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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 lipooligosaccharides, 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-Kdo_2 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),

86 and protection against oxidative stress in *Erwinia amylovora* (Berry *et al.*, 2009). O antigen
87 also contributes to swimming and swarming motility in *E. amylovora* (Berry *et al.*, 2009), *B.*
88 *cenocepacia* (Coutinho *et al.*, 2011), and *Pectobacterium atrosepticum* (Bowden *et al.*, 2013).
89 The immunogenicity of the O antigen polysaccharide elicits a robust antibody response, which
90 may cause selective pressure on bacteria to lose the ability to produce O antigen (King *et al.*,
91 2009); this is particularly common for chronic *P. aeruginosa* strains infecting the lungs of CF
92 patients (Hancock *et al.*, 1983). Conceivably, once the bacteria become mucoid (Govan &
93 Deretic, 1996), the nutrient burden is so high producing alginate and that the bacteria are
94 replicating in a “protected” niche in which O antigen becomes dispensable. However, this may
95 not be a universal notion since other bacteria chronically infecting the CF lung, such as
96 members of the *B. cepacia* complex, undergo different adaptive changes than those reported
97 for *P. aeruginosa* (Zlosnik *et al.*, 2014), including the observation of an inverse correlation
98 between the quantity of mucoid exopolysaccharide production and the rate of decline in CF
99 lung function (Zlosnik *et al.*, 2011).
100 Most *P. aeruginosa* strains produce two types of O antigen molecules ("A-band" and
101 "B-band"), which are structurally and serologically distinct and have different mechanisms of
102 biosynthesis (King *et al.*, 2009, Lam *et al.*, 2011). The "A-band" or "common polysaccharide
103 antigen" is a homopolymer of D-rhamnose that elicits a relatively weak antibody response
104 (King *et al.*, 2009). "B-band" or "O-specific antigens" are highly immunogenic heteropolymers
105 composed repetitive units of different sugars and form the basis for the AITS *P. aeruginosa*-
106 serotyping scheme that includes 20 serotypes (Knirel *et al.*, 2006). Structural data in several
107 *Pseudomonas* serotype strains (Sadovskaya *et al.*, 2000, Bystrova *et al.*, 2006) and genetic
108 experiments (Abeyrathne *et al.*, 2005) demonstrate that both common and O-specific
109 antigens are linked to the lipid A-core.
110 In this article, we review the literature on LPS variations occurring upon bacterial
111 adaptation to chronic infection, with special emphasis on chronic respiratory infections in
112 patients with CF and gastric infections. CF is a genetic disease that leads to ineffective
113 mucociliary clearance of the airways, resulting in chronic airways infection by several Gram-
114 negative bacterial opportunistic pathogens, such as *P. aeruginosa*, the *Burkholderia cepacia*
115 complex (Bcc), and *Achromobacter xylosoxidans* (Ciofu *et al.*, 2015, Cullen & McClean, 2015,

Parkins & Floto, 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-Kdo_2, 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 C_{55}-undecaprenyl phosphate (Und-P), which serves as an acceptor for O antigen

146 chain assembly (Valvano, 2011). Chain assembly occurs by the action of diverse
147 glycosyltransferases that synthesize the specific O antigen of each strain. Genes at the *wb**
148 (formerly *rfb*) locus encode most of the enzymes involved in O antigen assembly (Reeves *et*
149 *al.*, 1996). Because of the great diversity of O antigen structures, the *wb** loci are highly
150 polymorphic (Raetz & Whitfield, 2002, Lam *et al.*, 2011). The O antigen is initially assembled
151 on the cytoplasmic side of the membrane and then translocated to the periplasmic side and
152 ligated to lipid A-core (Valvano, 2015) (Figure 2). There are three pathways for O antigen
153 biosynthesis and export: (1) Wzy-dependent, (2) ABC-transporter-dependent, and (3)
154 synthase-dependent (Keenleyside & Whitfield, 1996, Lam *et al.*, 2011, Greenfield & Whitfield,
155 2012, Valvano, 2015). The mature LPS molecule is then transported across the periplasm
156 and inserted into the outer leaflet of the outer membrane by the conserved Lpt (LPS
157 transport) pathway (May *et al.*, 2015, Simpson *et al.*, 2015). Lpt proteins form a complex that
158 traverses the Gram-negative cell envelope to deliver LPS to the outer membrane and include
159 an ABC protein complex (LptBFG) that uses energy from ATP hydrolysis to extract LPS from
160 the periplasmic face of the inner membrane, several proteins that dock and promote the
161 transfer of LPS across the periplasm (LptCA and YhjD) and a complex of proteins on the
162 outer membrane (LptDE, YtfN, YfgH and YceK), responsible for the correct insertion of LPS in
163 the outer leaflet (Babu *et al.*, 2011, Sperandeo *et al.*, 2011, Sperandeo *et al.*, 2011, May *et*
164 *al.*, 2015, Simpson *et al.*, 2015). The Lpt system has not been investigated in Gram-negative
165 pathogens other than *E. coli* and sequence homology between *E. coli* and *P. aeruginosa*
166 genes is low, with the exception of LptB (66% sequence identity). Recently, it was shown that
167 *P. aeruginosa* LptA has a dimeric structure, unlike the oligomeric structure of *E. coli* LptA
168 (Shapiro *et al.*, 2014).
169 In *P. aeruginosa*, the common polysaccharide and the O-specific antigens are
170 synthesized *via* the ABC-transporter-dependent pathway and the Wzy-dependent pathway,
171 respectively (King *et al.*, 2009, Lam *et al.*, 2011). In both the synthesis is initiated by the same
172 glycosyltransferase, WbpL (homologous to the *E. coli* WecA), resulting the formation of an
173 Und-P-P-sugar intermediate (King *et al.*, 2009, Lam *et al.*, 2011). Four enzymes are required
174 for the biosynthesis of GDP-D-rhamnose, the nucleotide sugar precursor for the common
175 polysaccharide antigen: WbpW, AlgC, Gmd and Rmd (King *et al.*, 2009, Lam *et al.*, 2011).

176 The glycosyltransferases WbpX, WbpY and WbpZ are involved in the synthesis of the
177 common polysaccharide antigen (King *et al.*, 2009, Lam *et al.*, 2011), while genes *pa54-*
178 *55pa5459* have been suggested to encode proteins that play a role in controlling chain length
179 (Hao *et al.*, 2013). Once the common polysaccharide antigen is linked to the Und-P carrier,
180 the complex is exported across the membrane by the ABC-transport system Wzm-Wzt (King
181 *et al.*, 2009, Lam *et al.*, 2011). While the genes for the synthesis and assembly of the
182 common polysaccharide are conserved, different set of genes are responsible for the
183 biosynthesis of the O-specific antigen in each serotype strain. These genes are in a cluster
184 flanked by the highly conserved genes *himD/ihfB* and *wbpM* (King *et al.*, 2009, Lam *et al.*,
185 2011). While the *P. aeruginosa* O5, O6 and O11 O antigen clusters were studied to some
186 extent very little experimental work was conducted into the functions of genes in the
187 remaining O antigen loci (Lam *et al.*, 2011). The synthesized the Und-PP-linked O-repeat
188 units are translocated to the periplasmic side of the membrane and polymerized. The proteins
189 Wzy, Wzz and Wzx are required for this process, acting as polymerase, chain-length
190 regulator, and flippase, respectively (Lam *et al.*, 2011). Once on the periplasmic side, both the
191 common polysaccharide antigen and the O-specific antigen are independently linked to the
192 lipid A-core complex by the WaaL ligase (Figure 2) (Abeyrathne *et al.*, 2005, Valvano, 2011,
193 Ruan *et al.*, 2012).
194
195 **LPS VARIATION DURING CHRONIC RESPIRATORY INFECTIONS IN CF PATIENTS**
196
197 ***Pseudomonas aeruginosa* infection**
198 *P. aeruginosa* is the most common pathogen isolated from the respiratory tract of adult
199 patients with CF (Lipuma, 2010, Hauser *et al.*, 2011). Chronic airway infections caused by *P.*
200 *aeruginosa* are found in up to 80% of adult patients with CF (Aaron *et al.*, 2010, Lipuma,
201 2010) and are associated with increased morbidity and mortality (Hauser *et al.*, 2011).
202 Phenotypic changes suggesting *P. aeruginosa* adaptation to the CF lung have been reported
203 in several studies (Hogardt & Heesemann, 2010). They include loss of motility associated with
204 growth in microcolony (Sriramulu *et al.*, 2005), reduced expression of virulence factors, which
205 is presumably an adaptive strategy to escape detection by the host immune system (Smith *et*

206 *al.*, 2006), increased activity of efflux pumps associated with antibiotic resistance, especially
207 against those antibiotics used clinically (Poole, 2005), and a switch from non mucoid to
208 mucoid phenotypes (Ciofu *et al.*, 2010, Hogardt & Heesemann, 2010). The phenotypic
209 changes reflect point mutations accumulating in *P. aeruginosa* lineages that persist in CF
210 airways (Lorè *et al.*, 2012), and include mutations in alginate biosynthesis regulator genes
211 (Bragonzi *et al.*, 2006) and genes involved in the LPS modification (Cigana *et al.*, 2009),
212 motility (Mahenthiralingam *et al.*, 1994), quorum-sensing regulation (D'Argenio *et al.*, 2007,
213 Hoffman *et al.*, 2009), type 3 secretion system biosynthesis (Jain *et al.*, 2004), multidrug-
214 efflux pumps, and mutator genes (Oliver *et al.*, 2000).
215 The longitudinal course of chronic airway infection with *P. aeruginosa* in CF has been
216 followed in various studies (Smith *et al.*, 2006, Cigana *et al.*, 2009, Cramer *et al.*, 2011,
217 Mowat *et al.*, 2011, Warren *et al.*, 2011, Yang *et al.*, 2011, Lorè *et al.*, 2012, Dettman *et al.*,
218 2013). A study investigating over 1700 serial isolates obtained from 10 patients infected with
219 the same strain showed that within-patient diversity made the largest contribution to the
220 overall variation in the population and also that population compositions fluctuated over time
221 (Mowat *et al.*, 2011). The authors suggested that extensive diversity within the *P. aeruginosa*
222 population during chronic infection has the potential to provide a reservoir for antibiotic
223 resistant mutations and mutations in other virulence traits (Mowat *et al.*, 2011). Despite these
224 differences, certain traits were overrepresented in all isolates, most of which include
225 properties regulated by quorum sensing (Mowat *et al.*, 2011). *In silico* simulations reveal
226 virulence factor expression decline towards the end of chronic infections and adaptive
227 mutations that tend to improve metabolic fitness, which would optimize growth over the more
228 energetically expensive virulence factor production (Oberhardt *et al.*, 2010). *P. aeruginosa*
229 LPS modifications appear to be an important factor in the adaptation of this pathogen to
230 chronic infection (Cigana *et al.*, 2009). Indeed, chronic *P. aeruginosa* CF isolates have rough
231 colony phenotypes and contain few, short, or no O side chains, becoming non-typeable
232 (Hancock *et al.*, 1983). O antigen deficient isolates are sensitive to *in vitro* killing by serum
233 complement and become more tolerant to the antibiotic gentamicin (Kadurugamuwa *et al.*,
234 1993). Analysis of sequential variants of *P. aeruginosa* show O antigen loss (Lee *et al.*, 2005)
235 and lipid A modifications (Cigana *et al.*, 2009). Whole-genome analysis of two clinical *P.*

236 *aeruginosa* variants recovered from a chronic CF patient after 6 and 96 months of infection
237 also revealed non-synonymous mutations in the O antigen biosynthetic genes *wbpA* and
238 *pa5238* in the latter variant (Smith *et al.*, 2006). Another study of genes responsible for
239 modifying lipid A revealed one mutation in *pagL* in late variants, which abolish PagL
240 expression and leads to reduced TLR4-MD2-signalling (Cigana *et al.*, 2009). Thus, initial lipid
241 A modifications by addition of palmitate to the lipid A of *P. aeruginosa* make the LPS more
242 proinflammatory, but the subsequent modification through the loss of PagL activity decreases
243 its proinflammatory activity. Together, the results of these studies suggest that reduced LPS
244 immunostimulatory potential contributes to immune system evasion and survival over the
245 course of the chronic *P. aeruginosa* infection. Experimental data support this hypothesis since
246 a comparison of the pathogenicity of nine *P. aeruginosa* sequential clonal variants in the
247 infection models *Caenorhabditis elegans*, *Galleria mellonella*, *Drosophila melanogaster* and
248 two different mice backgrounds (C57Bl/6NCrl and BALB/cAnNCrl), show that early *P.*
249 *aeruginosa* variants were lethal in all infection models tested, while late strains exhibited
250 reduced or no virulence (Lorè *et al.*, 2012).
251 A microevolution analysis based on whole-genome sequencing of sequential *P.*
252 *aeruginosa* variants recovered from CF patients for more than 20 years (Cramer *et al.*, 2011)
253 identified codon changes in genes for lipid A biosynthesis (*lpxC*, *lpxO2* and *yciK*), core
254 biosynthesis (*rfaD* and *wapP*), and common polysaccharide antigen biosynthesis (*wbpZ*)
255 (Cramer *et al.*, 2011). Another genomic analysis taken over 200,000 bacterial generations of
256 12 selected *P. aeruginosa* DK2 variants recovered from six CF patients identified a total of
257 234 non-synonymous single nucleotide polymorphisms among the genomes in relation to
258 their common ancestor strain, suggesting that an initial period of rapid adaptation is followed
259 by a period of genetic drift in this lineage (Yang *et al.*, 2011). Three of the non-synonymous
260 single nucleotide polymorphisms occurred in genes needed for lipid A biosynthesis and
261 modification (*pagL* and *lpxO2*) and O-specific antigen synthesis (*wzz*) (Yang *et al.*, 2011). A
262 recent study analyzing whole-genome sequence data from *P. aeruginosa* clinical isolates
263 sampled from the sputum of 32 different patients reported that the O antigen ligase *waaL* is
264 one of the few hotspots of gene polymorphisms (Dettman *et al.*, 2013). To gain insight into the
265 role of mutator genes for generating adaptive variation, Warren *et al.* (2011) analysed the

266 genomes of two series of isolates recovered from two patients, similar in duration but different
267 in mutator incidence, and identified 15 LPS genes that lacked in multiple members both in
268 mutator and non-mutator series. All the identified genes are involved in the synthesis of
269 serogroup O2/O5/O26/O18/O20 O antigen (*wbpA*, *wbpB*, *wbpC*, *wbpD*, *wbpE*, *wbpG*, *wbpH*,
270 *wbpI*, *wbpJ*, *wbpK*, *wbpL*, *wzx*, *wzy*, *wzz* and *pa1385*) (Warren *et al.*, 2011).
271 In addition to changes in O antigen, adaption of *P. aeruginosa* to chronic lung
272 infection in CF patients involves the synthesis of various lipid A structures (Figure 3) (Ernst *et*
273 *al.*, 2007), which result in alteration of host innate immune responses and promote bacterial
274 persistence (Moskowitz & Ernst, 2010). These modifications involve deacylation of the lipid A
275 resulting in the loss of an acyl chain from the 3-position, which is catalyzed by PagL (Figure 3)
276 (Trent *et al.*, 2001, Geurtsen *et al.*, 2005, Ernst *et al.*, 2006). Under acylation of lipid A has
277 been associated with low inflammatory activity (Moskowitz & Ernst, 2010, Di Lorenzo *et al.*,
278 2015) and modulation of TLR4-MD2 receptor recognition (Ernst *et al.*, 2003). Also, *P.*
279 *aeruginosa* lipid A can acquire a secondary acyl chain into the 3'-position, which is catalyzed
280 by a divergent palmitoyltransferase functionally analogous to the *Salmonella* and *E. coli* PagP
281 enzyme (Figure 3) (Thaipisuttikul *et al.*, 2014). Further modifications involve the addition of
282 secondary acyl chains to the chains present at the 2- and 2'-positions, which is catalyzed by
283 HtrB and LpxO, respectively (Figure 3), as well as the incorporation of 4-amino-4-deoxy-L-
284 arabinopyranose (Ara*p*4N) to phosphate groups at the 1- and 4'-positions by the two-
285 component regulatory system PmrAB (Figure 3) (Moskowitz *et al.*, 2004). These lipid A
286 modifications contribute to *P. aeruginosa* adaptation to the CF airway (Moskowitz & Ernst,
287 2010). The addition of phosphoethanolamine to the *P. aeruginosa* lipid A via the ColRS two-
288 component system (Figure 3) in a Zn^{2+}-dependent manner was recently reported (Nowicki *et*
289 *al.*, 2015), but the role of this modification *in vivo* is not clear.
290 Collectively, the studies described above support the notion that chronically infecting
291 bacteria adapt to host immune responses by producing LPS lacking O antigen and by
292 introducing lipid A modifications in isolates recovered in late stages of CF chronic infection
293 (Table 1) (Lyczak *et al.*, 2002, Lee *et al.*, 2005, Smith *et al.*, 2006, Cigana *et al.*, 2009,
294 Moskowitz & Ernst, 2010, Cramer *et al.*, 2011, Yang *et al.*, 2011, Dettman *et al.*, 2013). This
295 conclusion is also supported from comparative studies using various host models

296 demonstrating that adaptation of different *P. aeruginosa* lineages within CF lungs selects
297 populations with reduced pathogenic potential in acute infections (Lorè *et al.*, 2012).
298
299 **Chronic Infections by other Gram-negative CF pathogens**
300 Bacteria from the *Burkholderia cepacia* complex (Bcc) emerged as significant CF pathogens
301 in the early 1980s, when a minority of infected patients exhibited rapid clinical deterioration,
302 resulting in early death (Mahenthiralingam *et al.*, 2005, Loutet & Valvano, 2010). Respiratory
303 infections with Bcc bacteria in CF patients generally lead to faster decline in lung function
304 and, in some cases to cepacia syndrome, a fatal necrotizing pneumonia frequently
305 accompanied by septicemia (Mahenthiralingam *et al.*, 2005, Coutinho *et al.*, 2011). Further,
306 Bcc bacteria are transmissible through social contacts and are intrinsically resistant to most
307 clinically used antibiotics, which renders their eradication from the CF lung very difficult, if not
308 virtually impossible (Mahenthiralingam *et al.*, 2005, Drevinek & Mahenthiralingam, 2010,
309 Coutinho *et al.*, 2011). Although transient infection of the respiratory tract may occur in some
310 patients, acquisition of Bcc most typically results in chronic infection (Mahenthiralingam *et al.*,
311 2005, Coutinho *et al.*, 2011). The same level of adaptation is not so clear cut in *B.*
312 *cenocepacia* infections, as studies using the various infection models (*C. elegans*, *G.*
313 *mellonella*, alfalfa, mice and rats) reported that most virulence factors are specific for one
314 infection model only and rarely essential for pathogenicity in multiple hosts (Uehlinger *et al.*,
315 2009, Lorè *et al.*, 2012). Furthermore, less is known about *Burkholderia* adaptation during CF
316 chronic infection; however, there has been an effort to characterize the evolution of
317 *Burkholderia* populations in the lung, including phenotyping (Coutinho *et al.*, 2011, Moreira *et*
318 *al.*, 2014) and genotyping of serial isolates (Lieberman *et al.*, 2011, Traverse *et al.*, 2013),
319 and comparative expression profiling of the transcriptome (Mira *et al.*, 2011) and the
320 proteome (Madeira *et al.*, 2011, Madeira *et al.*, 2013).
321 Lieberman *et al.* (2011) sequenced the genomes of 112 clinical *Burkholderia dolosa*
322 isolates that resulted from the evolution of a single strain in 14 CF patients over 16 years of
323 epidemic spread and discovered that genes involved in oxygen regulation, antibiotic
324 resistance, outer-membrane synthesis and secretion have recurrent mutation patterns
325 (Lieberman *et al.*, 2011). Interestingly, recurrent mutations in the same amino acid of the

326 glycosyltransferase WbaD were observed in nine patients, which resulted in production of O-
327 unit repeats that were absent in the ancestral phenotype (Lieberman *et al.*, 2011). The
328 ancestral *B. dolosa* genotype encodes a stop codon at this locus that prevents O antigen
329 synthesis. In some variants, two different mutations affecting the same amino acid were
330 detected, both of them restoring the full-length WbaD protein and leading to O antigen
331 production (Table 2) (Lieberman *et al.*, 2011). Although this gain-of-function mutation does
332 not follow the loss of O antigen tendency described in *P. aeruginosa*, these results underpin
333 the importance of the O antigen switch mechanism during chronic infection. Another
334 metagenomic analysis of six lineages evolved in biofilm mode of growth revealed an
335 extraordinary mutational parallelism, including genes known to affect LPS biosynthesis,
336 transcription, galactose metabolism, tricarboxylic acid cycle enzymes and altered metabolism
337 of cyclic diguanosine monophosphate (Traverse *et al.*, 2013). One commonly mutated locus,
338 showing twenty independent mutations in both *B. dolosa* and *Burkholderia cenocepacia*, was
339 *manC*, encoding a nucleotide mannose biosynthesis protein presumably involved in surface
340 polysaccharide biosynthesis that could be either an exopolysaccharide or LPS (Traverse *et*
341 *al.*, 2013). Interestingly, these authors showed that complementation of one of the *manC*
342 mutations dramatically reduced biofilm formation, and they speculated that the loss of
343 polysaccharide may be required for efficient biofilm formation rather than immune evasion
344 (Traverse *et al.*, 2013).
345 A comparison of the transcriptome and the proteome of three *B. cenocepacia* isolates
346 recovered at the beginning of the infection and later during the progress of the disease
347 suggests that the expression from genes involved in LPS biosynthesis is altered during
348 chronic infection (Madeira *et al.*, 2011, Mira *et al.*, 2011, Madeira *et al.*, 2013), in particular of
349 those required for O antigen biosynthesis. Indeed, recent analysis of the LPS structure of
350 these isolates revealed that, although the early-stage isolate has a complete LPS with the O-
351 chain moiety, the late-stage variants have a rough-type LPS, lacking O antigen (Maldonado *et*
352 *al.,* unpublished data).
353 Several studies at genome, transcriptome and proteome levels have contributed to a
354 better understanding of Bcc bacteria genome-wide adaptive mechanisms during chronic
355 infections. Together, they suggest that there is a high selective pressure on the O antigen

356 locus leading to alterations both at the structural, sequence and regulatory levels. Given the
357 exceptional parallelism found among the relatively few studies dedicated to Bcc bacteria and
358 *P. aeruginosa*, the LPS seems to play an important role during chronic infection, both in
359 immune system evasion and biofilm adaptation. Moreover, lack of O antigen in *B.*
360 *cenocepacia* leads to increased internalization into macrophages upon phagocytosis (Saldías
361 *et al.*, 2009), which may explain the higher invasiveness of epidemic strains, such as J2315,
362 which do not produce O antigen. O antigen loss could therefore facilitate access of Bcc
363 bacteria to macrophages, where intracellular bacteria could find a niche to persist, in
364 agreement with a recent study showing that in human lungs, Bcc bacteria but not *P.*
365 *aeruginosa* are found mainly inside macrophages (Schwab *et al.*, 2014). Other Gram-
366 negative opportunistic pathogens that cause CF chronic infections include *Stenotrophomonas*
367 *maltophilia*, *Achromobacter xylosoxidans* and *Haemophilus influenza*. Recently, some studies
368 characterizing the adaptive traits of sequential isolates of *S. maltophilia* (Vidigal *et al.*, 2014),
369 *A. xylosoxidans* (Trancassini *et al.*, 2014) and *H. influenza* (Watson *et al.*, 2004) recovered
370 from CF patients have been published, however the LPS characterization of these clinical
371 isolates is still lacking.
372
373 **LPS VARIATION DURING CHRONIC GASTRIC INFECTION**
374 The human gastric pathogen *Helicobacter pylori* is usually acquired during childhood by
375 colonizing the human gastric mucosa and producing a superficial gastritis, which may remain
376 asymptomatic during the lifetime of colonized individuals or eventually lead to gastric ulcer
377 and atrophic gastritis (Linz *et al.*, 2013, Otero *et al.*, 2014). This geographically wide-spread
378 bacteria infects more than half of the human population and is one of the most genetically
379 diverse bacterial species, being also one of the most ubiquitous infectious organisms (Linz *et*
380 *al.*, 2013). The genetic diversity of *H. pylori* is caused by a high mutation rate, presumably
381 due to the lack of several mutation repair genes (Kang & Blaser, 2006). Chronic infection with
382 *H. pylori* is recognized as the most common cause of gastric and duodenal ulcers (Brown,
383 2000). *H. pylori* chronic infection is also associated with the development of gastric
384 adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue (Otero *et al.*, 2014,

385 Mégraud *et al.*, 2015), for which this bacterium is considered to be a class 1 carcinogen
386 (WHO, 1994).
387 *H. pylori* produces several virulence factors of which the vacuolating toxin A (VacA),
388 the cytotoxin-associated gene A (CagA), and LPS play major roles in immunomodulation and
389 contribute to maintain chronic infection (Posselt *et al.*, 2013, Rubin & Trent, 2013, Chmiela *et*
390 *al.*, 2014, de Bernard & Josenhans, 2014, Hatakeyama, 2014). These factors contribute to
391 maintain the infection by preventing the clearance of *H. pylori* from the gastric mucosa and
392 interfering with innate and adaptive immune responses. Structural modifications of the lipid A
393 result in reduced endotoxicity, while expression and variation of Lewis determinants exposed
394 on the bacterial cell surface as a terminal O-specific oligosaccharide (Aspinall *et al.*, 1996,
395 Monteiro *et al.*, 1998) mimic host components expressed on the human gastric epithelium
396 (Moran *et al.*, 1996, Moran, 2008) and reduce detection by the immune system. *H. pylori* lipid
397 A presents a unique structure and shows remarkably lower biological activity compared with
398 lipid A from other bacteria (Muotiala *et al.*, 1992, Moran & Aspinall, 1998). Structural analysis
399 revealed that the lipid A acyl chains are longer (16 to 18 carbons) than those present in
400 enterobacterial lipid A (Moran *et al.*, 1997). The predominant form is tetra-acylated lipid A,
401 which is also underphosphorylated (Moran *et al.*, 1997, Cullen *et al.*, 2011).
402 Underphosphorylation and underacylation of *H. pylori* lipid A are responsible for reduced
403 endotoxicity (Ljungh *et al.*, 1996), as determined by its low reactivity against anti-lipid A
404 antibodies (Mattsby-Baltzer *et al.*, 1992), reduced ability to induce the production of cytokines,
405 nitric oxide and prostaglandin E2 (Pérez-Pérez *et al.*, 1995), and E-selectin expression
406 (Darveau *et al.*, 1995), as well as reduced activation of leukocytes (Baker *et al.*, 1994,
407 Semeraro *et al.*, 1996). Lipid A remodelling in *H. pylori* occurs mainly on the periplasmic side
408 of the inner membrane. A first set of modifications involves removal of the 1-phosphate group
409 by LpxE and the addition of a phosphorylethanolamine in its place by EptA (Tran *et al.*, 2004,
410 Tran *et al.*, 2006). These modifications increase bacterial resistance to antimicrobial peptides
411 (Tran *et al.*, 2006). Second, a two-protein Kdo-hydrolase complex removes the terminal Kdo
412 sugar, a modification that is critical to allow the ligation of the O-specific oligosaccharides to
413 the lipid A core (Stead *et al.*, 2010). Third, LpxF catalyses the removal of the 4'-phosphate
414 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 Le^X; Le^Y, Le^a, Le^b, sialyl-Le^X, 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 Le^X and Le^Y 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, Le^X *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 is several ways. For example, *H. pylori* can change its Lewis antigens in response to those present in the host, as demonstrated with Le^b-transgenic mice infected with Le^X-expressing *H. pylori,* which over time switched on Le^b expression (Pohl *et al.*, 2009). This change allowed better bacterial colonization than in the transgenic mice lacking Le^b expression, suggesting that Le^b *H. pylori* could survive better in a self-tolerant Le^b 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

445 the surface of gastric dendritic cells, which lead to a block in maturation of T-helper 1 cells
446 and reduced production of pro-inflammatory cytokines (Bergman *et al.*, 2004).
447 The first evidence that a single strain of *H. pylori* alters its LPS antigenic phenotype
448 during the course of infection was demonstrated by investigating the expression of Lewis
449 antigens in 127 isolates recovered from serial biopsies of 26 asymptomatic subjects (Rasko *et*
450 *al.*, 2000). This alteration of LPS biosynthesis in *H. pylori* occurs during host colonization in
451 response to several stimuli (Salaün *et al.*, 2005, Nilsson *et al.*, 2008) such as interaction with
452 T helper cells Bergman, 2004 #5473} and gastric pH (Skoglund *et al.*, 2009). More recently,
453 several studies have focused on the genomic changes occurring in *H. pylori* isolates that have
454 been recovered several years apart from patients with chronic infection (Falush *et al.*, 2001,
455 Israel *et al.*, 2001, Kraft *et al.*, 2006, Alvi *et al.*, 2007, Morelli *et al.*, 2010, Kennemann *et al.*,
456 2011).
457 A whole-genome analysis of 10 *H. pylori* sequential isolates recovered from 4 patients
458 over 16 years of chronic gastritis revealed 5 SNPs affecting LPS genes, including genes
459 involved in the biosynthesis of lipid A (phosphoethanolamine transferase), core (*kdsA* and
460 *waaF*) and O-specific oligosaccharide (*wecA*) synthesis, as well as in a putative
461 lipopolysaccharide biosynthetic protein (Kennemann *et al.*, 2011). A cluster of nucleotide
462 polymorphisms in the *fucT* (fucosyltransferase) gene, presumably facilitating its expression,
463 was identified in whole-genome analyses of two *H. pylori* strains isolated from spouses (Linz
464 *et al.*, 2013). Hyperexpression of *fucT* promotes posttranslational fucosylation of the O-
465 specific oligosaccharide, generating Lewis antigens (Ge *et al.*, 1997, Martin *et al.*, 1997,
466 Moran, 2008, Linz *et al.*, 2013). The alteration of *H. pylori* LPS during chronic gastric infection,
467 either by altering LPS biosynthesis or by adding fucosyl residues to O-specific
468 oligosaccharides, generates Lewis structures that mimic host antigens and contribute to
469 immune system evasion.
470
471 **MOLECULAR MECHANISMS OF LPS VARIATION**
472 Antigenic variation of surface structures is a powerful mechanism for pathogen evasion of
473 adaptive immune responses (Lerouge & Vanderleyden, 2002, van der Woude & Bäumler,
474 2004, Lukácová *et al.*, 2008). One of these adaptions involves phase variation, which is a

475 reversible, yet heritable form, of gene regulation that results in heterogeneous clonal
476 populations and can be mediated by various molecular mechanisms (van der Woude &
477 Bäumler, 2004). LPS phase variation can occur by addition of carbohydrates through the
478 activity of glycosyltransferases or sialyltransferases, or addition of phosphorylcholine (ChoP)
479 resulting in changes that affect antigenicity, serum sensitivity and adhesion (van der Woude &
480 Bäumler, 2004). Phase variation has been described for human pathogens such as *S.*
481 *enterica* serovar Typhimurium, *Campylobacter jejuni*, *Neisseria* spp. and *H. pylori* but
482 because variable LPS modification is not easily identified, it is possible that phase variation is
483 more widespread than currently known. Genetic and epigenetic mechanisms behind LPS
484 variation are discussed below.
485
486 **Adaptive mutagenesis and altered gene expression**
487 Acquisition of adaptive mutations is a common theme in microbial persistence. In CF patients
488 with chronic lung infection, *P. aeruginosa* strains accumulate a large proportion of mutator
489 strains (Oliver *et al.*, 2000) that contribute to selection of mucoid variants (Oliver *et al.*, 2000,
490 Mathee *et al.*, 2008, Ciofu *et al.*, 2010, Hogardt & Heesemann, 2010). The proinflammatory
491 microenvironment in the airways including polymorphonuclear cells, hydrogen peroxide
492 production, and antibiotics (Blázquez *et al.*, 2006) has been associated with mutagenesis and
493 mucoid conversion *in vitro* (Mathee *et al.*, 1999, Sanders *et al.*, 2006, Moyano *et al.*, 2007).
494 Cationic antimicrobial peptides can also exert a mutagenic inducing effect, as recently
495 demonstrated for human cathelicidin LL-37 (Limoli *et al.*, 2014). Mutagenesis depended on
496 LL-37 entering the bacterial cytosol and binding to DNA, which in turns promotes abnormal
497 DNA synthesis by the error-prone polymerase DinB (Sanders *et al.*, 2006, Limoli *et al.*, 2014).
498 Environmental cues, such as ionic concentration, can lead to O antigen structural
499 variations resulting from altered gene expression regulated by two-component signal
500 transduction systems. One of the best examples of this type of regulation is the PhoP/PhoQ
501 system in *Salmonella* (Prost & Miller, 2008, Needham & Trent, 2013). PhoQ is a membrane
502 sensor histidine kinase and PhoP is its cognate response regulator. Activation of the
503 PhoP/PhoQ system by acidic pH, specific antimicrobial peptides, and depletion of Mg^{2+} and
504 Ca^{2+} stimulates transcription of *pagP* and *pagL* (among other genes) and subsequent

505 upregulation of the encoded proteins, which acylate and deacylate lipid A, respectively (Prost
506 & Miller, 2008, Needham & Trent, 2013). Further, CF clinical isolates of *P. aeruginosa*
507 obtained from patients treated with inhaled colistin (polymyxin E) can develop resistance by
508 loss-of-function mutations in the *phoQ* gene (Miller *et al.*, 2011). Disruption of *phoQ* in the
509 presence of an intact *phoP* stimulated Ara*p*4N addition to lipid A by upregulated expression of
510 the Ara*p*4N synthesis operon. Therefore, this adaptive mutagenesis strategy results in high-
511 level polymyxin resistance clinical strains of *P. aeruginosa*.
512
513 **Slipped-strand mispairing**
514 One of the molecular mechanisms of phase variation involves slipping of one of the DNA
515 strands, which causes mispairing between daughter and parent strands during DNA
516 replication (slipped-strand mispairing) (Lukácová *et al.*, 2008). Short DNA repeats,
517 microsatellites and tandem repeats are particularly prone to slipped-strand mispairing (van
518 Belkum *et al.*, 1997, Torres-Cruz & van der Woude, 2003). In *H. pylori*, phase variation is
519 related to an increase in the number of poly-C tract repeats in the β-(1,3)-galactosyl
520 transferase (GalT), which leads to a switching on Le^b expression (Pohl *et al.*, 2009). Also,
521 repetitive poly-A and poly-C sequences in the fucosyltransferase *fucT* mediate slipped-strand
522 mispairing, which in turn results in production of Lewis antigens with different fucosylated
523 oligosaccharides (Wang *et al.*, 2000, Nilsson *et al.*, 2008). Further, the α-(1,2)-
524 fucosyltransferase gene *futC* contains an heptameric sequence (AAAAAAG) next to the
525 ribosome binding site, which may cause a phase shift in the reading frame during translation
526 (Wang *et al.*, 2000).
527
528 **Lateral gene transfer, recombination, and genetic rearrangements**
529 The heterogeneity of O antigens is mostly due to variation within the O antigen gene cluster,
530 but it is unclear how such variation was generated (Reeves *et al.*, 2013). Genes involved in O
531 antigen biosynthesis are generally arranged in large operons with low G+C content relative to
532 the average G+C characteristic of each species, which suggests that these clusters were
533 acquired by horizontal gene transfer from a species with low G+C content (Lerouge &
534 Vanderleyden, 2002). The G+C content within the O antigen clusters also greatly differs from

535 gene to gene, indicating that the gene clusters might have been assembled from multiple
536 horizontal transmission events and from several sources over a much longer time (Lerouge &
537 Vanderleyden, 2002). The role of lateral gene transfer in the evolution of O antigen clusters
538 and O antigen diversification has been well described in *Salmonella* (Perepelov *et al.*, 2011,
539 Reeves *et al.*, 2013), *Escherichia* (D'Souza *et al.*, 2005, Hu *et al.*, 2010, Azmuda *et al.*, 2012),
540 *Vibrio* (González-Fraga *et al.*, 2008, Wildschutte *et al.*, 2010), *Yersinia* (Cunneen & Reeves,
541 2007) and *Brucella* (Wattam *et al.*, 2014). Another mechanism of variation involves large
542 chromosomal rearrangements. For example, more than half of the *P. aeruginosa* clone C
543 isolates from CF lung infection exhibit large chromosomal inversions mediated an IS*6100*-
544 induced coupled insertion-inversion mechanism. This creates also a selective advantage by
545 insertion of IS*6100* into *wbpM*, *pilB* and *mutS*, which leads to common CF phenotypes such
546 as O-antigen and type IV pili deficiency and hyper mutability (Kresse *et al.*, 2003).
547
548 **CONCLUDING REMARKS**
549 The LPS is an abundant molecule of the outer membrane of most Gram-negative bacteria
550 and plays a key role during host-pathogen interaction and the establishment of chronic
551 infection. LPS-mediated virulence resides both in the endotoxic activity of lipid A and in the
552 ability of the core and O antigen to provide the bacterium with resistance to host defence
553 mechanisms. O antigen modification in general contributes to enhance the bacteria's ability to
554 establish infection. For example, *P. aeruginosa* O antigen modification directed by the D3
555 prophage promotes adhesion to epithelial cells (Vaca-Pacheco *et al.*, 1999), while in *H. pylori*,
556 expression of the Lewis antigen Le^X promotes bacterial adhesion to the gastric epithelia by
557 interacting with host lectins. Further, O antigen modification can contribute to host immune
558 evasion either by mimicry of host molecules (e.g., Lewis antigens in *H. pylori*) or by inhibiting
559 activation of the host complement system (Raetz & Whitfield, 2002). It is also well established
560 that during chronic infection there is an increase of mutator phenotypes (Oliver *et al.*, 2000),
561 which leads to a higher mutation rate and will consequently contribute to the accumulation of
562 modifications in LPS structure during colonization.
563 Several studies have shown alterations in the LPS molecule during chronic infection,
564 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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1081 **Table 1. LPS genes altered in *P. aeruginosa* during chronic infections.**

LPS metabolism	Genes	Reference
Lipid A biosynthesis and modification	*lpxO2*	Cramer *et al*., 2011; Yang *et al*., 2011
	lpxC, *yciK*	Cramer *et al*., 2011
	pagL	Cigana *et al*., 2009; Yang *et al*., 2011
Core biosynthesis and modification	*rfaD*, *wapP*	Cramer *et al*., 2011
Common polysaccharide antigen biosynthesis	*wbpZ*	Cramer *et al*., 2011
O-specific antigen biosynthesis	*wbpA*, *pa5238*	Smith *et al*., 2006
	wzz	Yang *et al*., 2011
	wbpA, *wbpB*, *wbpC*, *wbpD*, *wbpE*, *wbpG*, *wbpH*, *wbpI*, *wbpJ*, *wbpK*, *wbpL*, *wzx*, *wzy*, *wzz*, *pa1385*	Warren *et al*., 2011
O antigen ligase	*waaL*	Dettman *et al*., 2013

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1085 **Table 2. LPS genes altered in *B. dolosa* and *B. cenocepacia* during chronic infections.**
1086 The homologous gene in *P. aeruginosa* is also indicated.
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Gene or locus	Homologous gene in *P. aeruginosa* PAO1	Reference
wbaD	-	Lieberman *et al*., 2011
YP_834517	*rmlB*	Traverse *et al*., 2013
YP_834518	*rmlA*	Traverse *et al*., 2013
YP_834524	*migA*	Traverse *et al*., 2013
YP_834525	*wbpW*	Traverse *et al*., 2013
YP_834526	*gmd*	Traverse *et al*., 2013
YP_834528	-	Traverse *et al*., 2013
YP_834530	*wapR*	Traverse *et al*., 2013
YP_834532	*wbpL*	Traverse *et al*., 2013
YP_834533	*wbpM*	Traverse *et al*., 2013

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1094 Legend to Figures

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1096 **Figure 1. Cell envelope organization of Gram-negative bacteria.** The cell envelope of
1097 Gram-negative bacteria is characterized by the presence of two lipid bilayers: the outer
1098 membrane (OM) and the cytoplasmic membrane (CM), which are separated by the periplasm,
1099 containing hydrolytic enzymes, binding proteins, chemoreceptors and the peptidoglycan cell
1100 wall. The OM is an asymmetric lipid bilayer. The outer leaflet of the OM contains mainly LPS
1101 molecules, which form contacts with integral outer membrane proteins (OMPs). The inner
1102 layer of the OM and the lipid layers of the cytoplasmic membrane contain phospholipids and
1103 membrane proteins.

1104

1105 **Figure 2. Simplified overview of the LPS biosynthesis.** Lipid A-Kdo_2 is synthesized on the
1106 cytoplasmic surface of the cytoplasmic membrane. The rest of the core is assembled to the
1107 lipid A-Kdo_2 and MsbA flips the whole complex to the periplasmic side of the cytoplasmic
1108 membrane. The O antigen is synthesized by cytoplasmic membrane-associated enzyme
1109 complexes using C55-undecaprenol phosphate (Und-P) as an acceptor for chain assembly
1110 and is then flipped to the periplasmic face of the membrane by one of the three pathways: (1)
1111 Wzy-dependent, (2) ABC-transporter-dependent, or (3) synthase-dependent. For simplicity,
1112 only the ABC-transporter pathway is represented. Once on the periplasmic side, the O
1113 antigen is linked to the lipid A-core by the WaaL ligase and the mature LPS molecule is then
1114 transported across the periplasm and inserted into the outer leaflet of the outer membrane by
1115 the Lpt (LPS transport) system, a complex that spans the Gram-negative cell envelope to
1116 deliver LPS to the outer membrane (E). OM, Outer membrane; CM, Cytoplasmic membrane.

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1118 **Figure 3. Lipid A modifications occurring in *P. aeruginosa* during adaptation to long-**
1119 **term chronic infection.** The basic tetra-acylated lipid A structure can be modified by:
1120 deacylation by PagL; palmitoylation by PagP; acylation by HtrB; acylation by LpxO; addition of
1121 Ara*p*4N by PmrAB on position 1 or 4'; and addition of phosphoethanolamine by ColRS on
1122 position 1 or 4'.

1123

1124 **Figure 4. Lewis antigen structures.** *H. pylori* can produce type 1 (based on a β-(1,3)-linked
1125 galactose-GlcNAc sugar backbone) and type 2 (based on a β-(1,4)-linked galactose-GlcNAc
1126 sugar backbone) Lewis antigens. Le^a and Le^x are built by addition of a fucose residue to the
1127 GlcNAc sugar of the type 1 and type 2 backbone, through α-(1,4) or α-(1,3)
1128 linkages, respectively. Le^b and Le^Y are built by addition of a fucose residue through α-(1,2)
1129 linkage to Le^a and Le^x structures, respectively. Sialyl-Le^x (SLe^x) is built by addition of a sialyl
1130 group to the Le^x antigen by a α-(2,3) linkage.