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Lipopolysaccharide modification in Gram-negative bacteria during chronic infection

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One sentence summary: We review modifications of lipopolysaccharide structure and biosynthetic pathways that occur upon bacterial adaptation to chronic respiratory and gastrointestinal infections.

Keywords (6): adaptive mutation, O antigen, lipid A, Pseudomonas aeruginosa, Burkholderia cenocepacia, cystic fibrosis, Helicobacter pylori, gastric ulcer

Running title: Lipopolysaccharide modification during chronic infection
ABSTRACT

The Gram-negative bacterial lipopolysaccharide (LPS) is a major component of the outer membrane that plays a key role in host-pathogen interactions with the innate immune system. During infection, bacteria are exposed to a host environment that is typically dominated by inflammatory cells and soluble factors, including antibiotics, which provide cues about regulation of gene expression. Bacterial adaptive changes including modulation of LPS synthesis and structure are a conserved theme in infections, irrespective of the type or bacteria or the site of infection. In general, these changes result in immune system evasion, persisting inflammation, and increased antimicrobial resistance. Here, we review the modifications of LPS structure and biosynthetic pathways that occur upon adaptation of model opportunistic pathogens (Pseudomonas aeruginosa, Burkholderia cepacia complex bacteria, Helicobacter pylori and Salmonella enterica) to chronic infection in respiratory and gastrointestinal sites. We also discuss the molecular mechanisms of these variations and their role in the host-pathogen interaction.

INTRODUCTION

The lipopolysaccharide (LPS) is a central component of the outer membrane in Gram-negative bacteria and frequently plays a key role in pathogenesis (Figure 1) (Whitfield & Trent, 2014). LPS is the dominant glycolipid in the outer leaflet of the outer membrane, forming a layer that is stabilized by divalent cations and provides an effective permeability barrier against deleterious molecules such as antibiotics and cationic antimicrobial peptides (Nikaido, 2003). The classical LPS molecule has a tripartite structure comprising (i) lipid A, the hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane, (ii) core oligosaccharide (herein core), which together with lipid A, contributes to maintain the integrity of the outer membrane, and (iii) O antigen polysaccharide or O antigen, which is connected to the core and consists of a polymer made of repeating oligosaccharide units in direct contact with the external milieu (Figure 1) (Whitfield & Trent, 2014). LPS molecules only including lipid A and core are generally referred to as "rough" and often called lipoooligosaccharides, while the complete LPS capped with O antigen is called "smooth". The lipid A is embedded in the outer membrane and composed of acyl chains linked to
a backbone dimer of glucosamine by ester and/or amide linkages. The typically hexa-acylated lipid A elicits robust inflammatory responses upon recognition by the complex Toll-like receptor 4 and myeloid differentiation factor 2 (TLR4-MD2), which is predominantly found on macrophages, monocytes and dendritic cells (Park et al., 2009, Park & Lee, 2013).

Modification of the lipid A acylation patterns, or addition of positively-charged substituents to the lipid A phosphate groups (Raetz et al., 2007), confer protection against host innate defenses by reducing even further the permeability of the outer membrane to antimicrobial peptides and dampening inflammatory responses by the host (Raetz et al., 2007, Needham & Trent, 2013, Di Lorenzo et al., 2015).

Lipid A is glycosylated at the 6′-position with two residues of 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo); the inner Kdo serves as the point of attachment for the remaining core. Some bacterial species such as Burkholderia (Silipo et al., 2005, Silipo et al., 2007) produce a modified Kdo, which is converted into d-glycero-d-talo-oct-2-ulosonic acid (Ko) by a unique Kdo-3 hydroxylase (Chung & Raetz, 2011). The next sugars added to the Lipid A-Kdo₂ are typically two or more residues of L-glycero-d-manno-heptose, although in some species LPS molecules are devoid of heptose (Valvano et al., 2002). The rest of the core consists of a set of sugars that differs among species and even among strains of the same species (Mamat et al., 2011). Phosphorylation of the core sugars in P. aeruginosa has been associated with increased membrane impermeability and resistance to antibiotics (Walsh et al., 2000), and is also required for the transport of LPS to the outer membrane (Delucia et al., 2011). The P. aeruginosa core may also be a ligand for the cystic fibrosis (CF) transmembrane conductance regulator protein displayed on the apical surface of epithelial cells (Schroeder et al., 2002).

O antigens comprise repeating oligosaccharide units that may be linear or branched (Whitfield & Trent, 2014). The O-repeating unit is highly variable immunochemically giving rise to a vast number of different O-specific serotypes (Valvano et al., 2011, Whitfield & Trent, 2014). The O antigen contributes to evasion of host immune defenses, particularly evasion of the complement cascade in Salmonella enterica serovar Typhimurium (Murray et al., 2006), delay of recognition and internalization in epithelial cells in Salmonella Typhimurium and Burkholderia cenocepacia (Duerr et al., 2009, Saldías et al., 2009), enhanced intracellular survival in Shigella flexneri (West et al., 2005) and Brucella melitensis (Paixão et al., 2009),
and protection against oxidative stress in Erwinia amylovora (Berry et al., 2009). O antigen
also contributes to swimming and swarming motility in E. amylovora (Berry et al., 2009), B.
cenocepacia (Coutinho et al., 2011), and Pectobacterium atrosepticum (Bowden et al., 2013).
The immunogenicity of the O antigen polysaccharide elicits a robust antibody response, which
may cause selective pressure on bacteria to lose the ability to produce O antigen (King et al.,
2009); this is particularly common for chronic P. aeruginosa strains infecting the lungs of CF
patients (Hancock et al., 1983). Conceivably, once the bacteria become mucoid (Govan &
Deretic, 1996), the nutrient burden is so high producing alginate and that the bacteria are
replicating in a “protected” niche in which O antigen becomes dispensable. However, this may
not be a universal notion since other bacteria chronically infecting the CF lung, such as
members of the B. cepacia complex, undergo different adaptive changes than those reported
for P. aeruginosa (Zlosnik et al., 2014), including the observation of an inverse correlation
between the quantity of mucoid exopolysaccharide production and the rate of decline in CF
lung function (Zlosnik et al., 2011).

Most P. aeruginosa strains produce two types of O antigen molecules ("A-band" and
"B-band"), which are structurally and serologically distinct and have different mechanisms of
biosynthesis (King et al., 2009, Lam et al., 2011). The "A-band" or "common polysaccharide
antigen" is a homopolymer of D-rhamnose that elicits a relatively weak antibody response
(King et al., 2009). "B-band" or "O-specific antigens" are highly immunogenic heteropolymers
composed repetitive units of different sugars and form the basis for the AITS P. aeruginosa-
serotyping scheme that includes 20 serotypes (Knirel et al., 2006). Structural data in several
Pseudomonas serotype strains (Sadovskaya et al., 2000, Bystrova et al., 2006) and genetic
experiments (Abeyrathne et al., 2005) demonstrate that both common and O-specific
antigens are linked to the lipid A-core.

In this article, we review the literature on LPS variations occurring upon bacterial
adaptation to chronic infection, with special emphasis on chronic respiratory infections in
patients with CF and gastric infections. CF is a genetic disease that leads to ineffective
mucociliary clearance of the airways, resulting in chronic airways infection by several Gram-
negative bacterial opportunistic pathogens, such as P. aeruginosa, the Burkholderia cepacia
complex (Bcc), and Achromobacter xylosoxidans (Ciofu et al., 2015, Cullen & McClean, 2015,
Chronic gastric infection by *H. pylori* leads to a pre-cancerous state associated with loss of acid-producing parietal cells, which results in increased gastric pH, and pepsinogen-producing zymogenic cells. The gastric environment changes during disease progression and as a result, infecting *H. pylori* strains must adapt to persist in a gastric habitat with increased pH and different cell composition (Skoglund *et al.*, 2009, Rubin & Trent, 2013, Malnick *et al.*, 2014). Because both respiratory infections in CF patients and gastric infections by *H. pylori* remain during the lifetime of the patient, they provide natural human models of disease progression and microbial adaptation to the host environment.

**LPS BIOSYNTHESIS**

**Lipid A-core biosynthesis**

The biosynthesis of LPS has been reviewed in detail elsewhere (Raetz *et al.*, 2007) (King *et al.*, 2009, Lam *et al.*, 2011, Greenfield & Whitfield, 2012, Whitfield & Trent, 2014, Valvano, 2015). Briefly, the lipid A is synthesized on the cytoplasmic side of the inner membrane by a conserved pathway of nine enzymes catalyzing the sequential conversion of the precursor UDP-N-acetyl-glucosamine into lipid A-Kdo₂, which is the acceptor for the rest of the core sugars that are added from nucleotide sugar precursors via sequential glycosyl transfer reactions (Figure 2) (Mamat *et al.*, 2011, Whitfield & Trent, 2014). The complete lipid A-core is transported to the periplasmic face of the inner membrane by the ABC transporter MsbA (Whitfield & Trent, 2014). Diverse covalent modifications of lipid A may occur during its transit from the periplasmic side of the inner membrane to the outer leaflet of the outer membrane (Raetz *et al.*, 2007), which are important for niche adaptation and can influence the virulence of the pathogen (Needham & Trent, 2013). In bacteria that produce O antigen, the O polysaccharide is assembled by a separate biosynthesis pathway (see next section) and attached to the core at the periplasmic side of the inner membrane (Figure 2).

**O antigen biosynthesis**

The O antigen is synthesized by cytoplasmic membrane-associated enzyme complexes and requires C₅₅-undecaprenyl phosphate (Und-P), which serves as an acceptor for O antigen.
chain assembly (Valvano, 2011). Chain assembly occurs by the action of diverse glycosyltransferases that synthesize the specific O antigen of each strain. Genes at the wb* (formerly rfb) locus encode most of the enzymes involved in O antigen assembly (Reeves et al., 1996). Because of the great diversity of O antigen structures, the wb* loci are highly polymorphic (Raetz & Whitfield, 2002, Lam et al., 2011). The O antigen is initially assembled on the cytoplasmic side of the membrane and then translocated to the periplasmic side and ligated to lipid A-core (Valvano, 2015) (Figure 2). There are three pathways for O antigen biosynthesis and export: (1) Wzy-dependent, (2) ABC-transporter-dependent, and (3) synthase-dependent (Keenleyside & Whitfield, 1996, Lam et al., 2011, Greenfield & Whitfield, 2012, Valvano, 2015). The mature LPS molecule is then transported across the periplasm and inserted into the outer leaflet of the outer membrane by the conserved Lpt (LPS transport) pathway (May et al., 2015, Simpson et al., 2015). Lpt proteins form a complex that traverses the Gram-negative cell envelope to deliver LPS to the outer membrane and include an ABC protein complex (LptBFG) that uses energy from ATP hydrolysis to extract LPS from the periplasmic face of the inner membrane, several proteins that dock and promote the transfer of LPS across the periplasm (LptCA and YhjD) and a complex of proteins on the outer membrane (LptDE, YtfN, YfgH and YceK), responsible for the correct insertion of LPS in the outer leaflet (Babu et al., 2011, Sperandeo et al., 2011, Sperandeo et al., 2011, May et al., 2015, Simpson et al., 2015). The Lpt system has not been investigated in Gram-negative pathogens other than E. coli and sequence homology between E. coli and P. aeruginosa genes is low, with the exception of LptB (66% sequence identity). Recently, it was shown that P. aeruginosa LptA has a dimeric structure, unlike the oligomeric structure of E. coli LptA (Shapiro et al., 2014).

In P. aeruginosa, the common polysaccharide and the O-specific antigens are synthesized via the ABC-transporter-dependent pathway and the Wzy-dependent pathway, respectively (King et al., 2009, Lam et al., 2011). In both the synthesis is initiated by the same glycosyltransferase, WbpL (homologous to the E. coli WecA), resulting the formation of an Und-P-P-sugar intermediate (King et al., 2009, Lam et al., 2011). Four enzymes are required for the biosynthesis of GDP-α-rhamnose, the nucleotide sugar precursor for the common polysaccharide antigen: WbpW, AlgC, Gmd and Rmd (King et al., 2009, Lam et al., 2011).
The glycosyltransferases WbpX, WbpY and WbpZ are involved in the synthesis of the common polysaccharide antigen (King et al., 2009, Lam et al., 2011), while genes pa54-55pa5459 have been suggested to encode proteins that play a role in controlling chain length (Hao et al., 2013). Once the common polysaccharide antigen is linked to the Und-P carrier, the complex is exported across the membrane by the ABC-transport system Wzm-Wzt (King et al., 2009, Lam et al., 2011). While the genes for the synthesis and assembly of the common polysaccharide are conserved, different set of genes are responsible for the biosynthesis of the O-specific antigen in each serotype strain. These genes are in a cluster flanked by the highly conserved genes himD/ihfB and wbpM (King et al., 2009, Lam et al., 2011). While the P. aeruginosa O5, O6 and O11 O antigen clusters were studied to some extent very little experimental work was conducted into the functions of genes in the remaining O antigen loci (Lam et al., 2011). The synthesized the Und-PP-linked O-repeat units are translocated to the periplasmic side of the membrane and polymerized. The proteins Wzy, Wzz and Wzx are required for this process, acting as polymerase, chain-length regulator, and flippase, respectively (Lam et al., 2011). Once on the periplasmic side, both the common polysaccharide antigen and the O-specific antigen are independently linked to the lipid A-core complex by the WaaL ligase (Figure 2) (Abeyrathne et al., 2005, Valvano, 2011, Ruan et al., 2012).

LPS VARIATION DURING CHRONIC RESPIRATORY INFECTIONS IN CF PATIENTS

Pseudomonas aeruginosa infection

P. aeruginosa is the most common pathogen isolated from the respiratory tract of adult patients with CF (Lipuma, 2010, Hauser et al., 2011). Chronic airway infections caused by P. aeruginosa are found in up to 80% of adult patients with CF (Aaron et al., 2010, Lipuma, 2010) and are associated with increased morbidity and mortality (Hauser et al., 2011). Phenotypic changes suggesting P. aeruginosa adaptation to the CF lung have been reported in several studies (Hogardt & Heesemann, 2010). They include loss of motility associated with growth in microcolony (Sriramulu et al., 2005), reduced expression of virulence factors, which is presumably an adaptive strategy to escape detection by the host immune system (Smith et
increased activity of efflux pumps associated with antibiotic resistance, especially against those antibiotics used clinically (Poole, 2005), and a switch from non mucoid to mucoid phenotypes (Ciufu et al., 2010, Hogardt & Heesemann, 2010). The phenotypic changes reflect point mutations accumulating in \textit{P. aeruginosa} lineages that persist in CF airways (Lorè et al., 2012), and include mutations in alginate biosynthesis regulator genes (Bragonzi et al., 2006) and genes involved in the LPS modification (Cigana et al., 2009), motility (Mahenthiralingam et al., 1994), quorum-sensing regulation (D’Argenio et al., 2007, Hoffman et al., 2009), type 3 secretion system biosynthesis (Jain et al., 2004), multidrug-efflux pumps, and mutator genes (Oliver et al., 2000).

The longitudinal course of chronic airway infection with \textit{P. aeruginosa} in CF has been followed in various studies (Smith et al., 2006, Cigana et al., 2009, Cramer et al., 2011, Mowat et al., 2011, Warren et al., 2011, Yang et al., 2011, Lorè et al., 2012, Dettman et al., 2013). A study investigating over 1700 serial isolates obtained from 10 patients infected with the same strain showed that within-patient diversity made the largest contribution to the overall variation in the population and also that population compositions fluctuated over time (Mowat et al., 2011). The authors suggested that extensive diversity within the \textit{P. aeruginosa} population during chronic infection has the potential to provide a reservoir for antibiotic resistant mutations and mutations in other virulence traits (Mowat et al., 2011). Despite these differences, certain traits were overrepresented in all isolates, most of which include properties regulated by quorum sensing (Mowat et al., 2011). \textit{In silico} simulations reveal virulence factor expression decline towards the end of chronic infections and adaptive mutations that tend to improve metabolic fitness, which would optimize growth over the more energetically expensive virulence factor production (Oberhardt et al., 2010). \textit{P. aeruginosa} LPS modifications appear to be an important factor in the adaptation of this pathogen to chronic infection (Cigana et al., 2009). Indeed, chronic \textit{P. aeruginosa} CF isolates have rough colony phenotypes and contain few, short, or no O side chains, becoming non-typeable (Hancock et al., 1983). O antigen deficient isolates are sensitive to \textit{in vitro} killing by serum complement and become more tolerant to the antibiotic gentamicin (Kadurugamuwa et al., 1993). Analysis of sequential variants of \textit{P. aeruginosa} show O antigen loss (Lee et al., 2005) and lipid A modifications (Cigana et al., 2009). Whole-genome analysis of two clinical \textit{P.}
aeruginosa variants recovered from a chronic CF patient after 6 and 96 months of infection also revealed non-synonymous mutations in the O antigen biosynthetic genes wbpA and pa5238 in the latter variant (Smith et al., 2006). Another study of genes responsible for modifying lipid A revealed one mutation in pagL in late variants, which abolish PagL expression and leads to reduced TLR4-MD2-signalling (Cigana et al., 2009). Thus, initial lipid A modifications by addition of palmitate to the lipid A of P. aeruginosa make the LPS more proinflammatory, but the subsequent modification through the loss of PagL activity decreases its proinflammatory activity. Together, the results of these studies suggest that reduced LPS immunostimulatory potential contributes to immune system evasion and survival over the course of the chronic P. aeruginosa infection. Experimental data support this hypothesis since a comparison of the pathogenicity of nine P. aeruginosa sequential clonal variants in the infection models Caenorhabditis elegans, Galleria mellonella, Drosophila melanogaster and two different mice backgrounds (C57Bl/6NCrl and BALB/cAnNCrl), show that early P. aeruginosa variants were lethal in all infection models tested, while late strains exhibited reduced or no virulence (Lorè et al., 2012).

A microevolution analysis based on whole-genome sequencing of sequential P. aeruginosa variants recovered from CF patients for more than 20 years (Cramer et al., 2011) identified codon changes in genes for lipid A biosynthesis (lpxC, lpxO2 and yciK), core biosynthesis (rfaD and wapP), and common polysaccharide antigen biosynthesis (wbpZ) (Cramer et al., 2011). Another genomic analysis taken over 200,000 bacterial generations of 12 selected P. aeruginosa DK2 variants recovered from six CF patients identified a total of 234 non-synonymous single nucleotide polymorphisms among the genomes in relation to their common ancestor strain, suggesting that an initial period of rapid adaptation is followed by a period of genetic drift in this lineage (Yang et al., 2011). Three of the non-synonymous single nucleotide polymorphisms occurred in genes needed for lipid A biosynthesis and modification (pagL and lpxO2) and O-specific antigen synthesis (wzz) (Yang et al., 2011). A recent study analyzing whole-genome sequence data from P. aeruginosa clinical isolates sampled from the sputum of 32 different patients reported that the O antigen ligase waaL is one of the few hotspots of gene polymorphisms (Dettman et al., 2013). To gain insight into the role of mutator genes for generating adaptive variation, Warren et al. (2011) analysed the...
genomes of two series of isolates recovered from two patients, similar in duration but different
in mutator incidence, and identified 15 LPS genes that lacked in multiple members both in
mutator and non-mutator series. All the identified genes are involved in the synthesis of
serogroup O2/O5/O26/O18/O20 O antigen (wbpA, wbpB, wbpC, wbpD, wbpE, wbpG, wbpH,

In addition to changes in O antigen, adaption of *P. aeruginosa* to chronic lung
infection in CF patients involves the synthesis of various lipid A structures (Figure 3) (Ernst et
al., 2007), which result in alteration of host innate immune responses and promote bacterial
persistence (Moskowitz & Ernst, 2010). These modifications involve deacylation of the lipid A
resulting in the loss of an acyl chain from the 3-position, which is catalyzed by PagL (Figure 3)
(Trent et al., 2001, Geurtsen et al., 2005, Ernst et al., 2006). Under acylation of lipid A has
been associated with low inflammatory activity (Moskowitz & Ernst, 2010, Di Lorenzo et al.,
2015) and modulation of TLR4-MD2 receptor recognition (Ernst et al., 2003). Also, *P.
aeruginosa* lipid A can acquire a secondary acyl chain into the 3'-position, which is catalyzed
by a divergent palmitoyltransferase functionally analogous to the *Salmonella* and *E. coli* PagP
enzyme (Figure 3) (Thaipisuttikul et al., 2014). Further modifications involve the addition of
secondary acyl chains to the chains present at the 2- and 2'-positions, which is catalyzed by
HtrB and LpxO, respectively (Figure 3), as well as the incorporation of 4-amino-4-deoxy-L-
arabinopyranose (Arap4N) to phosphate groups at the 1- and 4'-positions by the two-
component regulatory system PmrAB (Figure 3) (Moskowitz et al., 2004). These lipid A
modifications contribute to *P. aeruginosa* adaptation to the CF airway (Moskowitz & Ernst,
2010). The addition of phosphoethanolamine to the *P. aeruginosa* lipid A via the ColRS two-
component system (Figure 3) in a Zn$^{2+}$-dependent manner was recently reported (Nowicki et
al., 2015), but the role of this modification in vivo is not clear.

Collectively, the studies described above support the notion that chronically infecting
bacteria adapt to host immune responses by producing LPS lacking O antigen and by
introducing lipid A modifications in isolates recovered in late stages of CF chronic infection
(Table 1) (Lyczak et al., 2002, Lee et al., 2005, Smith et al., 2006, Cigana et al., 2009,
Moskowitz & Ernst, 2010, Cramer et al., 2011, Yang et al., 2011, Dettman et al., 2013). This
conclusion is also supported from comparative studies using various host models
demonstrating that adaptation of different *P. aeruginosa* lineages within CF lungs selects populations with reduced pathogenic potential in acute infections (Lorè *et al.*, 2012).

**Chronic Infections by other Gram-negative CF pathogens**

Bacteria from the *Burkholderia cepacia* complex (Bcc) emerged as significant CF pathogens in the early 1980s, when a minority of infected patients exhibited rapid clinical deterioration, resulting in early death (Mahenthiralingam *et al.*, 2005, Loutet & Valvano, 2010). Respiratory infections with Bcc bacteria in CF patients generally lead to faster decline in lung function and, in some cases to cepacia syndrome, a fatal necrotizing pneumonia frequently accompanied by septicemia (Mahenthiralingam *et al.*, 2005, Coutinho *et al.*, 2011). Further, Bcc bacteria are transmissible through social contacts and are intrinsically resistant to most clinically used antibiotics, which renders their eradication from the CF lung very difficult, if not virtually impossible (Mahenthiralingam *et al.*, 2005, Drevinek & Mahenthiralingam, 2010, Coutinho *et al.*, 2011). Although transient infection of the respiratory tract may occur in some patients, acquisition of Bcc most typically results in chronic infection (Mahenthiralingam *et al.*, 2005, Coutinho *et al.*, 2011). The same level of adaptation is not so clear cut in *B. cenocepacia* infections, as studies using the various infection models (*C. elegans*, *G. mellonella*, alfalfa, mice and rats) reported that most virulence factors are specific for one infection model only and rarely essential for pathogenicity in multiple hosts (Uehlinger *et al.*, 2009, Lorè *et al.*, 2012). Furthermore, less is known about *Burkholderia* adaptation during CF chronic infection; however, there has been an effort to characterize the evolution of *Burkholderia* populations in the lung, including phenotyping (Coutinho *et al.*, 2011, Moreira *et al.*, 2014) and genotyping of serial isolates (Lieberman *et al.*, 2011, Traverse *et al.*, 2013), and comparative expression profiling of the transcriptome (Mira *et al.*, 2011) and the proteome (Madeira *et al.*, 2011, Madeira *et al.*, 2013).

Lieberman *et al.* (2011) sequenced the genomes of 112 clinical *Burkholderia dolosa* isolates that resulted from the evolution of a single strain in 14 CF patients over 16 years of epidemic spread and discovered that genes involved in oxygen regulation, antibiotic resistance, outer-membrane synthesis and secretion have recurrent mutation patterns (Lieberman *et al.*, 2011). Interestingly, recurrent mutations in the same amino acid of the
glycosyltransferase WbaD were observed in nine patients, which resulted in production of O-unit repeats that were absent in the ancestral phenotype (Lieberman et al., 2011). The ancestral \emph{B. dolosa} genotype encodes a stop codon at this locus that prevents O antigen synthesis. In some variants, two different mutations affecting the same amino acid were detected, both of them restoring the full-length WbaD protein and leading to O antigen production (Table 2) (Lieberman et al., 2011). Although this gain-of-function mutation does not follow the loss of O antigen tendency described in \emph{P. aeruginosa}, these results underpin the importance of the O antigen switch mechanism during chronic infection. Another metagenomic analysis of six lineages evolved in biofilm mode of growth revealed an extraordinary mutational parallelism, including genes known to affect LPS biosynthesis, transcription, galactose metabolism, tricarboxylic acid cycle enzymes and altered metabolism of cyclic diguanosine monophosphate (Traverse et al., 2013). One commonly mutated locus, showing twenty independent mutations in both \emph{B. dolosa} and \emph{Burkholderia cenocepacia}, was \emph{manC}, encoding a nucleotide mannose biosynthesis protein presumably involved in surface polysaccharide biosynthesis that could be either an exopolysaccharide or LPS (Traverse et al., 2013). Interestingly, these authors showed that complementation of one of the \emph{manC} mutations dramatically reduced biofilm formation, and they speculated that the loss of polysaccharide may be required for efficient biofilm formation rather than immune evasion (Traverse et al., 2013).

A comparison of the transcriptome and the proteome of three \emph{B. cenocepacia} isolates recovered at the beginning of the infection and later during the progress of the disease suggests that the expression from genes involved in LPS biosynthesis is altered during chronic infection (Madeira et al., 2011, Mira et al., 2011, Madeira et al., 2013), in particular of those required for O antigen biosynthesis. Indeed, recent analysis of the LPS structure of these isolates revealed that, although the early-stage isolate has a complete LPS with the O-chain moiety, the late-stage variants have a rough-type LPS, lacking O antigen (Maldonado et al., unpublished data).

Several studies at genome, transcriptome and proteome levels have contributed to a better understanding of Bcc bacteria genome-wide adaptive mechanisms during chronic infections. Together, they suggest that there is a high selective pressure on the O antigen
locus leading to alterations both at the structural, sequence and regulatory levels. Given the exceptional parallelism found among the relatively few studies dedicated to Bcc bacteria and P. aeruginosa, the LPS seems to play an important role during chronic infection, both in immune system evasion and biofilm adaptation. Moreover, lack of O antigen in B. cenocepacia leads to increased internalization into macrophages upon phagocytosis (Saldías et al., 2009), which may explain the higher invasiveness of epidemic strains, such as J2315, which do not produce O antigen. O antigen loss could therefore facilitate access of Bcc bacteria to macrophages, where intracellular bacteria could find a niche to persist, in agreement with a recent study showing that in human lungs, Bcc bacteria but not P. aeruginosa are found mainly inside macrophages (Schwab et al., 2014). Other Gram-negative opportunistic pathogens that cause CF chronic infections include Stenotrophomonas maltophilia, Achromobacter xylosoxidans and Haemophilus influenza. Recently, some studies characterizing the adaptive traits of sequential isolates of S. maltophilia (Vidigal et al., 2014), A. xylosoxidans (Trancassini et al., 2014) and H. influenza (Watson et al., 2004) recovered from CF patients have been published, however the LPS characterization of these clinical isolates is still lacking.

**LPS VARIATION DURING CHRONIC GASTRIC INFECTION**

The human gastric pathogen Helicobacter pylori is usually acquired during childhood by colonizing the human gastric mucosa and producing a superficial gastritis, which may remain asymptomatic during the lifetime of colonized individuals or eventually lead to gastric ulcer and atrophic gastritis (Linz et al., 2013, Otero et al., 2014). This geographically wide-spread bacteria infects more than half of the human population and is one of the most genetically diverse bacterial species, being also one of the most ubiquitous infectious organisms (Linz et al., 2013). The genetic diversity of H. pylori is caused by a high mutation rate, presumably due to the lack of several mutation repair genes (Kang & Blaser, 2006). Chronic infection with H. pylori is recognized as the most common cause of gastric and duodenal ulcers (Brown, 2000). H. pylori chronic infection is also associated with the development of gastric adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue (Otero et al., 2014,
Méraud et al., 2015), for which this bacterium is considered to be a class 1 carcinogen (WHO, 1994).

*H. pylori* produces several virulence factors of which the vacuolating toxin A (VacA), the cytotoxin-associated gene A (CagA), and LPS play major roles in immunomodulation and contribute to maintain chronic infection (Posselt et al., 2013, Rubin & Trent, 2013, Chmiela et al., 2014, de Bernard & Josenhans, 2014, Hatakeyama, 2014). These factors contribute to maintain the infection by preventing the clearance of *H. pylori* from the gastric mucosa and interfering with innate and adaptive immune responses. Structural modifications of the lipid A result in reduced endotoxicity, while expression and variation of Lewis determinants exposed on the bacterial cell surface as a terminal O-specific oligosaccharide (Aspinall et al., 1996, Monteiro et al., 1998) mimic host components expressed on the human gastric epithelium (Moran et al., 1996, Moran, 2008) and reduce detection by the immune system. *H. pylori* lipid A presents a unique structure and shows remarkably lower biological activity compared with lipid A from other bacteria (Muotiala et al., 1992, Moran & Aspinall, 1998). Structural analysis revealed that the lipid A acyl chains are longer (16 to 18 carbons) than those present in enterobacterial lipid A (Moran et al., 1997). The predominant form is tetra-acylated lipid A, which is also underphosphorylated (Moran et al., 1997, Cullen et al., 2011).

Underphosphorylation and underacylation of *H. pylori* lipid A are responsible for reduced endotoxicity (Ljungh et al., 1996), as determined by its low reactivity against anti-lipid A antibodies (Mattsby-Baltzer et al., 1992), reduced ability to induce the production of cytokines, nitric oxide and prostaglandin E2 (Pérez-Pérez et al., 1995), and E-selectin expression (Darveau et al., 1995), as well as reduced activation of leukocytes (Baker et al., 1994, Semeraro et al., 1996). Lipid A remodelling in *H. pylori* occurs mainly on the periplasmic side of the inner membrane. A first set of modifications involves removal of the 1-phosphate group by LpxE and the addition of a phosphorylethanolamine in its place by EptA (Tran et al., 2004, Tran et al., 2006). These modifications increase bacterial resistance to antimicrobial peptides (Tran et al., 2006). Second, a two-protein Kdo-hydrolase complex removes the terminal Kdo sugar, a modification that is critical to allow the ligation of the O-specific oligosaccharides to the lipid A core (Stead et al., 2010). Third, LpxF catalyses the removal of the 4′-phosphate group (Cullen et al., 2011). After ligation of the O-specific oligosaccharide (see below) the
complete LPS molecule is transported and displayed on the surface of the bacterial outer
membrane. Once in the outer membrane the lipid A undergoes a final modification that
consist on the removal of the 3'-linked acyl chains by LpxR, producing the characteristic tetra-
acylated lipid A structure (Stead et al., 2008).

The *H. pylori* O-specific oligosaccharide is initially formed as a lipid-linked
oligosaccharide resulting from the addition of monosaccharides, but does not form a
repeating oligosaccharide unit (Berg et al., 1997, Rubin & Trent, 2013). The O-specific
oligosaccharide has a common backbone that is further modified by fucosyltransferases
generating structures that mimic human Lewis antigen molecules and other related blood-
group antigens such as LeX; LeY, Leα, Leβ, sialyl-LeX, H-1 antigen, and blood groups A and B
antigens (Rubin & Trent, 2013) (Figure 4) This lipid-linked fucosylated oligosaccharide is
translocated across the inner membrane by Wzk, an ABC-transporter protein homologous to
PglK from *Campylobacter jejuni*, and subsequently ligated to the lipid A-core by the WaaL
ligase (Hug et al., 2010).

The presence of terminal fucosylated sugars on the outer surface of the bacterium, in
particular the most common LeX and LeY structures, is critical for colonization in mice models
(Logan et al., 2000, Moran et al., 2000). However, the diversity of Lewis antigen expression in
*H. pylori* hampers efforts to clearly define the role of these molecules in infection and disease
progression. In humans, LeX *H. pylori* O-specific oligosaccharide is recognized by galectin-3,
a β-galactoside-binding lectin that serves as a gastric receptor (Fowler et al., 2006). However,
the main role attributed to the Lewis antigens is that of molecular mimicry, which could be
manifested in several ways. For example, *H. pylori* can change its Lewis antigens in response
to those present in the host, as demonstrated with Leβ-transgenic mice infected with LeX-
expressing *H. pylori*, which over time switched on Leβ expression (Pohl et al., 2009). This
change allowed better bacterial colonization than in the transgenic mice lacking Leβ
expression, suggesting that Leβ *H. pylori* could survive better in a self-tolerant Leβ host (Pohl
et al., 2009). Alternatively, *H. pylori* expressing different Lewis antigens than those in the host
can induce production of autoantibodies that recognize gastric parietal cells leading to
disease (Negrini et al., 1996, Faller et al., 1997). Further, Lewis antigens can also dampen
host immune responses to *H. pylori* through interactions with the C-type lectin DC-SIGN on
the surface of gastric dendritic cells, which lead to a block in maturation of T-helper 1 cells and reduced production of pro-inflammatory cytokines (Bergman et al., 2004).

The first evidence that a single strain of *H. pylori* alters its LPS antigenic phenotype during the course of infection was demonstrated by investigating the expression of Lewis antigens in 127 isolates recovered from serial biopsies of 26 asymptomatic subjects (Rasko et al., 2000). This alteration of LPS biosynthesis in *H. pylori* occurs during host colonization in response to several stimuli (Salaün et al., 2005, Nilsson et al., 2008) such as interaction with T helper cells Bergman, 2004 #5473 and gastric pH (Skoglund et al., 2009). More recently, several studies have focused on the genomic changes occurring in *H. pylori* isolates that have been recovered several years apart from patients with chronic infection (Falush et al., 2001, Israel et al., 2001, Kraft et al., 2006, Alvi et al., 2007, Morelli et al., 2010, Kennemann et al., 2011).

A whole-genome analysis of 10 *H. pylori* sequential isolates recovered from 4 patients over 16 years of chronic gastritis revealed 5 SNPs affecting LPS genes, including genes involved in the biosynthesis of lipid A (phosphoethanolamine transferase), core (*kdsA* and *waaF*) and O-specific oligosaccharide (*wecA*) synthesis, as well as in a putative lipopolysaccharide biosynthetic protein (Kennemann et al., 2011). A cluster of nucleotide polymorphisms in the *fucT* (fucosyltransferase) gene, presumably facilitating its expression, was identified in whole-genome analyses of two *H. pylori* strains isolated from spouses (Linz et al., 2013). Hyperexpression of *fucT* promotes posttranslational fucosylation of the O-specific oligosaccharide, generating Lewis antigens (Ge et al., 1997, Martin et al., 1997, Moran, 2008, Linz et al., 2013). The alteration of *H. pylori* LPS during chronic gastric infection, either by altering LPS biosynthesis or by adding fucosyl residues to O-specific oligosaccharides, generates Lewis structures that mimic host antigens and contribute to immune system evasion.

MOLECULAR MECHANISMS OF LPS VARIATION

Antigenic variation of surface structures is a powerful mechanism for pathogen evasion of adaptive immune responses (Lerouge & Vanderleyden, 2002, van der Woude & Bäumler, 2004, Lukácová et al., 2008). One of these adaptions involves phase variation, which is a
reversible, yet heritable form, of gene regulation that results in heterogeneous clonal
populations and can be mediated by various molecular mechanisms (van der Woude &
Bäumler, 2004). LPS phase variation can occur by addition of carbohydrates through the
activity of glycosyltransferases or sialyltransferases, or addition of phosphorylcholine (ChoP)
resulting in changes that affect antigenicity, serum sensitivity and adhesion (van der Woude &
Bäumler, 2004). Phase variation has been described for human pathogens such as S.
enterica serovar Typhimurium, Campylobacter jejuni, Neisseria spp. and H. pylori but
because variable LPS modification is not easily identified, it is possible that phase variation is
more widespread than currently known. Genetic and epigenetic mechanisms behind LPS
variation are discussed below.

Adaptive mutagenesis and altered gene expression
Acquisition of adaptive mutations is a common theme in microbial persistence. In CF patients
with chronic lung infection, P. aeruginosa strains accumulate a large proportion of mutator
strains (Oliver et al., 2000) that contribute to selection of mucoid variants (Oliver et al., 2000,
Mathee et al., 2008, Ciofu et al., 2010, Hogardt & Heesemann, 2010). The proinflammatory
microenvironment in the airways including polymorphonuclear cells, hydrogen peroxide
production, and antibiotics (Blázquez et al., 2006) has been associated with mutagenesis and
mucoid conversion in vitro (Mathee et al., 1999, Sanders et al., 2006, Moyano et al., 2007).
Cationic antimicrobial peptides can also exert a mutagenic inducing effect, as recently
demonstrated for human cathelicidin LL-37 (Limoli et al., 2014). Mutagenesis depended on
LL-37 entering the bacterial cytosol and binding to DNA, which in turns promotes abnormal
DNA synthesis by the error-prone polymerase DinB (Sanders et al., 2006, Limoli et al., 2014).
Environmental cues, such as ionic concentration, can lead to O antigen structural
variations resulting from altered gene expression regulated by two-component signal
transduction systems. One of the best examples of this type of regulation is the PhoP/PhoQ
system in Salmonella (Prost & Miller, 2008, Needham & Trent, 2013). PhoQ is a membrane
sensor histidine kinase and PhoP is its cognate response regulator. Activation of the
PhoP/PhoQ system by acidic pH, specific antimicrobial peptides, and depletion of Mg$^{2+}$ and
Ca$^{2+}$ stimulates transcription of pagP and pagL (among other genes) and subsequent
upregulation of the encoded proteins, which acylate and deacylate lipid A, respectively (Prost & Miller, 2008, Needham & Trent, 2013). Further, CF clinical isolates of \textit{P. aeruginosa} obtained from patients treated with inhaled colistin (polymyxin E) can develop resistance by loss-of-function mutations in the \textit{phoQ} gene (Miller \textit{et al.}, 2011). Disruption of \textit{phoQ} in the presence of an intact \textit{phoP} stimulated \textit{Arap4N} addition to lipid A by upregulated expression of the \textit{Arap4N} synthesis operon. Therefore, this adaptive mutagenesis strategy results in high-level polymyxin resistance clinical strains of \textit{P. aeruginosa}.

\textbf{Slipped-strand mispairing}

One of the molecular mechanisms of phase variation involves slipping of one of the DNA strands, which causes mispairing between daughter and parent strands during DNA replication (slipped-strand mispairing) (Lukáčová \textit{et al.}, 2008). Short DNA repeats, microsatellites and tandem repeats are particularly prone to slipped-strand mispairing (van Belkum \textit{et al.}, 1997, Torres-Cruz & van der Woude, 2003). In \textit{H. pylori}, phase variation is related to an increase in the number of poly-C tract repeats in the $\beta$-(1,3)-galactosyltransferase (GalT), which leads to a switching on Le$^b$ expression (Pohl \textit{et al.}, 2009). Also, repetitive poly-A and poly-C sequences in the fucosyltransferase \textit{fucT} mediate slipped-strand mispairing, which in turn results in production of Lewis antigens with different fucosylated oligosaccharides (Wang \textit{et al.}, 2000, Nilsson \textit{et al.}, 2008). Further, the $\alpha$-(1,2)-fucosyltransferase gene \textit{futC} contains an heptameric sequence (AAAAAAG) next to the ribosome binding site, which may cause a phase shift in the reading frame during translation (Wang \textit{et al.}, 2000).

\textbf{Lateral gene transfer, recombination, and genetic rearrangements}

The heterogeneity of O antigens is mostly due to variation within the O antigen gene cluster, but it is unclear how such variation was generated (Reeves \textit{et al.}, 2013). Genes involved in O antigen biosynthesis are generally arranged in large operons with low G+C content relative to the average G+C characteristic of each species, which suggests that these clusters were acquired by horizontal gene transfer from a species with low G+C content (Lerouge & Vanderleyden, 2002). The G+C content within the O antigen clusters also greatly differs from
gene to gene, indicating that the gene clusters might have been assembled from multiple horizontal transmission events and from several sources over a much longer time (Lerouge & Vanderleyden, 2002). The role of lateral gene transfer in the evolution of O antigen clusters and O antigen diversification has been well described in Salmonella (Perepelov et al., 2011, Reeves et al., 2013), Escherichia (D’Souza et al., 2005, Hu et al., 2010, Azmuda et al., 2012), Vibrio (González-Fraga et al., 2008, Wildschutte et al., 2010), Yersinia (Cunneen & Reeves, 2007) and Brucella (Wattam et al., 2014). Another mechanism of variation involves large chromosomal rearrangements. For example, more than half of the P. aeruginosa clone C isolates from CF lung infection exhibit large chromosomal inversions mediated an IS6100-induced coupled insertion-inversion mechanism. This creates also a selective advantage by insertion of IS6100 into wbpM, pilB and mutS, which leads to common CF phenotypes such as O-antigen and type IV pili deficiency and hyper mutability (Kresse et al., 2003).

CONCLUDING REMARKS
The LPS is an abundant molecule of the outer membrane of most Gram-negative bacteria and plays a key role during host-pathogen interaction and the establishment of chronic infection. LPS-mediated virulence resides both in the endotoxic activity of lipid A and in the ability of the core and O antigen to provide the bacterium with resistance to host defence mechanisms. O antigen modification in general contributes to enhance the bacteria’s ability to establish infection. For example, P. aeruginosa O antigen modification directed by the D3 prophage promotes adhesion to epithelial cells (Vaca-Pacheco et al., 1999), while in H. pylori, expression of the Lewis antigen LeX promotes bacterial adhesion to the gastric epithelia by interacting with host lectins. Further, O antigen modification can contribute to host immune evasion either by mimicry of host molecules (e.g., Lewis antigens in H. pylori) or by inhibiting activation of the host complement system (Raetz & Whitfield, 2002). It is also well established that during chronic infection there is an increase of mutator phenotypes (Oliver et al., 2000), which leads to a higher mutation rate and will consequently contribute to the accumulation of modifications in LPS structure during colonization.

Several studies have shown alterations in the LPS molecule during chronic infection, which are thought to contribute to adhesion, host colonization, immune defenses evasion and
adaptation to the infection niche. Different mechanisms, both at the genetic and epigenetic
levels have been implied in LPS variation, creating LPS diversity and thus contributing to the
success of the infection.

Future progress in LPS research will require interdisciplinary experimental
approaches, combining the application of genome-wide approaches (such as genomics,
transcriptomics, proteomics and metabolomics), structural biology, animal knockout models,
enzymology, carbohydrate chemistry and membrane biochemistry. LPS phase variation has
been described for some human pathogens (*S. enterica* serovar Typhimurium, *C. jejuni,*
*Neisseria* spp. and *H. pylori*) and future research should address the investigation of these
mechanisms in other species as well. An in-depth understanding of LPS variation and its
effects on pathogenicity and virulence is of paramount importance in the understanding of
infection establishment and progression.

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<table>
<thead>
<tr>
<th>LPS metabolism</th>
<th>Genes</th>
<th>Reference</th>
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<tr>
<td>Lipid A biosynthesis and modification</td>
<td>lpxO2</td>
<td>Cramer et al., 2011; Yang et al., 2011</td>
</tr>
<tr>
<td></td>
<td>lpxC, yciK</td>
<td>Cramer et al., 2011</td>
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<td></td>
<td>pagL</td>
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<td>Core biosynthesis and modification</td>
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<td>Cramer et al., 2011</td>
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<td>Common polysaccharide antigen biosynthesis</td>
<td>wbpZ</td>
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<td>O-specific antigen biosynthesis</td>
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<td>Smith et al., 2006</td>
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<td></td>
<td>wzz</td>
<td>Yang et al., 2011</td>
</tr>
<tr>
<td>O antigen ligase</td>
<td>waaL</td>
<td>Dettman et al., 2013</td>
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</table>
Table 2. LPS genes altered in *B. dolosa* and *B. cenocepacia* during chronic infections. The homologous gene in *P. aeruginosa* is also indicated.

<table>
<thead>
<tr>
<th>Gene or locus</th>
<th>Homologous gene in <em>P. aeruginosa</em> PAO1</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>wbaD</em></td>
<td>-</td>
<td>Lieberman <em>et al.</em>, 2011</td>
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<td>YP_834517</td>
<td><em>rmlB</em></td>
<td>Traverse <em>et al.</em>, 2013</td>
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<td><em>rmlA</em></td>
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<td>YP_834524</td>
<td><em>migA</em></td>
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<td>YP_834525</td>
<td><em>wbpW</em></td>
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<td><em>gmd</em></td>
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<td>YP_834528</td>
<td>-</td>
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<tr>
<td>YP_834533</td>
<td><em>wbpM</em></td>
<td>Traverse <em>et al.</em>, 2013</td>
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</table>
Figure 1. Cell envelope organization of Gram-negative bacteria. The cell envelope of Gram-negative bacteria is characterized by the presence of two lipid bilayers: the outer membrane (OM) and the cytoplasmic membrane (CM), which are separated by the periplasm, containing hydrolytic enzymes, binding proteins, chemoreceptors and the peptidoglycan cell wall. The OM is an asymmetric lipid bilayer. The outer leaflet of the OM contains mainly LPS molecules, which form contacts with integral outer membrane proteins (OMPs). The inner layer of the OM and the lipid layers of the cytoplasmic membrane contain phospholipids and membrane proteins.

Figure 2. Simplified overview of the LPS biosynthesis. Lipid A-Kdo₂ is synthesized on the cytoplasmic surface of the cytoplasmic membrane. The rest of the core is assembled to the lipid A-Kdo₂ and MsbA flips the whole complex to the periplasmic side of the cytoplasmic membrane. The O antigen is synthesized by cytoplasmic membrane-associated enzyme complexes using C55-undecaprenol phosphate (Und-P) as an acceptor for chain assembly and is then flipped to the periplasmic face of the membrane by one of the three pathways: (1) Wzy-dependent, (2) ABC-transporter-dependent, or (3) synthase-dependent. For simplicity, only the ABC-transporter pathway is represented. Once on the periplasmic side, the O antigen is linked to the lipid A-core by the WaaL ligase and the mature LPS molecule is then transported across the periplasm and inserted into the outer leaflet of the outer membrane by the Lpt (LPS transport) system, a complex that spans the Gram-negative cell envelope to deliver LPS to the outer membrane (E). OM, Outer membrane; CM, Cytoplasmic membrane.

Figure 3. Lipid A modifications occurring in *P. aeruginosa* during adaptation to long-term chronic infection. The basic tetra-acylated lipid A structure can be modified by: deacylation by PagL; palmitoylation by PagP; acylation by HtrB; acylation by LpxO; addition of *Ara*p₄N by PmrAB on position 1 or 4'; and addition of phosphoethanolamine by ColRS on position 1 or 4'.
Figure 4. Lewis antigen structures. *H. pylori* can produce type 1 (based on a β-(1,3)-linked galactose-GlcNAc sugar backbone) and type 2 (based on a β-(1,4)-linked galactose-GlcNAc sugar backbone) Lewis antigens. Le\(^a\) and Le\(^x\) are built by addition of a fucose residue to the GlcNAc sugar of the type 1 and type 2 backbone, through α-(1,4) or α-(1,3) linkages, respectively. Le\(^b\) and Le\(^y\) are built by addition of a fucose residue through α-(1,2) linkage to Le\(^a\) and Le\(^x\) structures, respectively. Sialyl-Le\(^x\) (SLe\(^x\)) is built by addition of a sialyl group to the Le\(^x\) antigen by a α-(2,3) linkage.