REVIEW ARTICLE

Lipopolysaccharide modification in Gram-negative bacteria during chronic infection

Rita F. Maldonado¹, Isabel Sá-Correia¹ and Miguel A. Valvano²,³,*

¹Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Lisbon 1049-001, Portugal, ²Department of Microbiology and Immunology, University of Western Ontario, London, ON N6A 5C1, Canada and ³Centre for Infection and Immunity, Queen’s University Belfast, Belfast BT9 7BL, UK

*Corresponding author: Centre for Infection and Immunity, Queen’s University Belfast, 97 Lisburn Rd, Belfast BT9 7BL, UK. Tel: +44-28-9097-6025; E-mail: m.valvano@qub.ac.uk

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

Editor: Chris Whitfield

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.

Keywords: adaptive mutation; O antigen; lipid A; Pseudomonas aeruginosa; Burkholderia cepacia; cystic fibrosis; Helicobacter pylori; gastric ulcer

INTRODUCTION

The lipopolysaccharide (LPS) is a central component of the outer membrane in Gram-negative bacteria and frequently plays a key role in pathogenesis (Fig. 1) (Whitfield and 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 (Fig. 1) (Whitfield and 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’.

Received: 23 August 2015; Accepted: 10 March 2016
© FEMS 2016. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com
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 and Lee 2013). Modification of the lipid A acylation patterns, or addition of positively charged substituents to the lipid A phosphate can reduce 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 and Trent 2013; Di Lorenzo et al. 2015a).

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, 2007) produce a modified Kdo, which is converted into 3-glycero-3-talono-oct-2-ulosonic acid (Kt) by a unique Kdo-3 hydroxylase (Chung and 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, Messner and Kosma 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, Skurnik and Bengoechea 2011). Phosphorylation of the core sugars in Pseudomonas aeruginosa has been associated with increased membrane permeability and resistance to antibiotics (Walsh et al. 2000), and is also required for the transport of LPS to the outer membrane (Ducia 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 and Trent 2014). The O-repeating unit is highly variable immunologically giving rise to a vast number of different O-specific serotypes (Valvano, Patel and Furlong 2011; Whitfield and Trent 2014). The O antigen contributes to evasion of host immune defenses, particularly evasion of the complement cascade in Salmonella enterica serovar Typhimurium (Murray, Attridge and Morona 2006), delay of recognition and internalization in epithelial cells in Salmonella Typhimurium and Burkholderia cenocepacia (Duerr et al. 2009; Saldias, Ortega and Valvano 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. 2011a) 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 patients with CF (Hancock et al. 1983). Conceivably, once the bacteria become mucoid (Govan and 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 of 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 (Aberyraithne 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 and McClean 2015; Parks and Floto 2015). Chronic gastric infection by Helicobacter 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 and Trent 2013; Malnick et al. 2014). Because both respiratory infections in patients with CF 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 and Whitfield 2012; Whitfield and 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-acetylglucosamine 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 (Fig. 2) (Mamat, Skurnik and Bengoechea 2011; Whitfield and Trent 2014). The complete lipid A-core is transported to the periplasmic face of the inner membrane by the ABC transporter MsbA (Whitfield and 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 and Trent 2013). In bacteria that produce O antigen, the O polysaccharide is assembled by a separate biosynthesis pathway (see the next section) and attached to the core at the periplasmic side of the inner membrane (Fig. 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 

![Figure 2](https://academic.oup.com/femsre/article-abstract/40/4/480/2197984/4144682157384?download=true)
export: (1) Wzy dependent, (2) ABC transporter dependent and (3) synthase dependent (Keenleyside and Whitfield 1996; Lam et al. 2011; Greenfield and 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, YtN, YfG and YcEK), responsible for the correct insertion of LPS in the outer leaflet (Babu et al. 2011; Sperandeo, Déhö and Polissi 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 Escherichia 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, Gu and Gao 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 in 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-D-ribonose, 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/ibfB 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 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 polymerases, chain-length regulator and flipase, 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 (Fig. 2) (Abeyratne et al. 2005; Valvano 2011; Ruan et al. 2012).

**LPS VARIATION DURING CHRONIC RESPIRATORY INFECTIONS IN PATIENTS WITH CF**

**Pseudomonas aeruginosa infection**

Pseudomonas 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 and Heesemann 2010). They include loss of motility associated with growth in microcolony (Strimalu et al. 2005), reduced expression of virulence factors, which is presumably an adaptive strategy to escape detection by the host immune system (Smith, Buckley and Wu 2006), 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 (Ciofu et al. 2010; Hogardt and Heesemann 2010). The phenotypic changes reflect point mutations accumulating in 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, Campbell and Speert 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 P. aeruginosa in CF has been followed in various studies (Smith, Buckley and Wu 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 composition varied over time (Mowat et al. 2011). The authors suggested that extensive diversity within the 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). In silico simulations reveal that virulence factor expression declines 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). *Pseudomonas aeruginosa* LPS modifications appear to be an important factor in the adaptation of this pathogen to chronic infection (Cigana et al. 2009). Indeed, chronic 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 in vitro killing by serum complement and become more tolerant to the antibiotic gentamicin (Kadarugamwula, Lam and Beveridge 1993). Analysis of sequential variants of *P. aeruginosa* shows O antigen loss (Lee et al. 2005) and lipid A modifications (Cigana et al. 2009). Whole-genome analysis of two clinical *P. aeruginosa* variants recovered from a patient with chronic CF 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, Buckley and Wu 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-signaling (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 response.
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 patients with CF for more than 20 years (Cramer et al. 2011) identified codon changes in genes for lipid A biosynthesis (*ipaX*, *lpqO2* and *yqIK*), core biosynthesis (*rfaD* and *wagP*) 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 patients with CF identified a total of 234 non-synonymous single nucleotide polymorphisms (SNPs) 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 SNPs occurred in genes needed for lipid A biosynthesis and modification (*pagL* and *lpqO2*) 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) analyzed 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 02/05/026/018/02 O antigen (*wbpA, wbpB, wbpC, wbpD, wbpE, wbpG, wbpH, wbpI, wbpJ, wbpK, wbpL, wzx, wzy, wzz and pa1385*) (Warren et al. 2011).

In addition to changes in O antigen, adaption of *P. aeruginosa* to chronic lung infection in patients with CF involves the synthesis of various lipid A structures (Fig. 3) (Ernst et al. 2007), which result in alteration of host innate immune responses and promote bacterial persistence (Moskowitz and 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 (Fig. 3) (Trent et al. 2001; Geurtsen et al. 2005; Ernst et al. 2006). Underacylation of lipid A has been associated with low-inflammaty activity (Moskowitz and Ernst 2010; Di Lorenzo et al. 2015b) 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 (Fig. 3) (Thaipisutikul 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 (Fig. 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 (Fig. 3) (Moskowitz, Ernst and Miller 2004). These lipid A modifications contribute to *P. aeruginosa* adaptation to the CF airway (Moskowitz and Ernst 2010). The addition of phosphoethanolamine to the *P. aeruginosa* lipid A via the ColRS two-component system (Fig. 3) in a Zn²⁺-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, Cannon and Pier 2002; Lee et al. 2005; Smith, Buckley and Wu 2006; Ciganà et al. 2009; Moskowitz and 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 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, Urban and Goldberg 2005; Loutet and Valvano 2010). Respiratory infections with Bcc bacteria in patients with CF generally lead to faster decline in lung function and, in some cases to cepacia syndrome, a fatal necrotizing pneumonia frequently accompanied by septicemia (Mahenthiralingam, Urban and Goldberg 2005; Coutinho et al. 2011b). 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, Urban and Goldberg 2005; Drevinek and Mahenthiralingam 2010; Coutinho et al. 2011b). Although transient infection of the respiratory tract may occur in some patients, acquisition of Bcc most typically results in chronic infection (Mahenthiralingam, Urban and Goldberg 2005; Coutinho et al. 2011b). 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. 2011a; Moreira et al. 2014) and genotyping of serial isolates (Liebermann 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, 2013).

Lieberman et al. (2011) sequenced the genomes of 112 clinical *B. dolosa* isolates that resulted from the evolution of a single strain in 14 patients with CF 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 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 *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...
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 Ara4N by PmrAB on position 1 or 4; and addition of phosphoethanolamine by ColRS on position 1 or 4.

described in *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 20 independent mutations in both *B. dolosa* and *B. cenocepacia*, was *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 *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 *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, 2013; Mira et al. 2011), 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 (Saldias, Ortega and Valvano 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 (Vidalig et al. 2014), A. xylosoxidans (Trancassini et al. 2014) and H. influenza (Watson, Burns and Smith 2004) recovered from patients with CF have been published; however, the LPS characterization of these clinical isolates is still lacking.

**LPS VARIATION DURING CHRONIC GASTRIC INFECTION**

The human gastric pathogen H. 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, Ruiz and Perez Perez 2014). This geographically widespread 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 and Blaser 2006). Chronic infection with H. pylori is recognized as the most common cause of gastric and duodenal ulcers (Brown 2000). Helicobacter pylori chronic infection is also associated with the development of gastric adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue (Otero, Ruiz and Perez Perez 2014; Mégraud, Bessède and Varon 2015), for which this bacterium is considered to be a class 1 carcinogen (WHO 1994).

Helicobacter pylori produces several virulence factors of which the vaculating toxin A (VacA), the cytotoxin-associated gene A (CagA) and LPS play major roles in immunomodulation and contribute to maintain chronic infection (Possett, Backert and Wessler 2013; Rubin and Trent 2013; Chi, Miszczyk and Rudnicka 2014; de Bernard and Josenhans 2014; Hatakayama 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, Prendergast and Appelmelk 1996; Moran 2008) and reduce detection by the immune system. Helicobacter 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 and 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, Lindner and Walsh 1997). The predominant form is tetra-acetylated lipid A, which is also underphosphorylated (Moran, Lindner and Walsh 1997; Cullen et al. 2011). Underphosphorylation and underacylation of H. pylori lipid A are responsible for reduced endotoxicity (Lungh, Moran and Wadström 1996), as determined by its low reactivity against anti-lipid A antibodies (Mattsby-Baltzer et al. 1998), 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 leucocytes (Baker et al. 1994; Semeraro et al. 1996). Lipid A remodeling 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 phosphorylthanolamine in its place by EptA (Tran et al. 2004, 2006). These modifications increase bacterial resistance to

---

**Table 1.** LPS genes altered in P. aeruginosa during chronic infections.

<table>
<thead>
<tr>
<th>LPS metabolism</th>
<th>Genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid A biosynthesis and modification</td>
<td>lipO2</td>
<td>Cramer et al. (2011); Yang et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>lipC, yciK</td>
<td>Cramer et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>pagL</td>
<td>Cigana et al. (2009); Yang et al. (2011)</td>
</tr>
<tr>
<td>Core biosynthesis and modification</td>
<td>rfaD, wapP</td>
<td>Cramer et al. (2011)</td>
</tr>
<tr>
<td>Common polysaccharide antigen biosynthesis</td>
<td>wbpZ</td>
<td>Cramer et al. (2011)</td>
</tr>
<tr>
<td>O-specific antigen biosynthesis</td>
<td>wbpA, pa5238</td>
<td>Smith, Buckley and Wu (2006)</td>
</tr>
<tr>
<td></td>
<td>uzx</td>
<td>Yang et al. (2011)</td>
</tr>
</tbody>
</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 P. aeruginosa PAO1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wbaD</td>
<td>–</td>
<td>Lieberman et al. (2011)</td>
</tr>
<tr>
<td>YP_834517</td>
<td>rmbB</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834518</td>
<td>rmbA</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834524</td>
<td>migA</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834525</td>
<td>wbpW</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834526</td>
<td>gmd</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834528</td>
<td>–</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834530</td>
<td>wapR</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834532</td>
<td>wbpL</td>
<td>Traverse et al. (2013)</td>
</tr>
<tr>
<td>YP_834533</td>
<td>wbpM</td>
<td>Traverse et al. (2013)</td>
</tr>
</tbody>
</table>
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 catalyzes 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 consists 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 and Trent 2013). The O-specific oligosaccharide has a common backbone that is further modified by fusosyltransferases generating structures that mimic human Lewis antigen molecules and other related blood group antigens such as LeX, LeY, Lea, sialyl-Lea, H-1 antigen, and blood groups A and B antigens (Rubin and Trent 2013) (Fig. 4). This lipid-linked fusosylated oligosaccharide is translocated across the inner membrane by Wzk, an ABC-transporter protein homologous to FglK from Campylobacter jejuni, and subsequently ligated to the lipid A-core by the Waal ligase (Hug et al. 2010).

The presence of terminal fusosylated 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 Leb-transgenic mice infected with LeX-expressing H. pylori, which over time switched on Leb expression (Pohl et al. 2009). This change allowed better bacterial colonization than in the transgenic mice lacking Leb expression, suggesting that Leb H. pylori could survive better in a self-tolerant Leb 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 proinflammatory 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 (Salauin, Ayraud and Saunders 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 four patients over 16 years of chronic gastritis revealed five SNPs affecting LPS genes, including genes involved in the biosynthesis of lipid A (phosphoethanolamine transferase), core (kdsA and uwaF) and O-specific oligosaccharide (wecA) synthesis, as well as in a putative LPS biosynthetic protein (Kennemann et al. 2011). A cluster of nucleotide polymorphisms in the fucT (fusosyltransferase) 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 fusosylation 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 fusosyl 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 and Vanderleyden 2002; van der Woude and Bämmer 2004; Lukáčová, Barák and Kázar 2008). One of these adaptations

---

**Figure 4. Lewis antigen structures.** Helicobacter 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. Lea and LeX 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. Leb and LeY are built by addition of a fucose residue through α-(1,2) linkage to Leb and LeY structures, respectively. Sialyl-Lea (SLea) is built by addition of a sialyl group to the Lea antigen by a α-(2,3) linkage.
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 and Bäumler 2004). LPS phase variation can occur by addition of carbohydrates through the activity of glycosyltransferases or sialyltransferases, or addition of phosphocholine (ChoP) resulting in changes that affect antigenicity, serum sensitivity and adhesion (van der Woude and Bäumler 2004). Phase variation has been described for human pathogens such as S. enterica serovar Typhimurium, C. 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 patients with CF 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 and 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 depends 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 and Miller 2008; Needham and 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 and Miller 2008; Needham and Trent 2013). Further, CF clinical isolates of P. aeruginosa obtained from patients treated with inhaled colistin (polymyxin E) can develop resistance by loss-of-function mutations in the phoQ gene (Miller et al. 2011). Disruption of phoQ in the presence of an intact phoP stimulated Arap4N addition to lipid A by upregulated expression of the Arap4N synthesis operon. Therefore, this adaptive mutagenesis strategy results in high-level polymyxin resistance clinical strains of P. aeruginosa.

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, Barák and Kazár 2008). Short DNA repeats, microsatellites and tandem repeats are particularly prone to slipped-strand mispairing (van Belkum et al. 1997; Torres-Cruz and van der Woude 2003). In H. pylori, phase variation is related to an increase in the number of poly-C tract repeats in the $\beta$-(1,3)-galactosyl transferase (GalT), which leads to a switching on Le$^b$ expression (Pohl et al. 2009). Also, repetitive poly-A and poly-C sequences in the fuscsyltransferase fusC mediate slipped-strand mispairing, which in turn results in production of Lewis antigens with different fucosylated oligosaccharides (Wang et al. 2000; Nilsson et al. 2008). Further, the $\alpha$-(1,2)-fucosyltransferase gene fusC contains an heptameric sequence (AAAAAAG) next to the ribosome-binding site, which may cause a phase shift in the reading frame during translation (Wang et al. 2000).

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 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 and 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 and 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 (Perelov et al. 2011; Reeves et al. 2013), Escherichia (D’Souza, Samuel and Reeves 2005; Hu et al. 2010; Azmuda et al. 2012), Vibrio (González-Fraga et al. 2008; Wildschutte et al. 2010), Yersinia (Cunneen and Reeves 2007) and Brucella (Watam 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-reversion mechanism. This creates also a selective advantage by insertion of IS6100 into wbpM, pilR and mutS, which leads to common CF phenotypes such as O-antigen and type IV pili deficiency and hypermutability (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 defense 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 and 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.

**FUNDING**

Research in the authors’ laboratories has been supported the grants from the Canadian Institutes of Health Research, Cystic Fibrosis Canada, and the UK Cystic Fibrosis Trust (to M.A.V.) and from the Portuguese Foundation for Science and Technology (UID/BIO/04565/2013) and Programa Operacional Regional de Lisboa 2020 (Project N. 007317) to the Institute for Bioengineering and Biosciences (to I.S-C.). R.F.M. was supported by the PhD fellowship SFHR/BD/84233/2012. I.S-C. and M.A.V. were members of the EU COST Action BM1003: Microbial cell surface determinants of virulence as targets for new therapeutics in cystic fibrosis (http://www.cost-bm1003.info/).

**Conflict of interest** None declared.

**REFERENCES**

Aaron SD, Vandemheen KL, Ramotar K et al. Infection with transmissible strains of *Pseudomonas aeruginosa* and clinical outcomes in adults with cystic fibrosis. JAMA 2010;304: 2145–53.


Bowden SD, Hale N, Chung JC et al. Surface swarming motility by Pectobacterium atrosepticum is a latent phenotype that requires O antigen and is regulated by quorum sensing. *Microbiology* 2013;159:2375–85.


Delucia AM, Six DA, Caughlan RE et al. Lipopolysaccharide (LPS) inner-core sugars are required for complete LPS synthesis and transport to the outer membrane in Pseudomonas aeruginosa PA01. MBio 2011;2: e00142-11.


Hao Y, King JD, Hubschmanz S et al. Five new genes are important for common polysaccharide antigen biosynthesis in Pseudomonas aeruginosa. MBio 2013;4:e00631–12.


Schroeder TH, Lee MM, Yacono PW et al. CFTR is a pattern recognition molecule that extracts *Pseudomonas aeruginosa* LPS from the outer membrane into epithelial cells and activates NF-kappa B translocation. *P Natl Acad Sci USA* 2002;99:6907–12.


Silipo A, Molinaro A, Ierano T et al. The complete structure and pro-inflammatory activity of the lipooligosaccharide of the highly epidemic and virulent Gram-negative


Watson ME, Burns JL, Smith AL. Hypermutable Haemophilus influenzae with mutations in mutS are found in cystic fibrosis sputum. Microbiology 2004;150:2947–58.


