotide sequences in three of seven alleles (13). In earlier investigations it has been shown that the LPS of the strains contain Gly and Neu5Ac (38, 93). The lipid A structure has also been confirmed (46). In order to facilitate the analysis and to unambiguously determine the LPS structures of the two strains an *lpsA* mutant of strain 1268 was constructed.

4.2.2 Results

LPS from the wild-type and mutant strains was treated with anhydrous hydrazine under mild conditions to give LPS-OH which was subjected to ESI-MS experiments as well as compositional analyses by GLC-MS of the corresponding alditol acetate and 2-butyl glycoside derivatives. Additionally, LPS-OH samples were dephosphorylated prior to methylation analysis.

Mild acid hydrolysis of LPS with dilute aqueous acetic acid afforded insoluble lipid A and OS material. Purification by GPC gave leading fractions of higher molecular mass referred to as 1268OS and 1200OS. Strain 1268/*lpsA* gave *lpsA*OS. 1268OS, 1200OS and *lpsA*OS were investigated in detail using the same methods as described above and

also by sequence analysis on dephosphorylated and permethylated OS as well as by NMR analyses.

Methylation analysis performed on dephosphorylated 1268OS showed terminal Glc, terminal Gal, 4-substituted Gal, 4-substituted Glc, 3-substituted Gal, terminal Hep, 2-substituted Hep, 3,4-disubstituted Hep, terminal GalN, 2,3-disubstituted Hep and 4-substituted GlcN in the relative amounts of 18:8:7:16:6:5:5:16:1:17:1. Methylation analysis performed on phosphorylated 1268OS revealed the same sugars but with a decrease in terminal Glc, 4-substituted Glc and 2,3-disubstituted Hep, which indicated phosphorylation on those sugars. Sugar and methylation analyses from strain 1200 gave similar results. In contrast methylation analysis on dephosphorylated *lpsAOS* gave the same sugar derivatives as described above but showed a significant increase in terminal Hep and the absence of 2-substituted Hep, both observations indicating HepIII to be terminal.

ESI-MS performed on LPS-OH from the wild-type strains indicated the presence of heterogeneous mixtures of glycoforms. Major ions were observed and corresponded to glycoforms with respective compositions $PCho \cdot Hex_2 \cdot Hep_3 \cdot PEtn_{1,2} \cdot P \cdot Kdo \cdot LipidA-OH$. Ions corresponding to $HexNAc \cdot Hex_{4,5}$ and $Hex_{3,5}$ containing glycoforms were also indicated. ESI-MS data of LPS-OH from 1268*lpsA* showed less heterogeneity with no indications of Hex5 or $HexNAcHex5$ glycoforms.

In contrast ESI-MS on 1268OS and 1200OS revealed major $HexNAcHex4$ and $HexNAcHex5$ glycoforms in agreement with OS samples being leading fractions after GPC. The glycoforms observed in *lpsAOS* were in agreement with those found in the equivalent LPS-OH sample and showed major Hex2 glycoforms. All strains indicated glycylation in minor amounts. In addition, OS samples from NTHi 1200 showed ions corresponding to acetylated glycoforms. Information on the location of Ac in 1200OS was provided by ESI-MS¹⁻³ and indicated HepIII to be substituted with two acetates. The experiments also confirmed that *PCho* substituted the hexose linked to HepII and that *PEtn* substituted HepII.

Four major glycoforms ($Hex4$, $Hex5$, $HexNAcHex4$ and $HexNAcHex5$) were observed in the sequence analysis of dephosphorylated and permethylated 1268OS, consistent with compositional ESI-MS data. The two major isomeric $Hex4$ glycoforms and the single isoforms detected in $Hex5$, $HexNAcHex4$ and $HexNAcHex5$ are shown in **Figure 27**. Minor amounts of a $HexNAc2Hex4$ glycoform were also detected in the permethylated sample. This glycoform contained a $tHexNAc-Hex-HexNAc-Hex-Hex$ -unit elongating from HepI and one hexose substituting HepII. 1200OS contained virtually the same glycoforms as observed in 1268OS except for those glycoforms having elongations from HepI. However, traces of three other higher molecular mass forms; $HexNAcHex6$, $HexNAcHex7$ and $HexNAc2Hex7$ were observed. The $HexNAc2Hex7$ glycoform indicated one isomer substituted by a hexose at HepI and $tHexNAc-Hex-Hex-Hex$ units substituting both HepII and HepIII (**Figure 27**). The $HexNAcHex6$ and $HexNAcHex7$ glycoforms indicated truncated versions of this structure. ESI-MSⁿ data obtained from *lpsAOS* clearly indicated the absence of glycoforms expressing chain extension from HepIII. The major isoforms were otherwise equivalent to those found in the wild type strain, except for an extra $Hex1$ glycoform containing one hexose substituent on HepI.

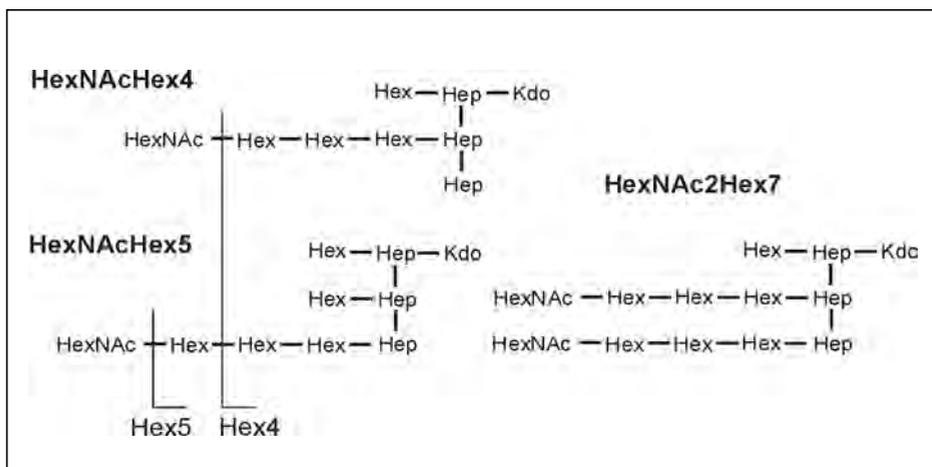


Figure 27. The major structures found in 1268OS and 1200OS. In addition the HexNAc2Hex7 glycoform detected in strain 1200 is shown.

The presence of ions corresponding to sialylated glycoforms were confirmed using LPS-OH material of 1268*lpsA* in precursor ion monitoring tandem mass spectrometry experiments. Importantly glycoforms with compositions corresponding to *P*Cho-Neu5Ac-HexNAc₂-Hex₅-Hep₃-PEtn_{1,2}-*P*-Kdo-LipidA-OH were proposed to have a [(PEtn→6)-α-D-GalpNAc-(1→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→)] unit linked to HepI and a sialyllactose unit substituting HepII (**Figure 28**). Sialyllactose has previously not been found to extend from this position.

Major structures were elucidated by detailed ¹H, ¹³C and ³¹P NMR analyses on deacylated 1268OS, 1200OS and *lpsA*OS using gradient chemical shift correlation techniques (COSY, TOCSY and HMQC experiments) and inter- and intra-NOE connectivities. Since *lpsA*OS did not express extensions from HepIII, this sample was used to unambiguously determine the HexNAcHex4 glycoform containing extensions from HepII. Furthermore the information obtained from methylation and sequence analyses was valuable for the interpretation of NMR spectra.

It was concluded that the HexNAcHex4 glycoform contained a β-D-GalNAcp-(1→3)-α-D-Galp-(1→4)-β-D-Galp-(1→4)-α-D-Glcp-(1→ “globotetraose like” epitope extending from O-3 on HepII and a β-D-Glcp-(1→ unit substituting O-4 at HepI. The HexNAcHex5 glycoform was proposed to contain a β-D-Glcp-(1→ and an α-D-Glcp-(1→ unit linked to HepI and HepII respectively and globotetraose (β-D-GalNAcp-(1→3)-α-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Glcp-(1→) linked to O-2 at HepIII. In the ¹H NMR spectrum anomeric resonances corresponding to the triheptosyl moiety (HepI-HepIII) of the HexNAcHex4 glycoform were identified at δ 5.05-5.16, 5.83 and 5.03 and for the HexNAcHex5 glycoform at δ 5.05-5.16, 5.71 and 5.13, respectively. In addition H-1 of GlcI was observed at δ 4.54 in both forms.

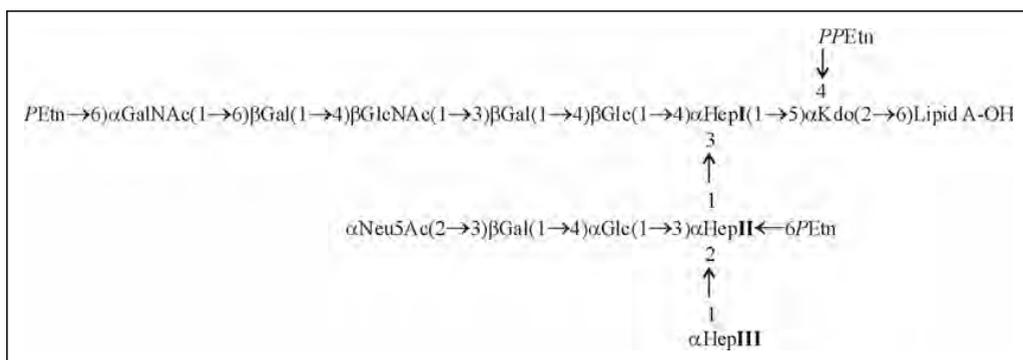


Figure 28. Proposed sialyllactose structure obtained from the *lpsA* mutant strain.

Spin systems corresponding to the novel β -D-GalNAcVIII p -(1 \rightarrow 3)- α -D-GalVII p -(1 \rightarrow 4)- β -D-GalVI p -(1 \rightarrow 4)- α -D-GlcV p -(1 \rightarrow) residue extending from HepII in the HexNAcHex4 glycoform were identified in the COSY and TOCSY spectra at δ 5.28 **V**, 4.57/4.64 **VI**, 4.92 **VII**, 4.66 **VIII**, respectively. In the HexNAcHex5 glycoform anomeric signals at δ 4.43 and δ 4.52 could be attributed to the [\rightarrow 4- β -D-Glcp] and [\rightarrow 4- β -D-Galp] residues of the globotetraose unit on HepIII. Additional spin systems corresponding to terminal GalNAc and 3-substituted Gal residues indicated by methylation and sequence analyses were overlapping with the resonances of the corresponding sugars extending from HepII.

The high H-6_{A/B} chemical shifts of the α -D-Glcp-(1 \rightarrow) residue (**V**) linked to O-2 at HepII ($\delta_{\text{HexNAcHex4}}$ 4.11/4.18 or $\delta_{\text{HexNAcHex5}}$ 4.10/4.28) indicated O-6 to be substituted by PCho, which was confirmed in ^1H - ^{31}P correlation experiments.

Signals originating from truncated versions of the HexNAcHex4 (Hex4) and HexNAcHex5 (Hex5) as well as a Hex2 glycoform were also assigned. The major detected glycoforms are shown in **Figure 29**.

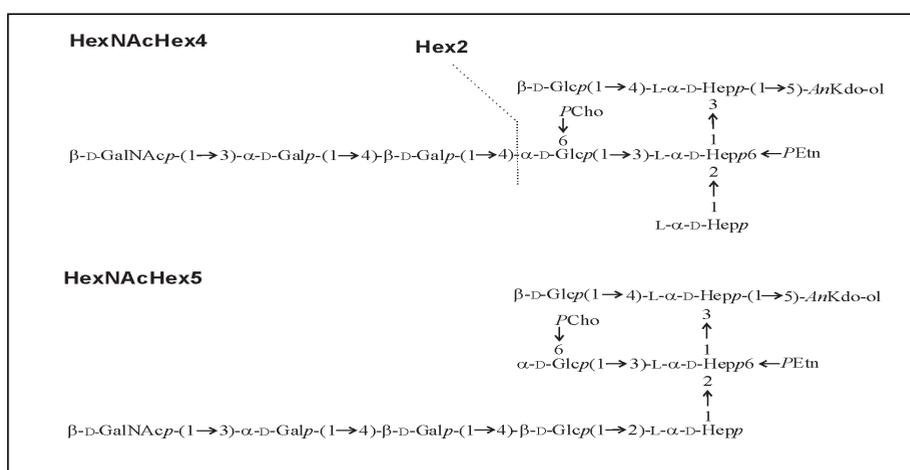


Figure 29. The major glycoforms observed in NTHi strain 1268 and 1200.

4.3 Paper III

Structural analysis of the lipopolysaccharide from non-typeable *Haemophilus influenzae* strain R2846

4.3.1 Background

NTHi strain R2846 is a clinical isolate taken from the middle ear of a patient with otitis media (121) and has been used in a genome sequencing project (GenBank NZ DQ007026). A candidate gene for a novel heptosyltransferase (LosB) that incorporates a fourth heptose in the LPS outer-core has recently been identified from the genome sequence of strain R2846 through homology to a gene in the related species *Haemophilus ducreyi* (122, 123).

In this study LPS from NTHi strain R2846 was structurally elucidated. In addition mutant strains R2846*losB1*, R2846*losB2* and R2846*losB1/losB2* were constructed in order to investigate differences in the LPS expression of non-core heptose (HepIV). A strain mutated in the *lpsA* gene was also investigated (R2846*lpsA*) to confirm its function.

4.3.2 Results

LPS from R2846 was treated with anhydrous hydrazine under mild conditions to give water soluble LPS-OH. In addition, mild acid hydrolysis of LPS with dilute aqueous acetic acid afforded insoluble lipid A and core OS material. The OS was further purified by GPC that resulted in oligosaccharide fractions OS1-4 of which OS3 was major. Lipid A was identical to the conserved features as described previously for Hi by using fatty acid analysis and ESI-MSⁿ experiments.

ESI-MS of LPS-OH revealed ions corresponding to a mixture of different glycoforms. Two major ions were observed with the respective compositions, Hex₃·Hep₄·PEtn_{1,2}·P·Kdo·lipidA-OH. Furthermore, sialylated glycoforms (Neu5Ac·HexNAc·Hex₅·Hep₄·PEtn_{1,2}·P·Kdo·lipid A-OH) were detected by precursor ion monitoring tandem mass spectrometry experiments.

The OS3 fraction contained a major glycoform with the composition Hex₃·Hep₄·PEtn·AnKdo-ol. OS4 gave virtually identical results to OS3. In contrast, glycoform populations observed in OS1 and OS2 were more heterogeneous and of higher molecular mass containing glycoforms with additional hexoses and hexoseamines and also glycoforms containing P, PCho and additional PEtn substituents. Of note were glycoforms with the respective compositions HexNAc·Hex₅·Hep₄·PEtn·P_{0,1}·AnKdo-ol and HexNAc₂·Hex₅·Hep₄·PEtn₂·P_{0,1}·AnKdo-ol. All fractions indicated a high content of O-acetates and minor content of ester linked glycine.

LPS-OH was dephosphorylated with 48% hydrogen fluoride prior to methylation analysis. Significant amounts of terminal Glc, terminal Gal, 6-substituted Glc, 4-substituted DD-Hep, 2-substituted Hep and 3,4-disubstituted Hep were detected. Methylation analysis on dephosphorylated OS3 showed the same major sugar derivatives as detected for LPS-OH. In contrast, methylation analysis performed on OS1 showed high content of terminal Glc, terminal Gal, 3-substituted Gal, 6-substituted Glc, 6-substituted Gal, 4,6-disubstituted Glc, 4-substituted DD-Hep, 2-substituted Hep, 3,4-disubstituted Hep, terminal GalN and 4-substituted GlcN. In addition, minor

amounts of 4-substituted Gal, 4-substituted Glc, terminal DD-Hep, terminal Hep and 4-substituted Hep were detected.

Sequence and branching details on the major Hex3Hep4 glycoform indicated one isomer in which HepI was substituted by a tHex-HepIV-Hex- unit and HepIII was substituted by one hexose. Glycoforms in OS1 corresponding to HexNAc₁₋₂-Hex₅-Hep₄-AnKdo-ol indicated the first hexose on HepI to be branched with a tHex-HepIV- unit and an additional tHex-HexNAc-Hex- or tHexNAc-Hex-HexNAc-Hex- unit. Similar to the Hex3Hep4 glycoform HepIII was substituted with one hexose. Notably, in addition to the tHexNAc-Hex-HexNAc-Hex- and tHex-HexNAc-Hex- units extending from the first hexose on HepI a minor tHexNAc-Hex-Hex- unit was identified.

The major Hex3Hep4 structure was also established using detailed ¹H, ¹³C and ³¹P NMR analyses on deacylated OS3. ¹H, ¹³C and ³¹P NMR resonances were assigned using gradient chemical shift correlation techniques (COSY, TOCSY and HMQC experiments). NOE data also served to confirm the anomeric configurations of the linkages and the monosaccharide sequence. Anomeric resonances corresponding to the triheptosyl moiety were identified at δ 5.04-5.15 (HepI), 5.68 (HepII) and 4.99 (HepIII). Subspectra corresponding to the hexose residues and the non-core heptose were identified at δ 4.50 (GlcI), 4.96 (HepIV), 4.56 (GlcII) and 4.40 (GalI), respectively. Inter-residue NOE were observed between the proton pairs of GlcI H-1/HepI H-4/H-6 HepIV H-1/GlcI H-6A and GlcII H-1/HepIV H-4 confirming a β-D-GlcIIp-(1→4)-α-D,D-HepIVp-(1→6)-β-D-GlcIp- unit extending from O-4 on HepI. Furthermore, inter-residue NOE between the proton pairs of GalI H-1/HepIII H-1/H2 confirmed β-D-GalI to be linked to position O-2 in HepIII.

Due to heterogeneity and small abundance, the OS structures extending from the O-4 position of GlcI could not unambiguously be established by NMR. However, the combined data from precursor ion scan CE-ESI-MS/MS and HPLC-ESI-MSⁿ analysis in addition to the significant amounts of t-GalNAc, 6-Gal, 4-GlcNAc, 3-Gal and 4,6-Glc detected in methylation analysis made it reasonable to suggest that strain R2846 expresses both extending [α-Neu5Ac-(2→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→)] (SiaT) and [(PEtn→6)-α-D-GalpNAc-(1→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→)] (GaT) units from HepI.

Mutant strains R2846*losB1*, R2846*losB2* and R2846*losB1/losB2* were constructed to investigate the function of the *losB1* and *losB2* heptosyltransferase genes detected in R2846. LPS from the strains were elucidated using the same methods as described above.

The results indicated that R2846*losB1* expressed a major Hex2Hex3 glycoform in which HepI and HepIII were substituted by one hexose each. The predominant Hex2Hep3 glycoform indicated inter-residue NOE between the proton pairs of GlcI H-1/HepI H-4/H-6 and GalI H-1/HepIII H-1/H2 which confirmed GlcI to substitute HepI at O-4 and GalI linked to O-2 of HepIII. Interestingly, a number of minor glycoforms (Hex_{2,3}-Hep₄-AnKdo-ol and HexNAc_{1,2}-Hex₅-Hep₄-AnKdo-ol) containing four heptoses were observed during the structural elucidation. Sequence analysis confirmed these glycoforms to contain non-core heptose at the same position as in the wild-type strain. However, in contrast to R2846, no DD-Hep was detected. Since 4-

substituted LD-Hep was observed in methylation analyses it was concluded to originate from non-core HepIV.

Analyses of LPS from R2846*losB2* gave virtually the same results as the wild-type strain. In contrast to R2846, however, no traces of 4-substituted LD-Hep could be detected.

The R2846*losB1/losB2* mutant strain showed the same major Hex2Hep3 glycoform as was present in R2846*losB1* but no glycoforms containing four heptoses were detected.

From these results it was concluded that the *losB1* gene directs the addition of outer-core DD-Hep to GlcI and that *losB2* directs LD-Hep to the same position.

In agreement with previously investigated *lpsA* mutant strains, the LPS from R2846/*lpsA* did not express any elongation from HepIII.

Information on the location of *P*Cho, *PEtn*, *P* and Ac was provided by CE-ESI-MS/MS of OS fractions derived from R2846, R2846*losB1* and R2846*losB2*. The results indicated *P*Cho to be substituted both to the Hex on HepIII and to HepIV. The results also indicated that *P* could substitute HepIII. A *PEtn* unit was assigned to the terminal GalNAc on the GaT unit extending from HepI. In addition, an acetate group was assigned to the GlcNAc unit on the same epitope. The position of glycine could not be determined. Attempts were made to obtain the exact location sites of the *P*Cho and *P* substituents on partially ethylated and methylated alditol acetates of originally phosphorylated sugars. Using this procedure *P*Cho was indicated to substitute the O-6 position of GalI. *P*Cho was also indicated to substitute either O-6 or O-7 of HepIV but due to possible migration between the 6- and 7-position of this residue no exact position could be determined. The analysis also resulted in indications of 2-substituted Hep with a phosphorylation site at the 7- and 6-position. Since HepII is substituted at O-6 with *PEtn* this alditol product was concluded to originate from this residue but also from the phosphate linked to HepIII. In the ^{31}P - ^1H HMQC spectrum of OS2 of R2846 a strong signal assigned to a phosphate was observed at (δ_{H} 3.91 / δ_{P} 4.55) which indicated *P* to be linked to O-7 of HepIII (**Figure 30**) (110).

The results are summarized in **Figure 31**.

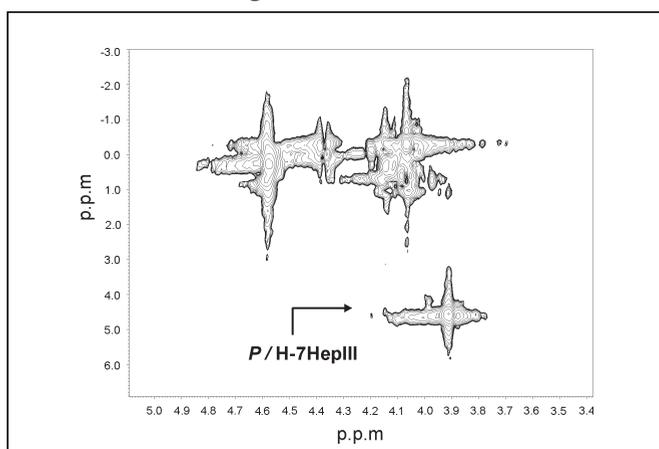


Figure 30. ^{31}P - ^1H HMQC spectrum of OS2 of R2846. The proposed cross peak between *P* and H-7 of HepIII is indicated. Other signals in the spectrum are corresponding to *PEtn* and *P*Cho connections.

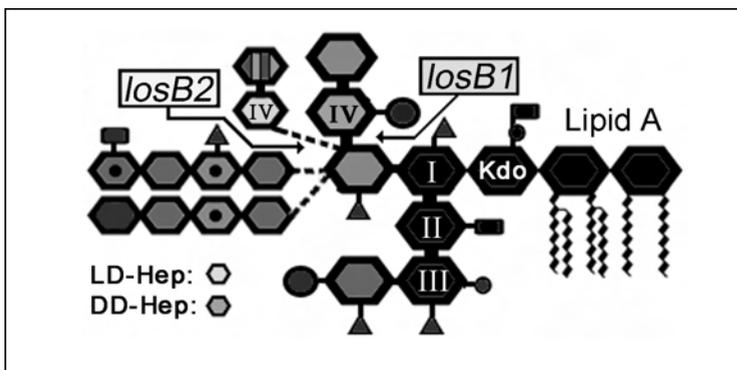


Figure 31. Summary of results, Paper III. Triangles indicate Ac, large circles indicate PCho, small circles indicate P and squares indicate PEtn.

4.4 Paper IV

Application of CE-ESI-MS and LC-ESI-MSⁿ to profile glycoform expression during *Haemophilus influenzae* pathogenesis in the chinchilla model of experimental otitis media

4.4.1 Background

Detailed structural LPS studies of *in vivo* grown samples have been limited due to the relatively small number of bacterial cells in the middle ear fluid (MEF) and the additional contamination of tissue and blood.

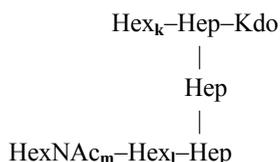
In this study we investigated MEF samples taken after seven days from 10 animals inoculated using NTHi strain 1003 as the challenge strain. Samples were analyzed directly using CE-ESI-MS experiments and compared to the same MEF samples cultured *ex vivo* in liquid brain-heart infusion (sBHI) broth and on unsupplemented chocolate agar (uCA). Following passage, the signal-to-noise ratio increased significantly and the amount of LPS material was also sufficient to determine the sequence of glycoforms by HPLC-ESI-MSⁿ. It was observed that LPS glycan profiles were essentially the same when the MEF samples of 7 of the 10 animals were passaged on uCA. This observation was used in a following experiment where changes in LPS glycoform distribution during the course of infection were investigated.

4.4.2 Results

In vitro grown NTHi 1003 had previously been investigated using NMR and ESI-MS on O-deacylated LPS and major core OS material obtained following mild acid hydrolysis of LPS (35). To obtain information on undetected minor glycoforms and glycoform isomerism dephosphorylated and permethylated oligosaccharide material from the *in vitro* grown strain was subjected to LC-ESI-MS²⁻³ (Table 3). The same method was later used for profiling LPS from material derived from the chinchilla ear (see below). Several glycoforms were observed and structurally determined that were not easily detected in underivatized samples. Only the Hex2 glycoform and the major Hex3 glycoform had been detected previously.

Ten chinchillas were inoculated in the left ear with NTHi strain 1003 and developed otitis media. MEF was taken from the animals 7 days after inoculation. The samples were analyzed directly or cultured *ex vivo* in liquid sBHI and on uCA. LPS was obtained by micro extraction and directly analyzed by CE-ESI-MS analysis following *O*-deacylation *in situ* to give LPS-OH. Samples from three animals were contaminated with *Staphylococcus aureas* and showed no interpretable data. Doubly charged ions corresponding to Hex2 glycoforms were detected in all other MEF samples and, in addition, ions corresponding to Hex1, Hex3 or Hex4 glycoforms were detected in the MEF from some of the animals. The spectra for LPS-OH from sBHI cultured bacteria were characterized by weak ion intensities corresponding to a very low abundance or an absence of Hex3 and Hex4 glycoforms with Hex1 and Hex2 glycoforms representing the major populations. However, the CE-ESI-MS spectra of samples obtained after *ex vivo* growth on solid medium (uCA) not only showed significant increased signal to noise ratio but for the majority of animals the distribution of glycoforms corresponded to that from MEF. These observations indicate that LPS glycoform populations obtained following single passage of bacteria derived from MEF on uCA best represent those glycoforms presented in bacteria directly from the MEF.

Table 3. Isomeric glycoforms detected in HPLC-ESI-MSⁿ on NTHi strain 1003. Isomer **B2** and **E** were not detected in the *in vitro* grown sample.



Glycoform	Isomer	k	l	m
		Hex1	A	1
	A2	0	1	0
Hex2	B1	1	1	0
	B2	2	0	0
Hex3	C1	1	2	0
	C2	2	1	0
Hex4	D1	1	3	0
	D2	2	2	0
	D3	3	1	0
Hex4HexNAc	E	1	3	1

The LPS-OH samples were sequence analyzed using LC-ESI-MSⁿ. To increase sensitivity, SIM and SRM experiments were employed using the information obtained from CE-ESI-MS. Singly charged sodiated ions corresponding to Hex1, Hex2, Hex3, Hex4 and HexNAcHex4 glycoforms were used for SIM and SRM analyses.

In the spectra of MEF samples and those passaged in sBHI, only low signal intensity ions corresponding to the Hex1-Hex3 glycoforms detected by CE-ESI-MS were observed. This precluded further MS/MS analyses other than MS². Spectra of samples obtained after passage on uCA correlated well with those from CE-ESI-MS and showed sufficient signal to noise ratios for MS²⁻³ experiments.

A1 was the predominant Hex1 glycoform in all samples. The Hex2 glycoform **B1** was dominant in all samples except one in which structure **B2** was predominant. **B2** was not observed in *in vitro* grown NTHi 1003. The Hex3 glycoform **C1** was the most abundant in all samples. The Hex4 glycoform was only detectable in four samples and in these, structure **D1** predominated. Interestingly, **D1** was minor when NTHi 1003 was grown *in vitro* in sBHI. A HexNAcHex4 glycoform that was only observed in one sample in LPS-OH, was detected in three of the methylated samples. Fragmentation on the molecular ion of this glycoform defined a structure (**E**) in which a HexNAcHex3 unit and a hexose substitute HepIII and HepI, respectively.

The previously not detected glycoform **B2** was proposed to have a [β -D-Glcp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)] unit linked to HepI based on the proposed function of *lex2* in this strain. HepIII of **E** is predicted to be elongated by a globotetraose [β -D-GalpNAc-(1 \rightarrow 3)- α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)] unit. This is because the genes *lgtD* and *lgtC* are both present in NTHi strain 1003 (**Figure 32**).

We concluded that LPS from MEF passaged on uCA was comparable to LPS from direct samples. Therefore this approach was used to evaluate changes in LPS glycoform expression during the course of otitis media in the chinchilla.

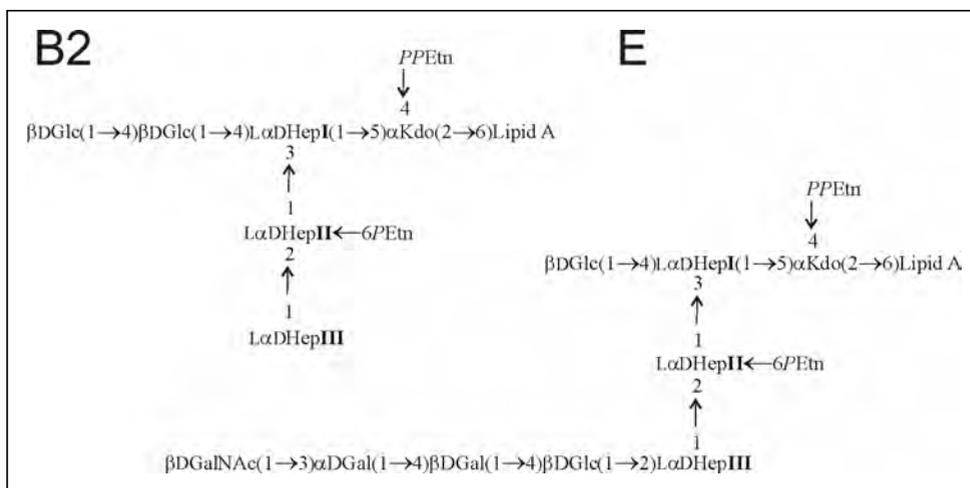


Figure 32. Proposed structures **B2** and **E**.

Exudates from the middle-ears of animals 2, 5 and 9 days after inoculation were passaged on chocolate agar plates with or without sialic acid and the LPS was analyzed by CE-ESI-MS following *O*-deacylation. Interestingly, there was no significant difference in glycoform distribution during four consecutive passages or between growths irrespective of whether sialic acid was added to the medium or not. A trend towards lower hexose containing (truncated) glycoforms was observed during the course of infection for eight out of the ten animals in this study.

Sialylated glycoforms were not evident in the full scan mass spectra of any of the samples, but could be detected by precursor ion monitoring. Sialic acid containing glycoforms were only present in LPS-OH samples obtained at 2 and 5 days; no sialic acid was detectable in the samples obtained at day 9. When grown *in vitro*, strain NTHi 1003, has been shown to express glycoforms having sialyllactose linked to HepIII but only in very low abundance (93).

4.5 Summary and Conclusion

In order to unambiguously determine the function of LPS biosynthesis genes, a structural elucidation of mutant strains with NMR and mass spectrometric methods has proved to be crucial. Additionally, the knowledge of LPS biosynthetic genes is an important tool used to facilitate the structural elucidation of heterogeneous strains. Paper I, Paper II and Paper III in this thesis are examples of this mutual relationship. Paper IV gives an insight in trends of changes in LPS glycoform expression during infection.

The *lpsA* gene is the first known example in which Hi LPS inter-strain variation is directed by a specific gene sequence that encodes one of four alternative sugar additions. Since a Glc- in contrast to a Gal-unit on HepIII will provide further saccharide extensions, the importance of allelic variants of *LpsA* goes beyond just the specificity of directing the hexose and its anomeric linkage. Thus, known virulence factors such as digalactoside and sialylated and disialylated lactose extensions will only be expressed when HepIII is substituted with a Glc.

Notably, strain 486 could be transformed to have HepIII O-2 substituted with Gal but not sufficiently with Glc. One suggestion is that the *LpsA* catalyzation of an O-2 substituted Glc is hindered sterically by a *PCho* linked to α -Glc on HepII in this strain. Interestingly, HepIII of strains 1268 and 1200 in Paper II, is O-2 substituted with a β -glucose in the same position and contain the same structural feature at HepII as in strain 486. Little is known about the 3D structure of Hi LPS, but it would be expected that different conformations and dynamics of the molecules will have great impact during the biosynthesis and also importantly during interactions with host receptors etc.

As predicted, the closely related NTHi strains 1268 and 1200 expressed virtually identical LPS glycoforms, the only difference being the presence of *O*-acetyl groups in strain 1200. Since *O*-acetylation has been shown to be a potential virulence factor of NTHi this minor difference may still be of importance. Both strains contained globotetraose as well as its truncated versions globoside and lactose linked to HepIII and the corresponding novel structures with an α -D-Glc_{6P} as the reducing sugar linked to HepII. It would be predicted that due to phase-variation two units of host mimicking

digalactoside [α -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow)] can be expressed simultaneously in the strains. The presence of two digalactoside units in the outer-core has previously been shown to be associated with increased virulence. In addition to facilitate the overall structural elucidation of the wild-type strains, the *lpsA* mutant strain importantly helped to elucidate a novel sialyllactose [α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- α -D-Glcp(1 \rightarrow)] unit substituting HepII. Similar to the digalactoside, sialyllactose is an important virulence factor that is regulated by phase-variable genes.

The LPS from NTHi R2846 was found to express a very heterogeneous mixture of glycoforms. In particular additions of non-carbohydrate substituents (*P*, *PEtn*, *PCho*, *Ac* and *Gly*) contributed to the observed structural diversity. The major LPS glycoform contained a β -D-Glcp-(1 \rightarrow 4)-D- α -D-Hepp-(1 \rightarrow 6)- β -D-Glcp-(1 \rightarrow 4) motif extending from HepI and a β -D-Galp-(1 \rightarrow 2) unit substituting HepIII. NTHi R2846 can also express minor glycoforms were GlcI is branched with additional extensions from O-4 with [α -Neu5Ac-(2 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow)] and the related structure [(*PEtn* \rightarrow 6)- α -D-GalpNAc-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)- β -D-GlcpNAc-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow)]. Analysis of LPS from mutant strains R2846*losB1*, R2846*losB2* and R2846*losB1/losB2* indicated that both *losB1* and *losB2* are involved in the addition of non-core heptose and that the expression of DD-Hep is catalyzed by *LosB1* and LD-Hep by *LosB2*. Unpublished data has shown a trend toward similar results in other NTHi strains. Of note is that the presence of *losB1* in pair with the gene *losA1* precludes the genes *lic2C* and *lic2B* being present. Since *lic2C* is responsible for the addition of the first hexose to HepII this will disable glycosyl extensions from HepII. Similarly *LpsA* in R2846 directs Gal to HepIII which will result in no further extensions from this position. The numerous acetylation sites and the expression of other non-carbohydrate substituents in R2846 may be a response due to this lack of important glycosyl extensions. Both the phase-variable expression of *PCho* and *Ac* in NTHi LPS are known virulence factors. In addition the expression of sialyllacto-*N*-neotetraose from HepI was observed in relatively high contents. Notably, trace amounts of a Hex3Hep3 glycoform containing extension of two hexoses from HepIII were detected in the HPLC-ESI-MSⁿ sequence analysis of the R2846*losB1* and R2846*losB1/losB2* mutants. Due to the limited amount of sample, these epitopes could however not be elucidated further. The development of the methods we use during a structural LPS analysis (in particular by using HPLC-MSⁿ) has increased the detection limit for what is possible to analyze. This has facilitated analyses of trace amounts as seen in Paper IV, but it also increases the complexity of what is possible to detect in *in vivo* grown samples. The biosynthesis of the elongation with a tHex-Hex- unit on HepIII could be due to a bifunctionality of *LpsA* where a majority (95< %) of the enzyme transfers Gal and a minority (5> %) transfers Glc to HepIII. Alternatively the *LgtC* enzyme that transfers a α -D-Gal to O-4 at a lactose (β -D-Galp-(1 \rightarrow 4)- β -D-Glcp(1 \rightarrow)) unit extending from HepIII can synthesize the same linkage to β -D-Galp(1 \rightarrow) directly linked to HeppIII in minor abundances. As described in Section 4.1.2 (Paper I), minor amounts of the same tHex-Hex- unit extending from HepIII were observed when *LpsA* was directing a Gal to HepIII.

The LPS glycan profiles from MEF samples passaged on solid media were essentially the same as those taken and analyzed directly. Notably, the LC-ESI-MSⁿ technique used in this study did provide two glycoforms which were not previously identified for

in vitro grown NTHi 1003. Since the strain contains the genes *lex2*, *lgtC* and *lgtD*, strain 1003 was proposed to express both a globotetraose unit extending from HepIII and a [β -D-Glcp-(1 \rightarrow 4)- β -D-Glcp-(1 \rightarrow)] unit extending from HepI.

In the follow-up study trends in LPS expression 2, 5 and 9 days post-infection indicated, as previously observed, that sialic acid containing glycoforms were only detected during the early stages of infection (87). A trend towards more truncated and less complex LPS glycoforms that lacked sialic acid was found as disease progressed. This suggests that sialic acid is necessary for initiation of infection in the middle ear but not necessary for continuation once established. From another perspective the observed phenotype of the LPS glycoforms could be a response to the metabolic adaptation of the bacteria to the host environment when large numbers of organisms are present within the middle ear. Evidence from *in vitro* assays also suggests that the more truncated the LPS glycoforms, the more susceptible would these bacteria be to innate host defense clearance mechanisms.

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