Lipopolysaccharide bioengineering in *Bordetella* species:

Path to vaccines with reduced reactogenicity

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Lipopolysaccharide bioengineering in *Bordetella* species: Path to vaccines with reduced reactogenicity.
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Lipopolysaccharide bioengineering in *Bordetella* species:

Path to vaccines with reduced reactogenicity

Bio-engineering van lipopolysacchariden in *Bordetella* soorten:
De weg naar vaccins met verminderde reactogeniciteit
(met een samenvatting in het Nederlands)

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General introduction

Bordetella and disease

The genus *Bordetella* currently comprises sixteen species of Gram-negative, pleomorphic, and aerobic coccobacilli. Some species, such as *Bordetella petrii* and *Bordetella tumbae*, have been isolated from various environmental sources, while others were found colonizing the respiratory tract of birds, i.e. *Bordetella avium* and *Bordetella hinzii*. However, the best studied group comprises those species able to cause infections in mammals and includes the "classical" species *Bordetella pertussis*, *Bordetella bronchiseptica* and *Bordetella parapertussis*. These classical species are able to colonize the respiratory tract of mammals, including humans, and are considered closely related subspecies that evolved from a common *B. bronchiseptica*-like ancestor (Nieves and Heininger, 2016; Hamidou Soumana et al., 2017).

B. bronchiseptica causes respiratory diseases in several mammalian species, such as infectious tracheobronchitis in dogs (a.k.a. kennel cough), snuffles in rabbits, or atrophic rhinitis in pigs (Goodnow, 1980). In addition, it has been related to zoonotic infections in immunocompromised humans and in healthy humans in close contact with infected animals (Mattoo and Cherry, 2005). Atrophic rhinitis, which is responsible for significant economic losses in animal production worldwide, is caused by co-infection with *B. bronchiseptica* and toxigenic isolates of *Pasteurella multocida*. The *P. multocida* toxin (PMT) is commonly produced by serogroup A and D strains and is a major virulence factor in atrophic rhinitis (Rajeev et al., 2003).

B. pertussis is a strictly human pathogen responsible for a highly contagious respiratory infection known as whooping cough or pertussis. It mainly affects young children below nine years old and is characterized by three phases: (i) the 1- to 2-weeks long catarrhal phase with mild symptoms similar to a common cold; (ii) the paroxysmal phase, which is characterized by intermittent spasmodic coughing followed by inspiratory whoops and can last for up to 10 weeks; and (iii) the convalescent phase, which corresponds to the bacterial clearance and gradual decline of symptoms and can persist for a month or even longer. In infants below 1 year old, the disease can be more aggressive, and bacteria can disseminate into the lungs resulting, in the most extreme cases, in respiratory failure and death (Melvin et al., 2014). For this reason, pertussis was a prominent cause of childhood morbidity and mortality prior to the introduction of vaccines against this pathogen.

B. parapertussis consists of two separate lineages that infect either humans or sheep. The lineage affecting humans produces pertussis-like infections with less severe symptoms. In addition, the frequency of pertussis cases caused by *B. parapertussis* is generally low compared to those caused by *B. pertussis* (Mattoo and Cherry, 2005). In this thesis, we focus on the two main pathogens of the genus, i.e. *B. pertussis* and *B. bronchiseptica*.

Vaccines against *Bordetella* infection

Around the 1940s, a rapid decline in pertussis incidence was experienced, which was due to the worldwide introduction of whole-cell pertussis (wP) vaccines consisting of inactivated bacteria (Mattoo and Cherry, 2005). The vaccination resulted, for example, in a 150-fold reduction in disease incidence in the first 30 years of use in the United States (Nieves and Heininger, 2016). However, local and systemic side effects upon vaccination, including fever, vomiting, anorexia, and convulsions, and particularly the unfounded association with brain damage, raised concerns about the safety of the wP vaccines and eventually resulted in their replacement by acellular (aP) formulations in many high-income countries in the 1990s (Melvin et al., 2014; Nieves and Heininger, 2016). These aP vaccines consist of one to five purified antigens (i.e. pertussis toxin, pertactin, filamentous haemagglutinin/adhesin (FHA), and fimbrial proteins 2 and 3), and they lack the main component responsible for wP vaccine reactogenicity, the endotoxin or lipopolysaccharide (LPS). Although the aP vaccines were proven to be effective and less reactogenic, the number of pertussis cases has been rising during the past decades in the countries using them. This seems to be related to three main factors: (i) the lack of protection against mucosal colonization resulting in increased asymptomatic carriage and transmission; (ii) the emergence of bacterial variants with mutations in genes encoding vaccine antigens that allowed for the spread of vaccine escape variants; and (iii) the fast waning of immune protection elicited by aP, which can be as short as 3.5 years. As a result, additional booster doses had to be incorporated to the vaccination schedules in many countries (Esposito et al., 2019).

During *B. pertussis* infection, innate and adaptive immune responses in the host cooperate to control the infection. Activation of innate immune cells, such as macrophages, results in the production of pro-inflammatory cytokines, e.g. interleukin (IL)-6, IL-8, IL-1β, and tumor necrosis factor (TNF)-α. It also results in the release of anti-inflammatory IL-10. The main cells involved in the adaptive response are B lymphocytes, which produce antibodies involved in bacterial opsonization and toxin neutralization, and CD4+ T cells, a.k.a. T-helper (Th) cells, which enhance microbicidal activity of innate immune cells and protect the lungs against reinfection. Th1 and Th17 responses seem to be of major importance in the control of *B. pertussis* infection and colonization (Kapil and Merkel, 2019).

The immune response elicited by wP vaccines is very similar to that of natural infections. It consists of a strong Th1 and a weaker Th17 reaction. On the other hand, aP vaccines induce mainly a Th2 response with low levels of Th1 and Th17, which prevents disease, albeit for a shorter period, but it does not avoid colonization and transmission of the pathogen (Esposito et al., 2019; Kapil and Merkel, 2019). Therefore, it is necessary to develop next-generation vaccines that provide long-term protection, avoid carriage and transmission, contain an abundant number of antigens, and present low reactogenicity.

Several approaches are being considered nowadays, including aP vaccines containing more antigens and adjuvants to increase durability and avoid colonization, outer membrane vesicle (OMV)-based formulations, and either live-attenuated or killed wP vaccines with reduced reactogenicity (Kapil and Merkel, 2019).

Since their development in the 1970s, vaccines against *B. bronchiseptica* are mostly applied in dogs and swine livestock. Adverse reactions evoked by whole-cell vaccines, such as fever, anorexia, and abortion, have a negative effect on animal welfare as well as on meat production. Despite being mostly disregarded in the past, these undesired effects have been a concern for the last decades (Deville et al., 2009; Ellis, 2015). LPS toxicity is again responsible for the undesired effects of these whole-cell vaccines in animals (Norimatsu et al., 1995; Garcia et al., 1998). The LPS of *B. bronchiseptica* can evoke intense inflammatory reactions (Mann et al., 2005) and, therefore, provide strong reactogenicity to the vaccine. To protect pigs against atrophic rhinitis, combined vaccines containing *B. bronchiseptica* and *P. multocida* toxin are under development in the last decades (Sakano et al., 1997; Rajeev et al., 2003). Commercially available injectable whole-cell vaccines consisting of inactivated bacteria are limited by their endotoxin content to avoid side effects (EMA website). Most of the currently licensed canine vaccines consist of live-attenuated strains and are commonly nasally administered. Such a route of administration is undesired in vicious and difficult to control animals and might result in accidental human infection during administration (Strausbaugh and Berkelman, 2003). In addition, immunocompromised pet owners, such as cystic fibrosis patients, can be infected by the live-attenuated vaccine strain (Moore et al., 2022). Since vaccination of companion animals with these formulations has to be repeated every 6 to 12 months, the risk of infection is very high for those individuals.

Gram-negative bacterial cell envelope

As bordetellae are Gram-negative bacteria, their cell envelope is composed of an inner membrane (IM), a cell wall made up of peptidoglycan, and an outer membrane (OM). The aqueous compartment between IM and OM containing the cell wall is called periplasm. In the periplasm, chaperones and proteases, such as survival protein A (SurA) and DegP, are involved in the correct folding or degradation of misfolded proteins (Rollauer et al., 2015). The IM is a phospholipid (PL) bilayer that contains proteins involved in energy production, lipid biosynthesis, protein export, and nutrient transport, amongst others. The OM is an asymmetric lipid bilayer, which contains PLs in the inner leaflet and LPS in the outer leaflet. The proteins present in the OM are mainly lipoproteins, which are usually embedded in the inner leaflet of the OM with their lipid moiety, and β-barrel proteins, which are transmembrane proteins commonly referred to as OM proteins (OMPs) (Silhavy et al., 2010). OMPs form closed cylinders of antiparallel β-strands integrated into the OM by the β-barrel assembly machinery (BAM). OMPs are translocated across the IM by the SecYEG translocon and inserted from the periplasm into the OM via

the BamA subunit of the BAM complex (Tommassen, 2010; Rollauer et al., 2015; Doyle and Bernstein, 2019). OMPs are implicated in nutrient uptake, protein secretion, cell adhesion, and membrane remodeling, amongst others. Some of them are considered as important virulence factors during bacterial infections. The most abundant OMPs found in Gram-negative bacteria are channel-forming proteins called porins. Porins allow for the passive diffusion of small hydrophilic compounds through the membrane (Nikaido, 2003). In *Bordetella*, OmpP is the dominant porin, and it appears to be expressed in all conditions (Armstrong et al., 1986; Finn et al., 1995). Another important group of OMPs is constituted of autotransporters. These proteins are synthesized as precursors consisting of a signal sequence for translocation across the IM via the Sec complex, a β domain that integrates as a β-barrel into the OM, and a passenger domain that is translocated to the cell surface or released into the extracellular environment to mediate its often virulence-related function (Dautin and Bernstein, 2007). Some relevant autotransporters found in bordetellae are the *Bordetella* resistance-to-killing protein A (BrkA), which protects the bacteria against the bactericidal activity of serum (Rambow et al., 1998; Passerini de Rossi et al., 1999), and pertactin, which mediates adhesion to mammalian cells and resistance to neutrophil-mediated clearance (Emsley et al., 1996). Other OMPs involved in virulence are the iron-regulated protein FauA, which functions as a receptor for the siderophore alcaligin (Brickman and Armstrong, 1999; Register et al., 2001), the major adhesin FHA (Locht et al., 1993), and the type I fimbriae (Willems et al., 1992).

Lipopolysaccharide (LPS)

The OM of Gram-negative bacteria functions as an effective permeability barrier for large and for small hydrophobic molecules, including many antibiotics. LPS is a major and usually essential component of the OM. It is an amphiphilic molecule and usually consists of three moieties, i.e. lipid A, a core oligosaccharide, and a polysaccharide known as O-antigen. The latter is absent in some bacterial species, including *B. pertussis*, and such LPS is also referred to as lipooligosaccharide (LOS). The hydrophobic character of lipid A inhibits hydrophilic compounds from crossing the bilayer, while the hydrophilic character of the core and the O-antigen as well as the dense packing of the acyl chains of lipid A in the membrane make the OM impermeable to hydrophobic molecules. LPS presents several negatively charged groups that create repulsive forces between the molecules. These charges are stabilized by divalent cations, which create crosslinks between the LPS molecules and thereby enhance the OM barrier function. Furthermore, the O-antigen reduces the accessibility of antibodies, bacteriocins, and bacteriophages to conserved receptors located deeper in the OM (van der Ley et al., 1986a, 1986b). Several Gram-negative bacteria, including *Borrelia burgdorferi* and *Sphingomonas* spp., produce other glycolipids instead of LPS. However, to our knowledge, only five species of Gram-negative bacteria that originally produce LPS have been shown to be viable after interference with LPS biosynthesis, i.e., *Neisseria meningitidis* (Steeghs et al., 1998), *Moraxella catarrhalis* (Peng et al., 2005), *A. baumannii* (Moffatt et al., 2010), *Yersinia ruckeri* (Altinok et al., 2016),

and *Caulobacter crescentus* (Zik et al., 2022). The absence of LPS in the outer leaflet of the OM is mainly compensated with PLs, but lipoprotein increments have been observed as compensatory method as well (Putker et al., 2015). To maintain the membrane asymmetry, mislocalized PLs are normally transported back to the IM by the maintenance-of-lipid-asymmetry (Mla) system or degraded by the OM phospholipase A (OMPLA, a.k.a. PldA). In *A. baumannii*, inactivation of the Mla system and OMPLA improved the fitness of an LPS-deficient mutant (Powers and Trent, 2018). OMPLA and the Mla system have recently been identified also in *B. pertussis*, and the combined inactivation of these mechanisms resulted in accumulation of PLs in the outer leaflet of the OM (de Jonge et al., 2022b).

LPS structure and biosynthesis

Lipid A

The lipid A moiety anchors the LPS into the membrane. It consists of a glucosamine (GlcN) disaccharide backbone phosphorylated at the 1 and 4′ positions and substituted at positions 2, 2′, 3, and 3′ with fatty acyl chains (Figure 1). These fatty acids are hydroxylated and can be substituted with secondary acyl chains. Additionally, the phosphate groups can be decorated with phosphoethanolamine (PEA) or sugar groups (Raetz and Whitfield, 2002).

Lipid A is synthesized in the cytoplasm and at the inner leaflet of the IM via a conserved pathway, which is known as the Raetz pathway and has been extensively described in *Escherichia coli* (Figure 2). It consists of nine steps catalyzed by nine enzymes in a well-determined order (Raetz and Whitfield, 2002). It starts with the reversible addition of an acyl chain to a uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) by the acyltransferase LpxA. Next, the first committed step of the lipid A synthesis takes place by the de-acetylation of the UDP-GlcNAc moiety by the UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase LpxC. After the introduction of another acyl chain by LpxD, a portion of the UDP-2,3-diacylglucosamine molecules generated is cloven by the pyrophosphatase LpxH resulting in 2,3-diacylglucosamine-1-phosphate. This product is condensed with an unmodified UDP-2,3-diacylglucosamine by LpxB, resulting in a tetra-acylated, singly phosphorylated GlcN disaccharide. The molecule resulting after 4′-phosphorylation by LpxK is known as lipid IV_A and is the intermediate to which the first residue of the core, i.e. 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), is added by the Kdo transferase KdtA (a.k.a. WaaA) (Raetz and Whitfield, 2002). In *E. coli*, KdtA is bi-functional inasmuch as it attaches two Kdo residues to lipid IV_A, but in some other species, including *Bordetella* spp., only a single Kdo is attached (Isobe et al., 1999; Raetz and Whitfield, 2002). After that, the secondary acyl chains are introduced by the late acyltransferases, which are denominated LpxL and LpxM in *E. coli*, where they introduce acyl chains to the hydroxyl groups of the primary chains at positions 2′ and 3′, respectively (Raetz and Whitfield, 2002). In bordetellae, the late acyltransferases are denominated LpxL1 and LpxL2 and introduce a secondary 2-hydroxy laureate at position 2 and a secondary myristate at position 2′, respectively (Geurtsen et al., 2007).

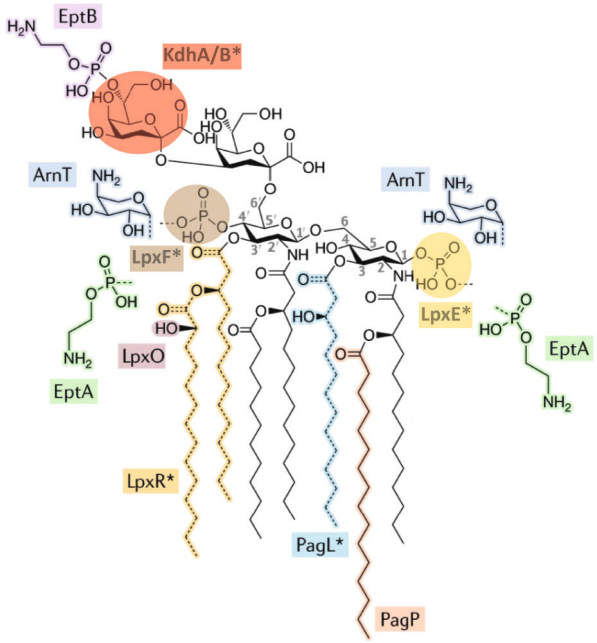

Figure 1. Schematic representation of the Kdo$_2$-lipid A structure in *E. coli* including an overview of common post-synthetic modifications found in various bacteria. The modified groups and the enzymes responsible for these modifications are color coded similarly. Enzymes catalyzing elimination reactions are indicated with asterisks; the others mediate substitutions. Carbon atoms in the GlcN disaccharide backbone are numbered. Adapted from Simpson and Trent (2019) and reproduced by permission of the publisher.

Core oligosaccharide

The LPS core is often divided into two regions, namely the inner and the outer core. The inner core is generally well conserved within a species and consists of Kdo and heptose residues, which are often modified with various substituents. While the Kdo residues are inserted during the Raetz pathway, the extension with heptose usually happens after the action of the late acyltransferases. The outer core is less conserved even within a species and is formed by the consecutive addition of different sugars, mainly hexoses, in a specific order. Once the lipid A-core molecule (depicted as LOS in Figure 2) is synthesized, it is flipped from the inner leaflet to the outer leaflet of the IM by the ATP-binding cassette (ABC) transporter MsbA (Figure 2), which is substrate specific and most efficient after the insertion of the secondary acyl chains and the complete core (Bertani and Ruiz, 2018).

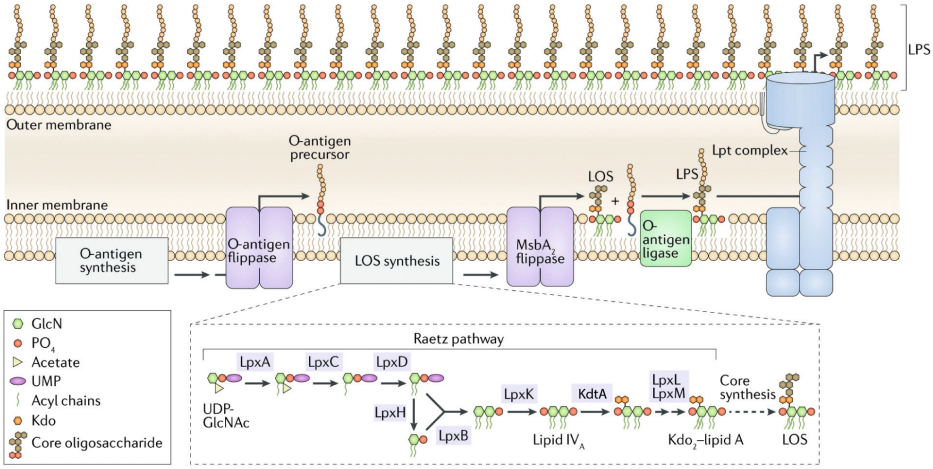

Figure 2. Representation of LPS biogenesis in *E. coli*. The Kdo2-lipid A fragment is synthesized at the inner leaflet of the IM during the Raetz pathway and, subsequently, the rest of the core region is supplemented. Then, MsbA flips the lipid A-core moiety (indicated as lipooligosaccharide, LOS) to the outer leaflet of the IM, where the separately synthesized O-antigen, if present, is ligated to the lipid A-core before transport of the mature LPS to the OM by the Lpt complex. Enzymes implicated in the Raetz pathway and proteins involved in LPS transport are indicated. Adapted from Simpson and Trent (2019) and reproduced by permission of the publisher.

In bordetellae, the branched dodecasaccharide core starts with two heptose residues attached to the single Kdo (Caroff et al., 2000). This Kdo residue is further non-stoichiometrically substituted with a phosphate group, which can be decorated with PEA (Figure 3). The genes responsible for the insertion of the sugars of the outer core have been characterized only partially (Geurtsen et al., 2009). The outer core is substituted with a terminal non-repeating trisaccharide consisting of α-*N*-acetyl-GlcN (GlcNAc), β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid (Man2NAc3NAcA), and β-L-2-acetamido-4-methylamino-fucose (Fuc2NAc2NMe) (Figure 3) (Caroff et al., 2000). The trisaccharide is synthesized on undecaprenyl phosphate and translocated across the IM before it is attached *en bloc* to the outer core. The genes responsible for the addition of these sugars appear in the *wlb* operon (Allen and Maskell, 1996; Allen et al., 1998). When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), *B. pertussis* LPS presents two bands: band A corresponds to the full lipid A-core molecules, while band B lacks the terminal trisaccharide and is usually far less abundant than band A (Figure 3) (Peppler, 1984).

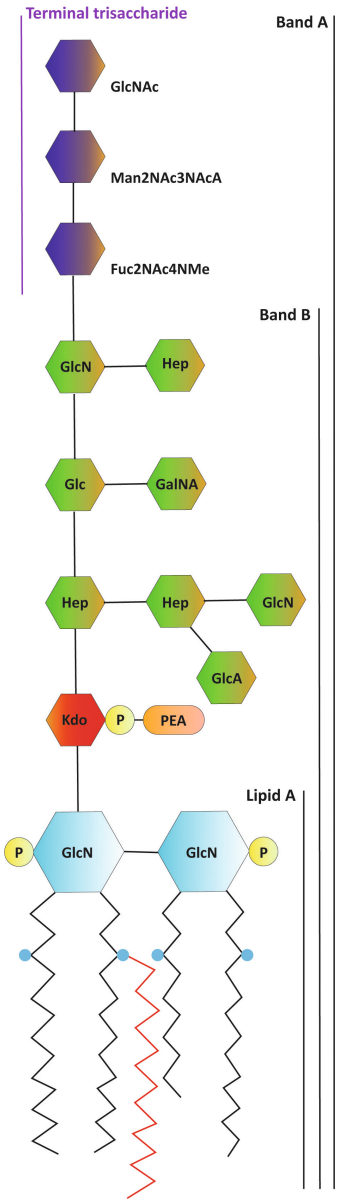

Figure 3. Schematic representation of *B. pertussis* LPS based on the study of Caroff et al. (2000). The various sugar units are depicted with hexagons, the phosphate groups with yellow circles, the PEA substitution with a rounded rectangle, and the hydroxylation of fatty acids with blue circles. The secondary C_{14} chain at position 2', which is inserted by the acylase LpxL2, is indicated in red. The portions of the LPS corresponding with lipid A, band A, band B, and the terminal trisaccharide are indicated with vertical lines. Abbreviations: GlcN, glucosamine; P, phosphate; Kdo, 3-deoxy-d-*manno*-oct-2-ulosonic acid; PEA, phosphoethanolamine; Hep, heptose; GlcA, glucuronic acid; Glc, glucose; GalNA, galactosaminuronic acid; Fuc2NAc4NMe, β-L-2-acetamido-4-methylamino-fucose; Man2NAc3NAcA, β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid; GlcNAc, α-*N*-acetyl-GlcN.

O-antigen

The outermost moiety of LPS is the O-antigen, a highly diverse polysaccharide formed by a variable number of repeating sugar or oligosaccharide units. These repeating units are assembled independently from the rest of the LPS at the inner leaflet of the IM, and eventually ligated as a polymer to the lipid A-core at the outer leaflet of the IM (Figure 2). Polymerization and transport of the O-antigen or its subunits generally follows one of two distinct pathways, the Wzy polymerase-dependent pathway or the ABC-transporter-dependent pathway (Kalynych et al., 2014; Bertani and Ruiz, 2018). In the first case, the O-antigen repeats are synthesized, flipped individually to the periplasmic side of the IM, and then polymerized by Wzy. After that, the complete O-antigen structure is connected to the rest of the LPS by the ligase WaaL. In the ABC-transporter-dependent pathway, the polymerization of the entire O-antigen occurs at the cytoplasmic side of the IM. Then, the molecule is flipped to the outer leaflet of the IM by a specialized ABC transporter and ligated to the lipid A-core moiety by WaaL (Kalynych et al., 2014; Bertani and Ruiz, 2018). The latter pathway is used by *Bordetella* spp. including *B. bronchiseptica*, and the genes involved in O-antigen biosynthesis are encoded in the *wbm* locus (Preston et al., 1999). However, this locus is entirely replaced by an insertion sequence in *B. pertussis*, which is the reason for the lack of O-antigen in this species (Preston et al., 1999). The length of the O-antigen present in surface-exposed LPS is heterogeneous, as reflected in the ladder-like pattern of LPS bands usually seen in SDS-PAGE gels. This is the result of the insertion of variable numbers of repeating units during the polymerization of the O-antigen. Due to post-synthetic modifications of the O-antigen structure, the banding pattern might vary as has been described in *B. bronchiseptica* (King et al., 2009). Variability in the O-antigen composition is required to escape from bactericidal antibodies and from phages that use O-antigen as primary receptor, inasmuch as the O-antigen is the outermost part of the bacteria. Thus, variation is needed to escape, as a species, from elimination by such selective forces. Furthermore, the O-antigen plays an important role in bacterial survival in the host since it helps to avoid phagocytosis, resists the action of the complement, mimics molecular structures of the host to avoid recognition, and facilitates colonization of epithelial surfaces or intracellular survival (Lerouge and Vanderleyden, 2002). In addition, the O-antigen is important for the vaccine efficacy for at least some species presenting complete LPS, including *Bordetella* spp., as has been reported for *B. parapertussis* (Zhang et al., 2009).

Once the O-antigen has been ligated to the lipid A-core moiety, the mature LPS is exported to the cell surface by the LPS transport (Lpt) machinery (Figure 2), which is considered an envelope-spanning translocating machine that recognizes the lipid A portion of the LPS regardless of the presence of O-antigen (Putker et al., 2015).

Regulation of LPS biosynthesis

As mentioned above, LpxC catalyzes the first committed step in LPS synthesis and, probably, that is why many of the regulatory mechanisms of LPS production operate

through control of LpxC. One of the mechanisms described for some enteric bacteria is conducted by the FtsH/YciM (a.k.a. LapB) protease complex that degrades LpxC (Guest et al., 2021) and, in turn, is antagonized by YejM (a.k.a. PbgA) (Figure 4). The latter protein detects increased periplasmic levels of LPS by recognition of a single phospho-GlcNAc unit of the lipid A. When periplasmic levels of LPS are low, YejM functions as a negative regulator of YciM. However, when LPS binds to YejM, YciM can promote FtsH-mediated degradation of LpxC (Clairfeuille et al., 2020; Fivenson and Bernhardt, 2020; Guest et al., 2020). Additionally, FtsH can regulate LPS biosynthesis through degradation of KdtA (Katz and Ron, 2008). Furthermore, reduced levels of LPS in the OM indirectly inhibit LpxC degradation through OMPLA (May and Silhavy, 2018). As a consequence of LPS deficiency, PLs will be mislocalized to the outer leaflet of the OM, where they are degraded by OMPLA. The released fatty acids are transported to the cytoplasm where they function as second messengers and eventually inhibit FtsH-mediated degradation of LpxC. Disparate LpxC regulatory systems have been described in other bacteria, for example in *N. meningitidis*, where inactivation of the YciM homolog, called Ght, did not result in increased, but in reduced LpxC activity and, therefore, lowered LPS levels (Putker et al., 2014; Guest et al., 2021), and in *Pseudomonas aeruginosa*, where LpxC doesn't seem to be prone to proteolytic degradation (Langklotz et al., 2011; Guest et al., 2021). Additionally, LPS levels are regulated by transcriptional control of genes involved in lipid A synthesis and LPS transport via the σ factor RpoE, which is activated as a stress response when LPS or misfolded OMPs accumulate in the periplasm (Putker et al., 2015).

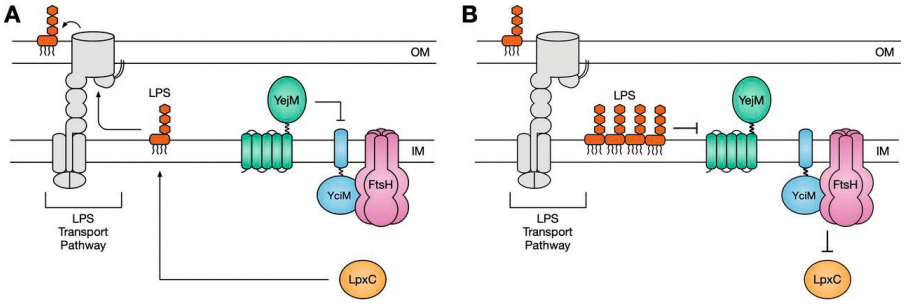

Figure 4. FtsH/YciM-mediated regulatory mechanism of LPS production. (**A**) With regular levels of LPS, LpxC degradation by the FtsH/YciM protease complex is inhibited by YejM, which allows for continued LPS biosynthesis and subsequent transport to the OM. (**B**) With high levels of LPS synthesis or defective LPS transport, accumulation of LPS in the inner membrane inhibits the antagonistic activity of YejM allowing for degradation of LpxC by FtsH/YciM, which then reduces LPS biosynthesis. Reproduced from Guest et al. (2020).

Lipid A-core modification systems

The variation that is observed when the lipid A structures of different bacteria are compared is in part caused by different substrate specificities of the enzymes of the Raetz pathway. For example, the acyltransferases can differ in their selectivity for the length, branching, and saturation of the fatty acids they incorporate, and the late acyltransferases

can also vary in the position where they attach the secondary acyl chain (Raetz et al., 2007). Before core synthesis is continued by the heptosyl transferases, the incorporated Kdo can be modified by enzymes as KdoO or KdkA, which mediate hydroxylation or phosphorylation, respectively, of the Kdo (Valvano, 2022). Furthermore, the production of enzymes that function at the same position may shift in response to environmental conditions. That is the case, for example, for the acyltransferases LpxL and LpxP in *E. coli*. While LpxL incorporates laurate as a secondary acyl chain to position 2′ of lipid A above 30 °C, its homologue LpxP transfers palmitoleate to the same position at low temperatures (Carty et al., 1999). Environmental conditions also regulate the expression of enzymes that modify the LPS structure after the Raetz pathway. Such enzymes, the presence of which strongly varies between different bacterial species, mainly target the acyl chains and the phosphate groups of lipid A (Figure 1) (Raetz et al., 2007; Simpson and Trent, 2019). The acyl chains of lipid A can be modified at the IM, for example by LpxO, which hydroxylates secondary acyl chains at their C-2 position. Modification of the number of fatty acids is usually catalyzed by OM-based deacylases, such as LpxR and PagL, which remove the (acyloxy)acyl chains from positions 3′ and 3, respectively, and acylases, such as PagP, which transfers a secondary acyl chain, i.e., a palmitate, to the primary chains at positions 2 or 3′. This palmitate is extracted from PLs mislocalized in the outer leaflet of the OM and transferred to lipid A usually in response to changes in environmental factors such as limitation of Mg^{2+} in the medium or the presence of antimicrobial peptides (Guo et al., 1998; Preston et al., 2003). The phosphate groups present at positions 1 and 4′ of lipid A can be removed by the phosphatases LpxE and LpxF, respectively. Alternatively, substituents can be attached to the phosphates. Some commonly found groups decorating the phosphates are 4-aminoarabinose, introduced by ArnT, and PEA, introduced by EptA. The phosphate group attached to the core Kdo can also be decorated with PEA, which is catalyzed then by EptB. Also, the number of Kdo sugars can be reduced by the Kdo hydrolase system KdhA/B, which was first characterized in *Francisella tularensis*, *Helicobacter pylori*, and *Legionella pneumophila* (Chalabaev et al., 2010). All these modifications are commonly used to adjust membrane fluidity and stability, and for immune evasion, host recognition, and resistance to antimicrobial peptides (Raetz et al., 2007; Simpson and Trent, 2019).

Like many other virulence pathways, expression of LPS modification systems is often regulated by two-component systems. These two-component systems consist of a sensor kinase, which senses environmental signals, and a response regulator, which regulates gene expression. The two-component systems PhoPQ and PmrAB have been extensively characterized in *Salmonella enterica* serovar Typhimurium, but homologs are found in many other bacteria. They affect the lipid A structure upon changes in environmental factors such as pH, concentration of divalent cations, and the presence of antimicrobial peptides. Transcriptional regulation of OM enzymes, such as PagP, PagL, and LpxR, is a relatively slow process because it requires production and incorporation of the enzymes

into the OM. Therefore, additional control of the enzymatic activity, usually at the level of recognition or availability of their substrates, is required (Simpson and Trent, 2019).

In *Bordetella*, the BvgAS two-component system regulates the expression of virulence factors. During the Bvg$^+$ phase, virulence-activated genes, such as those encoding fimbriae (*fim*), filamentous hemagglutinin/adhesin (*fha*), and pertactin (*prn*), are expressed (Moon et al., 2017). Also genes encoding enzymes involved in the modification of LPS are controlled by BvgAS, as is the case for *pagP* (Preston et al., 2003).

Bordetella lipid A structure and modification

Lipid A of *B. pertussis* is known as a penta-acylated molecule with four primary and one secondary fatty acids attached to the GlcN disaccharide (Figure 5A). All primary acyl chains are 3-OH-C_{14} fatty acids, except for that in position 3, which is 3-OH-C_{10} (Caroff et al., 1994). The secondary acylation is a C_{14} chain and is stoichiometrically introduced by LpxL2 (Geurtsen et al., 2007). No evidence of phosphatase activity has been found in *Bordetella*, but either one or both of the phosphate groups of lipid A can be decorated with GlcN, a reaction which is catalyzed by the glycosyltransferase LgmB (formerly ArnT) (Marr et al., 2008). Expression of the *lgmB* gene is Bvg dependent, and it seems to be a strain-specific modification that is usually absent or present in only small portions in *B. pertussis* cells (Marr et al., 2008). The phosphate group that decorates the single Kdo sugar of the core appears commonly substituted with PEA (Caroff et al., 2000).

Despite being two closely related bacterial species, the lipid A of *B. bronchiseptica* (Figure 5B) presents several structural differences compared to that of *B. pertussis*. The primary 3-OH-C_{10} acyl chain at position 3 is usually absent in *B. bronchiseptica* due to the activity of deacylase PagL (Geurtsen et al., 2005; 2006). Although a *pagL* homolog is present in *B. pertussis*, the gene is disrupted by a frameshift mutation and, thus, the acyl chain remains present in the LPS structure (compare Figure 5A and B) (Geurtsen et al., 2006). Besides the secondary acyl chain introduced by LpxL2, an additional secondary acylation (2-OH-C_{12}) is present in the lipid A of *B. bronchiseptica*, which is stoichiometrically inserted by acylase LpxL1 (Basheer et al., 2011; MacArthur et al., 2011). Although a copy of the gene encoding LpxL1 is present in *B. pertussis*, the acyl chain has been observed in its lipid A only when the gene was artificially overexpressed from a plasmid (Geurtsen et al., 2007). The reason for the lack of expression of the chromosomal gene remains unknown. The hydroxyl group in the secondary 2-OH-C_{12} attached by LpxL1 is introduced by LpxO (MacArthur et al., 2011). Another secondary acyl chain can be found in the lipid A of *B. bronchiseptica*, i.e., a palmitate (C_{16}), which is attached to the primary chain at position 3′ by PagP (Figure 5B), a Bvg-regulated enzyme (Preston et al., 2003). When this enzyme is activated, the lipid A of *B. bronchiseptica* becomes hexa-acylated. In *B. pertussis*, the *pagP* gene presents an insertion sequence in the promoter region, which prevents its expression (Preston et al., 2003). GlcN decoration of one or both phosphate groups also appears to be a common feature in *B. bronchiseptica* LPS (Marr et al., 2008; Rolin et al., 2014).

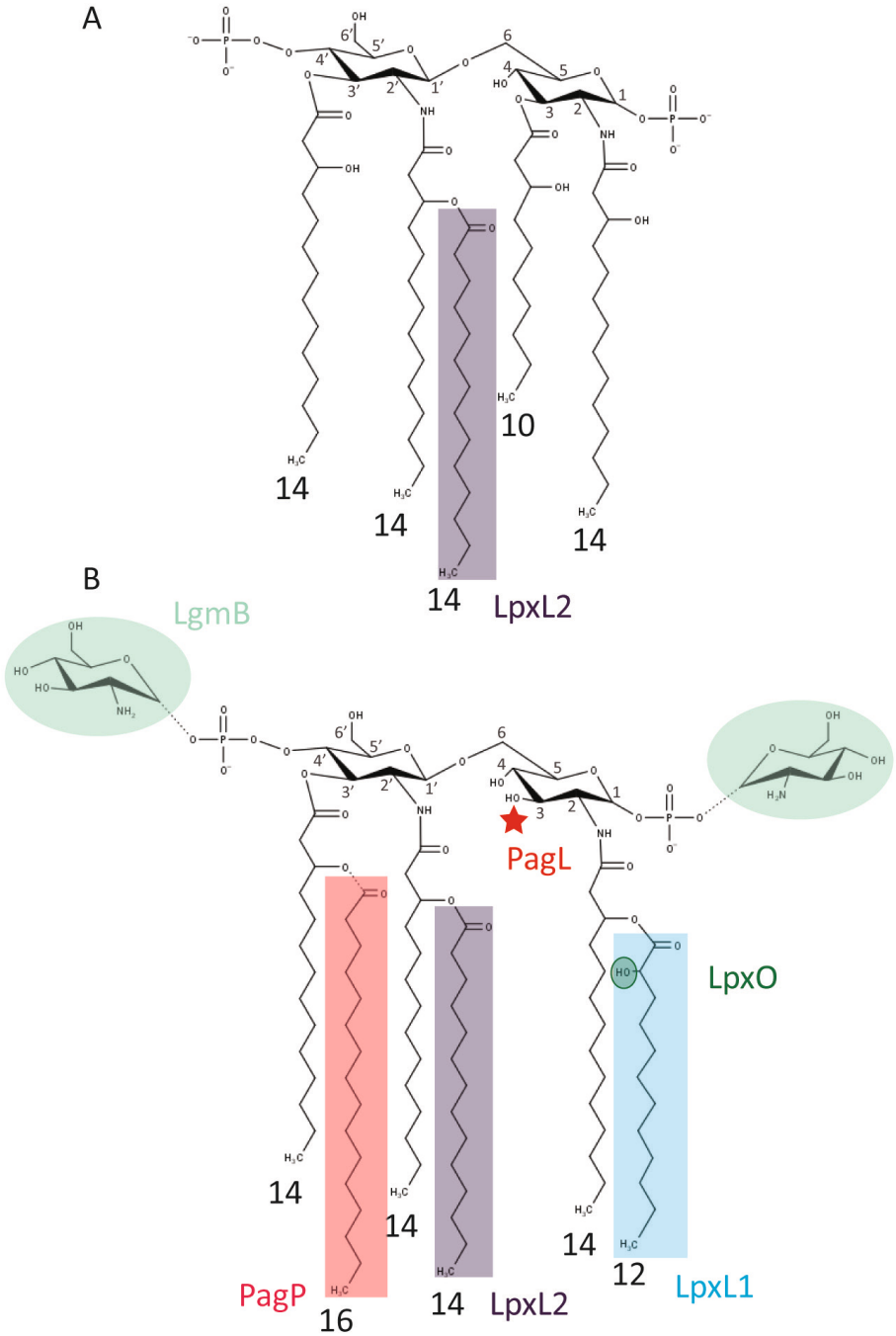

Figure 5. Structures of the lipid A of *B. pertussis* (A) and of *B. bronchiseptica* (B). The non-stoichiometric modifications are denoted by dashed bond lines. The enzymes responsible for insertions are color coded and the inserted chemical groups are indicated in the same color. The position of the fatty acid excised by PagL is indicated with a red star. Carbon atoms in the GlcN disaccharide backbone are numbered.

LPS and endotoxicity

LPS is responsible for the endotoxicity associated with infections by Gram-negative bacteria. At low concentrations, LPS facilitates protection of the infected host by stimulation of the immune system. At high concentrations, it can result in septic shock and death due to a systemic inflammatory response and multiorgan failure (Raetz and Whitfield, 2002; Maeshima and Fernandez, 2013; Matsuura, 2013). LPS is a so-called pathogen-associated molecular pattern (PAMP). The innate immune system of mammalians uses pathogen recognition receptors (PRRs) to identify PAMPs. One of the main PRR families is constituted by the Toll-like receptors (TLRs). LPS, typically its lipid A moiety, is one of the most potent PAMPs of the cell envelope of Gram-negative bacteria and is recognized by TLR4 and its co-receptor myeloid differentiation factor 2 (MD-2) of immune cells (Figure 6) (Raetz and Whitfield, 2002; Maeshima and Fernandez, 2013; Matsuura, 2013). LPS released from the bacterial surface forms complexes with LPS-binding protein (LBP) and either soluble or cell-surface-associated CD14, which delivers LPS to MD-2. LPS binding then triggers the dimerization of TLR4/MD-2 complexes. The receptor dimer formed consists of two copies of the TLR4–MD2–LPS complex. In the case of the hexa-acylated LPS of *E. coli*, lipid A is positioned with five acyl chains packed into the hydrophobic pocket of MD2 from one monomeric TLR4-MD-2 complex, while the remaining chain is located outside of the pocket and interacts with the TLR4 molecule of the opposite complex, thus triggering receptor dimerization (Park et al., 2009). In addition, the phosphate groups of lipid A contribute to receptor dimerization by interacting with positively charged clusters in MD2 and in both TLR4 molecules of the receptor dimer. The TLR4/MD-2 complex can signal through two distinct pathways: (i) the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway or (ii) the Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing interferon β (IFN-β) (TRIF)-dependent pathway. At the cell surface, TLR-4/MD-2 receptor dimerization activates the MyD88-dependent pathway, which results in the activation of the transcription factor nuclear factor kappa B (NF-κB) and the subsequent production of pro-inflammatory cytokines, such as IL-6 and TNFα. Additionally, the TLR4/MD-2 complex is internalized into endosomes and then, it activates the TRIF-dependent pathway, which is characterized by the production of IFN-β and several IFN-inducible proteins (Maeshima and Fernandez, 2013; Matsuura, 2013).

Additionally, an intracellular-LPS-sensing pathway called noncanonical inflammasome, which can cause sepsis without the involvement of TLR4, has been described in recent years (Pfalzgraff and Weindl, 2019). This pathway is triggered by the recognition of LPS by caspases in the cytosol and results in pyroptotic cell death and inflammasome activation followed by secretion of IL-1β and IL-18. While inflammasome activation seems to require simultaneous activation of TLR4 or other TLRs, pyroptosis is completely independent of PRRs. This pathway appears to be activated in the cytosol by free LPS and OMVs (Pfalzgraff and Weindl, 2019).

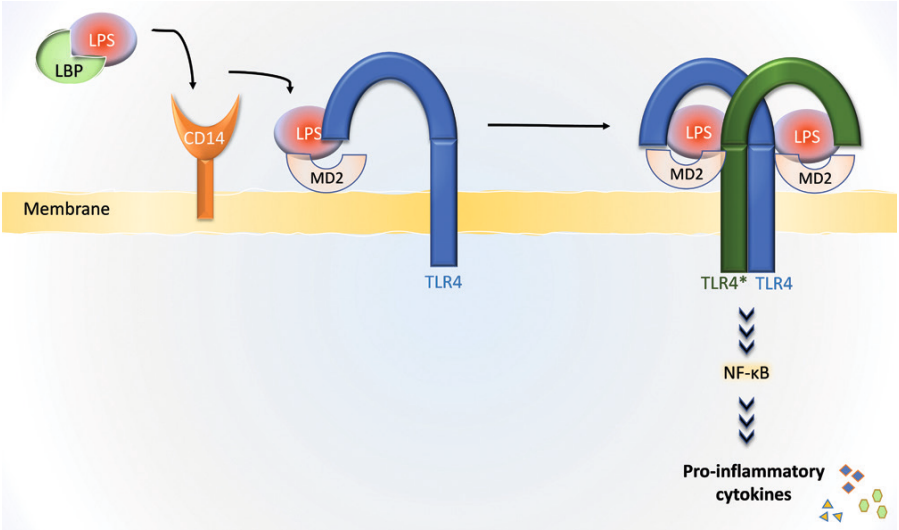

Figure 6. Schematic representation of LPS-induced activation of TLR4/MD-2 signaling. LPS collected by the LPS-binding protein (LBP) is transferred to the co-receptor CD14, which presents the LPS to the TLR4-MD-2 complex at the cell surface. Dimerization of the receptor complex triggers the activation of transcription factor NF-κB and, eventually, production of pro-inflammatory cytokines.

Lipid A engineering

As mentioned above, the endotoxic reaction elicited by LPS is one of the main reasons of the undesired effects evoked by whole-cell vaccines against Gram-negative bacteria. Structural modifications of lipid A can affect its TLR4-stimulating activity and, therefore, its endotoxicity. Usually, the number and the length of the fatty acids as well as the number and the substitutions of the phosphate groups determine lipid A endotoxicity (Raetz et al., 2007; Matsuura, 2013). Each bacterial species produces specific lipid A structures depending on constitutive and regulated production of enzymes. Genetic manipulation of these mechanisms can allow for directed modification of the LPS structure. Furthermore, heterologous gene expression facilitates structural modifications that are, otherwise, absent in the target species. These strategies have already been applied in several bacterial species and have proved to reduce endotoxicity (Simpson and Trent, 2019; Kawahara, 2021). Thus, this approach can be used in vaccine development. Reduction of endotoxicity using lipid A engineering has also been applied in *B. pertussis*. Increased lipid A acylation by artificial PagP expression (Geurtsen et al., 2006) or by overexpression of LpxL1 (Geurtsen et al., 2007) resulted, as expected, in increased TLR4 signaling, while deacylation by PagL reduced the TLR4-stimulating capacity of LPS (Geurtsen et al., 2006). However, the latter modification increased TLR4-stimulating activity of whole cells, which was probably the result of increased release of the deacylated LPS from the cell surface making it more accessible to the receptor on immune cells. Furthermore, the complete absence of GlcN decorations present at the phosphate groups of lipid A by inactivation

of *lgmB* resulted in reduced LPS endotoxicity, but only with human TLR4 and not with the murine receptor (Geurtsen et al., 2009; Marr et al., 2010). Similarly, *lgmB* inactivation in *B. bronchiseptica* showed no effect on endotoxicity with murine macrophages (Rolin et al., 2014). In *B. pertussis*, changes in the length of acyl chains achieved by heterologous expression of acyltransferases from other bacterial species can also drastically affect TLR4 signaling (Arenas et al., 2019, 2020).

A strategy alternative to those focusing on novel whole-cell vaccines is the use of OMVs. These blebs are spontaneously released from the OM depending on the environmental conditions. They contain bacterial surface antigens including LPS, have robust immunogenic capacity, and proved to be effective for vaccine development in *N. meningitidis* (Nøkleby et al., 2007; Gorringe and Pajon, 2012; Schwechheimer and Kuehn, 2015). In *B. pertussis*, an OMV-based vaccine can induce an immune protection similar to that induce by wP, at least in mouse models (Raeven et al., 2016). Similar observations were made with *B. bronchiseptica* OMVs (Bottero et al., 2018). Nevertheless, these OMVs might require additional reduction of endotoxicity to be used as safe vaccine alternatives (Asensio et al., 2011). Moreover, the spontaneous production of OMVs in *Bordetella* spp. seems to be low for a cost-effective production of vaccines and needs enhancement (Hozbor et al., 1999). Different approaches have been developed recently to increase the OMV production in *B. pertussis* and *B. bronchiseptica* (de Jonge et al., 2021, 2022a, 2022b). Additionally, structural modifications of lipid A can result in increased OMV formation as was demonstrated in *Salmonella* (Elhenawy et al., 2016). Thus, lipid A engineering could result in a combined detoxifying and OMV-enhancing effect.

Aims and outline of the thesis

Adverse reactions are the greater downside for vaccination safety and acceptance. Reliable next-generation vaccines are required to curb the resurgence of pertussis. Similarly, riskless and effective vaccines should be developed against *B. bronchiseptica*. Development of novel whole-cell vaccines that are genetically detoxified by LPS engineering has been on the rise over the last decades because it allows for the production of affordable and effective vaccines with reduced reactogenicity. In this thesis, we sought to reduce whole-cell endotoxicity in *B. bronchiseptica* (**chapters 2** and **3**) and *B. pertussis* (**chapters 4** and **5**) by genetic modifications. We have also investigated the consequences of the modifications on the biology of the bacteria.

In **chapter 2**, we inactivated the genes encoding the lipid A acylases PagP and LpxL1 in *B. bronchiseptica*. The consequences of the mutations on TLR4 activation as well as on various phenotypes, such as bacterial fitness in the presence of macrophages, susceptibility to antimicrobials, and biofilm formation, were explored. Structural analysis of lipid A of the *lpxL1* mutant revealed not only the expected loss of an acyl chain, but also of the GlcN decorations on the phosphates. To investigate the contribution of the

loss of GlcN decorations to the various phenotypes of the *lpxL1* mutant, we inactivated in **chapter 3** the gene encoding the glycosyltransferase LgmB and compared its phenotypes with those of the *lpxL1* mutant. Furthermore, we investigated in this chapter the suitability of the *Galleria mellonella* in vivo model for investigation of *B. bronchiseptica* virulence. In **chapter 4**, we explored the possibility of lipid A dephosphorylation in *B. pertussis* by heterologous expression of the phosphatases LpxE and LpxF from *Francisella novicida*. Moreover, we tested the ability of the bi-functional Kdo transferase from *E. coli* to introduce a second Kdo residue in *B. pertussis* LPS. Additionally, we inactivated the genes implicated in the insertion of the different residues at this level, i.e. *kdtA*, *kdkA*, and the newly identified *eptB*. The effect of these genetic modifications on the LPS structure, TLR4 activation, and cell morphology were evaluated. In **chapter 5**, we explored an alternative approach to reduce whole-cell endotoxicity in *B. pertussis*, i.e. by modulating the production of LPS. This was achieved by regulated expression of *lpxC*. The consequences of the diminished amounts of LPS on TLR4 activation, cell morphology, and OMV production were investigated. In **chapter 6**, the major findings of this thesis are summarized and discussed, together with the prospects of detoxified whole-cell vaccines against *B. pertussis* and *B. bronchiseptica*.

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2

Reduction of endotoxicity in *Bordetella bronchiseptica* by lipid A engineering: Characterization of *lpxL1* and *pagP* mutants

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Abstract

Whole-cell vaccines against Gram-negative bacteria commonly display high reactogenicity caused by the endotoxic activity of lipopolysaccharide (LPS), one of the major components of the bacterial outer membrane. Underacylation of the lipid A moiety of LPS has been related with reduced endotoxicity in several Gram-negative species. Here, we evaluated whether the inactivation of two genes encoding lipid A acylases of *Bordetella bronchiseptica*, i.e. *pagP* and *lpxL1*, could be used for the development of less reactogenic vaccines against this pathogen for livestock and companion animals. Inactivation of *pagP* resulted in the loss of the secondary palmitate chain at the 3' position of lipid A, but hardly affected the potency of the LPS to activate the Toll-like receptor 4 (TLR4). Inactivation of *lpxL1* resulted in the loss of the secondary 2-hydroxy laurate group present at the 2 position of lipid A and, unexpectedly, in the additional loss of the glucosamines that decorate the phosphate groups at 1 and 4' position and in an increase in LPS molecules carrying O-antigen. The resulting LPS showed greatly reduced potency to activate TLR4 in HEK-Blue reporter cells expressing human or mouse TLR4 as well as in porcine macrophages. Characterization of the *lpxL1* mutant revealed many pleiotropic phenotypes, including increased resistance to SDS and rifampicin, increased susceptibility to cationic antimicrobial peptides, decreased auto-aggregation and biofilm formation, and a tendency to decreased infectivity of macrophages, which are all related to the altered LPS structure. We suggest that the *lpxL1* mutant will be useful for the generation of safer vaccines.

Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is a major component of the outer membrane of Gram-negative bacteria. It contains a lipid A moiety, which anchors the molecule into the lipid bilayer, and a core oligosaccharide attached to the lipid A. In many species, LPS also contains a polysaccharide, known as O-antigen, with a strain-specific sugar composition. Lipid A is responsible for the endotoxic activity of LPS. It is recognized by the Toll-like receptor 4 (TLR4) and the co-receptor myeloid differentiation-2 (MD-2) of innate immune cells, which then triggers the production of pro-inflammatory cytokines, such as TNF-α and IL-1β. This response is essential for clearing local infections, but an overreaction can cause serious damage, including septic shock and death (Raetz and Whitfield, 2002).

Lipid A generally consists of a glucosamine disaccharide backbone substituted with phosphates at 1 and 4′ positions and acylated at the 2, 2′, 3 and 3′ positions with fatty acids. These acyl chains bear β-hydroxyl groups that allow for secondary acylation. The biosynthesis pathway of lipid A, known as the Raetz pathway, is conserved, yet differences in lipid A structure among species do exist. Furthermore, several lipid A variants may coexist in the same species and/or emerge under different environmental conditions (Raetz et al., 2007). Structural variation, resulting from the regulation of gene expression, may be introduced during the biosynthesis or through post-synthetic modification systems, and includes (de-)acylation, (de-)phosphorylation, or substitution of the phosphates with groups, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N), glucosamine, or phosphoethanolamine (Raetz et al., 2007). Endotoxicity is mainly determined by the presence or absence and the substitution of the phosphate groups along with the number, length and distribution of the acyl chains (Raetz et al., 2007; Matsuura, 2013). For instance, decrease in the number of fatty acids or phosphate groups is commonly related to diminished endotoxic responses.

Bordetella bronchiseptica is a Gram-negative bacterium that causes respiratory diseases in several mammalian species, such as infectious tracheobronchitis in dogs (a.k.a. kennel cough) or atrophic rhinitis in pigs (Goodnow, 1980). In addition, it has been related to zoonotic infections in immunocompromised and healthy humans in close contact with infected animals (Mattoo and Cherry, 2005). Prophylaxis is ordinarily achieved through vaccination, although booster doses seem to be necessary to keep long term protection (Ellis, 2015). Adverse reactions to whole-cell vaccines cause animal-welfare problems in livestock and companion animals and necessitate the development of new veterinary vaccines. Although undesired effects of whole-cell vaccines against *B. bronchiseptica* have been poorly studied, they have been a concern for the last decades (Deville et al., 2009; Ellis, 2015). *B. bronchiseptica* LPS is a very potent TLR4 agonist that produces stronger inflammatory responses than that of *B. pertussis* in vitro and in vivo (Mann et al., 2005). To reduce the potential for adverse reactions, parenteral acellular formulations and

intranasal vaccines were developed. Nevertheless, several studies showed that parenteral whole-cell vaccines produce faster, higher and more enduring antibody responses than the acellular and intranasal preparations (Ellis, 2015). By genetic engineering of the vaccine strain, changes in the lipid A structure can be induced to moderate TLR4 activation and minimize the risk of whole-cell vaccine reactogenicity.

B. bronchiseptica lipid A is generally known as a hexa-acylated structure with three primary acyl chains, each substituted with a secondary acyl chain (Figure 1). The primary acyl chain at the 3 position is lacking due to the post-synthetic activity of the deacylase PagL (MacArthur et al., 2011). However, several studies have shown extensive intraspecies variability in the ratio of hexa- to penta-acylated molecules (Zarrouk et al., 1997; Basheer et al., 2011; MacArthur et al., 2011). Two of the secondary acyl chains are introduced during the biosynthetic pathway by the acyl transferases LpxL1 and LpxL2. Homologues of these enzymes have been identified in *Bordetella pertussis* (Geurtsen et al., 2007), but their role in *B. bronchiseptica* has not been studied yet. In *B. pertussis*, LpxL2 mediates the introduction of a secondary myristate (C_{14}) at the 2' position of the lipid A structure, whereas LpxL1 is responsible for the addition of a secondary 2-hydroxy-laurate (2-OH C_{12}) in the 2 position (Figure 1). However, the latter was only observed when the *lpxL1* gene was overexpressed, adding an extra acyl chain to the normally penta-acylated lipid A structure. This modification resulted in higher TLR4 activation compared to the wild type (Geurtsen et al., 2007). In contrast, this 2-OH C_{12} chain is present in the lipid A of wild-type *B. bronchiseptica*, suggesting full activity of the LpxL1 enzyme in this species (Basheer et al., 2011; MacArthur et al., 2011). The hydroxylation commonly present in this fatty acid is dependent on the hydroxylase activity of an enzyme encoded by another gene, *lpxO* (MacArthur et al., 2011).

The third secondary acyl chain is a palmitate (C_{16}), which is attached at the 3' position by the outer membrane-based enzyme PagP (Figure 1). Expression of the *pagP* gene is regulated by the major virulence-regulatory system BvgAS and acylation takes place in the Bvg^+ phase (Preston et al., 2003). PagP-mediated acylation is reported to protect *B. bronchiseptica* against antibody-mediated complement lysis, which increases its persistence during respiratory infection (Preston et al., 2003; Pilione et al., 2004). At present, modulation of endotoxic activity by PagP has not been studied in *B. bronchiseptica*. However, the recombinant expression of the *pagP* gene in *B. pertussis*, where expression is otherwise abolished due to the insertion of an *IS* element in the promoter region, led to palmitoylation of the lipid A molecule and to increased TLR4 activation in macrophages stimulated with the LPS (Geurtsen et al., 2006).

Another modification featured among *Bordetella* spp. is the substitution of the phosphate groups with glucosamine (Figure 1). LgmB-mediated addition of glucosamine is induced in the Bvg^+ phase in *B. bronchiseptica* and *B. pertussis* (Marr et al., 2008; Shah et al., 2013; Rolin et al., 2014). In *B. pertussis*, this modification appears to be highly relevant for TLR4

activation in human but not in murine macrophages (Marr et al., 2010). Accordingly, *lgmB* inactivation in *B. bronchiseptica* did not affect TLR4 stimulation in murine macrophages (Rolin et al., 2014), but it decreased the resistance of the bacteria to cationic antibacterial peptides.

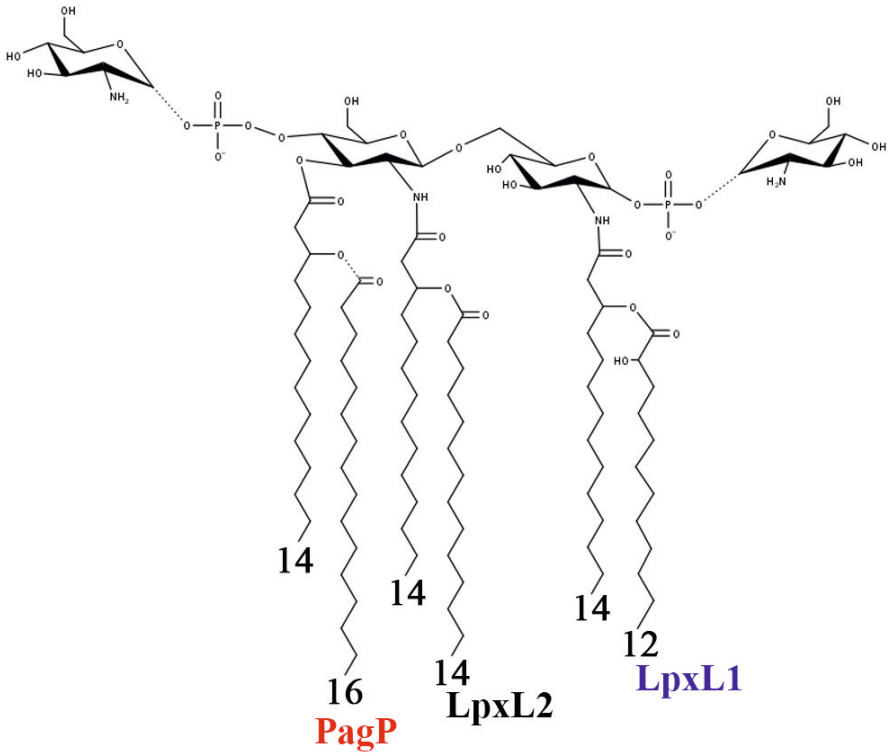

Figure 1. Representation of the lipid A structure of *B. bronchiseptica*. The non-stoichiometric glucosamine and C_{16} acyl chain modifications are denoted by dashed bond lines. The enzymes responsible for the addition of the secondary acyl chains are indicated.

With the eventual goal to construct a vaccine strain with limited reactogenicity, we investigated in this study the effect of *pagP* and *lpxL1* inactivation in *B. bronchiseptica* on lipid A structure and their consequences on TLR4 activation. We also explored the impact of the mutations on various phenotypes of the bacteria.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are described in Supplementary Table S1. *Escherichia coli* strains were grown at 37 °C in lysogeny broth (LB) while shaking or on LB agar plates. *B. bronchiseptica* strains were grown at 35 °C on Bordet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading) (BG-blood). For liquid

cultures, the bacteria were inoculated from plate in Verwey medium (Verwey et al., 1949) and grown at 35 ºC while shaking at 175 rpm. Alternatively, Stainer-Scholte (SS) medium (Stainer and Scholte, 1971) was used which was supplemented with 14 g/L Bacto casamino acids (BD biosciences) and then adjusted to pH 7.6. After overnight growth, the optical density at 600 nm (OD_{600}) was adjusted to 0.1, and cultures were grown for the time indicated. When needed for plasmid maintenance or strain selection, the following antibiotics were included in the medium: cefotaxime (5 µg/ml), streptomycin (300 µg/ml), gentamicin (10 µg/ml), ampicillin (100 µg/ml for *E. coli*, 200 µg/ml for *B. bronchiseptica*).

DNA manipulation and construction of mutants

All plasmids and PCR primers used in this study are listed in Supplementary Tables S1 and S2, respectively. Regular PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific), whilst PCR fragments generated for cloning were obtained using the Expand High Fidelity PCR system (Roche Diagnostics GmbH). For purification of PCR products, the commercial Wizard SV Gel and PCR Clean-Up System (Promega) was employed. Plasmids were isolated with the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). PCR products and plasmids were digested with the appropriate restriction enzymes (Thermo Scientific) according to manufacturer's instructions, purified, and ligated using T4 DNA ligase (5 U/µl) (Thermo Scientific).

To inactivate the *pagP* gene in *B. bronchiseptica*, we replaced the locus on the chromosome by a gentamicin-resistance cassette (gem^R). DNA fragments upstream (920 bp) and downstream (905 bp) of *pagP* were amplified by PCR from strain BB-P19, using a single colony from plate as DNA template. PCR products were introduced separately in the pCRII vector following the instructions of the manufacturer (Invitrogen), yielding plasmids pCRII-PagPup and pCRII-PagPdw, respectively. Upstream and downstream fragments were then combined into a single vector after digestion of both plasmids with endonucleases Eco81I and XbaI, obtaining pCRII-PagPup-PagPdw. A gem^R cassette was amplified from pYRC and inserted in between the two fragments after digestion of construct and PCR product with Eco81I, generating pCRII-PagPup-GemR-PagPdw. Hitherto, the DNA cloning was performed in *E. coli* strain DH5α by transformation using the $CaCl_2$ method. The construct obtained was then sub-cloned into the suicide vector pKAS32 after digestion of both plasmids with EcoRI, yielding pKAS-PagPup-GemR-PagPdw. The plasmid obtained was used to transform *E. coli* strain SM10(λpir) to allow for its replication and subsequent transfer to *B. bronchiseptica* by conjugation for inactivation of the chromosomal gene by allelic exchange. First, transconjugants were selected on plates containing gentamicin as well as cefotaxime for counter selection against *E. coli*. Subsequently, selection was made with streptomycin and gentamicin to ensure the loss of plasmid backbone together with the wild-type *pagP* gene.

To inactivate *lpxL1* gene, the *lpxL1* locus was amplified by PCR including 748 bp upstream of the ATG start codon and 315 bp downstream of the stop codon, and the PCR product

was introduced into the pCRII vector resulting in plasmid pCRII-upLpxL1dw. Then, the *gem*^{R} cassette was inserted into a PfoI site within the *lpxL1* coding sequence to disrupt the locus (pCRII-upLpxL1dw-GemR). The construct was subcloned into the pKAS32 vector after digestion with SacI and XbaI, yielding pKAS-upLpxL1dw-GemR, which was then used to inactivate the chromosomal gene in *B. bronchiseptica* as above.

LPS purification and analysis

LPS was isolated from bacterial cells grown in Verwey medium using the Tri-Reagent method (Yi and Hackett, 2000). Briefly, lyophilized cells were resuspended in TRIzol Reagent (Invitrogen) by intensive vortexing. After 10 min incubation at room temperature to allow for complete cell homogenization, 20 µl of chloroform (HPLC grade) per mg of cells were added. After vigorous vortexing, the mixture was incubated at room temperature for another 10 min and, subsequently, centrifuged for 10 min to separate phases. The aqueous phase was collected, and three additional extractions were performed by adding Milli-Q water to the organic phase, vortexing, incubation at room temperature and centrifugation. All the aqueous phases collected were combined, and the water was evaporated using a speed vacuum concentrator. The pellet was washed with 0.375 M $MgCl_2$ in 95% ethanol, pelleted again by centrifugation, and resuspended in Milli-Q water. The extracted LPS was lyophilized, weighed for quantification, and resuspended in endotoxin-free HyPure cell culture grade water (HyClone) for further use. The purity and integrity of purified samples were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with silver staining of LPS (Tsai and Frasch, 1982) or Coomassie staining of proteins.

Lipid A was isolated as previously described (Caroff et al., 1988), with slight modifications. Briefly, lyophilized LPS was dissolved in 100 mM ammonium acetate pH 4.5, heated at 80 °C for 2 h in 1% SDS and lyophilized overnight. Then, lipid A was suspended in water and washed three times with acidified ethanol to remove the detergent. Subsequently, it was solubilized in chloroform:methanol (2:1, v/v), dried under nitrogen stream, and finally solubilized again in methanol:water (1:1, v/v). Lipid A was analyzed by liquid chromatography–mass spectrometry (LC-MS) in the negative-ion mode (O'Brien et al., 2014).

TLR4 stimulation assays

HEK-Blue TLR4 cells co-expressing either human or murine TLR4, MD-2, and CD14 genes and an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter (Invivogen) were cultured according to the manufacturer's instructions. For TLR4 activation, 2.5 x 10^4 cells/well were incubated in a 96-well plate with serial dilutions of either purified LPS or whole bacterial cells previously killed by heat treatment at 56 °C for 1 h. After 17 h of incubation at 37 °C in a 5% saturated CO_2 atmosphere, supernatants were collected and incubated with 1 mg/ml of the SEAP substrate *p*-nitrophenyl phosphate in 1 M diethanolamine substrate buffer (pH 9.8) for 1 h, and the absorbance at 405 nm was

measured in a Biotek microplate reader. To analyze the LPS content of the heat-killed cells used in the assay, the cells were mixed 1:1 with 2x concentrated SDS-PAGE sample buffer, boiled for 5 min, and incubated for 1 h at 65 °C with 50 µg/ml of proteinase K (Thermo Scientific). The samples were then analyzed on SDS-PAGE gels with 14% acrylamide and the gels were stained with silver (Tsai and Frasch, 1982).

Porcine bone marrow-derived macrophages (PBMMs)

PBMMs were grown as previously described (Gao et al., 2018). Briefly, bone marrow was harvested from eight young adult pigs by puncture of the pelvis, from which mononuclear cells were isolated by Ficoll (GE Healthcare) density gradient centrifugation and subsequently frozen for storage. To differentiate towards macrophages, cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal calf serum (Corning) for 6 days exposed to either 1% granulocyte macrophage colony-stimulating factor (GM-CSF) (Bio-Rad) for pro-inflammatory M1 macrophages or 30 ng/ml macrophage colony-stimulating factor (M-CSF) (Peprotech) for anti-inflammatory M2 macrophages. Expression of M1 and M2 surface markers was determined by flow cytometry (Gao et al., 2018). All animals were used and kept under the approval and guidelines of the animal ethical committee of Utrecht University.

To quantify cytokine production, M1 macrophages were stimulated with 10 ng/ml of purified LPS for 24 h. Then, the supernatant was collected and stored at -20 °C until further use. For cytokine quantification, porcine-specific ELISAs (R&D Systems) were performed according to the manufacturer's recommendations.

To analyze the bacterial survival upon incubation with macrophages, bacteria were grown for 4 h in Verwey medium. Then, the bacterial cells were washed and resuspended in RPMI supplemented with 10% fetal calf serum. Mature porcine macrophages were exposed to the bacteria for 4 h at a multiplicity of infection (MOI) of 1 and, after removal of the supernatant, lysed with 1% Triton X-100 in phosphate-buffered saline (PBS). To measure internalized bacteria, the cells were exposed to 150 µg/ml colistin sulfate (Sigma Aldrich) in PBS for 1 h at 37 °C and then washed before lysis. Samples from supernatant, lysate (attached + internalized bacteria), or antibiotic-treated lysate (internalized bacteria) were plated on BG-blood agar and incubated for 72 h at 37 °C, and colonies were counted. Total growth was calculated from the colony-forming units (CFU) in the supernatant plus those in the lysate. Attached bacteria were calculated from the CFU in the lysate minus those in the lysate of cells treated with antibiotics. Data obtained with macrophages from one specific pig was identified as outlier using the ROUT test (GraphPad) with 1% aggressiveness and discarded.

Sensitivity to SDS and antimicrobials

Bacterial cultures were grown in Verwey medium for 4 h, and the OD_{600} was adjusted to 0.1 in fresh medium. Samples of 180 µl of this culture were incubated with 20 µl of either

10% SDS or, as a control, Milli-Q water at 35 °C in static conditions. After 2 h, 10-fold serial dilutions were prepared, and drops of 10 µl were spotted on BG-blood agar plates and incubated for 2 days at 35 °C.

Minimal inhibitory concentrations (MICs) of different antibiotics were determined by Etest (BioMerieux). Bacterial cultures were grown to an OD_{600} of 0.6 in Verwey medium, and 200-µl samples of the cultures were spread on BG-blood agar in square Petri dishes and allowed to dry for 30 min. Etest strips were placed on the plates, which were then incubated for 2 days at 35 °C before establishing MICs from the zone of growth inhibition.

Settling experiments and biofilm formation

For settling experiments, cultures grown for 48 h in SS medium supplemented with casamino acids were adjusted to an OD_{600} of 1 and incubated in test tubes at room temperature in static conditions. Samples from the top of the tubes were taken for OD_{600} measurements.

For biofilm formation, cultures grown for 24 h in SS medium supplemented with casamino acids were adjusted to an OD_{600} of 0.5. Then, 1-ml samples of the cultures were incubated for 24 h under static conditions at 35 °C in 24-well plates. To quantify the biofilm, supernatants were removed, the wells were washed with physiological salt solution, and biofilms were stained for 2 min with 1 ml of 0.5% crystal violet. After two washes with physiological salt solution, the stained biofilm was resuspended in 500 µl of 33% (v/v) acetic acid in water and then quantified by OD_{630} measurements of appropriate dilutions. The values measured for dilutions were multiplied with the dilution factor to obtain the OD_{630} of the original solution.

Bacterial adhesion to hydrocarbons (BATH)

BATH assay was performed as described (Rosenberg, 1984). Cultures were grown for 20 h in Verwey medium and bacterial cells were washed and resuspended in PBS, and the concentration was adjusted to an OD_{600} of 1. To test tubes containing 1.5 ml of bacterial solution, 1 ml of hexadecane was either added or not, and the suspensions were vigorously vortexed for 2 min. Aqueous and organic phases were allowed to separate for 15 min at room temperature, and the OD_{600} was measured from samples taken from the bottom of the tube (aqueous phase). BATH (%) was expressed as $(n-h)/n\times100$, where n is the OD_{600} in the tube without hexadecane and h is the OD_{600} from the tube with hexadecane.

Outer membrane isolation and analysis

To isolate outer-membrane fractions, overnight grown 25-ml cultures of *B. bronchiseptica* were inactivated by incubation for 1 h at 56 °C and the OD_{600} was determined. Cells were harvested by centrifugation at 10,000 rpm (Eppendorf 5920-R centrifuge, FA-6 ×50 rotor) for 5 min and washed with physiological salt solution. The cells were resuspended in 1

ml of 0.75 M sucrose, 10 mM Tris–HCl (pH 7.8). Then, 3 µl of 40 mg/ml lysozyme were added and 2 ml of 1.5 mM EDTA (pH 7.8). The suspension was incubated for 30 min at room temperature. Then, the cell preparations were diluted to OD_{600} of 5 by the addition of a solution of 0.25 M sucrose, 3.33 mM Tris–HCl, 1.0 mM EDTA (pH 7.8). The cells were frozen at − 80 °C, thawed, and lysed by ultrasonication. Unbroken cells and aggregates were removed by centrifugation for 1 h at 10,000 rpm in an Eppendorf 5920-R centrifuge (FA-6 ×50 rotor). The supernatant was then centrifuged for 1 h at 50,000 rpm (Beckman Coulter Optima TLX-120K, TLA 100.2 rotor), and the resulting pellet containing the outer membranes was resuspended in water.

Outer-membrane proteins were separated by SDS-PAGE and stained with Bradford reagent as described (Bos et al., 2015). Alternatively, the separated proteins were transferred to a 0.45-µm pore-size nitrocellulose membrane (GE Healthcare). For immunodetection, mouse antiserum directed against autotransporter BrkA, generously provided by Nathalie Devos (GlaxoSmithKline Biologicals SA) and rabbit antisera directed against the major porin OmpP and siderophore receptor FauA (de Jonge et al., 2021) were used. As secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antisera (ThermoFisher) were employed. Membranes were developed with the Clarity Western ECL Blotting Substrate (Bio-Rad).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (versions 6 and 8). For curve comparison, data from settling and TLR4 stimulation assays were used to calculate the mean area under the curve and standard error of the mean (SEM). In all cases, data was analyzed for statistical significance using one-way ANOVA (Dunnett's correction for multiple comparison). In addition, a repeated measures design was used during studies with macrophages.

Results

Lipid A structural analysis

As strain-dependent variation in lipid A structure may occur, we determined the structure of lipid A in the two *B. bronchiseptica* isolates that are routinely used in our laboratories. One of these strains was isolated from swine (strain BB-P19) and the other from dog (strain BB-D09-SR). Bacteria were grown under conditions in which they are expected to express the virulent Bvg^+ phase. The lipid A from these strains was isolated and analyzed by LC-MS. The spectra were similar for both isolates showing three major peaks at *m/z* 1586, 1748 and 1909 (Figures 2A,B). These peaks were attributed to the penta-acylated bis-phosphorylated species in which the lipid A backbone was substituted with three 3-OH C_{14}, one C_{14}, and one 2-OH C_{12} fatty acids (*m/z* 1586), with additional glucosamine substitutions on either one or both phosphate groups (*m/z* 1748 and 1909, respectively). Three additional minor peaks corresponding to the palmitoylated (C_{16}) form of the

aforementioned species were also present at *m/z* 1825, 1986 and 2145. These hexa-acylated forms represent a conjoined relative abundance of around 10% relative to the penta-acylated species. These results are consistent with the structure shown in Figure 1 and demonstrate that PagP-mediated acylation of lipid A is a minor event in our strains under the applied growth conditions, whilst substitution of the phosphate groups with glucosamine is rather abundant. The LpxO-mediated β-hydroxylation of the secondary acyl chain at the 2 position appears to be essentially complete.

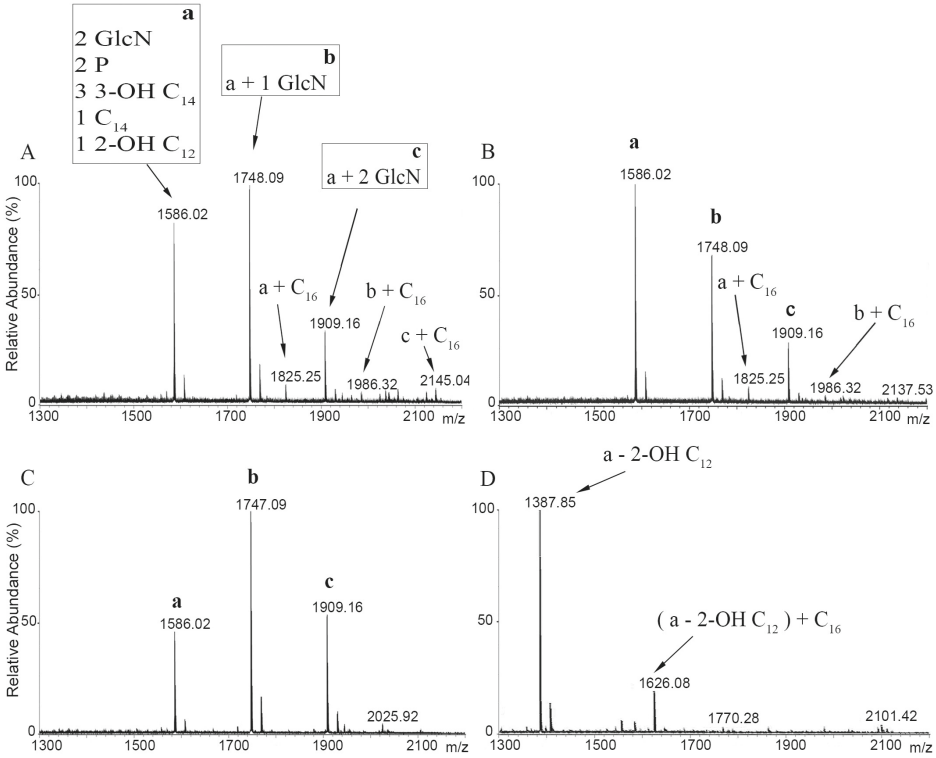

Figure 2. LC-MS analysis of *B. bronchiseptica* lipid A. Comparison of lipid A species from *B. bronchiseptica* strains BB-P19 (**A**) and BB-D09-SR (**B**), and the *pagP* (**C**) and *lpxL1* (**D**) mutant derivatives, respectively. Major peaks at *m/z* 1586, 1748 and 1909 were interpreted as the characteristic penta-acylated bis-phosphorylated species, and the corresponding species with one and two glucosamine substituents, respectively. Predicted divergence for the other peaks is indicated.

Inactivation of *lpxL1* and *pagP* and effects on lipid A structure

With the aim of reducing the number of secondary acyl chains in the lipid A structure of *B. bronchiseptica*, we inactivated the *lpxL1* and *pagP* genes (corresponding with locus tags BB0194 and BB4181, respectively, in reference strain RB50). Repeated attempts to similarly inactivate the *lpxL2* gene (locus tag BB0193) failed (data not shown), indicating that this

gene is essential in *B. bronchiseptica*, which is consistent with previous observations in *B. pertussis* (Geurtsen et al., 2007; Arenas et al., 2020). As expected, LC-MS analysis of lipid A from the *pagP* mutant strain presented the three major peaks also observed in the wild type (*m/z* 1586, 1748 and 1909) but the absence of the corresponding three palmitoylated species (*m/z* 1825, 1986 and 2145) (Figure 2C). Inactivation of *lpxL1* produced substantial differences in the spectrum compared to the wild-type strain (Figure 2D). The predominant peak at *m/z* 1388 corresponds to the molecular ion at *m/z* 1586 of wild-type lipid A with the expected loss of the 2-OH C_{12} chain. However, the expected additional peaks for the corresponding glycosylated forms are missing, suggesting that the LgmB-mediated addition of glucosamines is dependent on the presence of the secondary 2-OH C_{12} residue at the 2 position of the lipid A structure. The additional peak at *m/z* 1626 in the spectrum from the *lpxL1* mutant (Figure 2D) corresponds to the palmitoylated equivalent of the *m/z* 1388 ion. The relative abundance of the palmitoylated form, i.e. ~20%, was higher than in the wild type. Thus, *lpxL1* inactivation results in the loss of the secondary 2-OH C_{12} chain at the 2 position, but this modification also elicits loss of the glucosamine decorations as well as over-palmitoylation.

Purified LPS of the wild type and the mutants was also analyzed on SDS-PAGE gels, showing differences in banding patterns (Figure 3). At the position of LPS molecules consisting of lipid A and core sugars, the wild-type strain showed two bands presumably corresponding to the penta- (lower and more intense band) and hexa-acylated (upper and less intense band) forms and, accordingly, the upper band was completely absent in the LPS of the *pagP* mutant (Figure 3). LPS from the *lpxL1* mutant featured three bands, a lower band, probably corresponding to the LPS with the tetra-acylated lipid A that has lost the 2-OH C_{12} fatty acid, a band that could correspond with the former but including palmitoylation, and a third band that migrated more slowly on gel than the bands observed in the wild type. As we could not correlate the latter band with any of the peaks observed in the MS analysis, it probably represents LPS molecules with a substitution in the core moiety, perhaps the pentasaccharide linker that connects the O-antigen to the core (Preston et al., 2006). In all samples, we also observed complete LPS molecules substituted with O-antigen, but considerably increased amounts of O-antigen-bearing LPS appear to be present in the *lpxL1* mutant (Figure 3).

TLR4 activation by purified LPS and whole cells

To determine whether the modifications in the lipid A structure resulting from the inactivation of *pagP* and *lpxL1* can reduce the endotoxic activity of the LPS of *B. bronchiseptica*, TLR4 activation was tested in HEK-Blue reporter cells expressing either human or murine TLR4 (h- and m-TLR4, respectively) (Figure 4A). Both h- and m-TLR4 were activated to similar levels by LPS from the *pagP* mutant and from the wild-type strain. In contrast, purified LPS of *lpxL1* mutant strain showed significantly lower activity, with 10- to 100-fold higher concentrations being required to reach a similar signal as with the wild-type LPS (Figure 4A).

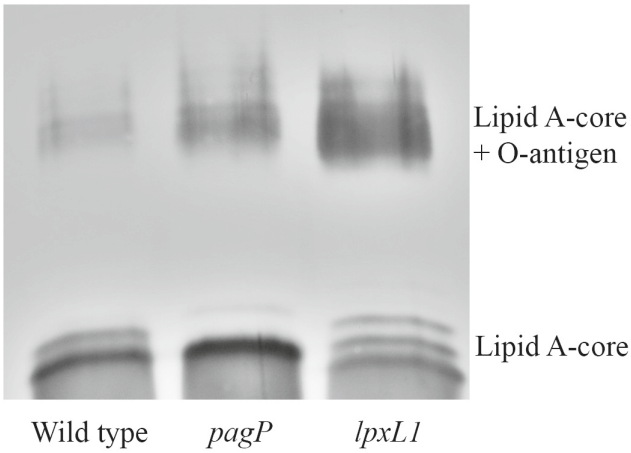

Figure 3. Analysis of LPS modifications by SDS-PAGE. Purified LPS of *B. bronchiseptica* strain BB-D09-SR and of the *pagP* and *lpxL1* mutants was analyzed by SDS-PAGE and visualized by silver staining. The identity of the various bands detected at the position of lipid A plus core sugars is discussed in the text. O-antigen-containing LPS appears as diffuse smear with lower electrophoretic mobility.

Our eventual goal is to produce novel whole-cell vaccines with reduced endotoxicity, but reduced endotoxicity of purified LPS preparations is not necessarily reflected in that of whole-cell preparations (Geurtsen et al., 2006). Therefore, we also tested the TLR4-stimulating activity of heat-killed whole-cell suspensions with the HEK-Blue cells. As observed with purified LPS, whole cells of the *lpxL1* mutant had a clearly reduced ability to activate m- and h-TLR4 (Figure 4B). Thus, consistent with the results obtained with purified LPS, whole-cell preparations of the *lpxL1* mutant showed reduced TLR4-stimulatory activity. Interestingly, also whole-cell preparations of the Δ*pagP* strain showed a slight, but statistically significant decrease in TLR4 activation compared to wild-type cells (Figure 4B). When we analyzed the LPS content of these cell preparations by SDS-PAGE, we noticed the presence of only a single band representing the lipid A substituted with core sugars in the wild-type cells, and this band migrated more slowly in the gel than the corresponding band in the *pagP* mutant (Supplementary Figure S1). This result can be explained by assuming that PagP is activated during the heat treatment that is used to kill the cells, resulting in fully palmitoylated LPS. Thus, also the presence of a secondary C_{16} chain bound to the primary 3OH-C_{14} at the 3′ position can contribute to the TLR4-stimulating activity of *B. bronchiseptica* LPS.

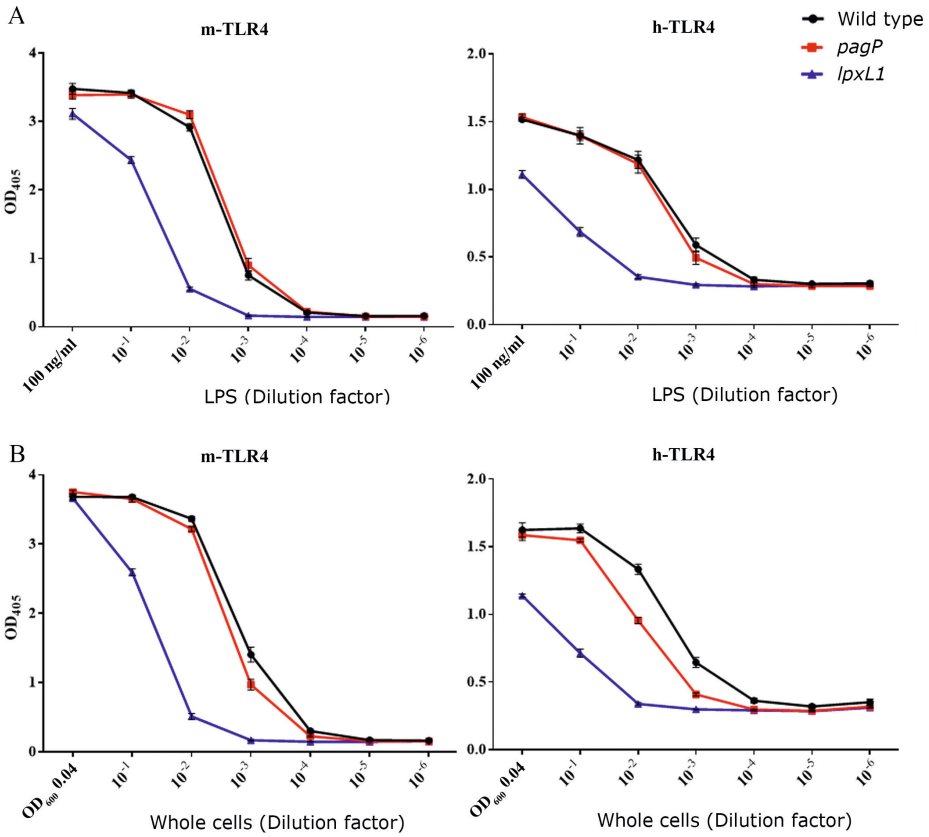

Figure 4. TLR4 activation by purified LPS and whole-cell preparations of strain BB-D09-SR and its *pagP* and *lpxL1* mutant derivatives. HEK-Blue cells expressing either m-TLR4 (left panels) or h-TLR4 (right panels) were incubated for 17 h with 10-fold serial dilutions of (**A**) purified LPS or (**B**) heat-inactivated whole cells. The concentrations of the undiluted samples are indicated in ng/ml and OD_{600} units in panels A and B, respectively. Graphs show mean ± SEM of SEAP activity measured at OD_{405} from the supernatants of three independent experiments performed in duplicate. In the stimulation assays with LPS, statistical comparison showed significant differences relative to the wild type only for the *lpxL1* mutant ($P < 0.0001$ for both m- and h-TLR4). When stimulated with whole cells, significant differences were found for the *lpxL1* mutant ($P < 0.0001$ for both m- and h-TLR4) but also for the *pagP* mutant ($P < 0.05$ for m-TLR4 and $P < 0.0001$ for h-TLR4).

Inactivation of *lpxL1* reduces the immune response in porcine macrophages

As swine livestock is one of the targets for a vaccine against *B. bronchiseptica* and the innate immune system of pigs might respond differently from that of humans and mice, we also investigated the immune response of PBMMs stimulated with LPS. To this end, PBMMs were incubated with purified LPS, and the production of several cytokines (TNFα, IL-10, IL-8, IL-1β and IL-6) was measured. In all cases, the IL-6 concentration was under the detection limit of the technique (data not shown). Stimulation of the PBMMs with LPS from the wild type or the *pagP* mutant resulted in similar production levels of the other

cytokines quantified (Figure 5). Although the concentration of IL-1β produced appeared lower upon incubation with LPS from Δ*pagP* strain (Figure 5D), this difference was not statistically significant (*P*= 0.2188). In contrast, PBMMs incubated with LPS from the *lpxL1* mutant produced significantly lower levels of TNFα and IL-10 (Figures 5A,B). Also the IL-1β concentration was lower (Figure 5D), yet not statistically significant (*P*= 0.0663). Production of IL-8 did not differ from the levels induced by wild-type LPS (Figure 5C). This data indicates that the reduced TLR4 activation observed by inactivation of *lpxL1* is consistent in all models tested here.

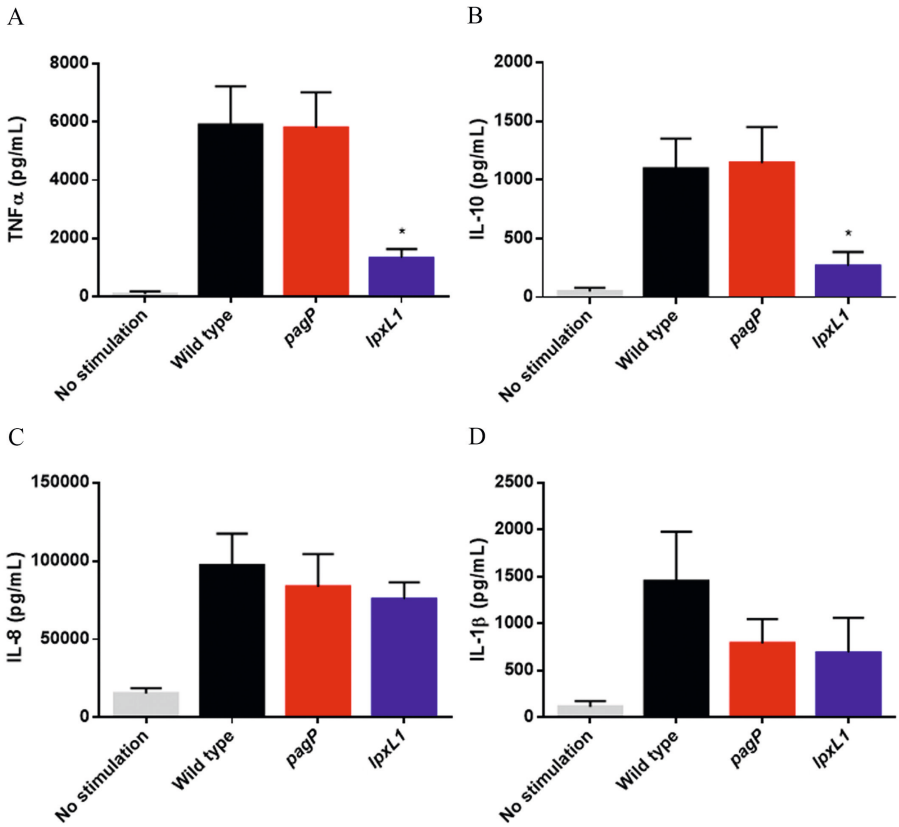

Figure 5. Cytokine secretion upon stimulation of PBMMs with LPS from the *B. bronchiseptica* strain BB-D09-SR and its *pagP*- and *lpxL1*-mutant derivatives. Secreted levels of TNF-α, IL-10, IL-8 and IL-1β by PBMMs of separate porcine individuals (n=5) were measured after 24 h incubation with 10 ng/ml of purified LPS. Values shown are means and standard deviations from three independent experiments. Statistically significant differences compared to the wild type are indicated with asterisks (*, *P*< 0.05).

Outer membrane permeability of the mutants

The outer membrane of Gram-negative bacteria protects these bacteria by forming a barrier for noxious compounds in the environment, including, for example, alkyl sulfates,

which are anionic surfactants (Cowles, 1938). Modifications in the lipid A structure may affect this barrier function. To test whether the *pagP* and *lpxL1* mutations affect membrane permeability, cultures were treated with the anionic surfactant SDS. Survival of the wild-type strain was drastically reduced upon incubation with SDS compared to the untreated control (Figure 6). Interestingly, the *pagP* and *lpxL1* mutants were more resistant to SDS as they showed 10-fold and 1000-fold increased survival, respectively, compared to the wild type (Figure 6). Thus, the mutations fortify the outer membrane of *B. bronchiseptica* by providing an improved barrier to SDS.

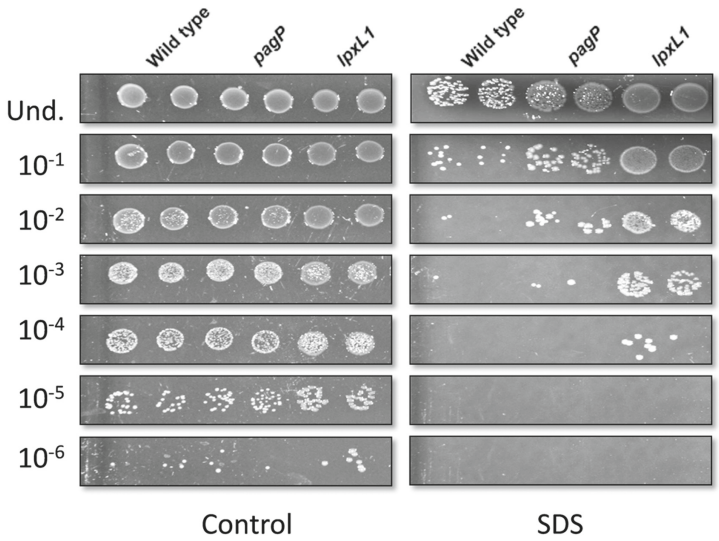

Figure 6. Bacterial sensitivity to SDS. Cultures of strain BB-D09-SR and its *pagP*- and *lpxL1*-mutant derivatives were incubated in duplicate with 1% SDS. After 2 h incubation, drops of 10-fold serial dilutions from these cultures were plated on BG-blood agar and incubated for 48 h. The control at the left shows the results for bacteria not exposed to SDS. A representative result of three independent experiments is shown.

Additionally, we tested if the susceptibility of the mutants to different antibiotics and cationic antimicrobial peptides (CAMPs) was affected (Table 1). The susceptibility of the Δ*pagP* strain was similar to that of its parental strain for all antimicrobials tested, except perhaps for polymyxin B that could be slightly decreased. On the other hand, the *lpxL1* mutant was less susceptible to the amphipathic antibiotic ciprofloxacin and it was resistant to the hydrophobic antibiotic rifampicin. In contrast, its susceptibility to polymyxin B and colistin, which are positively charged peptides that bind to the outer membrane of Gram-negative bacteria through electrostatic interactions, was increased. Its resistance to antibiotics that usually are ineffective against Gram-negative bacteria due to their large molecular size, i.e. the glycopeptide vancomycin and the lipopeptide daptomycin, remained unchanged.

Table 1. Antimicrobial susceptibility of strain BB-D09-SR and its mutant derivatives[a]

	Wild type	*pagP*	*lpxL1*
Polymyxin B	0.125	0.125 – 0.19	0.064 – 0.125
Colistin	0.125	0.125	0.047 – 0.094
Ciprofloxacin	0.19	0.19	0.38
Rifampicin	3 - 4	3 - 4	>32
Vancomycin	>256	>256	>256
Daptomycin	>256	>256	>256

[a]MICs reported are in µg/ml. Results were obtained in at least three independent experiments. In cases of discrepancy, data was inserted as range.

Inactivation of *lpxL1* reduces cell-surface hydrophobicity and affects biofilm formation

During our experiments, we noticed that the wild-type *B. bronchiseptica* cells formed aggregates that rapidly settled when the cultures were left standing under static conditions (Figure 7A). This effect was observed during growth in SS medium and, even more prominently, when SS was supplemented with casamino acids, but much less so in Verwey medium. Remarkably, this settling was not observed for the *lpxL1* mutant (Figure 7A). Monitoring of the OD_{600} of the top fractions of the bacterial suspensions confirmed these observations. Already after 45 min, a notable drop in the OD_{600} of the wild-type and the *pagP*-mutant cell suspensions was detected, while that of the *lpxL1* mutant remained unaffected even after 24 h (Figure 7B).

Bacterial auto-aggregation is often related to biofilm formation (Trunk et al., 2018). Indeed, a significant reduction in biofilm formation was observed for the *lpxL1* mutant strain (Figure 7C). Measurement of the OD_{600} of the supernatants obtained from the biofilms showed significantly higher values for the *lpxL1* mutant (data not shown), discarding the possibility that the reduced biofilm formation of this strain is due to lower growth.

Cell-surface hydrophobicity is one of the physicochemical properties that can determine the initial adhesion and aggregation of bacteria (Renner and Weibel, 2011). We hypothesized that the increased levels of hydrophilic O-antigen observed in the LPS of the *lpxL1* mutant (Figure 3) could result in reduced cell-surface hydrophobicity and thereby affect auto-aggregation and biofilm formation. Using the BATH method, we observed that the cell-surface hydrophobicity of the *lpxL1* mutant was indeed significantly reduced relative to that of the wild type, whilst that of the *pagP* mutant was unaffected (Figure 7D).

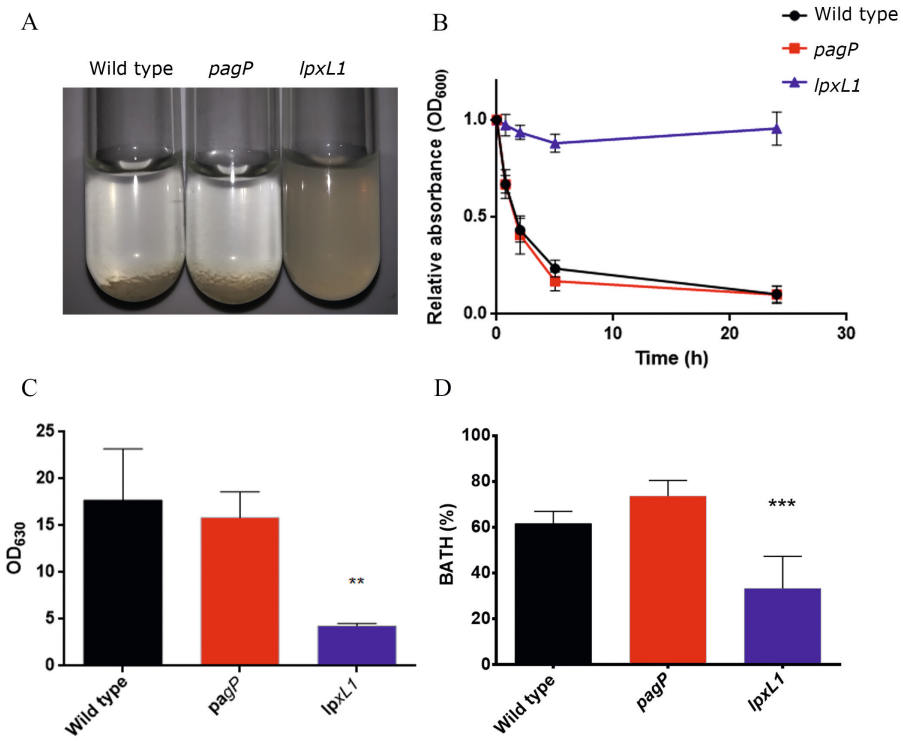

Figure 7. Inactivation of *lpxL1* reduces settling, biofilm formation and surface hydrophobicity. (**A**) Macroscopic view of bacterial settling. Cultures of strain BB-D09-SR and its mutant derivatives were grown in SS medium supplemented with casamino acids and adjusted to an OD_{600} of 1. A photograph was taken after 5 h incubation under static conditions. (**B**) Absorbance was measured from the same cultures as shown in panel A. Samples were taken at 0 and 45 min, and at 2, 5 and 24 h. Graph shows mean ± SEM of absorbance relative to the t=0 sample calculated from four independent experiments. Comparison with the parental strain showed statistically significant differences only for *lpxL1* mutant ($P < 0.0001$). (**C**) Biofilm formation under static conditions. After 24 h of incubation in SS medium supplemented with casamino acids, biofilms were stained with crystal violet and quantified by measuring the OD_{630}. Data represent means and standard deviations from three experiments performed in triplicate. Statistically significant difference compared to the wild type is indicated with asterisks (**, $P < 0.01$). (**D**) Surface hydrophobicity assessment using BATH method. Bacterial suspensions standardized at OD_{600} of 1 in PBS were mixed with hexadecane. Percentage hydrophobicity was calculated from OD measurements of samples from the water phase. The means and standard deviations from three experiments performed in duplicate are shown. Statistically significant difference compared to the wild type is indicated with asterisks (***, $P < 0.001$).

Influence of LpxL1 and PagP activity on the infection of macrophages

Bordetella spp. have been described to survive inside professional phagocytes (Saukkonen et al., 1991; Banemann and Gross, 1997). We tested whether the *lpxL1* and *pagP* mutations affect the survival of the bacteria when they are incubated with porcine macrophages. We used both M1 macrophages, which are pro-inflammatory and responsible for inflammatory signaling, and M2 macrophages, which are anti-inflammatory and participate in the resolution of the inflammatory process. The *lpxL1* mutant showed a

slight increase in growth when incubated with M1 macrophages, while the *pagP* mutant seemed unaffected relative to the wild type (Figure 8A). When the strains were incubated in RPMI medium without macrophages, no growth advantage of the *lpxL1* mutant was detected (data not shown). The increased amount of *lpxL1* bacteria was reflected in the supernatant (Figure 8B) and the adhesion to the macrophage surface (Figure 8C), but in contrast to the wild type, the *lpxL1* mutant was barely detected inside the macrophages (Figure 8D). Nevertheless, none of these differences were statistically significant. Similar tests were performed with M2 macrophages (Supplementary Figure S2), where the reduced infectivity of the *lpxL1* mutant was not observed (Figure S2D).

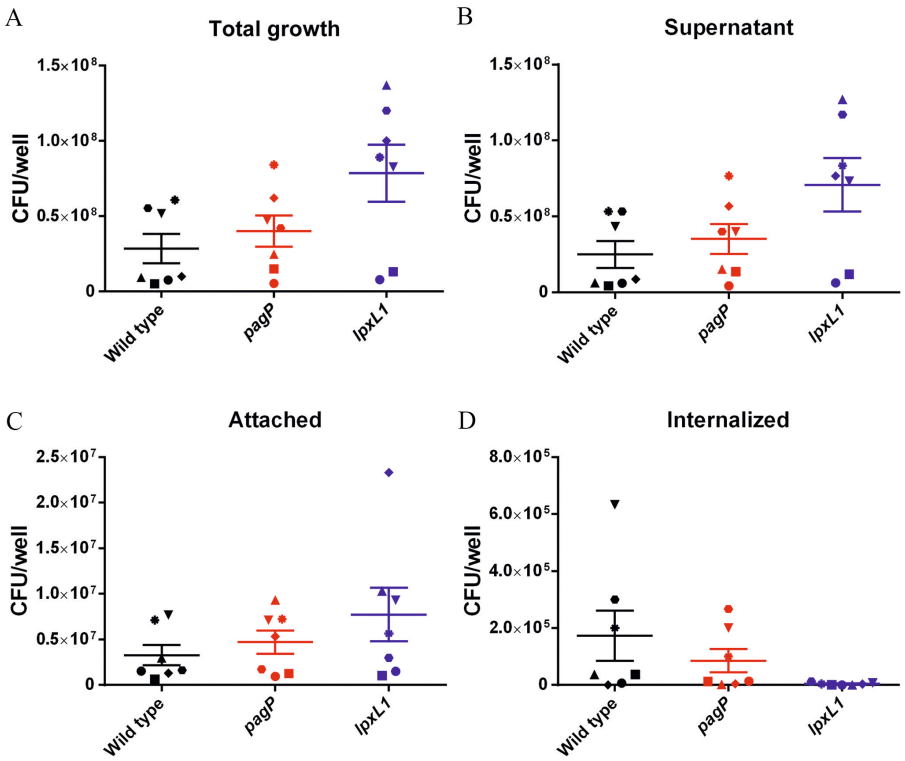

Figure 8. Bacterial survival with pro-inflammatory M1 macrophages. Suspensions of strain BB-D09-SR and its mutant derivatives were incubated with M1 macrophages at an MOI of 1 for 4 h, and CFU in different fractions were quantified. (**A**) Total bacterial growth (i.e. both inside and outside of the macrophages) expressed as CFU. (**B**) CFU in the supernatant. (**C**) CFU attached at the macrophage surface. (**D**) CFU inside macrophages. Symbols with different shapes correspond to PBMMs of separate porcine individuals (n=7). No statistical significance was found.

Presence of vaccine antigens in the outer membrane of the *lpxL1*mutant

As the LPS modifications in the *lpxL1* mutant could potentially affect the localization of relevant protective antigens at the cell surface, we compared the protein profiles of outer

membranes isolated from the parental strain and from its *lpxL1* mutant derivative. With the exception of an unidentified protein of ~40 kDa, which was present in diminished amounts in the *lpxL1* mutant, the protein profiles were almost identical (Figure 9A). In addition, the presence of several proteins that are considered as potential vaccine antigens, i.e. the iron-regulated siderophore receptor FauA (Brickman and Armstrong, 1999; Register et al., 2001) the major porin OmpP (Zhang et al., 2019), and the *Bordetella* resistance-to-killing autotransporter BrkA (Rambow et al., 1998), was investigated by Western blotting. All these proteins were equally well detectable in the outer-membrane preparations of both strains (Figure 9B).

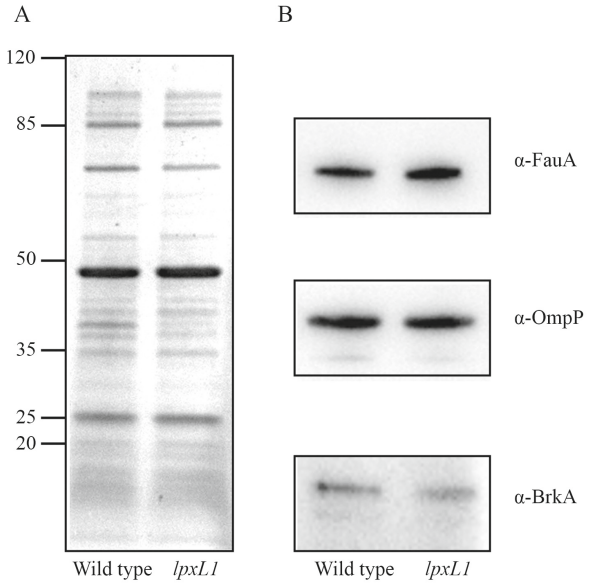

Figure 9. Comparison of protein content of outer-membrane preparations from *B. bronchiseptica* strain BB-D09-SR and its *lpxL1* mutant derivative. (**A**) SDS-PAGE analysis of isolated outer membranes of the wild-type strain and its *lpxL1* mutant derivative. Molecular weight markers are shown at the left. (**B**) Western blot analysis of relevant outer membrane antigens. Membranes were incubated with antibodies directed against the siderophore receptor FauA, major porin OmpP, and autotransporter BrkA.

Discussion

Modifications in the structure of the lipid A are associated with changes in the endotoxicity of the LPS, which can be used to develop less reactogenic whole-cell vaccines. In *B. bronchiseptica*, PagP and LpxL1 function as acylases attaching secondary acyl chains to primary fatty acids in the lipid A. For PagP, this function has previously been demonstrated in *B. bronchiseptica* (Preston et al., 2003), but the function of LpxL1 has only been validated in *B. pertussis*. In *B. pertussis*, *lpxL1* is normally not expressed under laboratory conditions, yet its overproduction resulted in secondary acylation (Geurtsen et

al., 2007). The consequences of *pagP* and *lpxL1* expression on the TLR4-stimulating activity of LPS of *B. bronchiseptica* were still unknown. Our mass spectrometry analysis confirmed the non-stoichiometric PagP-mediated palmitoylation in only ~10% of the molecules in our wild-type strains, the complete absence of this palmitoylation in a *pagP* mutant, and the loss of secondary 2-OH C_{12} in the 2 position in an *lpxL1* mutant. In the latter mutant, palmitoylated lipid A increased up to ~20% and, interestingly, we also observed that the abundant glucosamine substitution at the phosphate groups detected in our wild-type strains was fully abolished in the *lpxL1* mutant. This suggests that the absence of the secondary 2-OH C_{12} makes this structure a poor substrate for the introduction of glucosamines by the LgmB glycosyltransferase. A similar defect was observed in *E. coli* and *Salmonella* Typhimurium, where inactivation of *lpxM*, which mediates insertion of a secondary myristate group at 3' position, resulted in the loss of L-Ara4N addition to the phosphate groups of their lipid A (Tran et al., 2005). Moreover, glucosamine substitutions are rarely found in *B. pertussis* despite the presence of an intact *lgmB* locus (Marr et al., 2008), which might be due to the lack of LpxL1 activity. Additionally, inactivation of *lpxL1* resulted in an increased O-antigen substitution in the LPS (Figure 3).

Reduction of the number of acyl chains in lipid A is usually related to lower activation of TLR4 and, therefore, lower endotoxicity (Simpson and Trent, 2019). We showed here that inactivation of *pagP* did hardly or not affect TLR4 activation or any other phenotype of the cells tested. Since palmitoylation of LPS by PagP was quite low in our wild-type strains (Figures 2 and 3), it could be that the difference between wild type and mutant is too small to allow for a substantial effect on TLR4 activation. However, analysis of the LPS of the heat-killed cells that were used in the TLR4 activation assays by SDS-PAGE indicated that the LPS in the wild-type samples is fully palmitoylated (Figure S1), suggesting that PagP is activated during the heat treatment. This hypothesis is in line with our previous studies in which we used the same heat treatment to stimulate the release of outer-membrane vesicles (OMVs) (de Jonge et al., 2021). Lipidomic analysis showed a large increase in lysophospholipid content in heat-released OMVs compared to spontaneously released OMVs (Balhuizen et al., 2021). As this increase in lysophospholipid content was reduced when OMVs were isolated from a *pldA* mutant, it can be explained in part, but not completely, by the activation of the outer-membrane phospholipase A, which is encoded by the *pldA* gene, during the heat treatment. It was suggested that the remaining lysophospholipids could possibly be the result of activation of PagP (Balhuizen et al., 2021). The high level of palmitoylation of LPS in the heat-killed cells, suggested by the results in Figure S1, is consistent with this hypothesis. In addition, this explains the slightly, but significantly reduced TLR4 activation of the *pagP* mutant when heat-killed whole cells were used as TLR4 agonist (Figure 4B), but not when purified LPS was used (Figure 4A).

We observed a strong reduction of TLR4 signaling upon inactivation of *lpxL1*. In porcine macrophages, we observed a significant decrease in the production of the pro-inflammatory cytokine TNFα, but also of the anti-inflammatory cytokine IL-10. Induction

of the latter occurs concomitantly with the pro-inflammatory cytokines upon LPS-induced TLR4 activation, and its role is to provide immediate feedback to limit the immune response to pathogens (Saraiva and O'Garra, 2010). In our case, the reduced stimulation of TLR4 appears to influence both pro- and anti-inflammatory pathways. The main lipid A species in this *lpxL1* mutant is tetra-acylated but also non-glycosylated. Whether the reduced endotoxicity is a consequence of the loss of the acyl chain, the glucosamines, or both is unknown. In a previous study, *lgmB* inactivation in *B. bronchiseptica* showed no effect on TNFα production in murine macrophages (Rolin et al., 2014). However, the level of glucosamine substitutions in the wild-type strain used in that study appears to be rather low compared to that in the strains used in our study, which could be a reason for the absence of an effect of the *lgmB* mutation on TLR4 activation. Other studies performed in *B. pertussis* and *Bordetella parapertussis* have demonstrated that the substitution of the phosphate groups with glucosamine increases the TLR4-activating capacity. Geurtsen et al. (2009) showed that inactivation of *lgmB* in a *B. pertussis* strain with a low level of glucosamine substitution resulted in a small but statistically significant reduction in IL-6 production by human monocytes, whilst inactivation of *lgmB* in a *B. parapertussis* strain with a much higher level of lipid A glycosylation affected IL-6 production more drastically. Consistently, Marr et al. (2010) reported that inactivation of *lgmB* in a *B. pertussis* strain with a high abundance of glucosamines in the lipid A drastically reduced h-TLR4 activation. Interestingly, the *lgmB* mutation in that study did not affect m-TLR4 activation, suggesting species specificity in the role of the glucosamines. In our study, the reduced TLR4 activation by the *lpxL1* mutant was detected with both h- and m-TLR4 reporter cells, as well as with porcine macrophages. This difference indicates that the loss of the 2-OH C_{12} chain in the *lpxL1* mutant contributes to the reduced activity observed at least for the m-TLR4. Alternatively, the glucosamines could have a different impact on the LPS activity in *B. bronchiseptica* compared to *B. pertussis*.

It is important to note that the inactivation of *lpxL1* in *B. bronchiseptica* led to reduced TLR4 activation by both purified LPS and whole-cell preparations. An identical tetra-acylated lipid A structure was obtained by the heterologous expression of the de-acylase PagL in *B. pertussis* (Geurtsen et al., 2006). The resulting LPS showed reduced TLR4 activation in vitro, but whole cells of the recombinant strain showed even higher activity than wild-type cells (Geurtsen et al., 2006). This was reported to be due to the increased release of the tetra-acylated LPS from the cell surface in *B. pertussis* and the higher potency of released LPS relative to membrane-bound LPS in activating TLR4. Apparently, the LPS in the *B. bronchiseptica lpxL1* mutant is still stably anchored in the outer membrane.

The *lpxL1* mutant generated showed many pleiotropic phenotypes, including a less hydrophobic cell surface probably due to a raised portion of LPS molecules bearing O-antigen. Possibly, the under-acylated and non-glycosylated structure is less efficiently recognized by the Lpt machinery, which mediates the transport of the LPS molecules to the outer membrane (Putker et al., 2015). The prolonged residence time at the periplasmic

leaflet of the inner membrane might allow the WaaL ligase to substitute the lipid A-core subunits more efficiently with O-antigen. This change is possibly responsible for the reduced bacterial auto-aggregation and biofilm formation observed. We noticed an increased susceptibility of the *lpxL1* mutant to CAMPs. This phenotype is presumably associated with the rise of negative charges in the lipid A as a result of the loss of glucosamine decorations and is in agreement with previous studies which showed that inactivation of *lgmB* increases susceptibility to CAMPs in *B. bronchiseptica* (Rolin et al., 2014) and *B. pertussis* (Shah et al., 2014). Additionally, the reduced number of acyl chains might be contributing to the increased susceptibility to CAMPs, as increased acylation has previously been reported to represent a defense mechanism against CAMPs in other species (Joo et al., 2016). The more negatively charged LPS binds more divalent cations, which results in an increased integrity of the outer membrane and, therefore, a reduction of permeability to amphipathic and hydrophobic antimicrobials (Zgurskaya et al., 2015), as evinced here by the reduced susceptibility to SDS and ciprofloxacin, and rifampicin, respectively. In addition, the increased amount of O-antigen could be boosting the protection against these antimicrobials.

LPS can facilitate adhesion, but it can also act as physical impediment for other adhesins (Murray et al., 2006; Kline et al., 2009). Inactivation of *lpxL1* did not affect adhesion of the bacteria to pro-inflammatory porcine macrophages, but the infection of these cells appeared to be reduced although the difference was not significantly different (Figure 8). This observation is consistent with a previous report which showed that inactivation of *lpxL1* in *B. pertussis* impaired infection of human macrophages although adhesion was even enhanced (Geurtsen et al., 2007). We found a substantial reduction of TLR4 activation by the *lpxL1* mutation, which could lead to downsized phagocytosis (Anand et al., 2007; Skjesol et al., 2019) and may explain the reduced numbers of bacteria detected inside macrophages. However, this explanation cannot hold for *B. pertussis*, where LpxL1 does not acylate lipid A during in vitro growth and, hence, its inactivation cannot affect TLR4 activation. Therefore, the reduced infection of human macrophages by the *B. pertussis lpxL1* mutant suggests *lpxL1* expression is induced inside the macrophages and is important for intracellular survival.

Overall, our results point at the *B. bronchiseptica lpxL1* mutant as a potential candidate for the development of novel whole-cell vaccines with reduced reactogenicity. In addition, this strain presents increased amounts of O-antigen, which can be beneficial for an enhanced immune protection (Wolfe et al., 2007). Furthermore, we demonstrated that the outer-membrane protein profile is barely affected by the mutation, and we confirmed the presence of several potential vaccine antigens at similar levels in the outer membrane of the mutant as in the wild type, which suggests its capability of inducing an appropriate immune response. In future studies, we will investigate the reactogenicity of the modified bacteria in vivo and study the protective immunity induced in target animals.

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Supplementary material

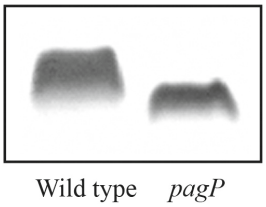

Figure S1. Analysis of LPS in heat-killed whole-cell preparations. Whole-cell lysates of heat-killed *B. bronchiseptica* strain BB-D09-SR and its *pagP* mutant derivative were analyzed by SDS-PAGE, and LPS was stained with silver. Only the part of the gel showing the position of lipid A plus core sugars is shown.

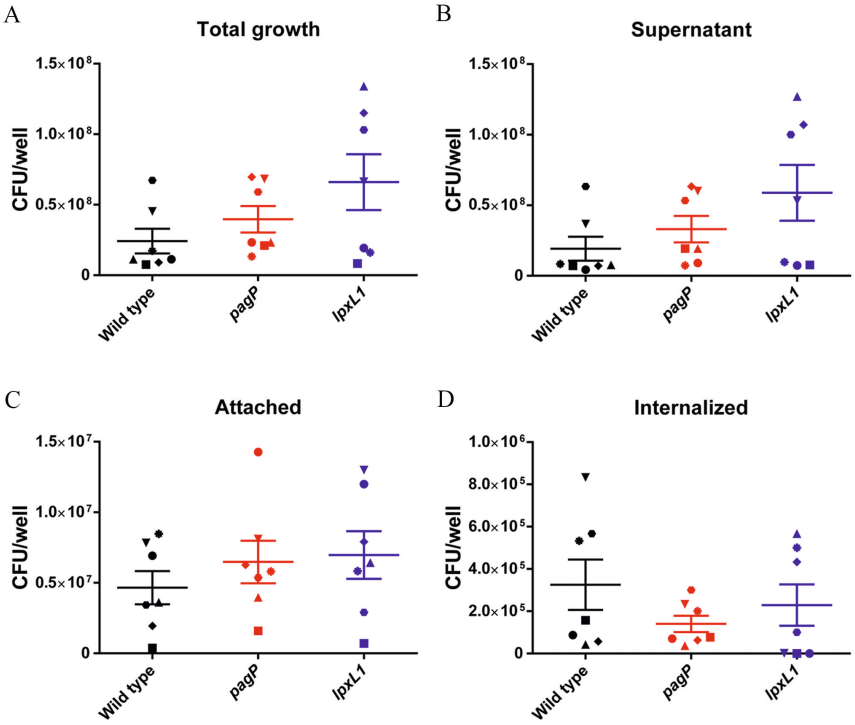

Figure S2. Bacterial survival with anti-inflammatory M2 macrophages. Suspensions of strain BB-D09-SR and its derivatives were incubated with M2 macrophages at an MOI of 1 for 4 h, and CFU in different fractions were quantified. (**A**) Total bacterial growth (i.e., both inside and outside the macrophages) expressed as CFU. (**B**) CFU in the supernatant. (**C**) CFU attached at the macrophage surface. (**D**) CFU inside macrophages. Symbols with different shapes correspond to PBMMs of separate porcine individuals (n=7). Data was analyzed for statistical significance using one-way ANOVA with repeated measures (Dunnett's correction for multiple comparison). No significant differences were found.

Table S1. Bacterial strains and plasmids

Strain / plasmid	Description [a]	Reference [b]
B. bronchiseptica		
BB-P19	Clinical isolate from pig (i. n. 2130108058), Cef^R, Str^R	UU
BB-D09	Clinical isolate from dog (i. n. 2170524052), Cef^R	UU
BB-D09-SR	Spontaneous Str^R derivative of BB-D09	This study
BB-P19 Δ*pagP*	*pagP* mutant of BB-P19, Cef^R, Str^R, Gem^R	This study
BB-D09-SR Δ*pagP*	*pagP* mutant of BB-D09-SR, Cef^R, Str^R, Gem^R	This study
BB-D09-SR *lpxL1*	*lpxL1* mutant of BB-D09-SR, Cef^R, Str^R, Gem^R	This study
E. coli		
DH5α	F^-, Δ(lacZYA-argF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15	(Grant et al., 1990)
SM10(λpir)	thi thr leu fhuA lacY supE recA::RP4-2-Tc::Mu λpir R6K. Kan^R	(Simon et al., 1983)
Plasmids		
pCRII	*E. coli* cloning vector, Amp^R, Kan^R	Invitrogen
pYRC	pBBR1MCS-5 *lacI* cloning vector, Gem^R	(Arts et al., 2007)
pCRII-PagPup	pCRII derivative harboring *pagP*-upstream sequence, Amp^R, Kan^R	This study
pCRII-PagPdw	pCRII derivative harboring *pagP*-downstream sequence, Amp^R, Kan^R	This study
pCRII-PagPup-PagPdw	pCRII derivative harboring *pagP*-up/down-stream sequence, Amp^R, Kan^R	This study
pCRII-PagPup-GemR-PagPdw	pCRII-PagPup-PagPdw harboring gentamicin-resistance cassette, Amp^R, Kan^R, Gem^R	This study
pCRII-upLpxL1dw	pCRII derivative harboring *lpxL1*-locus and up/down-stream sequence, Amp^R, Kan^R	This study
pCRII-upLpxL1dw-GemR	pCRII-upLpxL1dw harboring gentamicin-resistance cassette, Amp^R, Kan^R, Gem^R	This study
pKAS32	Allelic exchange suicide vector, Amp^R	(Skorupski and Taylor, 1996)
pKAS-PagPup-GemR-PagPdw	pKAS32 derivative harboring *pagP* knockout construct, Amp^R, Gem^R	This study
pKAS-upLpxL1dw-GemR	pKAS32 derivative harboring *lpxL1* knockout construct, Amp^R, Gem^R	This study

[a] Cef, cefotaxime; Str, streptomycin; Gem, gentamicin; Amp, ampicillin; Kan, kanamycin; i. n., isolate number.
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Table S2. PCR primers used in this study

Primer	Sequence (5'→3') [a]	Description
Fw-PagPup	ACAAGCTGCAAGGCGTCCTG	Primers for amplification of region upstream *pagP*, introducing an Eco81I restriction site
Rv-PagPup-Eco81I	GCGCGCCCTCAGGGTCATATGCTGCGCTAACGG	
Fw-Eco81I-PagPdw	GCGCGCCCTGAGGTTCATGTTTGGCCGCTGGG	Primers for amplification of region downstream *pagP*, introducing an Eco81I restriction site
Rv-PagPdw	CATCGAAGCGGTCGACTTGC	
Fw-Eco81I-GemR	GCGCGCCCTGAGGGACGCACACCGTGGAAA	Primers for amplification of *gem*^{R}, introducing Eco81I restriction sites at both flanks
Rv-GemR-Eco81I	GCGCGCCCTCAGGGCGGCGTTGTGACAATTT	
Fw-upLpxL1	AATTCGCTCTGGCGCTGCAC	Primers for amplification of *lpxL1* locus with partial upstream and downstream sequence
Rv-LpxL1dw	ATCAGGGCATTGATGCGTTC	
Fw-PfoI-GemR	GCGCGCTCCTGGAGACGCACACCGTGGAAA	Primers for amplification of *gem*^{R}, introducing PfoI restriction sites at both flanks
Rv-GemR-PfoI	GCGCGCTCCAGGAGCGGCGTTGTGACAATTT	

[a] Restriction sites are underlined.

3

Physiological consequences of inactivation of *lgmB* and *lpxL1*, two genes involved in lipid A synthesis in *Bordetella bronchiseptica*

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Abstract

To develop a *Bordetella bronchiseptica* vaccine with reduced endotoxicity, we previously inactivated *lpxL1*, the gene encoding the enzyme that incorporates a secondary 2-hydroxy-laurate in lipid A. The mutant showed a myriad of phenotypes. Structural analysis showed the expected loss of the acyl chain but also of glucosamine (GlcN) substituents, which decorate the phosphates in lipid A. To determine which structural change causes the various phenotypes, we inactivated here *lgmB*, which encodes the GlcN transferase, and *lpxL1* in an isogenic background and compared the phenotypes. Like the *lpxL1* mutation, the *lgmB* mutation resulted in reduced potency to activate human TLR4 and to infect macrophages and in increased susceptibility to polymyxin B. These phenotypes are therefore related to the loss of GlcN decorations. The *lpxL1* mutation had a stronger effect on hTLR4 activation and additionally resulted in reduced murine TLR4 activation, surface hydrophobicity, and biofilm formation, and in a fortified outer membrane as evidenced by increased resistance to several antimicrobials. These phenotypes, therefore, appear to be related to the loss of the acyl chain. Moreover, we determined the virulence of the mutants in the *Galleria mellonella* infection model and observed reduced virulence of the *lpxL1* mutant but not of the *lgmB* mutant.

Introduction

Gram-negative bacteria are covered by an outer membrane (OM) that primarily consists of lipopolysaccharide (LPS, a.k.a. endotoxin) in the outer leaflet of the bilayer. LPS is generally composed of three moieties, lipid A, a core oligosaccharide, and an O-antigen (Raetz and Whitfield, 2002). The lipid A domain is typically responsible for the endotoxicity associated with infections by Gram-negative bacteria. It interacts with a receptor composed of the Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) on innate immune cells, thereby triggering a response, which eventually results in the release of pro-inflammatory cytokines, such as TNFα and IL-1β (Raetz and Whitfield, 2002). Lipid A consists of a β-1,6-disaccharide of glucosamine (GlcN), which is phosphorylated and acylated with β-hydroxylated acyl chains. These primary fatty acids can be substituted with secondary acyl chains. The lipid A structure varies between species and can be modified in response to environmental conditions. The final structure is defined by the specificity of the enzymes involved in lipid A biosynthesis and the regulated expression of post-synthetic modification systems, which include acylation, de-acylation, dephosphorylation, and substitution of the phosphates with groups like phosphoethanolamine, 4-amino-4-deoxy-L-arabinose (L-Ara4N), or GlcN (Raetz et al., 2007). The endotoxicity of lipid A varies and depends on the number and the length of the fatty acids, as well as on the number and the substitutions of the phosphate groups (Raetz et al., 2007; Matsuura, 2013).

LPS toxicity is also responsible for undesired side effects of whole-cell vaccines against Gram-negative bacteria (Norimatsu et al., 1995; Garcia et al., 1998). This reactogenicity has been intensively studied in human vaccines and, although sometimes ignored in formulations for animals, it is also increasingly becoming a concern for the immunization of livestock and companion animals. This is the case for vaccines against *Bordetella bronchiseptica* (Deville et al., 2009; Ellis, 2015), a Gram-negative bacterium involved in respiratory diseases in mammalian species, such as tracheobronchitis (a.k.a. kennel cough) in dogs and atrophic rhinitis in pigs (Goodnow, 1980). The LPS of this bacterium triggers strong inflammatory responses after TLR4 activation (Mann et al., 2005), which makes whole-cell vaccine formulations highly reactogenic.

Structural modification of lipid A can be used to reduce TLR4 activation and diminish the reactogenicity of a vaccine. Genetic engineering has been applied to reduce endotoxicity in several bacterial species (Simpson and Trent, 2019; Arenas et al., 2020; Kawahara, 2021), including *B. bronchiseptica* (Pérez-Ortega et al., 2021). In the latter study, we showed the effect of inactivation of two genes encoding lipid A acylases, i.e. *pagP* and *lpxL1*, on LPS endotoxicity in a *B. bronchiseptica* dog isolate. PagP mediates the insertion of a secondary palmitate (C_{16}) to the primary acyl chain at the 3' position of lipid A. This enzyme is localized in the OM, and the regulation of its synthesis depends on the major virulence-regulatory system BvgAS (Preston et al., 2003). Inactivation of *pagP* resulted in

only a limited reduction of endotoxicity, possibly because the PagP-dependent acylation was found to be non-stoichiometric and present in only ~10% of the LPS molecules in the parental strain (Pérez-Ortega et al., 2021). LpxL1 is responsible for the insertion of a secondary 2-hydroxy-laurate (2-OH C_{12}) at the 2 position of lipid A. In *B. bronchiseptica*, lipid A is stoichiometrically acylated by LpxL1 (Basheer et al., 2011; MacArthur et al., 2011; Pérez-Ortega et al., 2021). Although the *lpxL1* gene is also present in the closely related human pathogen *Bordetella pertussis*, the corresponding acylation of lipid A was only observed after its artificial overexpression, which resulted then in an increased TLR4-stimulating activity (Geurtsen et al., 2007). Accordingly, inactivation of *lpxL1* in *B. bronchiseptica* drastically reduced TLR4-stimulating activity (Pérez-Ortega et al., 2021).

Further phenotypic characterization of the *B. bronchiseptica lpxL1* mutant revealed many pleiotropic effects of the mutation, including decreased susceptibility to rifampicin and sodium dodecyl sulfate (SDS), increased susceptibility to polymyxin B, and decreased surface hydrophobicity, auto-aggregation, biofilm formation, and infectivity of macrophages. Structural analysis of lipid A showed that *lpxL1* inactivation did not only result in the expected loss of the 2-OH C_{12} chain but also of the GlcN residues that non-stoichiometrically decorate the phosphate groups in lipid A (Pérez-Ortega et al., 2021). This result suggests that the decoration of the phosphates, which is mediated by the glycosyltransferase LgmB in bordetellae (Marr et al., 2008), is prevented in the absence of the 2-OH C_{12} chain. Since also the loss of these GlcN residues could affect TLR4 activation (Geurtsen et al., 2009; Marr et al., 2010), it was not clear whether the decreased TLR4-stimulating activity of the *lpxL1* mutant, or any of its other phenotypes, is due to the loss of the acyl chain, the GlcN residues, or both.

In this study, we aimed to elucidate the role of the absence of GlcN in the aforementioned phenotypes of the *lpxL1* mutant by constructing *lgmB* and *lpxL1* mutants in an isogenic background and comparing their phenotypes. In addition, we studied the effect of the lipid A structural changes on bacterial virulence using the *Galleria mellonella* in vivo model.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are described in Supplementary Table S1. *Escherichia coli* strains were grown at 37 °C in lysogeny broth (LB) while shaking or on LB agar plates. *B. bronchiseptica* strains were grown at 35 °C on Bordet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading) (BG-blood). For liquid cultures, bacteria were scraped from plate and grown in Verwey medium (Verwey et al., 1949) at 35 °C while shaking at 175 rpm. For biofilm formation assays, Stainer-Scholte (SS) medium (Stainer and Scholte, 1971) supplemented with 14 g/L Bacto casamino acids (BD biosciences) and then adjusted to pH 7.6 was used. The optical density at 600 nm

(OD_{600}) of the bacterial suspensions was adjusted to 0.1 with fresh medium, and cultures were grown for 17 h, unless otherwise indicated. When needed for plasmid maintenance or strain selection, the following antibiotics were included in the medium: cefotaxime (5 µg/mL), streptomycin (300 µg/mL), gentamicin (10 µg/mL), kanamycin (50 µg/mL), and ampicillin (100 µg/mL).

DNA manipulation and construction of mutants

All plasmids used in this study are listed in Table S1. PCR fragments used for cloning were generated using the Expand High Fidelity PCR system (Roche Diagnostics GmbH), while regular PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific). PCR products were purified using the commercial Wizard SV Gel and PCR Clean-Up System (Promega). Plasmids were isolated with the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). PCR products and plasmids were digested with the appropriate restriction enzymes (Thermo Scientific) according to manufacturer's instructions, purified, and ligated using T4 DNA ligase (5 U/µL) (Thermo Scientific).

To inactivate the *lgmB* gene (locus tag BB4268 in *B. bronchiseptica* reference strain RB50), a DNA fragment was synthesized at BaseGene that included segments of 800 bp of the sequences upstream and downstream of *lgmB* in strain RB50. The entire *lgmB* gene in between, with the exception of 4 bp overlapping with the upstream gene, was replaced in the synthetic sequence by a gentamicin-resistance (*gem*^{R}) cassette flanked by Eco81I restriction sites. In addition, the entire synthetic fragment was flanked by XbaI restriction sites. The artificial sequence was introduced in pUC57-Kan by blunt-end cloning into the SmaI restriction site of the vector. The resulting plasmid was introduced in *E. coli* strain DH5α by transformation using the $CaCl_2$ method. Strain selection was performed on LB plates containing either kanamycin alone or both kanamycin and gentamicin. No colonies were obtained on the plates with both antibiotics, suggesting that the *gem*^{R} cassette was not expressed. Therefore, an alternative *gem*^{R} cassette was PCR-amplified from plasmid pYRC using forward primer Fw-Eco81I-GemR (GCGCGC<u>CCTGAGG</u>GACGCACACCGTGGAAA) and reverse primer Rv-GemR-Eco81I (GCGCGC<u>CCTCAGG</u>GCGGCGTTGTGACAATTT), which both introduced Eco81I restriction sites (underlined). Then, the amplicon was used to replace the synthetic *gem*^{R} cassette by digestion of the plasmid and PCR product with Eco81I and ligation. After transformation of strain DH5α, colonies were obtained on LB plates containing both antibiotics, and the orientation of the new *gem*^{R} cassette in the direction of transcription of the *lgm* operon was confirmed by PCR on isolated colonies. From the plasmid obtained, named pUC57-Kan Δ*lgmB*, the knockout construct was then subcloned into the suicide vector pKAS32 after digestion of both plasmids with XbaI and ligation, yielding pKAS-Δ*lgmB*. This plasmid was used to transform *E. coli* strain SM10(λpir), which allowed for plasmid replication and subsequent transfer to *B. bronchiseptica* by conjugation. Chromosomal knockouts were obtained by allelic exchange. First, transconjugants were selected on plates containing gentamicin and cefotaxime for selection of plasmid integration and counterselection against *E. coli*,

respectively. Subsequently, selection was made with streptomycin and gentamicin to ensure the loss of the plasmid backbone together with the wild-type *lgmB* gene. Transconjugants were screened by PCR.

TLR4 stimulation assays

TLR4 stimulation assays were performed as previously described (Pérez-Ortega et al., 2021). Briefly, HEK-Blue TLR4 cells co-expressing either human (h-) or murine (m-)TLR4, MD-2, and CD14 genes and an NF-κB-inducible gene for a secreted embryonic alkaline phosphatase (SEAP) reporter (Invivogen) were incubated with serial dilutions of whole bacterial cells, which were killed by incubation for 1 h at 56 °C. After 17 h of incubation at 37 °C in a 5% saturated CO_2 atmosphere, supernatants were incubated with *p*-nitrophenyl phosphate solution for 1 h, and the absorbance at 405 nm was measured in a Biotek microplate reader.

Sensitivity to SDS and antimicrobials

Sensitivity to SDS and antimicrobials was determined as described previously (Pérez-Ortega et al., 2021). In short, exponentially growing bacterial cultures were adjusted to an OD_{600} of 0.1 in fresh medium and incubated with either SDS or, as a control, Milli-Q water. After 2 h incubation, 10-fold serial dilutions were prepared, and 10-μL drops of each dilution were spotted on BG-blood agar plates. Growth was evaluated after 72 h of incubation at 35 °C. Minimal inhibitory concentrations (MICs) of different antibiotics were determined by Etest (BioMerieux). Briefly, 200 μL of exponentially growing bacterial cultures were spread on BG-blood agar, and Etest strips were placed on the plates. After two days of incubation, MICs were calculated from the zone of growth inhibition.

Bacterial adhesion to hydrocarbons (BATH)

BATH assays (Rosenberg, 1984) were performed with previously described modifications (Pérez-Ortega et al., 2021). Briefly, bacterial cultures grown for 20 h were washed and resuspended in phosphate-buffered saline (PBS), and the OD_{600} was adjusted to 1. The bacterial solution was added to test tubes with or without hexadecane and, after vigorous vortexing, the aqueous and organic phases were allowed to separate during 15 min. Samples were taken from the aqueous phase and their OD_{600} was measured. BATH (%) was expressed as $(n-h) \times 100/n$, with n being the OD_{600} in the tube without hexadecane and h the OD_{600} in the tube with hexadecane.

Biofilm formation

Biofilms were formed and quantified as previously described (Pérez-Ortega et al., 2021). In short, cultures grown for 24 h in SS medium supplemented with casamino acids were adjusted to an OD_{600} of 0.5 and incubated for 24 h under static conditions at 35 °C in 24-well plates. Then, the biofilms formed were washed and stained for 2 min with 0.5% crystal violet. After two washes, the stained biofilm was resuspended in 500 μL of 33% (v/v) acetic acid, and the OD_{630} was quantified.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
Whole cells were mixed with sample buffer (Laemmli, 1970), boiled for 10 min, and analyzed on 8–16% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad). After electrophoresis, LPS was stained with silver (Tsai and Frasch, 1982).

Porcine bone marrow-derived macrophages (PBMMs)
PBMMs were grown as previously described (Gao et al., 2018) with modifications (Pérez-Ortega et al., 2021). Briefly, bone marrow was harvested from six young adult pigs, and mononuclear cells were isolated by Ficoll (GE Healthcare) density gradient centrifugation. For M1-macrophage differentiation, cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal calf serum (Corning) for 6 days exposed to 40 ng/mL granulocyte macrophage colony-stimulating factor (Bio-Rad). Expression of M1 surface markers was verified by flow cytometry (Gao et al., 2018). All animals were used and kept under the approval and guidelines of the animal ethical committee of Utrecht University.

Bacterial cultures were washed and resuspended in RPMI supplemented with 10% fetal calf serum. Bacterial suspensions were added to M1-macrophages at a multiplicity of infection (MOI) of 1 and incubated for 4 h. After removal of the supernatant, macrophages were lysed with 1% Triton X-100 in PBS. To measure only internalized bacteria, the cells were exposed to 150 µg/mL colistin sulfate (Sigma Aldrich) in PBS for 1 h at 37 °C and then washed before lysis. Samples from supernatant, lysate (attached + internalized bacteria), or lysate obtained after antibiotic treatment (internalized bacteria) were plated on BG-blood agar and incubated for 72 h at 37 °C, and colonies were counted. Total growth was calculated from the colony-forming units (CFU) in the supernatant plus those in the lysate. Attached bacteria were calculated from the CFU in the lysate minus those in the lysate of cells treated with antibiotics. After that, the CFU quantified for each fraction were normalized relative to CFU quantified in wells without macrophages.

***G. mellonella* in vivo test of virulence**
Bacterial cultures grown for 17 h in Verwey medium were washed and resuspended in Dulbecco's PBS (DPBS) (Sigma Aldrich) to an OD_{600} of 10. Then, 10 µL of bacterial suspension were injected in the last proleg of larvae purchased at a pet shop using a NovoPen 5 insulin pen (Novo Nordisk) combined with BD Micro-Fine Ultra (4 mm) needles. Ten larvae of approximately 180 to 350 mg were injected per group. After injection, larvae were placed in the dark at 35 °C, and their survival was monitored every 24 h. Death was assessed by absence of response to physical stimuli. A control group was injected with DPBS to discard death due to mechanical trauma.

Statistical analysis
All statistical analyses were performed using the GraphPad Prism software (version 6). In all cases, data was analyzed for statistical significance using either one- or two-way ANOVA (Dunnett's correction for multiple comparison).

Results

TLR4 activation by whole cells

To study the contribution of the loss of the GlcN decorations of lipid A to the various phenotypes of an *lpxL1* mutant, the *lpxL1* and *lgmB* genes were inactivated in strain BB-CHN-1P, a *B. bronchiseptica* isolate from pig. This strain was chosen as swine livestock is one of the main targets for *B. bronchiseptica* vaccines. Then, the TLR4-stimulating activities of heat-killed whole-cell suspensions of the wild type (WT) and the *lpxL1* and *lgmB* mutants were compared in HEK-Blue reporter cells expressing either h- or m-TLR4 (Figure 1). As reported before in the genetic background of dog isolate BB-D09-SR (Pérez-Ortega et al., 2021), inactivation of *lpxL1* resulted in drastically decreased activation of both h-TLR4 (Figure 1A) and m-TLR-4 (Figure 1B) as compared with WT cell preparations. Also preparations of the *lgmB* mutant strain showed a statistically significant decrease in h-TLR4 activation compared to WT cells, but the difference was much smaller than in the case of the *lpxL1* mutant (Figure 1A). In contrast, m-TLR4 stimulation was unaffected by *lgmB* inactivation (Figure 1B). These results demonstrate that the reduced potency of the *lpxL1* mutant to activate TLR4 is mainly due to the loss of the 2-OH C_{12} chain in lipid A, whilst the GlcN decorations also contribute to h-TLR4 but not to m-TLR4 activation.

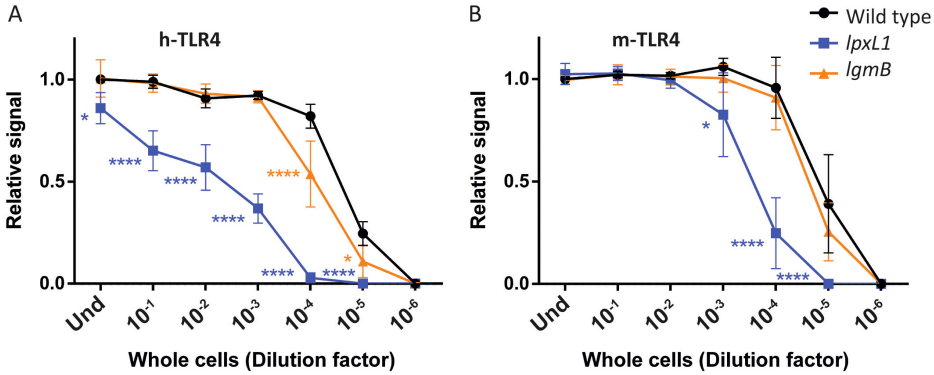

Figure 1. TLR4 activation by whole-cell preparations of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives. HEK-Blue cells expressing either h-TLR4 (**A**) or m-TLR4 (**B**) were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole bacterial cells. The OD_{600} of the undiluted bacterial suspensions (Und) was 0.04. Graphs show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution of each strain and the signal measured for the undiluted wild-type sample. Three independent experiments were performed in duplicate. Dilutions of the mutants with activities statistically different from those of the wild type are indicated with asterisks (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

OM barrier function

To test the integrity of the OM barrier function, SDS-sensitivity assays were performed. Consistent with previous results (Pérez-Ortega et al., 2021), the *lpxL1* mutant of strain BB-CHN-1P survived exposure to SDS much better than the WT did (Figure 2). On the other

hand, the *lgmB* mutant strain showed similar sensitivity to SDS as the WT, demonstrating that the higher SDS sensitivity of the WT compared with the *lpxL1* mutant is due to the presence of the 2-OH C_{12} chain in lipid A and not to the GlcN decorations.

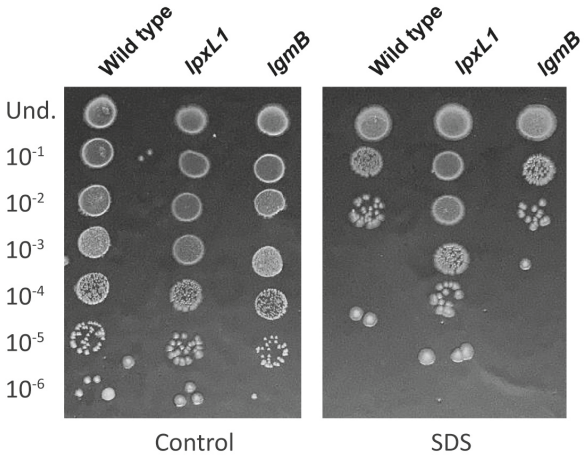

Figure 2. Bacterial sensitivity to SDS. Cultures of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were incubated with 1% SDS. After 2 h incubation, 10-µL drops of 10-fold serial dilutions from these cultures were plated on BG-blood agar and incubated for 72 h. The control (left panel) shows the results for bacteria not exposed to SDS. A representative result of three independent experiments is shown.

Furthermore, we compared the susceptibility of the WT and the mutants to the cationic antimicrobial peptides (CAMPs) polymyxin B and colistin (Table 1). Like the *lpxL1* mutant, the *lgmB* mutant strain showed a two-fold lower MIC for polymyxin B than the parental strain, indicating that the GlcN substitutions protect the WT against polymyxin B. In contrast, whilst the *lpxL1* mutant is slightly more sensitive to colistin than the WT, inactivation of *lgmB* did not affect colistin susceptibility (Table 1). Similarly, the susceptibility of the mutants to other antimicrobials was tested. Whilst susceptibility to the amphipathic antibiotic ciprofloxacin was hardly or not affected in the *lpxL1* or *lgmB* mutants, the *lpxL1* mutant was resistant to the hydrophobic antibiotic rifampicin in contrast to the *lgmB* mutant whose susceptibility to rifampicin was barely affected as compared with the WT (Table 1). Thus, the loss of the 2-OH C_{12} chain protects the *lpxL1* mutant against rifampicin.

Table 1. Antimicrobial susceptibility of strain BB-CHN-1P and its mutant derivatives[a]

	Wild type	***lpxL1***	***lgmB***
Polymyxin B	0.38	0.19	0.19
Colistin	0.125	0.094	0.125
Ciprofloxacin	0.38	0.50	0.38
Rifampicin	3	>32	4

[a]MICs reported are in µg/mL. Results were obtained in at least three independent experiments.

Cell-surface hydrophobicity and biofilm formation

Effects of the mutations on cell-surface hydrophobicity were tested by employing the BATH method. In concord with previous results (Pérez-Ortega et al., 2021), *lpxL1* inactivation in strain BB-CHN-1P strongly reduced cell-surface hydrophobicity, which dropped from 91% for the WT to ~32% for the mutant (Figure 3A). A statistically significant reduction in surface hydrophobicity was also observed for the *lgmB* mutant, but, as it dropped only to 85%, this reduction in hydrophobicity is negligible compared to the effect of *lpxL1* inactivation (Figure 3A).

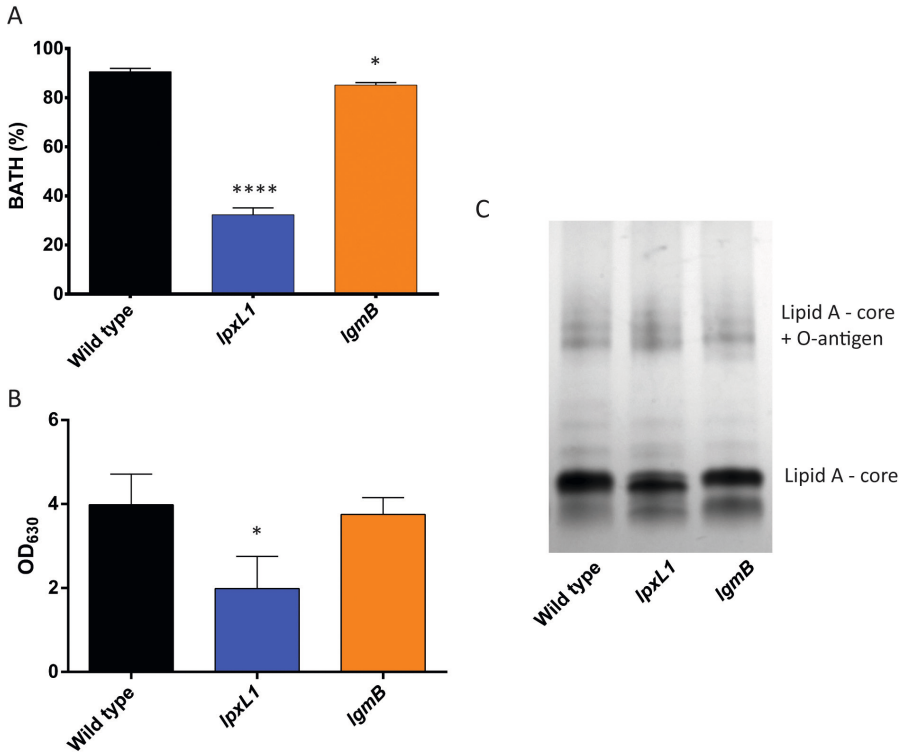

Figure 3. Effect of the inactivation of *lgmB* on surface hydrophobicity, biofilm formation, and LPS structure. (**A**) Surface hydrophobicity was assessed using the BATH method. Bacterial suspensions of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were mixed with hexadecane, and the percentage of hydrophobicity was calculated from OD_{600} measurements of samples from the hydrophilic phase. Data represent means and standard deviations from three independent experiments performed in duplicate. (**B**) Biofilm formation. Biofilms were grown for 24 h under static conditions in SS medium supplemented with casamino acids, stained with crystal violet, and quantified by measuring the OD_{630}. Data represent means and standard deviations from three experiments performed in triplicate. In both panels A and B, statistically significant differences compared to the wild type are indicated with asterisks (*, $P \leq 0.05$; ****, $p \leq 0.0001$). (**C**) Analysis of LPS modifications by SDS-PAGE. Whole-cell lysates of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were analyzed by SDS-PAGE, and LPS was stained with silver. O-antigen-containing LPS appears as diffuse smear with lower electrophoretic mobility than the lipid A-core forms.

Since changes in cell-surface hydrophobicity can affect biofilm formation (Renner and Weibel, 2011), also biofilm formation was measured. As compared to the WT, no significant reduction in biofilm formation was observed in the *lgmB* mutant strain, in contrast to the *lpxL1* mutant strain, which showed much lower production of biofilm (Figure 3B), consistent with previous results (Pérez-Ortega et al., 2021). These results suggest that the reduction in surface hydrophobicity and in biofilm formation of the *lpxL1* mutant is mainly a consequence of the loss of the 2-OH C_{12} chain.

We previously reported that the *lpxL1* mutant of dog isolate BB-D09-SR produced increased amounts of O-antigen-containing LPS, which could potentially explain the reduction in cell-surface hydrophobicity (Pérez-Ortega et al., 2021). To examine O-antigen production in strain BB-CHN-1P and its mutant derivatives, whole-cell lysates were treated with proteinase K and analyzed by SDS-PAGE. The majority of the LPS detected does not contain O-antigen and consists of lipid A and the core oligosaccharide (Figure 3C). Consistent with previous results in strain BB-D09-SR (Pérez-Ortega et al., 2021), a slightly increased electrophoretic mobility of this form of the LPS was observed in the *lpxL1* mutant (Figure 3C). Since a similar increase in electrophoretic mobility was not observed in the *lgmB* mutant, it is mainly due to the loss of the 2-OH C_{12} chain and not of the GlcN residues. In all three strains, similar amounts of O-antigen-containing LPS were detected (Figure 3C). This suggests that the increased amount of O-antigen previously observed in the *lpxL1* mutant of strain BB-D09-SR is a strain-specific consequence of the *lpxL1* mutation and not (solely) responsible for the observed reduction in surface hydrophobicity and biofilm formation.

Infection of macrophages

To determine whether the mutations affect the viability of *B. bronchiseptica* in the presence of macrophages, the bacteria were incubated for 4 h with porcine macrophages, and bacterial survival was quantified. The *lpxL1* mutant of BB-CHN-1P showed a slightly increased viability in the presence of macrophages, which is in agreement with previous results (Pérez-Ortega et al., 2021), but the *lgmB* mutant showed a slightly reduced viability relative to the WT (Figure 4A). These differences were reflected in the supernatant (Figure 4B), but not in the bacterial adhesion to macrophages (Figure 4C). The *lgmB* mutant showed increased ability to attach to the immune cells compared with the WT and *lpxL1* mutant strains. However, none of these differences was statistically significant. Interestingly, however, the amount of both the *lgmB* mutant and of the *lpxL1* mutant detected inside the macrophages was significantly lower than that of the WT (Figure 4D). Thus, the reduced infectivity of macrophages of the *lpxL1* mutant, also previously reported in strain BB-D09-SR (Pérez-Ortega et al., 2021), appears to be related to the loss of the GlcN decorations of lipid A, rather than to the loss of the 2-OH C_{12} chain.

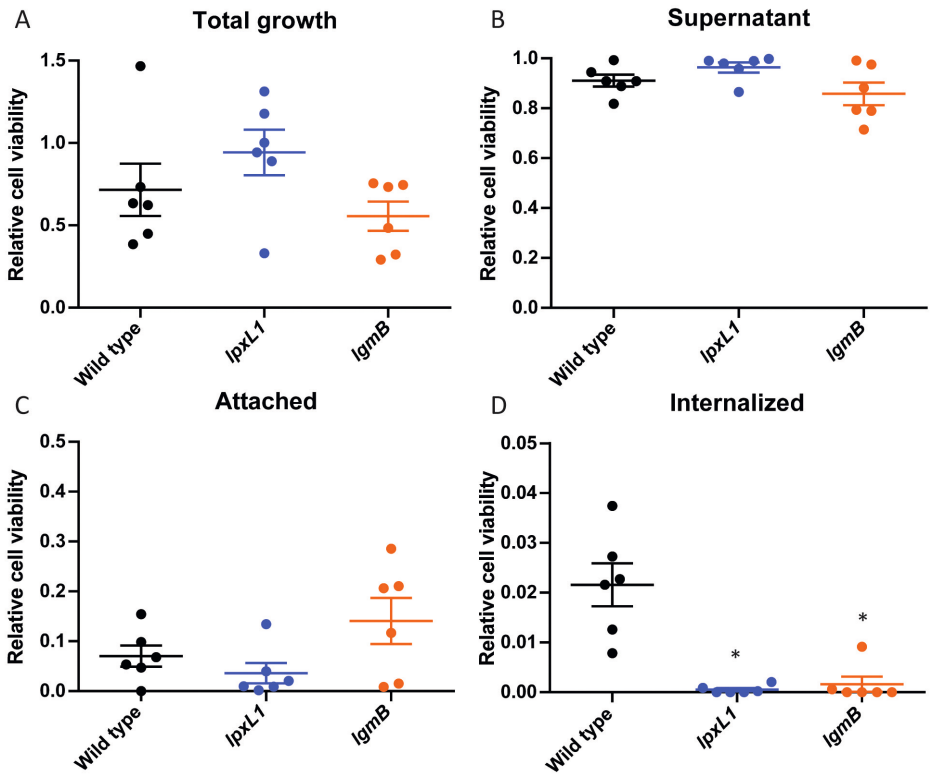

Figure 4. Bacterial survival with porcine macrophages. Suspensions of strain BB-CHN-1P and its *lgmB*- and *lpxL1*-mutant derivatives were incubated for 4 h with M1 macrophages at an MOI of 1. Data represent cell viability calculated from the CFU in different fractions expressed relative to CFU after incubation in the absence of macrophages. (**A**) Total relative bacterial count (i.e. both inside and outside of the macrophages). (**B**) Relative bacterial count in the supernatant. (**C**) Relative count of bacteria attached at the macrophage surface. (**D**) Relative bacterial count inside macrophages. Symbols correspond to PBMMs of six separate pigs. Statistical significance compared to the wild type was found for the internalized bacteria and is indicated with asterisks (*, $P \leq 0.05$).

Virulence of *B. bronchiseptica* mutants in the *G. mellonella* infection model

To determine the effect of different lipid A structural modifications on virulence, we injected equivalent amounts of bacterial suspensions of the WT strain BB-CHN-1P and its *lpxL1* and *lgmB* mutant derivatives into *G. mellonella* larvae and monitored larval mortality over time. Independent experiments consistently showed increased survival of the larvae infected with the *lpxL1* mutant compared to those infected with the WT or the *lgmB* mutant (see representative example in Supplementary Figure S1A). However, combination of the data from all replicate experiments resulted in large standard deviations and, consequently, differences were not statistically significant. This is probably due to the use of larvae from a pet shop, which are known to present large batch-to-batch variation due to differences in breeding and storage conditions. Such variation may affect the status of the immune system of the model at the time of the assay (Pereira and

Rossi, 2020). Therefore, we decided to express the combined results of the experiments as the difference in survival of mutant-infected larvae relative to that of WT-infected larvae per day in percentage points. In this way, we observed a small but significant effect on virulence by the *lpxL1* mutation (Figure 5A). To verify this result, we also tested the effect of *lpxL1* inactivation in the genetic background of strain BB-D09-SR. Of this strain, we also had a *pagP* mutant at our disposal, which we also tested. Again, the *lpxL1* mutation resulted in reduced virulence compared to the WT and the *pagP* mutant (see representative example in Figure S1B). When data from all replicate experiments were combined (Figure 5B), the effect on virulence of the *lpxL1* mutation was significant and even more prominent than in the genetic background of strain BB-CHN-1P (compare Figure 5A and B). As the virulence of the *lgmB* mutant was unaffected (Figure 5A and Figure S1A), the reduced virulence of the *lpxL1* mutants appears to be due to the loss of the 2-OH C_{12} chain, rather than to the loss of the GlcN modifications. Interestingly, the *pagP* mutant showed slightly increased virulence (Figure 5B and Figure S1B), but this difference was not statistically significant.

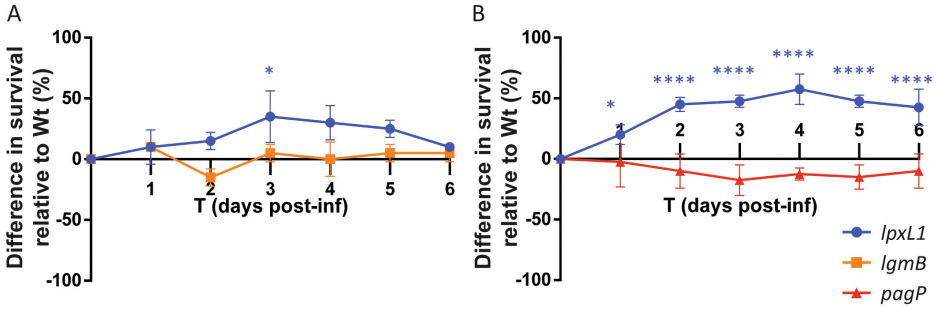

Figure 5. Survival of *G. mellonella* larvae infected with *B. bronchiseptica* WT strains and *lgmB*-, *lpxL1*-, and *pagP*-mutant derivatives. The *G. mellonella* larvae were infected with OD-adjusted bacterial suspensions of strains BB-CHN-1P (**A**) or BB-D09-SR (**B**) and their mutant derivatives. Survival was monitored every 24 h over 6 days post infection. Graphs show the difference in survival of larvae injected with mutant derivatives relative to those infected with the corresponding parental strain in percentage points. Data represent means and standard deviations from three independent experiments. For the mutants, statistically significant differences compared to the WT infections are indicated with asterisks (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

Discussion

Inactivation of the *lpxL1* gene in *B. bronchiseptica* strain BB-D09-SR resulted in many pleiotropic effects (Pérez-Ortega et al., 2021). Structural analysis of lipid A showed the expected loss of the 2-OH C_{12} acyl chain and, unexpectedly, also of the GlcN residues that decorate the phosphates in lipid A. In this study, we wanted to determine which structural alteration is responsible for the different phenotypes observed. Therefore, we constructed an *lgmB* mutant in strain BB-CHN-1P and compared its phenotypes with those of an isogenic *lpxL1* mutant. A caveat of this study is that we didn't perform mass

spectrometric analysis of lipid A to confirm that the *lpxL1* mutation also leads to the loss of the GlcN decorations in strain BB-CHN-P1. However, the myriad of phenotypes described for the *lpxL1* mutant of strain BB-D-09-SR was also found in that of strain BB-CHN-P1, suggesting the presence of identical structural changes in the LPS. Moreover, some of these phenotypes were also found in the *lgmB* mutant demonstrating that they can be attributed to the loss of the GlcN residues. In addition, it has been reported that mutation of *lpxM*, the gene encoding an acyltransferase responsible for the attachment of a secondary acyl chain to lipid A, in *E. coli* and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) prevents the decoration of the phosphates with L-Ara4N (Tran et al., 2005). Thus, a complete acylation pattern appears to be a common prerequisite for the glycosylation of the phosphates of lipid A, and, therefore, strain-specific differences in *B. bronchiseptica* in this respect seem to be very unlikely.

We found that *lgmB* deletion reduces the h-TLR4 stimulating activity but to a much lesser extent than *lpxL1* inactivation did. Furthermore, in contrast to *lpxL1* inactivation, it had no effect on m-TLR4-stimulating activity. It is also noteworthy that the *lpxL1* mutation has a considerably stronger effect on h-TLR4 activation than on m-TLR4 activation (Figure 1, compare panels A and B). This data points to an additive effect of the loss of the GlcN residues and of the acyl chain on h-TLR4 activation, while m-TLR4 activation is only affected by the loss of the acyl chain. With respect to the relevance of the GlcN residues, our results are in agreement with previous observations in *B. pertussis*, where it was demonstrated that *lgmB* inactivation resulted in drastic decline of h-TLR4 stimulation but had no effect on m-TLR4 activity (Marr et al., 2010). In another study, reduced production of pro-inflammatory cytokine IL-6 was observed after stimulation of human monocytes with *lgmB* mutants of *B. pertussis* or *Bordetella parapertussis* (Geurtsen et al., 2009). In addition, it has been reported that the production of the cytokine TNFα in murine macrophages upon stimulation with *B. bronchiseptica* cells was not affected by *lgmB* inactivation (Rolin et al., 2014). Altogether, it appears that the loss of the secondary 2-OH C_{12} acyl chain in the *lpxL1* mutant strongly reduces TLR4 activation, whereas the effect of the loss of GlcN residues on TLR4 stimulation is smaller and species specific as it additively reduces the activation of h-TLR4 but not of m-TLR4.

The OM of the *lpxL1* mutant is fortified as appears from the reduced susceptibility to hydrophobic (rifampicin) and amphipathic (SDS) antimicrobial compounds (Table 1 and Figure 2). We expected this to be related to the loss of the GlcN residues (Pérez-Ortega et al., 2021), since it should enable the phosphates in lipid A to bind divalent cations, which in turn should promote cross linking between LPS molecules and boost OM integrity (Zgurskaya et al., 2015). However, inactivation of *lgmB* hardly affected the susceptibility to these antimicrobials. Only a slight decrease in susceptibility to rifampicin was observed, which, indeed, could be due to improved cross linking between LPS molecules. Thus, the loss of the 2-OH C_{12} chain appears to be much more relevant for the decrease in OM permeability of the *lpxL1* mutant. Probably the loss of this hydroxylated acyl chain

improves the packing of the remaining acyl chains in the outer leaflet of the OM, thus preventing the penetration of the antimicrobial compounds into the hydrophobic interior of the bilayer.

It is well established that the decoration of the phosphate groups of lipid A can reduce bacterial susceptibility to CAMPs (Raetz et al., 2007), but also the insertion of acyl chains can have a similar effect, as has been demonstrated for example in *E. coli* and *Klebsiella pneumoniae* (Li et al., 2013; Velkov et al., 2013). Inactivation of *lgmB* indeed resulted in increased susceptibility to the CAMP polymyxin B (Table 1), which matches previous observations (Rolin et al., 2014). However, this increment was similar to that of the *lpxL1* mutant, suggesting that the reduced lipid A acylation in the latter had no additional impact on polymyxin B sensitivity. Although CAMPs are supposed to have a common mechanism of action, no effect of *lgmB* inactivation was observed on the susceptibility to colistin (a.k.a. polymyxin E), which has a chemical structure very similar to that of polymyxin B. Such contrasting results have also been reported for the susceptibility of an *lgmB* mutant to the CAMPs porcine β-defensin 1 (pBD1) and the closely related mouse β-defensin 3 (mBD3) (Rolin et al., 2014). While the mutant showed increased susceptibility to pBD1, no differences in susceptibility were detected for mBD3. Interestingly, the *lpxL1* mutant did show slightly increased sensitivity to colistin (Table 1) indicating that the loss of the acyl chain facilitates the activity of colistin but not of polymyxin B. Again, this discrepancy suggests that the two polymyxins do not have an identical mechanism of action.

Previously, we reported a strong decrease in surface hydrophobicity of an *lpxL1* mutant of strain BB-D09-SR (Pérez-Ortega et al., 2021). We hypothesized this decrease to be related to the increase in the amount of LPS containing O-antigen, which was observed in this mutant. However, this increase in O-antigen seems to be strain specific, inasmuch as it was not observed in the genetic background of strain BB-CHN-1P, which was used in the present study (Figure 3C). Nevertheless, reduced surface hydrophobicity and a concomitant decrease in biofilm production were also observed in the *lpxL1* mutant of this strain, but not in the *lgmB* mutant. Thus, apparently, the decreased hydrophobicity observed in the *lpxL1* mutant is related to the loss of the acyl chain, but how this structural change in lipid A exactly affects this phenotype is not immediately clear.

The capacity of *B. bronchiseptica* to invade macrophages was reduced upon inactivation of *lpxL1* (Pérez-Ortega et al., 2021). As *lgmB* inactivation similarly affected macrophage invasion (Figure 4D), the loss of the GlcN residues seems to be responsible for this defect. Previously, we suggested that the absence of the secondary 2-OH C_{12} chain was likely responsible for this phenotype (Pérez-Ortega et al., 2021). This hypothesis was based on earlier observations in an *lpxL1* mutant of *B. pertussis*, whose capacity to invade macrophages was similarly affected as was the *lpxL1* mutant of *B. bronchiseptica* (Geurtsen et al., 2007). However, GlcN decorations can also be found in *B. pertussis* lipid A (Marr

et al., 2008; Geurtsen et al., 2009), and it is likely that inactivation of *lpxL1* in *B. pertussis* hinders lipid A glycosylation similarly as in *B. bronchiseptica*. Thus, the loss of the GlcN residues in lipid A is probably also responsible for the reported defect in macrophage invasion in *B. pertussis* (Geurtsen et al., 2007). Interestingly, it was recently reported that also the decoration of lipid A with L-Ara4N in a colistin-resistant *Klebsiella pneumoniae* strain enhances the survival of the bacteria in human monocytes (Avendaño-Ortiz et al., 2023). Defense mechanisms of macrophages against intracellular bacteria include the production of CAMPs (Weiss and Schaible, 2015). Since the susceptibility of the *lgmB* mutant to polymyxin B is increased (Table 1), the CAMPs produced by the macrophages could be responsible for the reduced presence of intracellular bacteria lacking the GlcN decorations in lipid A.

The *G. mellonella* infection model has been established as a suitable in vivo model to study host–pathogen interactions due to its similarity to mammalian models with respect to the innate immune response, including the presence of a cellular response (i.e. hemocytes) and a humoral response (i.e. antimicrobial peptides, opsonins, and melanization). This model is easy to manipulate and reduces the use of mammalian models and costs (Pereira and Rossi, 2020). Unfortunately, the commercial *G. mellonella* TruLarv, which was specifically bred for scientific purposes, is not available anymore, as the producer has gone out of business because of trading problems due to the Brexit and the COVID-19 pandemic. Since another supplier of similar larvae could not be found, we had to rely on larvae obtained from a pet shop, which are known to yielded considerable variation between experiments (Pereira and Rossi, 2020). Nevertheless, we were able to show that inactivation of *lpxL1* significantly reduces the virulence of *B. bronchiseptica*, which could be related to an increased susceptibility of the mutant to the antimicrobial peptides produced by the larvae. Similarly, a mutant of *S.* Typhimurium with a defective *phoQ* gene, which is part of the two-component virulence regulatory system PhoPQ and regulates, amongst others, the decoration of the phosphate groups in lipid A with L-Ara4N, showed reduced virulence in the *G. mellonella* model and was less resistant to polymyxins (Bender et al., 2013). However, the *lgmB* mutant did not show reduced virulence despite being as susceptible to polymyxin B as the *lpxL1* mutant (Figure 5 and Table 1). As the *lgmB* mutant, in contrast to the *lpxL1* mutant, is not affected in colistin susceptibility, the mutants might also respond differently to the antimicrobial peptides produced by the larvae, and the presence of the secondary 2-OH C_{12} chain in lipid A might confer protection against these peptides. Alternatively, the LPS of *B. bronchiseptica* might be toxic to the *G. mellonella* larvae as has been demonstrated for LPS from *Caulobacter* spp. [35]. If that is the case, the loss of the 2-OH C_{12} chain might result in reduced LPS toxicity.

Taken together, our results indicate that the loss of the secondary 2-OH C_{12} chain in *B. bronchiseptica* lipid A strongly reduces TLR4-stimulating activity, cell-surface hydrophobicity, and biofilm formation, and it fortifies the OM barrier function as

evidenced by increased resistance to SDS and rifampicin. All these modifications have been observed in two different genetic backgrounds, discarding accidental secondary mutations as the reason for these changes. The GlcN decorations also contribute to the h-TLR4-stimulating activity but much less so than the secondary acyl chain, and they do not contribute to the m-TLR4-stimulating activity. Therefore, *lpxL1* mutants of *B. bronchiseptica* might be more valuable in the development of new whole-cell- or OMV-based vaccines with reduced endotoxicity than *lgmB* mutants. Furthermore, the GlcN residues confer protection against polymyxin B and improve macrophage invasion. We also demonstrate that *G. mellonella* can be a valuable model to investigate the effect of genetic modifications on the virulence of *B. bronchiseptica*, and the results obtained indicate that the secondary 2-OH C_{12} chain in lipid A contributes to the virulence of the bacteria. However, breeding conditions of the larvae should be meticulously controlled to avoid large differences between experiments.

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Supplemental material

Table S1. Bacterial strains and plasmids

Strain / plasmid	Description [a]	Reference
B. bronchiseptica		
BB-CHN-1P	Clinical isolate from pig, Cef^R	Pulike Biological Engineering Inc
BB-CHN-1P *lpxL1*	*lpxL1::gem* mutant of BB-CHN-1P, Cef^R, Str^R, Gem^R	This study
BB-CHN-1P *lgmB*	Δ*lgmB::gem* mutant of BB-CHN-1P, Cef^R, Str^R, Gem^R	This study
BB-D09-SR	Spontaneous Str^R derivative of BB-D09	(Pérez-Ortega et al., 2021)
BB-D09-SR *lpxL1*	*lpxL1::gem* mutant of BB-D09-SR, Cef^R, Str^R, Gem^R	(Pérez-Ortega et al., 2021)
BB-D09-SR *pagP*	Δ*pagP::gem* mutant of BB-D09-SR, Cef^R, Str^R, Gem^R	(Pérez-Ortega et al., 2021)
E. coli		
DH5α	F$^-$, Δ(lacZYA-argF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15	(Grant et al., 1990)
SM10(λpir)	thi thr leu fhuA lacY supE recA::RP4-2-Tc::Mu λpir R6K, Kan^R	(Simon et al., 1983)
Plasmids		
pUC57-Kan Δ*lgmB*	pUC57-Kan derivative harboring *lgmB* knockout construct, Kan^R, Gem^R	This study
pYRC	pBBR1MCS-5 *lacI* cloning vector, Gem^R	(Arts et al., 2007)
pKAS32	Allelic exchange suicide vector, Amp^R	(Skorupski and Taylor, 1996)
pKAS-Δ*lgmB*	pKAS32 derivative harboring *lgmB* knockout construct, Amp^R, Gem^R	This study
pKAS-upLpxL1dw-GemR	pKAS32 derivative harboring *lpxL1* knockout construct, Amp^R, Gem^R	(Pérez-Ortega et al., 2021)

[a] Cef, cefotaxime; Str, streptomycin; Gem, gentamicin; Amp, ampicillin; Kan, kanamycin.

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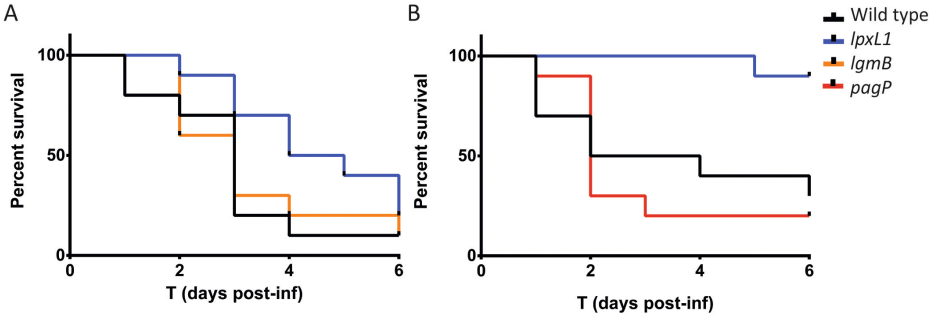

Figure S1. Survival of *G. mellonella* larvae infected with *B. bronchiseptica* WT strains and *lgmB*-, *lpxL1*-, and *pagP*-mutant derivatives. The *G. mellonella* larvae were infected with OD-adjusted bacterial suspensions of strains BB-CHN-1P (**A**) or BB-D09-SR (**B**) and their mutant derivatives. Survival was monitored every 24 h over 6 days post-infection. Both graphs show a representative experiment.

4

Biogenesis of the inner core of *Bordetella pertussis* LPS: Effect of mutations on LPS structure, cell division, and TLR4 activation

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Abstract

Structural modifications of the lipid A moiety of the lipopolysaccharide (LPS) influence the endotoxicity of many Gram-negative bacterial pathogens. The LPS of *B. pertussis*, the causative agent of whooping cough in humans, contains a penta-acylated lipid A with two phosphate groups. In this study, we initially aimed at dephosphorylating the lipid A by the heterologous production of the phosphatases LpxE and LpxF from *Francisella novicida*. Whereas the synthesis of LpxF did not result in significant lipid A dephosphorylation, production of LpxE drastically reduced phosphorylation, but Toll-like receptor 4 (TLR4)-stimulating activity remained unaffected. Therefore, we focused further on the inner core region of the LPS, which might also affect TLR4 signaling. *B. pertussis* lipid A is linked to the inner core sugars of the LPS via a single 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue, which is substituted with a phosphate that is non-stoichiometrically decorated with phosphoethanolamine (PEA). Insertion of a second Kdo was possible by the heterologous production of *Escherichia coli* Kdo transferase and resulted in a large loss of the terminal trisaccharide in the outer core region. The presence of the secondary Kdo also prevented dephosphorylation of the lipid A by *F. novicida* LpxE and caused cell filamentation. Additionally, we identified the *eptB* gene responsible for the introduction of PEA in *B. pertussis* LPS and showed that its inactivation not only prevented PEA insertion but also hindered lipid A dephosphorylation by *F. novicida* LpxE. Interestingly, these modifications at the Kdo level affected TLR4-stimulating activity of *B. pertussis* LPS. Although endotoxicity studies mainly focus on the configuration of the lipid A moiety, we present evidence that structural changes in the inner core can also affect TLR4-stimulating activity. In addition, such modifications affect the final structure of other moieties of the LPS.

Introduction

Lipopolysaccharide (LPS, a.k.a. endotoxin) is the major component of the outer leaflet of the outer membrane (OM) of Gram-negative bacteria (Silhavy et al., 2010). It consists of three domains, i.e., lipid A, a core oligosaccharide, and a polysaccharide known as O-antigen (Raetz and Whitfield, 2002). The latter is absent in some species, including *Bordetella pertussis*, and the LPS of these bacteria is also referred to as lipooligosaccharide (LOS). Innate immune cells recognize LPS during infection by Gram-negative bacteria and release pro-inflammatory cytokines, such as TNFα and IL-1β, which activate immune defenses. However, overstimulation of the immune system can cause serious damage with sometimes fatal consequences (Parrillo, 1993). This endotoxic response is also one of the main reasons for the adverse reactions observed after immunization with whole-cell vaccines against Gram-negative bacteria. LPS, mainly its lipid A moiety, is recognized by a heterodimeric receptor composed of the Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) (Raetz and Whitfield, 2002). Binding of LPS induces dimerization of the receptor, with LPS being the bridge between the two dimers in the resulting tetramer (Park et al., 2009). Lipid A is a glucosamine (GlcN) disaccharide substituted with phosphates at positions 1 and 4′ and acylated at positions 2, 2′, 3, and 3′ with β-hydroxylated fatty acids, which can be substituted with secondary acyl chains (Figure 1) (Raetz et al., 2007). Structural modification of lipid A can affect the activation of the TLR4 receptor and, thereby, of cytokine production. Such modifications naturally take place in many bacteria, often depending on the environmental conditions (Raetz and Whitfield, 2002). Usually, a reduction in the number of fatty acids or of the phosphates results in reduced endotoxicity (Raetz et al., 2007; Matsuura, 2013).

Some bacterial species, including *Francisella novicida*, *Helicobacter pylori*, and *Rhizobium leguminosarum*, produce the phosphatases LpxE and/or LpxF, which remove the phosphates at positions 1 and 4′, respectively, of lipid A. These phosphatases are located in the inner membrane and present their active site towards the periplasm. Their activity generates LPS forms with reduced endotoxicity (Raetz et al., 2007), since these phosphates contribute to receptor binding and dimerization by forming ionic interactions with clusters of positively charged residues in TLR4 and MD-2 (Park et al., 2009). LpxE of *F. novicida* ($LpxE_{Fn}$) has been shown to be effective in removing the phosphate at position 1 in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) lipid A (Wang et al., 2004). However, the *F. novicida* LpxF ($LpxF_{Fn}$) selectively dephosphorylates tetra- and penta-acylated lipid A molecules and, therefore, has no effect on wild-type lipid A from *E. coli*, which is hexa-acylated (Wang et al., 2006). Heterologous expression of $lpxE_{Fn}$ has proved to efficiently reduce endotoxicity in, for example, *S*. Typhimurium (Kong et al., 2011). Such strategies are currently used to reduce the endotoxicity of bacterial cells by genetic engineering for their use in vaccine development (Simpson and Trent, 2019; Kawahara, 2021).

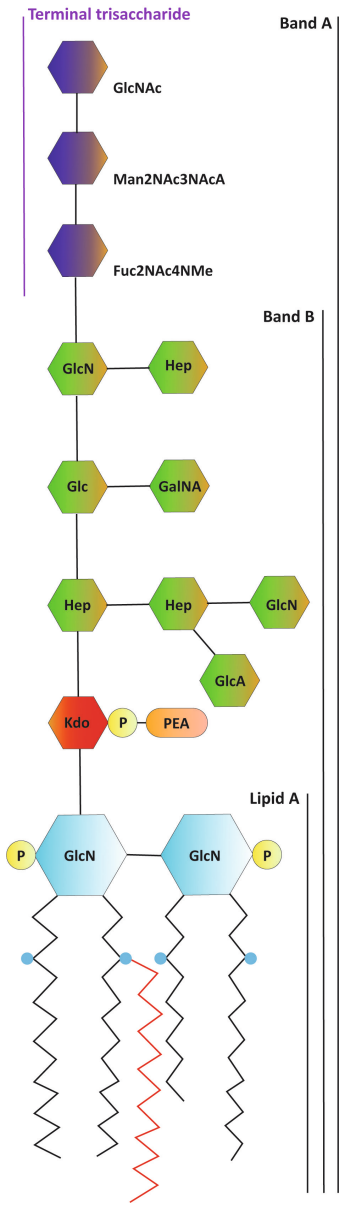


Figure 1. Schematic representation of *B. pertussis* LPS based on the study of Caroff et al. (2000). The various sugar units are depicted with hexagons, the phosphate groups with yellow circles, the PEA substitution with a rounded rectangle, and the hydroxylation of fatty acids with blue circles. The secondary C_{14} chain at position 2′, which is inserted by the acylase LpxL2, is indicated in red. The portions of the LPS corresponding with lipid A, band A, band B, and the terminal trisaccharide are indicated with vertical lines. Abbreviations: GlcN, glucosamine; P, phosphate; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; PEA, phosphoethanolamine; Hep, heptose; GlcA, glucuronic acid; Glc, glucose; GalNA, galactosaminuronic acid; Fuc2NAc4NMe, β-L-2-acetamido-4-methylamino-fucose; Man2NAc3NAcA, β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid; GlcNAc, α-*N*-acetyl-GlcN.

B. pertussis is a strictly human pathogen responsible for the respiratory infection known as whooping cough or pertussis. Introduction of the whole-cell pertussis vaccines (wP) around the 1940s led to a drastic reduction of pertussis cases. However, in the 1990s, these vaccines were replaced by acellular formulations (aP) because of safety concerns caused by the reactogenicity of wP, whose main culprit is the LPS (Mattoo and Cherry, 2005). In countries where aP vaccination was introduced, the number of pertussis cases is on the rise. This can at least partially be attributed to the rapid waning of the immune protection evoked by these vaccines as well as by their failure to prevent mucosal colonization and transmission (Esposito et al., 2019). In the last decades, genetic engineering has been applied in *B. pertussis* for the development of novel whole-cell vaccines with reduced endotoxicity (Geurtsen et al., 2006; Arenas et al., 2020). These studies focused on the number and length of the acyl chains of the LPS. *B. pertussis* lipid A presents two phosphate groups (Figure 1), which can be substituted with GlcN residues. The glycosyltransferase responsible for the introduction of this sugar is LgmB (**l**ipid A **G**lcN **m**odification **B**). In *B. pertussis*, *lgmB* expression seems to vary between strains, but its activity is usually low or even absent (Marr et al., 2008). To our knowledge, no genetic engineering efforts to reduce endotoxicity in *B. pertussis* have ever been targeting the phosphates in lipid A with the LpxE and/or LpxF phosphatases. Although an *lpxE* homolog has been found in the *B. pertussis* genome sequence (Geurtsen et al., 2006), the encoded enzyme appears to be a pyrophosphatase rather than a phosphatase (Zariri et al., 2016).

Aside from the phosphates in lipid A, negatively charged residues in the inner core seem to facilitate TLR4 signaling at least in some cases (Ittig et al., 2012). In *B. pertussis* LPS, a phosphate is present at position 4 of the single 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) present in the inner core region (Figure 1). Initially, this phosphate was considered as a minor non-stoichiometric substitution (Caroff et al., 2001) but, later, it was reported that the phosphate is often lost during the mild acid hydrolysis that is frequently used to isolate LPS fragments for mass-spectrometric analysis and that this phosphate actually is a stoichiometric substitution in the LPS (Novikov et al., 2019). On its turn, this phosphate can be substituted with a phosphoethanolamine (PEA) (Caroff et al., 2000) (Figure 1). The kinase responsible for the insertion of the phosphate group in Kdo and the specific PEA transferase are generically called KdkA and EptB, respectively (White et al., 1999; Reynolds et al., 2005). However, these enzymes have not been characterized so far in *B. pertussis*, and the *eptB* gene has not even been identified. As mentioned above, the *B. pertussis* LPS presents a single Kdo (KdoI), which is transferred by the monofunctional Kdo transferase KdtA (a.k.a. WaaA) (Isobe et al., 1999). In contrast, KdtA of *E. coli* ($KdtA_{Ec}$) and of many other Gram-negative bacteria introduces two Kdo residues (Belunis and Raetz, 1992; Raetz and Whitfield, 2002). The location of the second Kdo (KdoII) is position 4 of KdoI, i.e., the same position as where the phosphate substitution takes place in *B. pertussis*. In *F. novicida*, KdtA is also bi-functional but, at a later stage in LPS biosynthesis, a Kdo hydrolase trims off KdoII, thereby producing LPS molecules with only one, non-phosphorylated Kdo (Zhao and Raetz, 2010).

The outer core of *B. pertussis* LPS presents a terminal non-repeating trisaccharide (Figure 1) consisting of α-N-acetyl-GlcN (GlcNAc), β-2,3-diacetamido-2,3-dideoxy-mannuronic acid (Man2NAc3NAcA), and β-2-acetamido-4-N-methyl-2,4-dideoxy-fucose (Fuc2NAc4NMe) (Caroff et al., 2000), all synthesized and attached by enzymes encoded in the *wlb* operon (Allen and Maskell, 1996; Allen et al., 1998). This trisaccharide seems to be added en bloc to the core and is responsible for the detection of two bands when *B. pertussis* LPS is analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Band A corresponds to the entire LPS structure, i.e., lipid A and a branched dodecasaccharide core, while band B lacks the terminal trisaccharide (Figure 1).

In this study, we aimed at dephosphorylating the *B. pertussis* lipid A by the heterologous production of the phosphatases $LpxE_{Fn}$ and $LpxF_{Fn}$. In addition, we modified the environment of the core Kdo sugar by inactivation of *eptB* and by the heterologous production of the bi-functional KdtA of *E. coli*.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used are described in Supplementary Table S1. *E. coli* was grown at 37°C in lysogeny broth (LB) while shaking or on LB agar plates. *B. pertussis* was grown at 35°C on Bordet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading) (BG-blood). For liquid cultures, bacteria were scraped from BG-blood plates on which they had grown for three days, inoculated in Verwey medium (Verwey et al., 1949) to an optical density at 600 nm (OD_{600}) of 0.05, and grown for 24 h at 35°C while shaking at 175 rpm. When appropriate, the media were supplemented with nalidixic acid (50 µg/mL), streptomycin (300 µg/mL), gentamicin (10 µg/mL), kanamycin (50 µg/mL), and/or ampicillin (100 µg/mL) for plasmid maintenance or strain selection. To induce gene expression from plasmids, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium.

DNA manipulation and plasmid construction

All plasmids and relevant PCR primers used are listed in Supplementary Tables S1 and S2, respectively. Regular PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific), whilst PCR fragments generated for cloning were obtained using the Expand High Fidelity PCR system (Roche Diagnostics GmbH). For purification of PCR products, the commercial Wizard SV Gel and PCR Clean-Up System (Promega) was employed. Plasmids were isolated with the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). PCR products and plasmids were digested with the appropriate restriction enzymes according to manufacturer's instructions (Thermo Scientific), purified, and ligated using T4 DNA ligase (5 U/µL) (Thermo Scientific). All plasmids constructed were verified by DNA sequencing (Macrogen), and their presence in the selected clones was confirmed by PCR.

To express the $lpxE_{Fn}$ (GenBank accession number AY713119) and $lpxF_{Fn}$ (GenBank accession number DQ364143) genes in *B. pertussis*, codon-optimized sequences of these genes were synthesized (PriorityGENE service, GENEWIZ, Azenta Life Sciences). The $lpxF_{Fn}$ synthetic fragment included Acc65I and HindIII restriction sites at the flanks and was inserted in pMMB67EH via Acc65I-HindIII digestion and subsequent ligation. The codon-optimized $lpxE_{Fn}$ fragment was amplified using the forward primer Fw-XbaI-LpxE, which introduces an XbaI restriction site at the 5′ end of the gene, and the reverse primer Rv-LpxE-ApaI, which introduces an ApaI restriction site at the 3′ end. The resulting fragment was then cloned using these restriction sites into a pUC19-based plasmid containing *lgmB* upstream and downstream sequences flanking the *ompP* (BP0840) promoter region followed by XbaI and ApaI sites (sequences from *B. pertussis* genome sequence, accession number CP039022.1; plasmid provided by Gianmarco Gasperini, GSK). The resulting construct was subcloned in pSORTP1 using EcoRI and HindIII restriction sites from the pUC19 multiple cloning site. *E. coli* strain DH5α was transformed with the pMMB67EH and pSORTP1 constructs by electroporation. Plasmid DNA was isolated from transformants and introduced in *E. coli* strain SM10λ*pir* for conjugation into *B. pertussis*. For pMMB67EH, transconjugants were selected on plates containing ampicillin for plasmid selection and nalidixic acid for counterselection against *E. coli*. For integration of the pSORPT1 construct into the *B. pertussis* genome, transconjugants were selected on plates containing gentamicin for plasmid selection and nalidixic acid for counterselection against *E. coli*. Subsequently, the removal of the plasmid backbone was selected by cultivation on plates containing streptomycin. The presence of the $lpxE_{Fn}$ sequence inside the genomic *lgmB* locus was confirmed by PCR and DNA sequencing.

The $kdtA_{Ec}$ gene (locus tag JW3608) was PCR-amplified from *E. coli* strain W3110 using the forward primer Fw-XbaI-KdtA-Ec, which introduces an XbaI restriction site upstream of the gene, and the reverse primer Rv-KdtA-His-RBS-NdeI-HindIII, which introduces a DNA segment encoding a C-terminal His-Tag, a ribosome-binding site (RBS), an NdeI restriction site (included for future co-expression of other genes), and a HindIII restriction site at the 3′ end of the gene. The amplified fragment was inserted in pMMB67EH via XbaI-HindIII digestion and subsequent ligation. The resulting plasmid was used to transform *E. coli* strain DH5α using the $CaCl_2$ method. Plasmid DNA was extracted from transformants and introduced in *E. coli* strain SM10λ*pir*, which allowed for transfer to *B. pertussis* by conjugation as described above. Expression was confirmed by Western blotting with anti-His antibodies.

To inactivate the *kdtA* (locus tag BP0095), *kdkA* (locus tag BP2349), BP2327, and BP3136 (*eptB*) genes of *B. pertussis*, synthetic DNA fragments were designed that included 800-bp fragments upstream and downstream of the target genes in reference strain Tohama I. The entire sequence of each target gene, except for base pairs overlapping with other genes, was replaced in the synthetic sequence with a gentamicin-resistance cassette (gem^R) flanked by Eco81I restriction sites. In addition, the complete synthetic

fragments were flanked by XbaI restriction sites. The artificial sequences were introduced in pUC57-Kan by blunt-end ligation into the SmaI restriction site of the vector (services provided by BaseGene). The plasmids were used to transform *E. coli* strain DH5α. Strain selection was performed on LB plates containing either kanamycin or a combination of kanamycin and gentamicin. No growth was observed on the plates with both antibiotics, suggesting the gem^R cassette was not expressed. Therefore, another gem^R cassette was PCR-amplified from plasmid pYRC using primers Fw-Eco81I-GemR (forward) and Rv-GemR-Eco81I (reverse), which introduced at both flanks Eco81I restriction sites. This amplicon was used to replace the synthetic gem^R cassette by digestion of the plasmid and PCR product with Eco81I and subsequent ligation. Transformants of DH5α were obtained on LB plates containing the combination of both antibiotics, and the orientation of the cassette in the direction of transcription of the operon of the target genes was confirmed by PCR on isolated colonies. The knockout construct obtained was then subcloned into the suicide vector pKAS32 via XbaI digestion and ligation. The resulting plasmids were used to transform *E. coli* strain SM10λ*pir* and subsequently transferred to *B. pertussis* by conjugation. Chromosomal knockouts were obtained by allelic exchange. First, transconjugants were selected on plates containing gentamicin for selection and nalidixic acid for counterselection against *E. coli*. Subsequently, selection was made with streptomycin and gentamicin to ensure the loss of the plasmid backbone together with the wild-type gene.

Mass spectrometry

Bacterial cultures, grown for 24 h, were centrifuged at 5,000 x *g* for 15 min at 4°C, and the resulting pellet was resuspended in 10 mL of purification buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.6). Then, the cells were inactivated for 30 min at 56°C and sonicated for 20 min in cool water. After treatment with Benzonase Nuclease (50 U/mL, Millipore) for 1 h, unbroken cells and aggregates were removed by centrifugation for 30 min at 20,000 x *g* and 4°C. To the supernatant, purification buffer was added up to 16.5 mL, and the resulting suspension was centrifuged at 40,000 rpm for 2 h at 4°C (Beckman Coulter Optima LE-80K, Type 70 Ti rotor). The resulting pellet was resuspended in phosphate-buffered saline (PBS).

The resuspended pellet was subjected to ethanol precipitation by adding 300 µL of 95% ethanol, which was precooled at -20°C, to 100 µL of sample, vortexing, and incubation for 1 h at -20°C. Then, the samples were centrifuged at 13,000 rpm for 15 min at 4°C, and the pellet was dissolved in 50 µL of 50% (v/v) aqueous methanol solution. After subjecting the samples to vortexing and ultrasonic bath for 10 min, they were further dissolved with an ultrasonic microprobe for 1 min and centrifuged at 13,000 rpm for 10 min at 4°C. For LC-MS analysis in the negative-ion mode, the supernatant was injected in a Thermofisher Scientific Dionex U3000 RSLC with an Acquity UPLC BEH130 C18 (either 1 mm X 50 mm, 1.7 µm or 300 µm X 50 mm, 1.7 µm) column and analyzed as previously described (O'Brien et al., 2014). All mass spectra reported are monocharged ($[M-H]^-$) deconvoluted.

OM isolation

OMs were isolated as previously described (Pérez-Ortega et al., 2022). Briefly, the bacterial pellet collected from liquid cultures was resuspended to an OD_{600} of 7.5 in 2 mL of physiological salt solution, and inactivated by incubation for 30 min at 56°C. Then, cells were harvested, and spheroplasts were made by the addition of 2 mL of 0.75 M sucrose in 10 mM Tris-HCl (pH 7.8), 10 µL of 40 mg/mL lysozyme, and 4 mL of 1.5 mM EDTA (pH 7.5) (Osborn et al., 1972). After freezing the spheroplasts at -80°C, they were lysed by ultrasonication and centrifuged at 10,000 x *g* for 1 h at 4°C. To collect the OM, the supernatant was centrifuged at 40,000 rpm for 1 h at 4°C (Beckman Coulter Optima LE-80K, Type 70 Ti rotor), and the resulting pellet was resuspended in PBS.

Kdo assay

Secondary Kdo units were quantified in isolated OMs as previously described (Sunayana and Reddy, 2015) with slight modifications. Briefly, 50 µL of OM sample was mixed with 50 µL of 0.25 M H_2SO_4 and boiled for 20 min. After cooling down to room temperature, 50 µL of 0.1 M H_5IO_6 was added, and the mixture was incubated for 10 min before the addition of 200 µL of 4% (w/v) $NaAsO_2$. After the yellow color had vanished, 800 µL of 0.3% (w/v) thiobarbituric acid was added, and the mixture was boiled for 10 min. While the solution was still hot, 250 µL of dimethyl sulfoxide (DMSO) was added, and the OD_{550} was measured immediately afterwards. Known concentrations of 2-keto-3-deoxyoctonate ammonium salt (Sigma-Aldrich) were used to plot a standard curve.

SDS-PAGE and Western blotting

OM preparations were mixed with sample buffer (Laemmli, 1970), boiled for 10 min, and analyzed on Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) containing either 10 or 4-15% polyacrylamide for protein or LPS analysis, respectively. After electrophoresis, protein profiles were visualized by staining with Bradford reagent (Bos et al., 2015), and LPS was stained with silver (Tsai and Frasch, 1982). Alternatively, the proteins were transferred from gel to a 0.45-µm pore-size nitrocellulose membrane (GE Healthcare). For immunodetection, mouse antiserum directed against autotransporter BrkA and rabbit antisera directed against the major porin OmpP and the OM-associated protein RmpM (de Jonge et al., 2021) were used. As secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antisera (ThermoFisher) were employed. Membranes were developed with the Clarity Western ECL Blotting Substrate (Bio-Rad).

Microscopy

Bacterial cultures were fixed with 1% formaldehyde for at least 30 min at 4°C and stained for 15 min in the dark with either 5 *µg/mL* of FM4-64 (Invitrogen) or a mixture of SYTO 9 (5 *µM) and propidium iodide (30 µM)* (Live/Dead BacLight kit, Invitrogen). Then, 5-*µL samples of the stained suspensions were pipetted onto* 1%-agarose pads placed on microscopy slides, and cells were visualized with a Zeiss Axioskop 2 fluorescence microscope with a 100x objective.

LPS isolation and heptose quantification

Bacterial cultures were adjusted to an OD_{600} of 0.05 in fresh medium and grown for another 24 h. Then, cells were pelleted for 10 min at 10,000 x *g* and washed with Milli-Q water. After centrifugation, the washed pellet was resuspended in Milli-Q water and lyophilized. LPS was isolated from dry cells as described (Galanos et al., 1969) with slight modifications. Briefly, extractions were performed using a solution of phenol/chloroform/petroleum ether (PCP, 2:5:8, v/v/v). After extraction with 1 mL of PCP per ~100 mg of dry cells and vortexing for 15 min, samples were centrifuged for 20 min at 10,000 x *g*. Supernatants were collected and chloroform and petroleum ether were evaporated using alternate incubation in a water bath at 50°C and air stream, until remaining phenol crystalized. Then, 1.5 mL of acetone was added per mL of PCP originally used. Precipitated LPS was collected by centrifugation for 20 min at 10,000 x *g*. The resulting pellet was allowed to dry completely, and LPS was resuspended in Milli-Q water by alternating incubation at 60°C and vortexing. LPS was quantified based on heptose content, which was determined with the cysteine-sulfuric acid method (Dische, 1953) as modified by Osborn (Osborn, 1963).

TLR4 stimulation assays

TLR4 stimulation assays were performed as previously described (Pérez-Ortega et al., 2021). Briefly, HEK-Blue TLR4 reporter cells co-expressing human (h-)TLR4, MD-2, and CD14 genes (Invivogen) were incubated with serial dilutions of either whole bacterial cells that were killed by heat treatment at 56°C for 30 min or isolated LPS. After 17 h of incubation at 37°C in a 5%-saturated CO_2 atmosphere, supernatants were incubated with *p*-nitrophenyl phosphate solution for 1 h, and the absorbance at 405 nm was measured in a Biotek microplate reader.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software version 6. TLR4 stimulation data was analyzed for statistical significance using two-way ANOVA (Dunnett's correction for multiple comparisons) when more than two strains were compared, while multiple t test (Holm-Sidak correction) was used for comparison of two strains. One-way ANOVA (Tukey's correction for multiple comparisons) was performed on Kdo quantification studies.

Results

Targeting the phosphate groups of *B. pertussis* lipid A

Removal of the phosphate groups of lipid A was tackled by the heterologous production of the phosphatases $LpxE_{Fn}$ and $LpxF_{Fn}$ in a *B. pertussis* strain Tohama I GSK. A codon-optimized version of the $lpxE_{Fn}$ gene under the control of the strong, constitutive *B. pertussis ompP* promoter was inserted in the genome of this strain in place of the *lgmB* locus, while $lpxF_{Fn}$ was introduced into the strain on a plasmid, pMMB67EH, under the

control of the *lac* promoter. To evaluate the alterations in LPS composition, the LPS was analyzed by liquid chromatography–mass spectrometry (LC-MS) in the negative-ion mode. This analysis revealed the presence of three major LPS species in the parental strain (Figure 2A, see structural details in Table 1). The predominant peak at *m/z* 4053.7 was attributed to the characteristic penta-acylated and bis-phosphorylated lipid A species that is typically found in *B. pertussis* (Caroff et al., 1994) substituted with a complete core structure and containing a PEA substitution on the Kdo-bound phosphate (Figure 1). The peak at *m/z* 3861.7 corresponds to the same structure but with the loss of a heptose, indicating that the heptose in the outer core region (Figure 1) is a non-stoichiometric substitution. The third peak at *m/z* 4214.8 represents the major peak with a GlcN substitution on one of the phosphate groups of lipid A. A minor peak at *m/z* 3392.5 represents the predominant peak (*m/z* 4053.7) but lacking the terminal trisaccharide.

LPS of the strain producing $LpxF_{Fn}$, designated TI GSK $LpxF_{Fn}$, showed no relevant modifications compared to the wild-type LPS except that the peak representing the GlcN modification is substantially smaller (Figure 2B). Apparently, this phosphatase is not active on *B. pertussis* LPS as a substrate. However, expression of $LpxE_{Fn}$ resulted in a major peak at *m/z* 3973.8 which corresponds to the molecular ion at *m/z* 4053.7 of wild-type LPS with the expected loss of a phosphate (Figure 2C). Similarly, the new peak at *m/z* 3781.7 represents the LPS missing one heptose (*m/z* 3861.7 in wild-type LPS) with the additional loss of a phosphate. A peak reflecting the additional GlcN substitution could not be observed. This was expected because the $lpxE_{Fn}$ gene was introduced into the bacterial chromosome by allelic exchange with the *lgmB* locus. Only a small portion of wild-type LPS (*m/z* 4053.7) could be detected (Figure 2C).

To determine whether the dephosphorylation of the LPS structure reduces the TLR4-stimulating activity of *B. pertussis* whole cells, TLR4 activation was tested in HEK-Blue reporter cells expressing h-TLR4. Unexpectedly, heat-killed whole cells of TI GSK $LpxE_{Fn}$ and of its parental strain stimulated h-TLR4 to similar levels (Supplementary Figure S1), despite the efficient LPS dephosphorylation observed in the LC-MS analysis.

Table 1. Main ions detected in LC-MS data and proposed compositions for LPS of the *B. pertussis* strains analyzed in this study

Figure	Strain	Measured monoisotopic mass [M-H]-	Calculated monoisotopic mass [M-H]-	Mass error (ppm)	Proposed composition
2A	Tohama I GSK	4053,7067	4053,7570	-12	Wt (TerTri[a]· GalNA· Glc· $GlcN_2$· GlcA· Hep_3· PPEA· Kdo· lipid A)
		3861,6673	3861,6936	-7	Wt -Hep
		4214,7967	4214,8258	-7	Wt +GlcN
		3392,4523	3392,4764	-7	Wt -TerTri
2B	TI GSK $LpxF_{Fn}$	4053,7248	4053,7570	-8	Wt
		3861,6625	3861,6936	-8	Wt -Hep
		4214,7915	4214,8258	-8	Wt +GlcN
2C	TI GSK $LpxE_{Fn}$	3973,7829	3973,7907	-2	Wt-P
		3781,7040	3781,7273	-6	Wt -Hep -P
		4053,7363	4053,7570	-5	Wt
4A	B213	4053,6929	4053,7570	-16	Wt
		4214,7700	4214,8258	-13	Wt +GlcN
		3861,6441	3861,6936	-13	Wt -Hep
		3392,4317	3392,4764	-13	Wt -TerTri
4B	B213 $pKdtA_{Ec}$ Δ*kdtA*	3409,5180	3409,5598	-12	Wt -PPEA -TerTri +Kdo
		4070,7891	4070,8405	-13	Wt -PPEA +Kdo
5A	TI GSK $LpxE_{Fn}$ $pKdtA_{Ec}$ Δ*kdtA*	3409,5187	3409,5598	-12	Wt -PPEA -TerTri +Kdo
		3199,3224	3199,3615	-12	Wt -PPEA -TerTri +Kdo -C14
		3860,5950	3860,6421	-12	Wt -PPEA +Kdo -C14
		4070,7889	4070,8405	-13	Wt -PPEA +Kdo
5B	TI GSK $LpxE_{Fn}$ $pKdtA_{Ec}$ Δ*kdkA*	3409,5183	3409,5598	-12	Wt -PPEA -TerTri +Kdo
		4070,7872	4070,8405	-13	Wt -PPEA +Kdo
		3860,5930	3860,6421	-13	Wt -PPEA +Kdo -C14
		3199,3227	3199,3615	-12	Wt -PPEA -TerTri +Kdo -C14
8A	B213 ΔBP2327	4053,7128	4053,7570	-11	Wt
		4214,7791	4214,8258	-11	Wt +GlcN
		3861,6504	3861,6936	-11	Wt -Hep
8B	TI GSK $LpxE_{Fn}$ ΔBP2327	3973,7300	3973,7907	-15	Wt -P
		3781,6708	3781,7273	-15	Wt -Hep -P
8C	B213 ΔBP3136	3930,7000	3930,7485	-12	Wt -PEA
		4091,7665	4091,8173	-12	Wt +GlcN -PEA
8D	TI GSK $LpxE_{Fn}$ ΔBP3136	3930,7030	3930,7485	-12	Wt -PEA
		3850,7389	3850,7822	-11	Wt -PEA -P
		3738,6415	3738,6851	-12	Wt -Hep -PEA

[a] TerTri, terminal trisaccharide sugars (GlcNAc · Man2NAc3NAcA · Fuc2NAc4NMe).

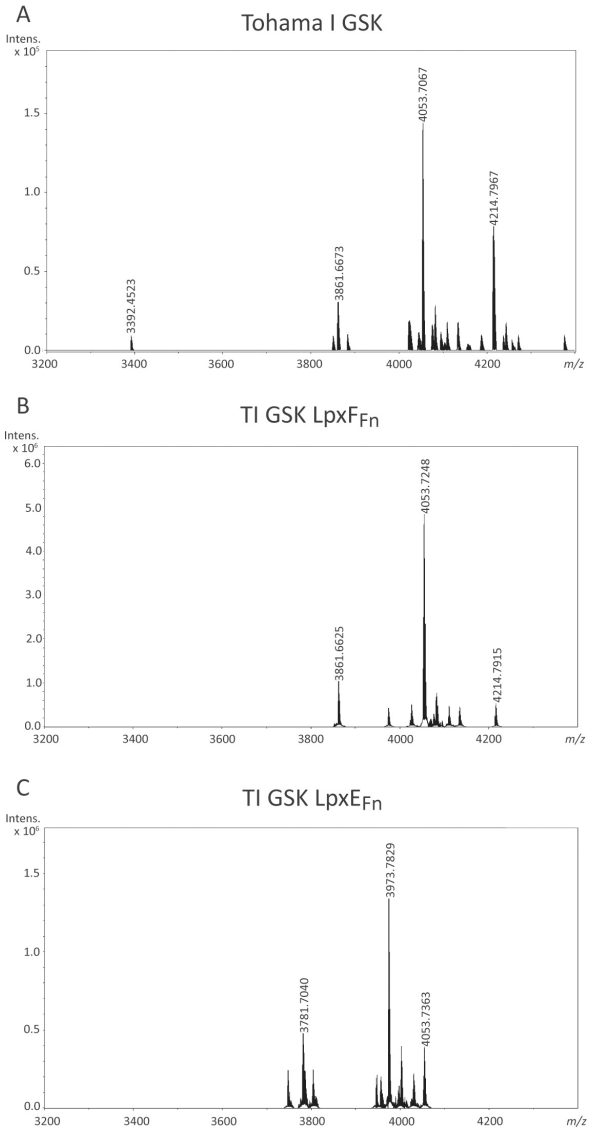


Figure 2. LC-MS analysis of *B. pertussis* LPS of strain Tohama I GSK (A) and its $LpxF_{Fn}$- (B) and $LpxE_{Fn}$- (C) producing derivatives. Measured monoisotopic mass of the peaks characterized is indicated. The peak at *m/z* 4053.7 corresponds to the expected structure of wild-type LPS. For interpretation of the other peaks, see Table 1.

Modification of the inner core of *B. pertussis* LPS by KdtA$_{Ec}$

As the removal of the 1-phosphate did apparently not affect TLR4 activation, we shifted our focus to the inner core region. Previously, it has been suggested that the negative charges in the inner core of the LPS of *Capnocytophaga canimorsus* are important for TLR4 activation (Ittig et al., 2012). The LPS of this species presents a single Kdo and lacks one of the phosphates of lipid A, while the other phosphate is replaced by PEA. Similarly, the inner core of *B. pertussis* LPS could affect TLR4 signaling. Therefore, we decided to change the structure of this region. Unfortunately, inactivation of the *kdtA* (locus tag BP0095) and *kdkA* (BP2349) genes failed, indicating that these genes are essential. Consequently, we first tried to change the inner core by introducing a KdoII in *B. pertussis* LPS. To this end, plasmid pKdtA$_{Ec}$ containing the gene for the bi-functional KdtA from *E. coli* was constructed and introduced into strain B213, another Tohama I derivative, by conjugation. Quantification of the lateral Kdo residues in the OMs of the resulting transconjugants showed a 4-fold increase in the signal (Figure 3A). We considered the possibility that the mono-functional KdtA of *B. pertussis*, expressed from the chromosome, or the physical hindrance resulting from the phosphate at position 4 of KdoI, i.e., where KdoII is expected to be inserted, could reduce the efficiency of KdoII insertion. Therefore, we tried to delete *kdtA* and *kdkA* in strain B213 carrying pKdtA$_{Ec}$. Although these genes could not be knocked out in strain B213, they could be knocked out in the strain carrying the plasmid. Kdo quantification, however, did not evidence a further increase in the incorporation of KdoII (Figure 3A).

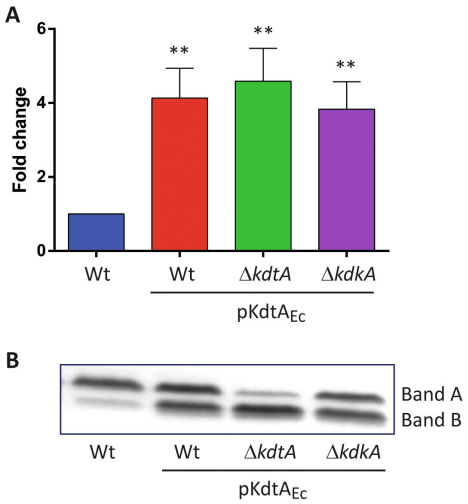


Figure 3. KdtA$_{Ec}$ expression affects the structure of *B. pertussis* LPS. (**A**) The amount of lateral Kdo units introduced by KdtA$_{Ec}$ was quantified in OMs isolated from strain B213 and its pKdtA$_{Ec}$-containing derivatives. Fold changes relative to the wild type (Wt) were calculated from the Kdo concentrations obtained. Data represent means and standard deviations from three experiments performed in duplicate. Statistically significant differences compared to the wild type are indicated with asterisks (**, $P < 0.01$). (**B**) LPS from one of the OM samples of each strain was analyzed by SDS-PAGE and visualized by silver staining. The positions of band A and band B are indicated.

Analysis of the LPS by SDS-PAGE showed structural modifications, reflected in altered electrophoretic mobility, in all the *B. pertussis* strains producing $KdtA_{Ec}$ (Figure 3B). Interestingly, the new LPS forms migrated faster than the wild-type structure, appearing at the level of band B. The $KdtA_{Ec}$-expressing *kdtA* mutant showed the most extensive switching to the form with higher electrophoretic mobility. The faster migration of the new LPS form suggests a reduction of the size of the LPS despite the KdoII insertion.

LC-MS analysis of strain B213 (Figure 4A) presented the four peaks as described above for strain Tohama I GSK (Figure 2A, compare in Table 1). The *kdtA* mutant derivative of B213 $pKdtA_{Ec}$ showed a major peak at *m/z* 3409.5 corresponding to the molecular ion at *m/z* 4053.7 of the parental strain (wild-type structure) and containing the additional KdoII but lacking the phosphate-PEA (PPEA) substitution and the terminal trisaccharide (Figure 4B and Table 1). A smaller peak at *m/z* 4070.8 corresponds to the major peak at *m/z* 3409.5 but including the terminal trisaccharide.

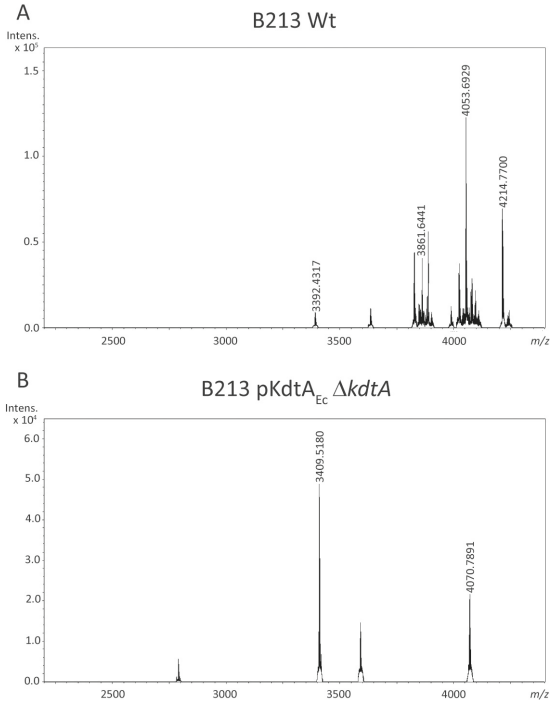


Figure 4. LC-MS analysis of LPS of *B. pertussis* strain B213 (A) and its Δ*kdtA* derivative containing $pKdtA_{Ec}$ (B). Measured monoisotopic mass of the peaks characterized is indicated. The peak at *m/z* 4053.7 corresponds to the wild-type LPS structure. For interpretation of the other peaks, see Table 1.

After evidencing the ability of KdoII insertion in *B. pertussis* LPS, we proceeded to combine this modification with $LpxE_{Fn}$ production. First, $pKdtA_{Ec}$ was introduced in strain TI GSK $LpxE_{Fn}$ and, subsequently, *kdtA* and *kdkA* mutants were generated. As previously

observed in the genetic background of B213 (Figure 3B), production of $KdtA_{Ec}$ in TI GSK $LpxE_{Fn}$ resulted in modification of the LPS structure in all the strains as evidenced by SDS-PAGE (Supplementary Figure S2), be it that in this case also the *kdkA* mutation strongly reinforced the relatively mild effect observed after introduction of $pKdtA_{Ec}$.

LC-MS analysis of the LPS from the *kdtA* mutant of strain TI GSK $LpxE_{Fn}$ containing $pKdtA_{Ec}$ (Figure 5A) showed prominent peaks at *m/z* 3409.5 and 4070.8, which correspond to those detected in the *kdtA* mutant of strain B213 $pKdtA_{Ec}$ (Figure 4B, compare in Table 1). Apparently, the introduction of KdoII prevented the dephosphorylation of lipid A by $LpxE_{Fn}$. Two additional peaks at *m/z* 3199.4 and 3860.6 (Figure 5A) represent the same structures as *m/z* 3409.5 and 4070.8, respectively, but with the additional loss of a C_{14} acyl chain (Table 1). Similarly, the *kdkA* mutant of strain TI GSK $LpxE_{Fn}$ containing $pKdtA_{Ec}$ showed the same peaks as identified in the *kdtA* mutant, although the peaks representing the deacylated form were less abundant in this case (Figure 5B).

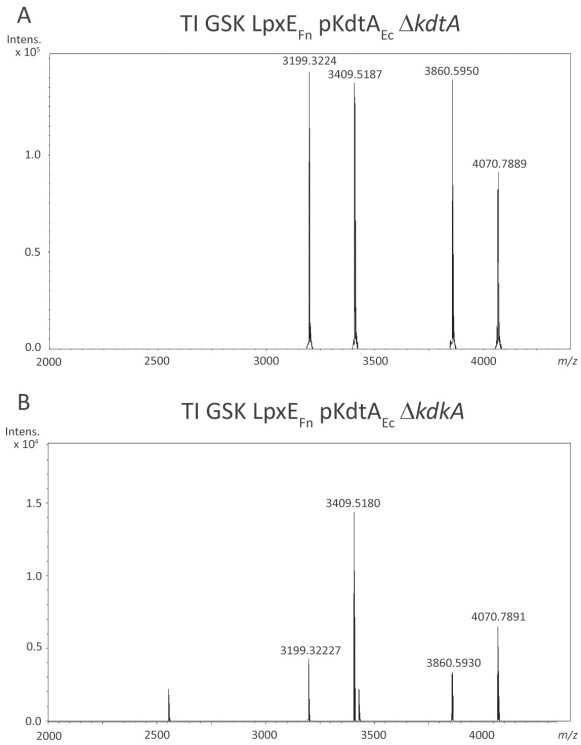


Figure 5. LC-MS analysis of LPS of strains (A) TI GSK $LpxE_{Fn}$ $pKdtA_{Ec}$ Δ*kdtA* and (B) TI GSK $LpxE_{Fn}$ $pKdtA_{Ec}$ Δ*kdkA*. Measured monoisotopic mass of the peaks characterized is indicated. Major peaks at *m/z* 3409.5 and *m/z* 3199.3 correspond to the LPS form with the additional KdoII but lacking the PPEA substitution and the final trisaccharide, and a form with the additional loss of a C_{14} acyl chain, respectively. Peaks corresponding to dephosphorylated forms were not detected.

Phenotypic characterization of $KdtA_{Ec}$-producing strains

All strains producing $KdtA_{Ec}$ showed reduced growth kinetics in liquid medium compared with their parental strain (Supplementary Figure S3). The OM protein profiles of strain B213 and derivatives were very similar except for the $pKdtA_{Ec}$-containing *kdtA* mutant (Figure 6A). In the latter, two prominent bands of around 36-38 kDa (indicated with arrowheads in Figure 6A) were lost. These bands probably correspond to the β-barrel domains of autotransporters such as BrkA, Prn, and Tcf (Hamstra et al., 1995; Passerini de Rossi et al., 1999). Notable differences were also observed in the banding pattern around

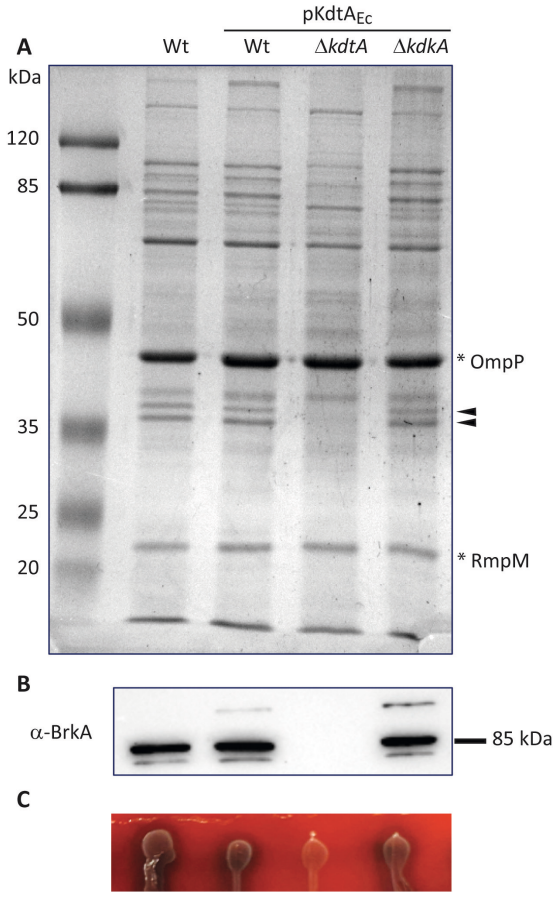


Figure 6. Comparison of protein content of OM preparations from *B. pertussis* strain B213 (Wt) and its $pKdtA_{Ec}$-containing derivatives. (**A**) Isolated OMs were analyzed by SDS-PAGE and protein profiles were visualized by Bradford staining. The major OM protein, porin OmpP, and the porin-associated periplasmic protein RmpM are indicated with asterisks at the right. Molecular weight markers are shown at the left. Arrowheads indicate two protein bands that are lost in the $pKdtA_{Ec}$-containing *kdtA* mutant and that probably represent the β-barrels of autotransporters. (**B**) OM proteins were separated by SDS-PAGE, and Western blot analysis was performed with antibodies directed against the autotransporter BrkA. The position of a molecular weight marker protein is indicated at the right. Only the relevant part of the blot is shown. (**C**) Hemolytic capacity of the strains was studied on BG-blood agar. Drops from bacterial cultures were plated on BG-blood agar and incubated for 48 h. The dark halo indicates hemolytic activity.

80-115 kDa (Figure 6A); these bands are probably related to the passenger domains of autotransporters. The production of specific OM proteins was further analyzed by Western blotting. The autotransporter BrkA could be detected in strain B213 and its derivatives except in the pKdtA$_{Ec}$-containing *kdtA* mutant (Figure 6B). In contrast, other OM proteins such as the major porin OmpP and the porin-associated periplasmic protein RmpM, a.k.a. OmpA (de Jonge et al., 2022), appeared to be present in similar amounts in all strains (Supplementary Figure S4).

As *brkA* expression is dependent on the two-component regulatory system Bvg (Passerini de Rossi et al., 1999), we considered the possibility of a phase switch from Bvg$^+$ to Bvg$^-$ in the pKdtA$_{Ec}$-containing *kdtA* mutant strain. To investigate this possibility, we studied another Bvg-dependent property, i.e., hemolysis (Weiss et al., 1983). While the wild-type strain showed hemolysis on BG-blood plates, which indicates that it was growing in the Bvg$^+$ phase, the *kdtA* mutant did not show this phenotype (Figure 6C), suggesting a switch to the Bvg$^-$ phase. The other two strains showed similar hemolysis as their parental strain. In the genetic background of the LpxE$_{Fn}$-expressing derivative of strain Tohama I GSK containing pKdtA$_{Ec}$, production of BrkA (Supplementary Figure S5A) and hemolysis on BG-blood plates (Figure S5B) were lost in both the *kdtA* and in the *kdkA* mutants.

The reduced growth of the pKdtA$_{Ec}$-containing strains could be due to impaired cell division. To investigate this possibility, cell morphology was studied by fluorescence microscopy. To this end, bacteria were stained with either the fluorescent membrane-dye FM4-64 or the Live/Dead BacLight kit, which contains two different nucleic acid stains. As expected, wild-type strain B213 displayed short rods (Figure 7A**,** left panel). Upon production of KdtA$_{Ec}$ and inactivation of the chromosomal copy of *kdtA*, many remarkably elongated cells were detected (indicated with yellow arrowheads in Figure 7B, left panel). As the nucleoids appear to be separated in these filaments (Figure 7B, right panel), but the membrane dye does not indicate the presence of septa in such elongated cells (Figure 7B, left panel), these results indicate a defect early in cell division probably at the level of the invagination of the cell membranes.

Cells producing LpxE$_{Fn}$ appeared shorter and more rounded than the classic wild-type structure of Tohama I and, occasionally, short chains of cells were observed (purple and blue arrowheads, respectively, in Supplementary Figure S6A). Cells producing both LpxE$_{Fn}$ and KdtA$_{Ec}$ were elongated and formed chains (yellow arrowheads in Figure S6B), like the phenotype observed when only KdtA$_{Ec}$ was produced (Figure 7B), which was to be expected as LpxE$_{Fn}$-mediated lipid A dephosphorylation does not take place when KdoII is introduced (Figure 5). However, invaginations of the membranes could generally be observed in these cells. The nucleoids also showed irregularities on the edge, suggesting defective compaction (white arrowheads in Figure S6B, right panel), which was not observed in the parental strain (Figure S6A, right panel).

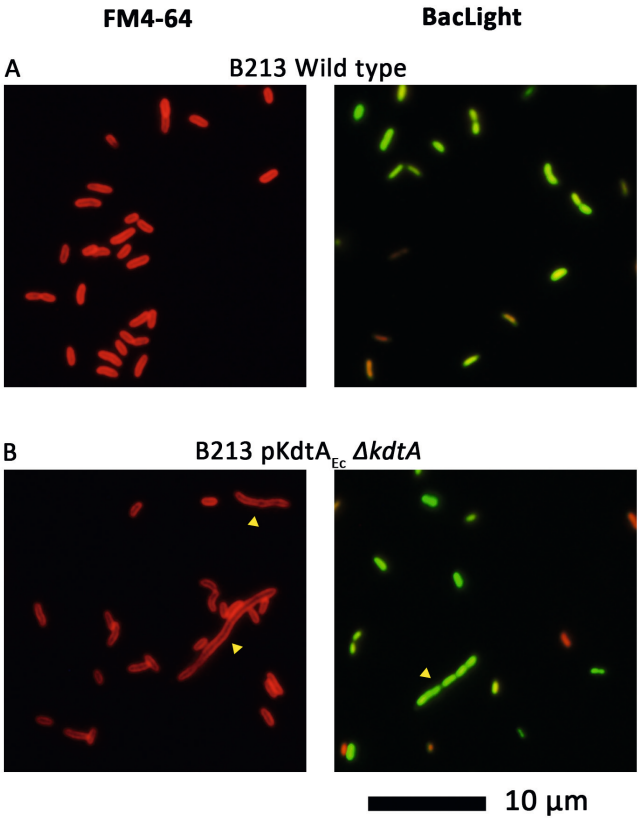
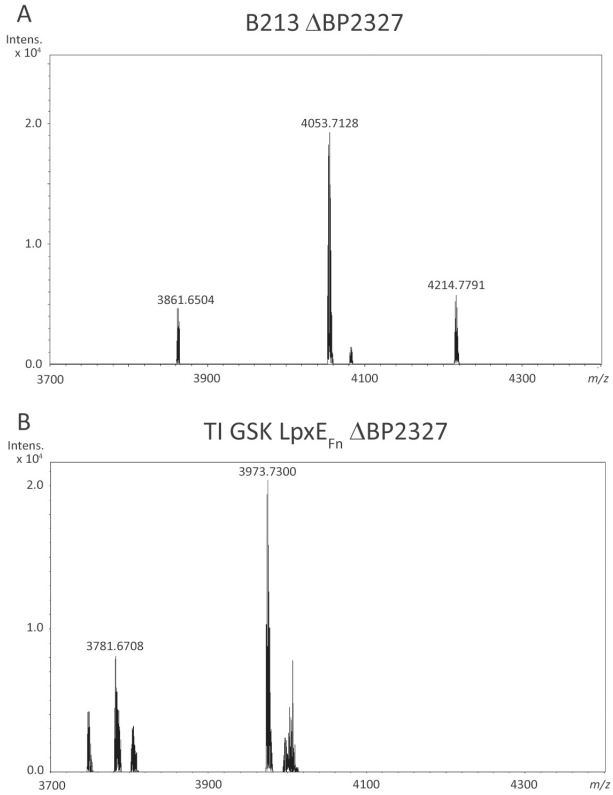


Figure 7. KdtA$_{Ec}$ production affects cell division. Bacterial cultures of strain B213 (**A**) and its pKdtA$_{Ec}$ Δ*kdtA* derivative (**B**) were stained with either the fluorescent dye FM4-64 (left panel) or Dead/Live BacLight kit (right panel) and studied by fluorescence microscopy. Scale bar represents 10 µm. Examples of cells showing elongated cell chains are indicated with yellow arrowheads.

Identification and characterization of the *eptB* gene in *B. pertussis*

Our mass spectrometry analysis showed that the phosphate group attached to the Kdo of the LPS is effectively substituted with PEA (Figures 2A and 4A). However, the gene encoding EptB has not yet been described in *B. pertussis*. We selected two possible candidates for being *eptB*, i.e., BP2327 and BP3136. BP2327 is located in a cluster of genes involved in core biosynthesis, next to BP2328-BP2331 (Geurtsen et al., 2009), and the encoded protein shows some homology to lipid A PEA transferases (EptA). However, PEA substitutions at the phosphate groups of *B. pertussis* lipid A have never been described. Like the BP2327 product, the BP3136 protein was classified as a protein with EptB-like structure including a sulfatase domain and, thus, it may have a similar function (de Gouw et al., 2014; Harper et al., 2017).

Both genes were inactivated in strains B213 and TI GSK $LpxE_{Fn}$, and the LPS structure was analyzed by LC-MS. Upon inactivation of BP2327, no relevant differences in the spectrum were observed compared to the LPS of their parental strains (Table 1). Thus, for B213 ΔBP2327, peaks corresponding to the main wild-type LPS species (*m/z* 4053.7) and either loss of heptose (*m/z* 3861.7) or inclusion of GlcN decoration (*m/z* 4214.8) were obtained (Figure 8A), whilst the spectrum for strain TI GSK $LpxE_{Fn}$ ΔBP2327 showed peaks at *m/z* 3973.7 and 3781.7 (Figure 8B), which correspond to the dephosphorylated forms of *m/z* 4053.7 and 3861.7, respectively. In contrast, inactivation of BP3136 in strain B213 resulted in LPS forms at *m/z* 3930.7 and 4091.8 (Figure 8C), which correspond to the molecular ions at *m/z* 4053.7 and 4214.8, respectively, with the loss of the PEA decoration. The LPS of strain TI GSK $LpxE_{Fn}$ ΔBP3136 showed peaks at *m/z* 3930.7 and 3738.6 (Figure 8D), which represent LPS forms derived from the wild-type LPS species with the loss of PEA and the same structure with the additional loss of heptose, respectively (Table 1).



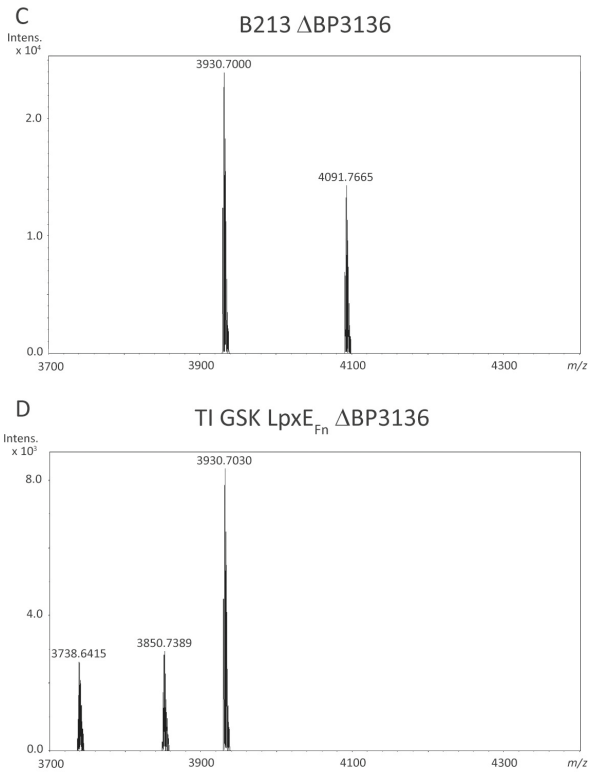


Figure 8. LC-MS analysis of LPS of *B. pertussis* BP2327 and BP3136 mutants. Shown are the spectra from B213 ΔBP2327 (**A**), TI GSK $LpxE_{Fn}$ ΔBP2327 (**B**), B213 ΔBP3136 (**C**), and TI GSK $LpxE_{Fn}$ ΔBP3136 (**D**). Measured monoisotopic mass of the peaks characterized is indicated. The peak at *m/z* 3930.7 corresponds to the structure of wild-type LPS with loss of PEA decoration. For interpretation of the other peaks, see Table 1.

In these LPS species, dephosphorylation by $LpxE_{Fn}$ had not taken place. An additional peak at *m/z* 3850.7, however, corresponds with the dephosphorylated form of *m/z* 3930.7 (Figure 8D, Table 1). These results demonstrate that BP3136 encodes the EptB protein that decorates the phosphate on Kdo with PEA and that $LpxE_{Fn}$ activity in *B. pertussis* is impaired in the absence of the PEA decoration.

TLR4 activation by inner core-modified derivatives of *B. pertussis*

The effect of $KdtA_{Ec}$ production on the TLR4-stimulating activity was tested with whole-cell preparations of B213 and derivatives. All the recombinant strains showed significantly higher h-TLR4-stimulating activity than the wild type, requiring around 100-fold higher concentrations of wild-type suspension than of the mutants to reach a similar signal (Figure 9A). Comparable results were obtained with the $KdtA_{Ec}$-producing derivatives from strain TI GSK $LpxE_{Fn}$ (Supplementary Figure S7).

The increased TLR4-stimulating activity of the cells could be a direct effect of the modifications in the LPS structure on TLR4 recognition or a consequence of unstable anchoring of the LPS at the cell surface resulting in increased LPS release and availability for the receptor (Geurtsen et al., 2006). To discriminate between these possibilities also purified LPS were tested. Activation of h-TLR4 was significantly higher with the LPS from the $KdtA_{Ec}$-producing derivative than with that of the wild type (Figure 9B), confirming the effect of the structural alterations on the activation of TLR4.

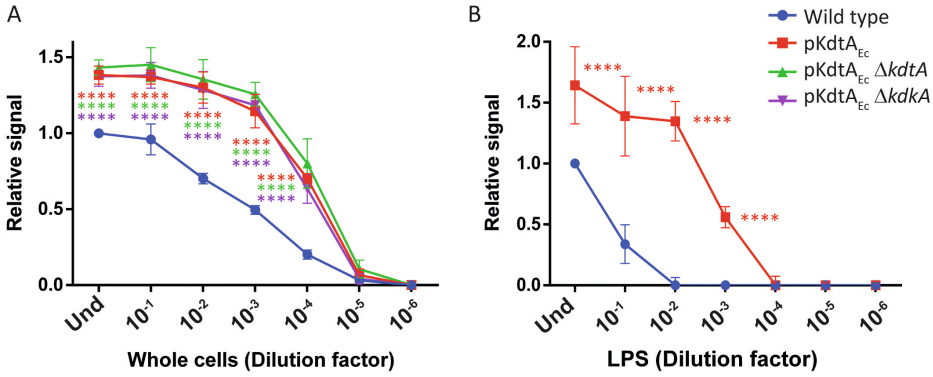


Figure 9. TLR4 activation by whole-cell preparations and purified LPS of strain B213 (Wild type) and its $pKdtA_{Ec}$-containing derivatives. HEK-Blue cells expressing h-TLR4 were incubated for 17 h with 10-fold serial dilutions of either heat-inactivated whole cells, of which the OD_{600} of the undiluted suspensions (Und) was 0.15 (**A**), or purified LPS (Und.: 20 nmol heptose/mL) (**B**). Graphs show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution with each strain or LPS preparation and the signal measured for Und. parental strain or LPS preparation. Three independent experiments were performed in duplicate (whole cells) or singular (LPS). Dilutions of the mutants with activities statistically different from those of the parental strain are indicated with asterisks (****, $p \leq 0.0001$).

The possible effect of the loss of the PEA group on TLR4 signaling was also studied with suspensions of bacterial cells. Whole cells of the *eptB* mutant of B213 had a significantly reduced ability to activate h-TLR4 (Figure 10A). This reduction was even more prominent in strain TI GSK $LpxE_{Fn}$ (Figure 10B), which might be the consequence of the combined loss of PEA and phosphate in a portion of the LPS produced by this strain (Figure 8D).

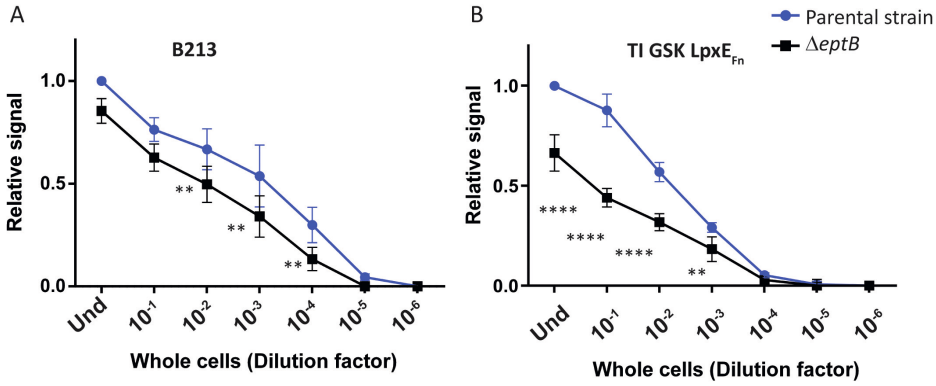


Figure 10. TLR4 activation by whole-cell preparations of (A) strain B213 and (B) strain TI GSK $LpxE_{Fn}$ (Parental strains) and their *eptB* mutant derivatives. HEK-Blue cells expressing h-TLR4 were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole cells. The OD_{600} of the undiluted suspensions (Und) was 0.15. Graphs show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution with each strain and the signal measured for Und. parental strain. Three independent experiments were performed in duplicate. Dilutions of the mutant with activities statistically different from those of the parental strain are indicated with asterisks (**, $p \leq 0.01$; ****, $p \leq 0.0001$).

Discussion

Dephosphorylation is generally known to reduce the TLR4-stimulating activity of lipid A (Maeshima and Fernandez, 2013). After synthesis of the lipid A phosphatases $LpxE_{Fn}$ and $LpxF_{Fn}$ in *B. pertussis*, only the production of $LpxE_{Fn}$ resulted in the loss of a phosphate. Interestingly, despite the very efficient removal of the 1-phosphate upon expression of $LpxE_{Fn}$, TLR4 activation by whole cell preparations was unaffected. This could be related to the presence of a phosphorylated Kdo in the inner core of *B. pertussis* LPS, which might functionally replace the lipid A 1-phosphate in the interaction with the TLR4-MD2 receptor complex. In a previous study in *C. canimorsus*, which presents LPS with a single Kdo substituted with PEA at position 4, the importance of the core in TLR4 activation was shown (Ittig et al., 2012). In contrast to the lipid A-core fraction of *C. canimorsus* LPS or *E. coli* lipid A, the purified lipid A of *C. canimorsus*, which is dephosphorylated at position 4′ and presents a PEA instead of phosphate at position 1, was unable to activate the receptor. The authors hypothesized that the negative charge of the carboxylic group of the Kdo facilitates the binding of LPS to MD2 and, thereby, TLR4 activation.

We hypothesized that modifications in the inner core could similarly affect TLR4 activation in *B. pertussis*. Our attempts to inactivate the inner core-related genes *kdtA* and *kdkA* were futile, which is in line with their previously predicted essentiality (Gonyar et al., 2019; Belcher et al., 2020). Nevertheless, we succeeded in inactivating these genes in a recombinant strain producing the bi-functional KdtA of *E. coli*. Apparently, the presence of a Kdo substituted with either phosphate or a second Kdo in the inner core is essential

for the viability of *B. pertussis*. This requirement seems to be similar for *E. coli*, whose bi-functional KdtA could only be replaced by a mono-functional homolog when a KdkA was co-expressed and, thereby, the single Kdo was phosphorylated (Brabetz et al., 2000). Interestingly, the presence of KdoII in *B. pertussis* LPS appeared to impair the substitution of the outer core with the terminal trisaccharide. Differences in the core structure due to the presence of either a phosphate substitution or a second Kdo attached to KdoI naturally occur in other species such as *Pasteurella multocida* (Harper et al., 2007). The depletion of the trisaccharide in *B. pertussis* producing $KdtA_{Ec}$ could be due to steric hindrance of the transferase that attaches the terminal trisaccharide by KdoII. This transferase has not been characterized yet but is likely encoded by the *wlbI* gene (Allen and Maskell, 1996; Preston et al., 2002). Another possibility is that the phosphate group attached to the KdoI facilitates the activity of the transferase. Alternatively, the loss of the terminal trisaccharide could be the result of the partial switch to the Bvg^- phase. Lack of band A, i.e., the absence of the trisaccharide, has been reported in Bvg mutant strains (Turcotte et al., 1997; Schaeffer et al., 2004). We observed a switch of Bvg phase in some of the mutants containing KdoII, although all the Kdo-modified strains showed reduced amounts of trisaccharide. However, according to the SDS-PAGE analysis, there is a strong correlation between the extent of the loss of the trisaccharide (Figures 3 and S2) and the observed Bvg switch (Figures 4 and S5). Despite this plausible connection, other factors may also be implicated as suggested by a previous study in which the author could not directly associate the Bvg phase to the presence or absence of the trisaccharide (Van den Akker, 1998). The situation in *B. pertussis* is reminiscent of that in *E. coli*, where the Kdo disaccharide in the inner core can be decorated with a third Kdo, PEA, or rhamnose. These decorations affect the substitution of the outer core with a disaccharide (Klein and Raina, 2015). Kdo decoration and attachment of the terminal disaccharide is controlled by a complex regulatory network that involves the two-component system PhoP/Q, which is similar to the BvgA/S system of *B. pertussis* (Park and Groisman, 2014), as well as the RpoE-dependent envelope stress response and the two-component systems PhoB/R and BasS/R (Klein and Raina, 2015).

In KdoII-containing strains, also a variable loss of the secondary C_{14} chain at the 2' position of lipid A was observed (Figures 5A, B). This acyl chain is inserted by the acylase LpxL2 (Geurtsen et al., 2007). The *B. pertussis* LpxL2 enzyme was previously shown *in vitro* to be optimally active on substrates with a phosphorylated Kdo in the inner core (Hankins and Trent, 2009). The presence of a KdoII instead of the phosphate decoration reduced but did not fully prevent its activity (Hankins and Trent, 2009), which explains that LPS molecules with the secondary C_{14} chain were still detected in the spectra (Figures 5A, B) and that the strains were viable even though the *lpxL2* gene and, therefore, the secondary C_{14} chain are essential for viability (Gonyar et al., 2019). Nevertheless, the growth reduction observed in the KdoII-containing strains (Figure S3) might reflect the reduced incorporation of this acyl chain. Moreover, defects in cell division were observed (Figure 7), which most likely reflect cellular stress caused by the underacylated LPS. The presence of KdoII in

B. pertussis LPS also prevented lipid A dephosphorylation in the strain producing $LpxE_{Fn}$ perhaps due to steric hindrance. However, this contrasts to the situation in *F. novicida*, where $LpxE_{Fn}$ dephosphorylates lipid A in the presence of KdoII (Wang et al., 2004).

The gene responsible for decorating the KdoI-linked phosphate with PEA, i.e., *eptB*, was hitherto not identified in *B. pertussis*. We selected two possible candidates for being *eptB*. Inactivation of BP2327, which shows homology to *eptA* of other Gram-negative bacteria, did not have any effect on LPS structure (Figure 8A), even though the gene is located in a cluster of genes involved in core biosynthesis (Geurtsen et al., 2009). Inactivation of BP3136, which shows similarity to the *eptB* gene of other species (de Gouw et al., 2014; Harper et al., 2017), however, resulted in the loss of PEA substituent. Interestingly, like in the strains decorated with KdoII on KdoI, lipid A dephosphorylation was diminished upon inactivation of *eptB* in the strain producing $LpxE_{Fn}$. Apparently, the presence of PEA is a prerequisite for the optimal activity of the phosphatase on *B. pertussis* LPS. On the other hand, production of the 4'-phosphatase $LpxF_{Fn}$ in *B. pertussis* had no impact on the LPS structure. The inability of $LpxF_{Fn}$ to dephosphorylate hexa-acylated LPS has previously been documented (Wang et al., 2006). However, its activity on penta-acylated molecules, such as present in *B. pertussis* LPS, is commonly observed. LpxF of *H. pylori* is hindered by the presence of KdoII (Cullen et al., 2011) and could, therefore, also be hindered by the PPEA decoration of KdoI in *B. pertussis*. However, this does not seem to be the case for $LpxF_{Fn}$, which has been shown to be active in absence of Kdo as well as in the presence of either one or two Kdo residues (Wang et al., 2006; Okan et al., 2013), discarding any influence of other core features on $LpxF_{Fn}$ activity as well. Nevertheless, it could be that the phosphatase encounters a physical impediment in the wild-type LPS of *B. pertussis* similar to, but opposite of that described for $LpxE_{Fn}$. While the KdoII-modified LPS might be interfering with the access of $LpxE_{Fn}$ to the 1-phosphate, the wild-type LPS organization might block the access to the 4'-phosphate instead. If this hypothesis is correct, the production of $LpxF_{Fn}$ in the *eptB* mutant could facilitate 4'-dephosphorylation. This modification could affect TLR4 activation and is a strategy that could be considered in the future.

Production of $KdtA_{Ec}$ increased the h-TLR4-activating capacity of *B. pertussis* cells. In contrast, inactivation of *eptB* reduced TLR4 activation, and inactivation of *eptB* in the $LpxE_{Fn}$-producing strain reduced the TLR4-stimulating capacity even further, even though the phosphatase activity of $LpxE_{Fn}$ was limited by the *eptB* mutation. Thus, *eptB* inactivation, especially in combination with $LpxE_{Fn}$ production, seems a promising genetic approach for the development of novel whole-cell vaccines with reduced endotoxicity against pertussis, and it is worthwhile to investigate the immunogenicity and reactogenicity of such vaccines further *in vivo*.

Overall, we have described the *eptB* gene in *B. pertussis* and shown that the PEA substitution attached by the encoded enzyme to the Kdo-linked phosphate is involved

in TLR4 activation. Furthermore, insertion of a second Kdo in the inner core affects the structure of the outer core by inhibiting the attachment of the terminal trisaccharide and of lipid A by limiting the attachment of a secondary acyl chain. These modifications result in defective cell division and, surprisingly considering the partial loss of an acyl chain, in increased TLR4-stimulating capacity of these cells. In addition, the lipid A dephosphorylation accomplished by $LpxE_{Fn}$ production, which shows no effect on TLR4 stimulation on its own, is abolished by the introduction of a second Kdo and remarkably reduced by the absence of the inner-core PEA. To our knowledge, this is the first study focusing on structural modifications of the inner core of *B. pertussis* and their effect on endotoxicity.

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Supplemental material

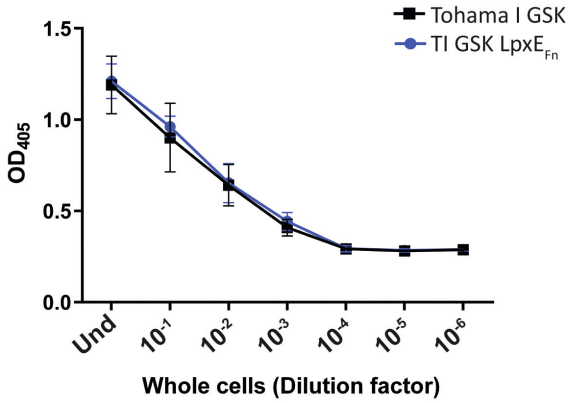

Figure S1. TLR4 activation by whole-cell preparations of strain Tohama I GSK and its LpxE$_{Fn}$-producing derivative. HEK-Blue cells expressing h-TLR4 were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole cells. The OD_{600} of the undiluted suspensions (Und) was 1. Graph shows means and standard deviations of SEAP activity measured at OD_{405} from the supernatants of a single experiment performed in duplicate.

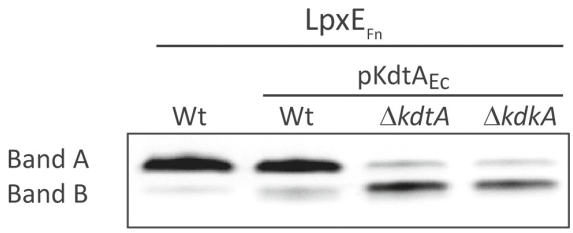

Figure S2. Comparison of LPS from *B. pertussis* strain TI GSK LpxE$_{Fn}$ and its pKdtA$_{Ec}$-containing derivatives. OM preparations were analyzed by SDS-PAGE, and LPS was visualized by silver staining. Wt refers to the parental strain Tohama I GSK. The positions of band A and band B are indicated.

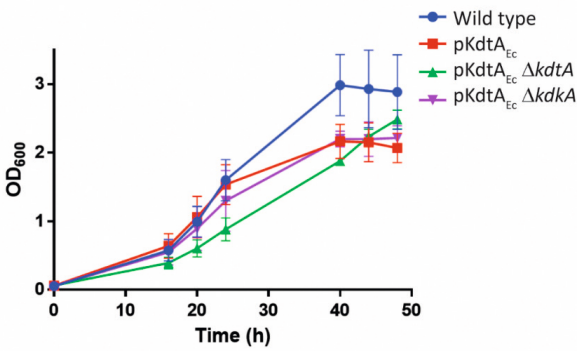

Figure S3. Growth of *B. pertussis* strain B213 (Wild type) and its pKdtA$_{Ec}$-containing derivatives. Optical densities of the cultures were measured at OD$_{600}$ for 48 h. Graph shows values of a single experiment performed in duplicate.

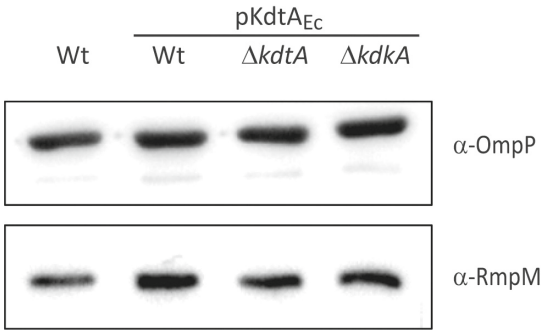

Figure S4. Western blot analysis of relevant OM antigens in strain B213 (Wt) and its pKdtA$_{Ec}$-containing derivatives. Isolated OMs were analyzed by SDS-PAGE and Western blotting with antisera directed against the major porin OmpP and OM-associated protein RmpM.

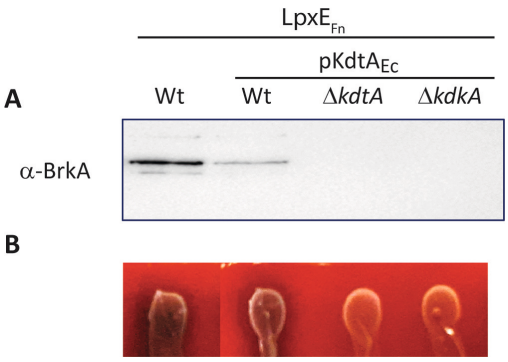

Figure S5. Comparison of Bvg-dependent properties of *B. pertussis* strain TI GSK LpxE$_{Fn}$ and its pKdtA$_{Ec}$-containing derivatives. (**A**) Isolated OMs were analyzed by SDS-PAGE and Western blot analysis was performed with antibodies directed against the autotransporter BrkA. (**B**) Hemolytic capacity was studied on BG-blood agar. Drops from bacterial cultures of the strains were plated on BG-blood agar and incubated for 48 h. The dark halo indicates hemolytic activity. Wt refers to the parental strain Tohama I GSK.

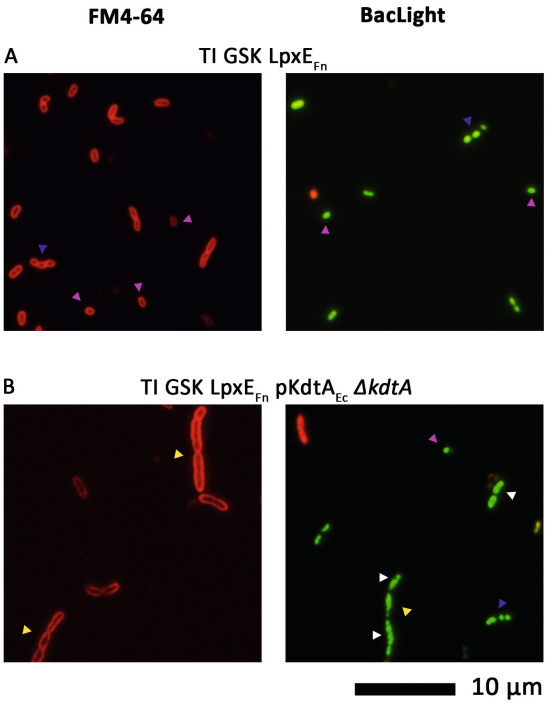

Figure S6. Morphology of *B. pertussis* cells visualized by fluorescence microscopy. Bacterial cultures of strain TI GSK LpxE$_{Fn}$ and its pKdtA$_{Ec}$ Δ*kdtA* derivative were stained with either the fluorescent dye FM4-64 (left panel) or Dead/Live BacLight kit (right panel). Scale bar represents 10 µm. Examples of cells showing shorter and more rounded shape than the wild-type cell (purple), short chains (blue), elongated cell chains (yellow), or uneven dye distribution (white) are indicated with colored arrowheads.

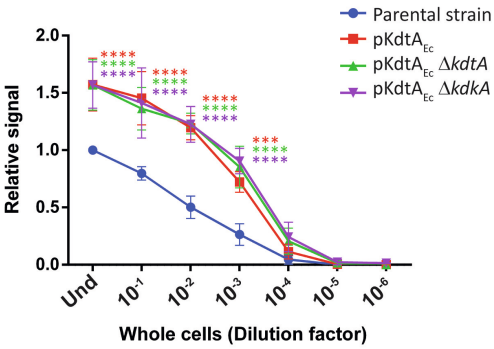

Figure S7. TLR4 activation by whole-cell preparations of strain TI GSK LpxE$_{Fn}$ (Parental strain) and its pKdtA$_{Ec}$-containing derivatives. HEK-Blue cells expressing h-TLR4 were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole cells. The OD_{600} of the undiluted suspensions (Und) was 0.15. Graphs show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution with each strain and the signal measured for the undiluted parental strain. Three independent experiments were performed in duplicate. Dilutions of the mutants with activities statistically different from those of the parental strain are indicated with asterisks (***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Table S1. Bacterial strains and plasmids

Strain / plasmid	Description [a]	Reference
B. pertussis		
B213	Str^R derivative of strain Tohama I	(King et al., 2001)
B213 pKdtA$_{Ec}$	B213 carrying pKdtA$_{Ec}$, Str^R, Amp^R	This study
B213 pKdtA$_{Ec}$ Δ*kdtA*	*ΔkdtA::gem* mutant of B213 pKdtA$_{Ec}$, Str^R, Amp^R, Gem^R	This study
B213 pKdtA$_{Ec}$ Δ*kdkA*	*ΔkdkA::gem* mutant of B213 pKdtA$_{Ec}$, Str^R, Amp^R, Gem^R	This study
B213 Δ*BP2327*	*ΔBP2327::gem* mutant of B213, Str^R, Gem^R	This study
B213 Δ*BP3136*	*ΔBP3136*(*eptB*)*::gem* mutant of B213, Str^R, Gem^R	This study
Tohama I GSK	Str^R derivative of strain Tohama I	GSK[b]
TI GSK LpxF$_{Fn}$	Tohama I GSK carrying pLpxF$_{Fn}$, Str^R, Amp^R	This study
TI GSK LpxE$_{Fn}$	Tohama I GSK carrying *lpxE$_{Fn}$* replacing *lgmB*, Str^R	This study
TI GSK LpxE$_{Fn}$ pKdtA$_{Ec}$	TI GSK LpxE$_{Fn}$ carrying pKdtA$_{Ec}$, Str^R, Amp^R	This study
TI GSK LpxE$_{Fn}$ pKdtA$_{Ec}$ Δ*kdtA*	*ΔkdtA::gem* mutant of TI GSK LpxE$_{Fn}$ pKdtA$_{Ec}$, Str^R, Amp^R, Gem^R	This study
TI GSK LpxE$_{Fn}$ pKdtA$_{Ec}$ Δ*kdkA*	*ΔkdkA::gem* mutant of TI GSK LpxE$_{Fn}$ pKdtA$_{Ec}$, Str^R, Amp^R, Gem^R	This study
E. coli		
DH5α	F^-, Δ(*lacZYA-argF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	(Grant et al., 1990)
SM10λ*pir*	*thi thr leu fhuA lacY supE recA*::RP4-2-Tc::Mu *λpir* R6K, Kan^R	(Simon et al., 1983)
W3110	K-12 derivative, F^-, λ^-, INV *(rrnD$^-$ rrnE)1*	ATCC 27325
Plasmids		
pUC57-Kan Δ*kdtA*	pUC57-Kan derivative harboring *kdtA* knockout construct, Kan^R, Gem^R	This study
pUC57-Kan Δ*kdkA*	pUC57-Kan derivative harboring *kdkA* knockout construct, Kan^R, Gem^R	This study
pUC57-Kan Δ*BP2327*	pUC57-Kan derivative harboring *BP2327* knockout construct, Kan^R, Gem^R	This study
pUC57-Kan Δ*BP3136*	pUC57-Kan derivative harboring *BP3136* knockout construct, Kan^R, Gem^R	This study
pYRC	pBBR1MCS-5 cloning vector containing *lacI*, Gem^R	(Arts et al., 2007)
pKAS32	Allelic exchange suicide vector, *rpsL*, Amp^R	(Skorupski and Taylor, 1996)
pKAS-Δ*kdtA*	pKAS32 derivative harboring *kdtA* knockout construct, Amp^R, Gem^R	This study
pKAS-Δ*kdkA*	pKAS32 derivative harboring *kdkA* knockout construct, Amp^R, Gem^R	This study
pKAS-Δ*BP2327*	pKAS32 derivative harboring *BP2327* knockout construct, Amp^R, Gem^R	This study
pKAS-Δ*BP3136*	pKAS32 derivative harboring *BP3136* knockout construct, Amp^R, Gem^R	This study
pSORTPI	Gem^R derivative of pRTPI, *colE1*, *oriT*, *rpsL*, Amp^R, Gem^R	(Stibitz et al., 1986)
pSORTPI-Δ*lgmB*-LpxE$_{Fn}$	pSORTPI harboring *F. novicida lpxE* under *ompP* promoter; construct for insertion as *lgmB* knockout, Amp^R, Gem^R	This study

Table S1. Continued.

Strain / plasmid	Description [a]	Reference
pMMB67EH	Broad-host-range expression vector, *tac* promoter, Amp^R	(Fürste et al., 1986)
$pKdtA_{Ec}$	pMMB67EH carrying *E. coli kdtA* with a 3′ sequence encoding a His-Tag, Amp^R	This study
$pLpxF_{Fn}$	pMMB67EH carrying *F. novicida lpxF*, Amp^R	This study

[a] Str, streptomycin; Gem, gentamicin; Amp, ampicillin; Kan, kanamycin.
[b] GlaxoSmithKline laboratory stocks.

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King, A. J., Berbers, G., van Oirschot, H. F. L. M., Hoogerhout, P., Knipping, K., and Mooi, F. R. (2001). Role of the polymorphic region 1 of the *Bordetella pertussis* protein pertactin in immunity. *Microbiology* 147, 2885–2895. doi:10.1099/00221287-147-11-2885.

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Skorupski, K., and Taylor, R. K. (1996). Positive selection vectors for allelic exchange. *Gene* 169, 47–52. doi:10.1016/0378-1119(95)00793-8.

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Table S2. PCR primers used in this study

Primer	Sequence (5′→3′) [a]	Description
Fw-XbaI-KdtA-Ec	GCGCGCTCTAGATGCTCGAATTGCTTTACACCGCCC	Primers for amplification of *kdtA E. coli*, introducing 5′-XbaI and 3′-HindIII restriction sites
Rv-KdtA-His-RBS-NdeI-Hind	GCGCGCAAGCTTCATATGTATATCTCCTTCTTAAAGT T**TCA***GTGGTGGTGGTGGTGGTG*ATGCGTTTTCGGTG GCAGGTAAGGTTCCAG	
Fw-Eco81I-GemR	GCGCGCCCTGAGGGACGCACACCGTGGAAA	Primers for amplification of *gem*^{R}, introducing Eco81I restriction sites at both flanks
Rv-GemR-Eco81I	GCGCGCCCTCAGGGCGGCGTTGTGACAATTT	
Fw-XbaI-LpxE	TAATCCTCTAGAATGCTGAAGCAGACCCTCCA	Primers for amplification of codon-optimized *lpxE F. novicida*, introducing 5′-XbaI and 3′-ApaI restriction sites
Rv-LpxE-ApaI	TAAGCTGGGCCC**TTA**GATGATCTCGCGATTGCGCA	

[a] Restriction sites are underlined; stop codon (in reverse primer) is in bold; sequence coding for a His-Tag is in italics.

5

Regulated expression of *lpxC* allows for reduction of endotoxicity in *Bordetella pertussis*

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Abstract

The Gram-negative bacterium *Bordetella pertussis* is the causative agent of a respiratory infection known as whooping cough. Previously developed whole-cell pertussis vaccines were effective but appeared to be too reactogenic mainly due to the presence of lipopolysaccharide (LPS, also known as endotoxin) in the outer membrane (OM). Here, we investigated the possibility of reducing endotoxicity by modulating the LPS levels. The promoter of the *lpxC* gene, which encodes the first committed enzyme in LPS biosynthesis, was replaced by an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter. The IPTG was essential for growth, even when the construct was moved into a strain that should allow for the replacement of LPS in the outer leaflet of the OM with phospholipids by defective phospholipid transporter Mla and OM phospholipase A. LpxC depletion in the absence of IPTG resulted in morphological changes of the cells and in overproduction of outer-membrane vesicles (OMVs). The reduced amounts of LPS in whole-cell preparations and in isolated OMVs of LpxC-depleted cells resulted in lower activation of Toll-like receptor 4 in HEK-Blue reporter cells. We suggest that, besides lipid A engineering, also a reduction in LPS synthesis is an attractive strategy for the production of either whole-cell- or OMV-based vaccines, with reduced reactogenicity for *B. pertussis* and other Gram-negative bacteria.

Introduction

The cell envelope of Gram-negative bacteria consists of an inner membrane (IM) and an outer membrane (OM), separated by the periplasm where the peptidoglycan sacculus is located. While the IM is a bilayer of phospholipids (PLs), the OM shows an asymmetric organization with the PLs and lipopolysaccharides (LPS, also known as endotoxin) located in the inner and the outer leaflet, respectively (Silhavy et al., 2010). The LPS consists of three moieties, i.e., lipid A, a core oligosaccharide, and a polysaccharide known as O-antigen (Raetz and Whitfield, 2002). In some bacteria, including *Bordetella pertussis*, an O-antigen is lacking, and their LPS is also referred to as lipooligosaccharide, or LOS. The LPS, or at least its lipid A moiety, appears essential for the viability of many Gram-negative bacteria, including *Escherichia coli*; however, three species, *Neisseria meningitidis* (Steeghs et al., 1998), *Moraxella catarrhalis* (Peng et al., 2005), and *Acinetobacter baumannii* (Moffatt et al., 2010), which all synthesize LPS without the O-antigen, have been reported to survive in its absence.

LPS, particularly its lipid A moiety, is responsible for the endotoxicity associated with infections by Gram-negative bacteria. This molecule is recognized by a receptor on the innate immune cells consisting of the Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2), which triggers the production of pro-inflammatory cytokines, such as TNFα and IL-1β (Raetz and Whitfield, 2002). The endotoxic reaction elicited by the LPS is one of the main reasons for the adverse reactions evoked by whole-cell vaccines against various Gram-negative bacteria, including *B. pertussis*. This pathogen, which is responsible for the human respiratory disease known as whooping cough or pertussis, was brought under control by the worldwide introduction of whole-cell pertussis vaccines (wP) around the 1940s (Mattoo and Cherry, 2005). However, because of the reactogenicity of wP, mainly caused by its endotoxin content, acellular (aP) replacement vaccines were developed to counteract the aversion that had risen against the use of wP. These aP vaccines, which consist of one to five purified antigens, were introduced in the 1990s and proved to be effective and less reactogenic (Mattoo and Cherry, 2005). Nevertheless, during the past few decades, the number of pertussis cases has been rising in the countries using aP (Esposito et al., 2019). The apparently lower efficacy of the aP vaccines is related to the lack of protection against mucosal colonization, the fast waning of the vaccine-induced immune protection, and the emergence of bacterial variants with mutations in the genes for vaccine antigens (Esposito et al., 2019). The differences in the immune response upon vaccination with wP versus aP seem to be one of the main reasons for the difference in efficacy. While wP elicits particularly strong T-helper (Th)1 and, to a lesser extent, Th17 responses, aP elicits a mixed Th2 and Th17 reaction. A Th1 response appears to be crucial for protection against pertussis colonization (Esposito et al., 2019; Kapil and Merkel, 2019). These data point towards the need for a new Th1- and Th17-response-inducing pertussis vaccine with low reactogenicity, preferably one with

an abundant number of bacterial antigens to avoid the vaccine-induced selection of escape mutants.

A solution could be the development of a novel whole-cell vaccine, but a prerequisite for this option is that its endotoxicity should be diminished. Another approach gaining ground in the protection against Gram-negative bacteria is the use of vaccines based on OM vesicles (OMVs). OMVs are blebs derived from the OM that are spontaneously released from the bacterial surface in multiple environmental conditions (Schwechheimer and Kuehn, 2015). These vesicles present bacterial surface antigens in combination with LPS and have robust immunogenic capacity. They have been shown to be an effective alternative in vaccine development against Gram-negative pathogens, in particular *N. meningitidis* (Nøkleby et al., 2007; Gorringe and Pajon, 2012). For *B. pertussis*, immunization studies in mice with an OMV-based vaccine showed a lower production of pro-inflammatory cytokines compared to wP immunization. In addition, the OMV vaccine induced an immune protection similar to wP, including a Th1 response, which was not elicited by aP (Raeven et al., 2016). Nevertheless, whether the reduction in endotoxicity in these OMVs is enough to produce acceptably safe vaccines for human use remains unknown.

A prevalent strategy to reduce the endotoxicity of bacterial cells, which could also be applied to OMVs, is through the genetic engineering of the lipid A structure to weaken its interaction with TLR4 (Simpson and Trent, 2019; Kawahara, 2021), an approach that has also been applied in *B. pertussis* (Geurtsen et al., 2006; Arenas et al., 2020). However, a reduction in the total amount of LPS might be an alternative approach to accomplish the appropriate endotoxin levels.

The amount of LPS produced in bacteria is meticulously regulated at different levels. The biosynthesis pathway of lipid A is known as the Raetz pathway (Raetz and Whitfield, 2002). The first committed reaction in this process is catalyzed by the UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase LpxC and is a crucial point for the regulation of LPS production. The cellular LpxC levels directly correlate with the LPS production and are governed, at least in some enteric bacteria, by the FtsH/LapB (also known as YciM) protease complex, which degrades LpxC (Klein et al., 2014; Guest et al., 2021). In addition, YejM (also known as PbgA) prevents the FtsH/LapB-mediated LpxC proteolysis and, therefore, stabilizes the LpxC levels (Klein and Raina, 2019; Biernacka et al., 2020; Clairfeuille et al., 2020; Fivenson and Bernhardt, 2020; Guest et al., 2020). However, the LpxC regulatory systems might work differently in other bacteria, as seems to be the case for *Pseudomonas aeruginosa* (Langklotz et al., 2011; Guest et al., 2021) and *N. meningitidis* (Putker et al., 2014; Guest et al., 2021), for example, and therefore, the mechanism remains unknown for many other species, including *B. pertussis*. Additionally, the LpxC levels can be indirectly controlled in the OM by the OM phospholipase A (OMPLA), encoded by the *pldA* gene, which degrades the mislocalized PLs that may appear in the outer leaflet

of the OM when the LPS levels are deficient. The OMPLA, then, releases fatty acids from these PLs, which form a signal to inhibit the LpxC proteolysis and, thereby, increase the LPS production (May and Silhavy, 2018). Consistently, the inactivation of the maintenance of lipid asymmetry (Mla) system, which mediates the retrograde transport of aberrantly localized PLs from the outer leaflet of the OM to the IM, prompts the accumulation of mislocalized PLs, which activates OMPLA and eventually stimulates LPS production (Sutterlin et al., 2016). Accordingly, the combined inactivation of OMPLA and the Mla system in *B. pertussis* results in an accumulation of PLs in the outer leaflet of the OM (de Jonge et al., 2022). In this study, we investigated whether it is possible to reduce the amount of LPS in *B. pertussis* whole-cell preparations and OMVs by regulating the *lpxC* expression.

Materials and Methods

Bacterial strains and growth conditions

All of the bacterial strains used are described in Supplementary Table S1. The *E. coli* strains were grown at 37 °C in lysogeny broth (LB) while shaking or on LB agar plates. The *B. pertussis* strain B213 and its derivatives were grown at 35 °C on Bordet–Gengou agar (Difco), supplemented with 15% defibrinated sheep blood (Biotrading, Mijdrecht, The Netherlands) (BG-blood). For the liquid cultures, the bacteria were scraped from BG-blood plates after three days of growth and used to inoculate Verwey medium (Verwey et al., 1949) to an optical density at 600 nm (OD_{600}) of 0.05. The bacteria were then grown at 35 °C while shaking at 175 rpm. Further details regarding the growth conditions are indicated in the Results section. When required for plasmid maintenance or strain selection, 100 µg/mL of ampicillin was included in the medium. To induce gene expression, the media were supplemented with 1 mM IPTG.

DNA manipulation and plasmid construction

All of the plasmids and PCR primers used are listed in Supplementary Tables S1 and S2, respectively. The regular PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific, Waltham, MA, USA), whilst the PCR fragments generated for cloning were obtained using the Expand High Fidelity PCR system (Roche Diagnostics GmbH, Mannheim, Germany). For the purification of the PCR products, the commercial Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) was employed. The plasmids were isolated with the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA). The PCR products and plasmids were digested with the appropriate restriction enzymes (Thermo Scientific), according to the manufacturer's instructions, purified, and ligated using T4 DNA ligase (5 U/µL) (Thermo Scientific).

The cloning procedures were performed in *E. coli* strain DH5α by transformation, using the $CaCl_2$ method. For the construction of the regulated *lpxC*-expression plasmid, a DNA fragment, containing the dual *lac* and *tac* promoters, the *lacI*^{q} regulatory gene, and the

erythromycin-resistance cassette from pEN11-NMB0338, was amplified by PCR using the primers pENP_ery_Fw and pENP_Rev_NdeI. The PCR product obtained and the vector pUC18 were digested with the endonucleases PstI and NdeI and, subsequently, ligated together, generating plasmid pUCK. The nucleotide sequence of the insert was validated by DNA sequencing. The primers RegLpxC-FW-NdeI and RegLpxC-RV-AatII were used to amplify an 807-bp truncated *lpxC* gene, with a premature stop codon introduced via the reverse primer. The amplicon was inserted into pUCK after digestion of the vector and PCR product with NdeI and AatII, resulting in pUCK-*lpxC*. After the validation of the insert by DNA sequencing, the plasmid pUCK-*lpxC* was introduced into *B. pertussis* by electroporation. The electrocompetent cells were prepared from cultures grown for 72 h, which were harvested by centrifugation for 10 min at 10,000× *g*, and the cells were washed twice with MilliQ water, which was precooled at 4 °C, in a volume equal to that of the discarded medium. The cells were subsequently washed in precooled 300 mM sucrose with one fourth of the original volume, resuspended in precooled 300 mM sucrose to an OD_{600} of 50, and stored in aliquots at −80 °C till further use. After thawing the electrocompetent cells, 3 µL of plasmid DNA (~1 µg) was added to 40 µL of the cell suspension in a 0.1-cm cuvette (Bio-Rad, Hercules, CA, USA), and electroporation was executed at 200 Ω, 25 µF, and 2.25 kV. The electroporated cells were mixed with prewarmed Verwey medium and incubated at 35 °C for 75 min before spreading them on BG-blood plates containing ampicillin for selection and IPTG for inducing gene expression. The integration of the construct in the genome was confirmed by PCR, using primers pENP-Sh-Fw-XbaI and Rv-LpxC-dw400.

OM isolation

The bacterial cells were collected from the liquid cultures by centrifugation at 10,000× *g* for 10 min at 4 °C, resuspended in 2 mL of physiological salt solution to an OD_{600} of 7.5, and inactivated by incubation for 30 min at 56 °C. Then, the cells were harvested by centrifugation at 10,000× *g* for 10 min at 4 °C. The spheroplasts were made, as previously described (Osborn et al., 1972). Briefly, the cells were resuspended in 2 mL of 0.75 M sucrose and 10 mM Tris-HCl (pH 7.8). Then, 10 µL of 40 mg/mL lysozyme was added, followed by 4 mL of 1.5 mM EDTA (pH 7.5). The suspension was incubated for 30 min at room temperature. The spheroplasts were frozen at −80 °C, thawed, and lysed by ultrasonication. The unbroken cells and aggregates were removed by centrifugation at 10,000× *g* for 1 h at 4 °C. The supernatant was then centrifuged for 1 h at 40,000 rpm (Beckman Coulter Optima LE-80K, Type 70 Ti rotor, Brea, CA, USA) at 4 °C, and the resulting pellet was resuspended in phosphate-buffered saline (PBS).

OMV isolation

The OMVs were isolated, as previously described (de Jonge et al., 2021) with slight modifications. Briefly, the bacterial cells from the liquid cultures were pelleted by centrifugation at 10,000× *g* for 10 min, and the supernatants were passed through a 0.45-µm pore-size filter (Sarstedt, Nümbrecht, Germany). The OMVs were pelleted by

ultracentrifugation for 2 h at 40,000 rpm and 4 °C (Beckman Coulter Optima LE-80K, Type 70 Ti rotor) and resuspended in PBS. The OMVs were quantified based on protein content, using a Lowry DC protein assay (Bio-Rad), according to the manufacturer's instructions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The whole cells, OM preparations, or isolated OMVs were mixed with the sample buffer (Laemmli, 1970), boiled for 10 min, and analyzed on 8–16% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad). After electrophoresis, the protein profiles were visualized by staining with Bradford reagent (Bos et al., 2015), and the LPS was stained with silver (Tsai and Frasch, 1982).

Microscopy

The bacterial cultures were fixed with 1% formaldehyde for at least 30 min at 4 °C and stained with 5 *µg/mL* of FM4-64 (Invitrogen, Waltham, MA, USA) for 15 min in the dark. Then, 5 *µL of the stained suspension was pipetted onto* 1%-agarose pads placed on microscopy slides, and cells were visualized with a Zeiss Axioskop 2 fluorescence microscope with a 100× objective (Oberkochen, Germany).

TLR4 stimulation assays

The TLR4 stimulation assays were performed as previously described (Pérez-Ortega et al., 2021). Briefly, the HEK-Blue TLR4 reporter cells (Invivogen) were incubated with serial dilutions of either whole bacterial cells that were killed by heat treatment at 56 °C for 1 h or the isolated OMVs. After 17 h of incubation at 37 °C in a 5% saturated CO_2 atmosphere, supernatants were incubated with *p*-nitrophenyl phosphate solution for 1 h, and the absorbance at 405 nm was measured in a Biotek microplate reader (Winooski, VT, USA).

Statistical analysis

All the statistical analyses were performed using the GraphPad Prism software version 6. The TLR4 stimulation data were analyzed for statistical significance using two-way ANOVA (Dunnett's correction for multiple comparisons), while one-way ANOVA (Tukey's correction for multiple comparisons) was applied on OMV quantification studies.

Results

Regulated expression of *lpxC* and its implication on growth

Since *lpxC* (locus tag BP3017) was suggested to be an essential gene in *B. pertussis* (Gonyar et al., 2019; Belcher et al., 2020), we set out to regulate the expression of the *lpxC* gene and, consequently, the production of LPS in the cells. To this end, plasmid pUCK-*lpxC* was constructed (Supplementary Figure S1). This plasmid, which doesn't replicate in *Bordetella*, contains an *lpxC* allele that is truncated because of a premature stop codon. It can integrate into the bacterial chromosome via homologous recombination into the *lpxC* gene. As a result of integration, the chromosomal gene is disrupted and an additional,

IPTG-regulated, intact copy of the gene is created, which is controlled by the dual *tac-lac* promoter included in the plasmid (Figure S1). The presence of an ampicillin-resistance marker in the plasmid allowed for the selection of recombinants containing the construct integrated into the chromosome.

After obtaining a *B. pertussis* strain containing the *lpxC* regulatory construct (Bp. RegC), we tested whether it could grow in the absence of IPTG and, thus, in the absence of LPS production. After growing the strain Bp. RegC on a Bordet–Gengou agar plate, supplemented with sheep blood (BG-blood), ampicillin, and IPTG, the cells were scraped from the plate and resuspended in a liquid medium with ampicillin and either with or without the inducer. Without IPTG, a slight increase in the OD_{600} of the culture was observed in the first hours, after which the OD_{600} remained static for the next 48 h (Figure 1, left panel), indicating the inability of the bacteria to grow in the absence of *lpxC* expression.

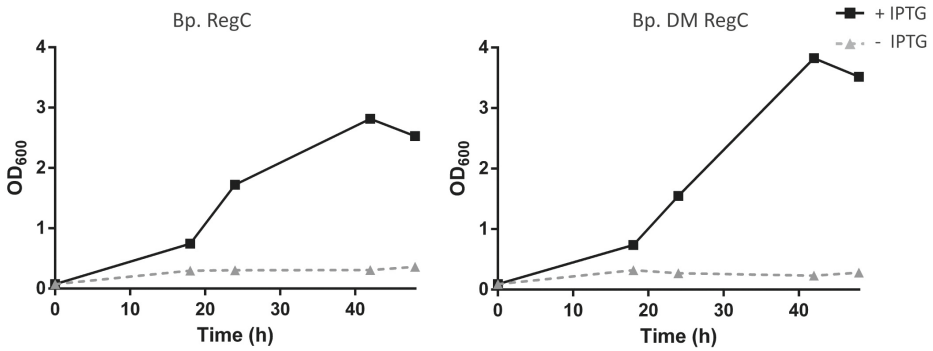

Figure 1. Growth of *B. pertussis* strains with regulatable *lpxC* in the presence or absence of 1 mM IPTG. Growth curves of Bp. RegC (left panel) and Bp. DM RegC (right panel) were measured at OD_{600} for 48 h in the presence or absence of IPTG. Each graph shows values of a single experiment.

In the absence of LPS synthesis, the PLs are presumed to reach the outer leaflet of the OM to replace the LPS. However, such mislocalized PLs are degraded by activated OMPLA, or transported back to the inner membrane by the Mla system. Such activities would prevent the formation of a stable phospholipid bilayer in the OM. Accordingly, although *A. baumannii* is viable in the absence of the LPS synthesis, the growth of such mutants is severely restricted but can be restored by the inactivation of OMPLA and the Mla system (Powers and Trent, 2018; Nagy et al., 2019). Thus, we decided to test whether we could rescue a *B. pertussis* strain lacking LPS in the same way. To that end, the *lpxC*-regulatory construct was introduced into a *B. pertussis* strain in which OMPLA and the Mla system were inactivated by *pldA* and *mlaF* mutations, respectively. However, these modifications didn't allow for the growth of the resulting strain, Bp. DM RegC, in the absence of IPTG (Figure 1, right panel).

LPS-depletion protocol

Since the constructed mutants can apparently not grow in the absence of IPTG, we wanted to develop a protocol for the growth of Bp. RegC that allows for obtaining sufficient bacterial cells but with reduced amounts of LPS. In this protocol (Figure 2A), Bp. RegC cells were grown on BG-blood plates containing IPTG and resuspended in liquid medium supplemented with IPTG. After 17 h of growth (T_1), the cells were diluted into fresh medium with IPTG to an OD_{600} of 0.05. After 24 h of growth of this preculture (T_2), the cells were washed twice with medium without IPTG by centrifugation for 3 min at 7000× *g*, adjusted to an OD_{600} of 0.2, and grown either with or without IPTG for another 24 h (T_3). In the absence of IPTG, an apparent, but statistically not significant, reduction in the growth was observed (Figure 2A, left panel). Interestingly, such a reduction in the growth was not observed for Bp. DM RegC (Figure 2A, right panel), suggesting that the accumulation of PLs in the outer leaflet of the OM can compensate to some extent for the downregulation of *lpxC*, even though the *lpxC* expression is also essential for viability in this strain (Figure 1).

The whole cells collected at the end of the LPS-depletion protocol (T_3) were analyzed by SDS-PAGE. A silver staining of the gel showed reduced LPS levels in strains Bp. RegC and Bp. DM RegC grown in the absence of IPTG compared to the cells grown with IPTG (Figure 2B). The protein profiles of the isolated OMs of the parental strains and their *lpxC*-regulated derivatives were comparable, even after growth in the LPS-depletion conditions (Figure 2C).

LPS depletion affects cell shape

We next wanted to study the effect of the LPS depletion on cell morphology. To this end, the bacteria were stained with the fluorescent membrane dye FM4-64 and examined by fluorescence microscopy (Figure 3; details of the frames shown in Figure 3 are shown enlarged in Supplementary Figure S2). Whereas the wild-type strain showed, as expected, short rods (Figures 3A and S2A), the Bp. RegC cells appeared shorter and more rounded than the wild type when the bacteria were grown in the absence of IPTG (examples indicated with green arrowheads in Figures 3B and S2B), and this phenotype was suppressed after growth of the strain in the presence of IPTG (Figures 3C and S2C). In addition, we observed an uneven distribution of the dye on the surface of the LPS-depleted mutant cells (blue arrowheads in Figures 3B and S2B), which suggests alterations in the membrane composition and/or integrity.

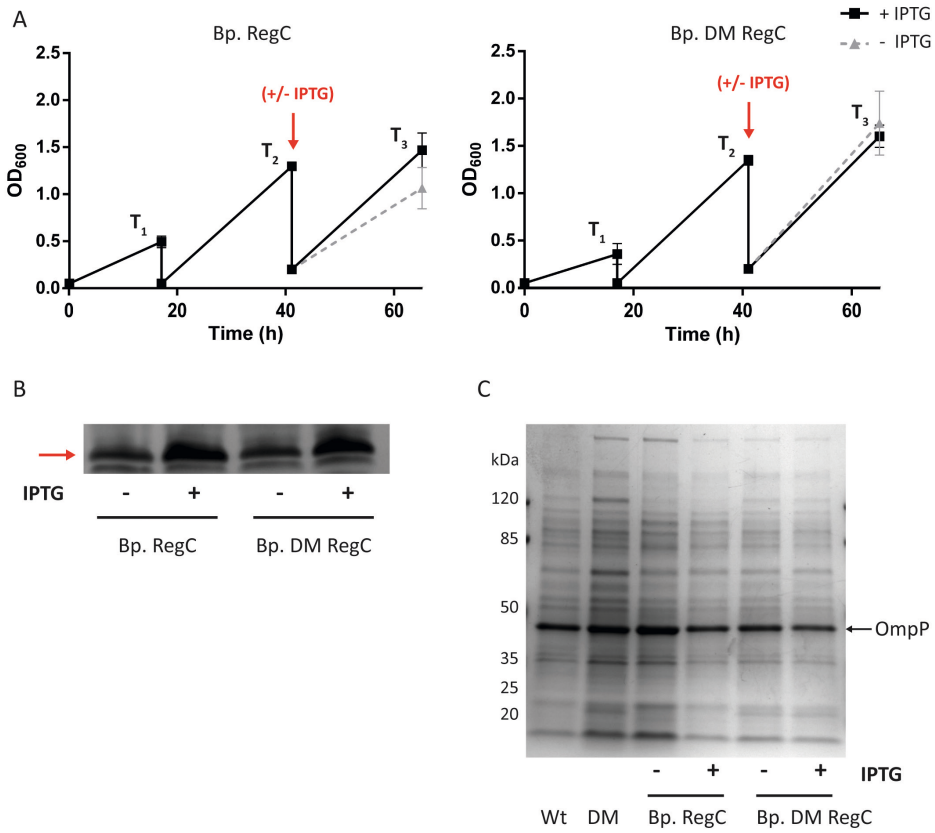

Figure 2. Growth of *B. pertussis* strains with regulatable *lpxC* during LPS-depletion conditions and characterization of the cell content. (**A**) Growth curves of Bp. RegC (left panel) and Bp. DM RegC (right panel) were measured at OD_{600} following the LPS-depletion growth protocol discussed in the text. At T_2, bacterial cultures were washed and grown further either without IPTG (grey dashed line) or with 1 mM IPTG (black solid line). Graphs show mean values with standard deviations of three independent experiments. No significant differences were found with a paired *t*-test; (**B**) Analysis of the LPS content of cells by SDS-PAGE. Samples taken at T_3 in panel A were adjusted based on OD, analyzed by SDS-PAGE, and LPS was visualized by silver staining. Only the relevant part of the gel is shown. The band corresponding to LPS is indicated with an arrow; (**C**) The protein content of OM preparations from Bp. RegC and its wild-type parental strain (Wt) and from Bp. DM RegC and its parental strain (DM) was analyzed by SDS-PAGE. The major OM protein, porin OmpP, is indicated with an arrow at the right. Molecular-weight markers are indicated at the left.

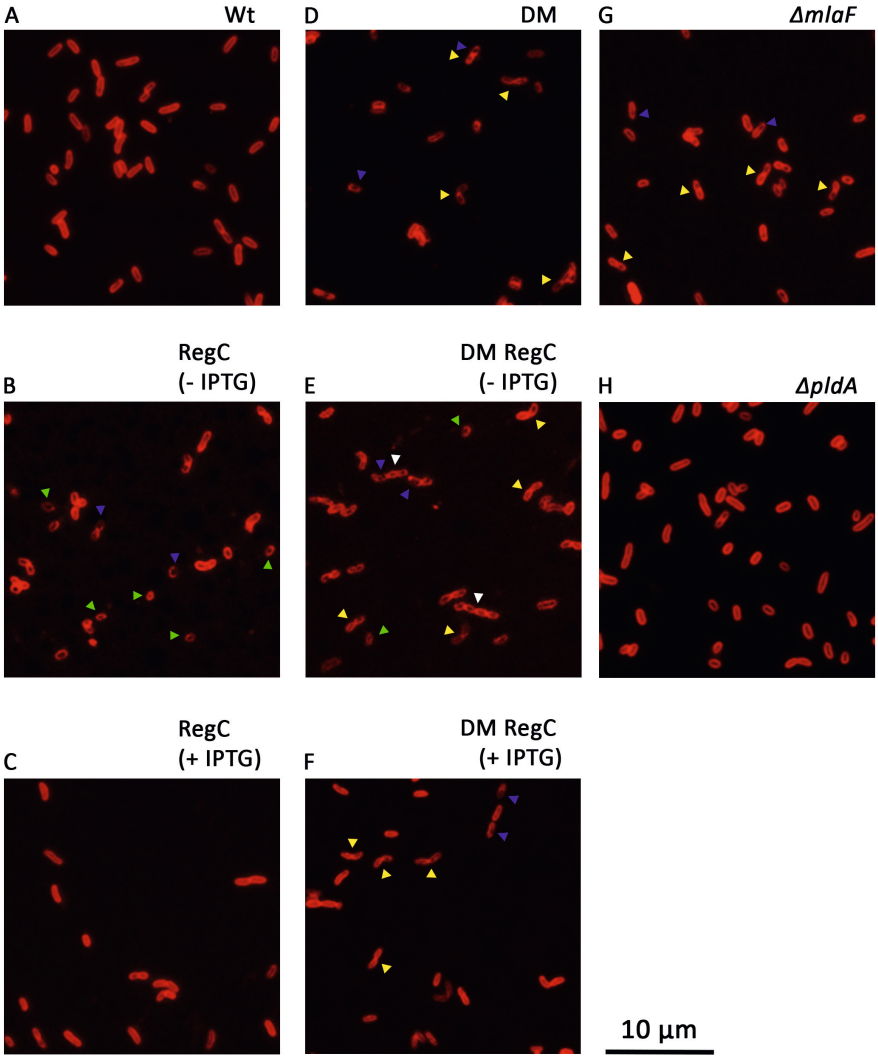

Figure 3. Morphology of *B. pertussis* cells visualized by fluorescence microscopy. Representative captions of the wild-type (Wt) (**A**), Bp. RegC (**B**, **C**) grown in absence (-) or presence (+) of IPTG, DM mutant (**D**), Bp. DM RegC (**E**, **F**) grown in absence (-) or presence (+) of IPTG, and the *mlaF* (**G**) and *pldA* (**H**) mutant strains are included. Wt and the Δ*mlaF*, Δ*pldA*, and DM mutant strains were grown in liquid medium for 17 h, and strains Bp. RegC and Bp. DM RegC were grown with 1 mM IPTG or without IPTG following the LPS-depletion protocol and analyzed at T_3 as defined in Figure 2A. The cells were stained with the fluorescent dye FM4-64. Scale bar represents 10 µm. Examples of cells showing a shorter and more rounded shape than the wild-type cells (green), uneven dye distribution (blue), or appearing in pairs (yellow) or short chains (white) are indicated with colored arrowheads. Enlargements of details of these figures are shown in Figure S2.

While the cells observed in the wild type were mostly singular cells (Figures 3A and S2A), the *mlaF pldA* double mutant Bp. DM more frequently showed pairs of cells (examples indicated by yellow arrowheads in Figures 3D and S2D) suggesting a defect in the cell division. We hypothesized that this defect is due to the inactivation of either OMPLA or the Mla system in Bp. DM. Interestingly, many pairs of the cells were also detected in an *mlaF* single mutant (yellow arrowheads in Figures 3G and S2G) but not in a *pldA* single mutant (Figures 3H and S2H). These results indicate a novel role of the Mla system in cell division. When Bp. DM RegC was deprived of *lpxC* expression, we observed many short chains of cells (white arrowheads in Figures 3E and S2E), which suggests that the defect in the cell division is exacerbated by the lack of LPS. The cell chaining was reduced again when this mutant was grown with IPTG, and many pairs of cells were observed under these conditions (Figures 3F and S2F).

LPS depletion affects OMV production

To determine whether the OMV production was affected by the reduction in the LPS content, we isolated the OMVs from the liquid cultures grown following the LPS-depletion protocol (at T_3 in Figure 2A), and the amounts of the isolated OMVs were quantified on

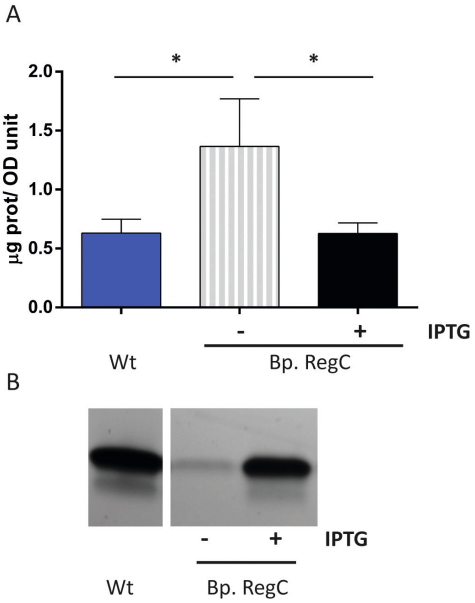

Figure 4. Influence of LPS depletion on OMV production. OMVs were isolated from the supernatant of the same volumes of cultures of the wild-type strain (Wt) and of the Bp. RegC strain obtained at T_3 as defined in Figure 2A following the LPS-depletion protocol with or without IPTG. (**A**) OMVs were quantified based on protein content using a Lowry assay. Protein content is depicted as the amount of protein in the OMV fractions per liter of bacterial culture per OD_{600} unit. Values shown are means and standard deviations from three independent experiments, and significant differences are indicated with asterisks ($p \leq 0.05$); (**B**) LPS in the isolated OMVs was analyzed by SDS-PAGE after adjusting the samples to similar protein content, and the LPS was visualized in the gel by silver staining.

the basis of protein content. As shown in Figure 4A, the OMV production was significantly enhanced (~two-fold increase) relative to the wild type when Bp. RegC was grown in the absence of IPTG, and this overproduction was suppressed when the strain was grown in the presence of IPTG. To examine the LPS content of the OMVs, the OMV preparations were adjusted to protein content and analyzed by SDS-PAGE. Remarkably, the intensity of the LPS band was much lower in the OMV preparations from the mutant cells lacking *lpxC* expression than in those from the cells grown with IPTG or from the parental strain (Figure 4B), and the difference in the LPS content in the OMVs appeared considerably larger than in the whole-cell lysates of the strains (Figure 2B).

LPS depletion reduces TLR4 activation by whole cells or OMVs

To determine if the reduced LPS content in the cells and OMVs of strain Bp. RegC grown in the absence of IPTG reduces TLR4 signaling, we tested TLR4 activation in the HEK-Blue reporter cells expressing either human or murine TLR4 (h- and m-TLR4, respectively)

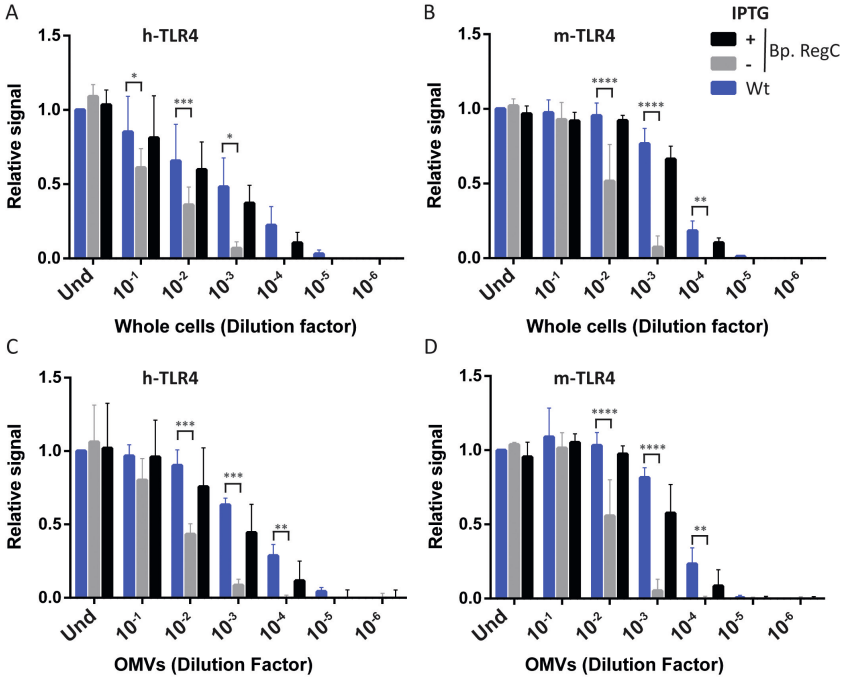

Figure 5. TLR4 activation by whole-cell and OMV preparations. HEK-Blue cells expressing either h-TLR4 (**A**,**C**) or m-TLR4 (**B**,**D**) were incubated for 17 h with 10-fold serial dilutions of heat-inactivated whole cells (the OD_{600} of the undiluted cell suspensions (Und) was 0.15) (**A**,**B**) or isolated OMVs (the protein concentration in the undiluted preparations (Und) was 1 µg/mL) (**C**,**D**). Diagrams show means and standard deviations of relative SEAP activity calculated as the ratio between the signal measured for each dilution of each strain and the signal measured for Und. Wt (parental strain). Three independent experiments were performed in duplicate (whole cells) or singularly (OMVs). Statistical comparisons showed significant differences relative to the wild type for Bp. RegC grown in absence of IPTG but not for Bp. RegC grown with IPTG. Dilutions with statistically different results are indicated with asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

after their stimulation with whole-cell or OMV preparations (Figure 5). The whole-cell preparations of the parental strain and of strain Bp. RegC induced with IPTG showed similar TLR4 activation, while the preparations of strain Bp. RegC grown in the absence of IPTG showed significantly reduced h- and m-TLR4 stimulation, with 10- to 100-fold higher cell concentrations being required for a similar activation of the receptors (Figure 5A,B). Similarly, the OMV preparations were tested. The OMV preparations used were adjusted to a similar protein content. Following the trend observed for the whole-cell preparations, the OMVs obtained from Bp. RegC after growth in the absence of IPTG showed a drastically reduced activation of both h-TLR4 (Figure 5C) and m-TLR4 (Figure 5D).

Discussion

Immunization with wP vaccines was discontinued in the last 30 years in industrialized countries because of their high reactogenicity, which was mainly due to the presence of LPS in these vaccines. They were replaced by aP vaccines, which proved to be safer, but also seemed to be less effective, considering the resurgence of pertussis in the last few decades (Esposito et al., 2019; Kapil and Merkel, 2019). Thus, new pertussis vaccines are needed. Such new vaccines should preferably be based on whole cells because of the multitude of antigens they contain and the Th1/Th17-type response that such vaccines induce. However, a prerequisite is that the reactogenicity is reduced relative to the original wP vaccines.

An alternative approach is the use of OMV-based vaccines, which also contain a multitude of relevant antigens and have been proven to be effective for another Gram-negative bacterium, i.e., *N. meningitidis* (Micoli and MacLennan, 2020). The OMV-based vaccines that have been licensed to date consist of detergent-extracted OMVs, a procedure that reduces the LPS content. However, this treatment can also reduce the content of relevant surface antigens (Micoli and MacLennan, 2020). Preliminary in vivo studies indicated that the OMV-based vaccines can also be used for *B. pertussis* (Raeven et al., 2016). In that study, it was demonstrated that these vaccines can provide comparable protection in mice to whole-cell formulations, but they showed lower endotoxicity, which makes them a safer alternative. However, the reason for the lower reactogenicity of these OMV preparations is unknown, and it remains to be established whether reactogenicity is sufficiently reduced for application in humans. Hence, additional measures to reduce endotoxicity may be required.

A commonly applied method to reduce the endotoxicity of whole-cell preparations is lipid A engineering (Zariri et al., 2016; Simpson and Trent, 2019; Kawahara, 2021; Pérez-Ortega et al., 2021). The endotoxicity of lipid A is determined by the number and the length of its fatty acyl chains, as well as by its phosphate groups (Teghanemt et al., 2005; Park et al., 2009), which can all be modified by genetic engineering. In *B. pertussis*, the

genetically induced modifications of lipid A have successfully been applied to reduce the endotoxicity. This was achieved by using different approaches, i.e., by shortening the length (Arenas et al., 2020), or reducing the number of acyl chains (Geurtsen et al., 2006). The latter approach, however, which was achieved by the expression of the lipid A deacylase PagL in the cells, resulted in less toxic LPS, but the whole-cell preparations showed increased endotoxicity, probably as a result of an increased release of LPS from the bacterial surface (Geurtsen et al., 2006).

Here, we present an alternative strategy to reduce endotoxicity, i.e., by genetically interfering with the LPS synthesis, which was achieved by controlling *lpxC* expression. This strategy can be applied to reduce the endotoxicity of both whole-cell and OMV preparations and is potentially also applicable in the development of vaccines against other bacteria. Previously, whole-cell formulations with a reduced LPS content have also been developed (Zorzeto et al., 2009; Dias et al., 2013). However, this reduction was commonly achieved by the treatment of whole cells with organic solvents, which can cause the loss of other OM components, e.g., hydrophobic (lipo)proteins (Molloy et al., 1999). With our strategy, we reduced the amount of LPS, while avoiding harsh treatments that could cause the loss of surface-exposed antigens that are relevant for optimal immune protection.

Although LPS is essential for the viability of many Gram-negative bacteria, some of them, including *A. baumannii*, can survive without it (Steeghs et al., 1998; Peng et al., 2005; Moffatt et al., 2010). Such mutant strains of *A. baumannii* grow poorly, but growth could be considerably improved by the inactivation of OMPLA and the Mla system, which degrade or remove PLs showing up in the outer leaflet of the OM, respectively (Powers and Trent, 2018; Nagy et al., 2019). Our results demonstrate that the LPS synthesis is essential for the viability of *B. pertussis*. We considered the possibility that the formation of a stable bilayer of PLs in the OM in the absence of LPS synthesis is prevented by the activity of OMPLA and the Mla system. The combined inactivation of the OMPLA and the Mla system was previously demonstrated to allow for the accumulation of PLs in the outer leaflet of the OM of *B. pertussis* (de Jonge et al., 2022). However, the combined inactivation of the *pldA* and *mlaF* genes appeared to be ineffective or, at least, insufficient to sustain the growth of *B. pertussis* in the absence of *lpxC* expression. Thus, we did not succeed in constructing a strain totally devoid of LPS. However, besides its adverse effects, LPS is also a potent adjuvant. The activation of TLR4 by LPS seems to play an important role in the induction of the appropriate cellular immune responses, i.e., Th1 and Th17 (Higgins et al., 2006), and even the addition of the LPS analogs as adjuvant to an aP vaccine has been reported to be effective (Geurtsen et al., 2007). Our *lpxC*-regulated strain could allow for the fine-tuning of the LPS amount to the needs for a safe and effective vaccine formulation with sufficiently retained adjuvant activity.

Additionally, de Jonge et al. (2022) showed that the disrupted lipid asymmetry in the OM of an *mlaF pldA* double mutant results in increased OMV production, which is in line with the results of a previous study in other species (Roier et al., 2016). Likewise, we observed the overproduction of OMVs in the LpxC-depleted strain, probably as a consequence of the increased amounts of PLs in the outer leaflet of the OM. The LpxC depletion resulted in a stronger reduction in the LPS content in OMVs than in the whole cells (compare Figures 2B and 4B), suggesting that increased blebbing serves to shed accumulated PLs from the OM and to restore the LPS/PL stoichiometry. A similar observation was made in a YejM/PbgA-depletion strain of *E. coli*, which produced OMVs highly enriched in PLs, apparently to compensate for the decreased levels of LPS in the OM (Clairfeuille et al., 2020). The overproduction of OMVs in the LpxC-depletion strain could improve the cost efficiency of an OMV-based vaccine against *B. pertussis*.

The depletion of LPS also resulted in morphological defects as the cells became smaller and coccoid in the absence of *lpxC* expression. Interestingly, such morphological alteration was also reported for LPS-depleted *A. baumannii* (Nagy et al., 2019). Additionally, in the strain in which the Mla system and OMPLA were inactivated, chains of cells were formed, especially in the absence of *lpxC* expression (Figures 3E and S2E). Interestingly, similar chains of cells can be observed in the micrographs of an *A. baumannii* Δ*mlaA* Δ*pldA* Δ*lpxC* triple mutant strain, but not in those of a Δ*lpxC* single mutant (Nagy et al., 2019). This analogy suggests that chaining occurs as a consequence of one or a combination of these mutations, and that this phenotypic deviation is shared by different bacterial species. We suggest that the inactivation of the Mla system is responsible for this morphological change, since our *mlaF* single mutant already showed a division defect. The additional absence of *lpxC* expression may then exacerbate the division defect. Thus, these observations suggest a direct or indirect role of the Mla system in cell division.

Overall, we present a new strategy to reduce the endotoxicity of the whole-cell- or OMV-based vaccines against Gram-negative bacteria. The strain we created allows for the production of *B. pertussis* cells with a substantial reduction in the LPS content and, consequently, a reduced TLR4-stimulating activity. In addition, we did not observe evident modifications of the protein content of the OM. In the same vein, this approach could be used for the development of OMV-based vaccines, in as much as it allows for a drastic reduction in the LPS content in OMVs, while increasing the OMV production. However, further strain development may be needed for commercial vaccine manufacturing. The presence of the antibiotic-resistance cassettes and vector sequences is undesirable in a vaccine strain, and they should be removed. Furthermore, the use of an authentic inducible *Bordetella* promoter instead of the dual *tac-lac* promoter and a switch to a more recent *B. pertussis* isolate instead of the Tohama I derivative used in this study may be considered. Additionally, the LPS-depletion protocol will probably have to be adapted to large-scale culturing in fermenters, and the immunogenicity and reactogenicity of preparations still have to be tested in vivo. Nevertheless, our study already provides proof-

of-principle for a strategy by which the LPS levels in whole-cell and OMV formulations are genetically controlled to generate novel vaccines with reduced reactogenicity.

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Supplementary material

Table S1. Bacterial strains and plasmids

Strain / plasmid	Description [a]	Reference
B. pertussis		
B213	Str^R derivative of strain Tohama I	(King et al., 2001)
B213 Δ*mlaF*	*ΔmlaF::gem* mutant of B213, Str^R, Gem^R	(de Jonge et al., 2022)
B213 Δ*pldA*	*ΔpldA::gem* mutant of B213, Str^R, Gem^R	(de Jonge et al., 2022)
Bp. DM	*ΔpldA::gem* mutant of B213 Δ*mlaF*, Str^R, Gem^R	(de Jonge et al., 2022)
Bp. RegC	B213 with integrated pUCK-lpxC, Str^R, Amp^R, Ery^R	This study
Bp. DM RegC	Bp. DM with integrated pUCK-lpxC, Str^R, Gem^R, Amp^R, Ery^R	This study
E. coli		
DH5α	F^-, Δ(lacZYA-argF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15	(Grant et al., 1990)
Plasmids		
pEN11-NMB0338	pEN11 [4] harboring NMB0338, Ery^R, Cam^R	Unpublished
pUC18	Cloning vector, Amp^R	(Norrander et al., 1983)
pUCK	pUC18 derivative harboring dual *lac* and *tac* promoter, *lacI*^{q} and Ery^R cassette from pEN11-NMB0338, Amp^R, Ery^R	This study
pUCK-*lpxC*	pUCK derivative harboring truncated *lpxC* gene from *B. pertussis* B213, Amp^R, Ery^R	This study

[a] Str, streptomycin; Gem, gentamicin; Amp, ampicillin; Ery, erythromycin; Cam, chloramphenicol.

5

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Table S2. PCR primers used in this study

Primer	Sequence (5'→3') [a]	Description
pENP_ery_Fw	GCGCGCGGTACCGGTATCAACACTGCAGAA	Primers for amplification of pEN11 fragment including p*lac*, p*tac*, *lacI*^{q}, and erythromycin-resistance cassette, introducing PstI and NdeI restriction sites
pENP_Rev_NdeI	CGCGCGCATATGCAGTTCCTTGTGGTGCGGA	
RegLpxC-FW-NdeI	GCGCGCCATATGTTCCGACAGCGCAGTATTC	Primers for amplification of *B. pertussis lpxC* introducing premature stop codon and NdeI and AatII restriction sites
RegLpxC-RV-AatII	GCGCGCGACGTC**CTA**ATGGCCCGATTTGTAGGCAAC	
pENP-Sh-Fw-XbaI	GCGCGCTCTAGATGTGGAATTGTGAGCGGATA	Primers for confirmation of construct integration aligning at the promoter of the vector and the chromosomal sequence downstream the target gene.
Rv-LpxC-dw400	GAACCAGCATCTGCAGTTG	

[a] Restriction sites are underlined; stop codon (in reverse primer) is in bold.

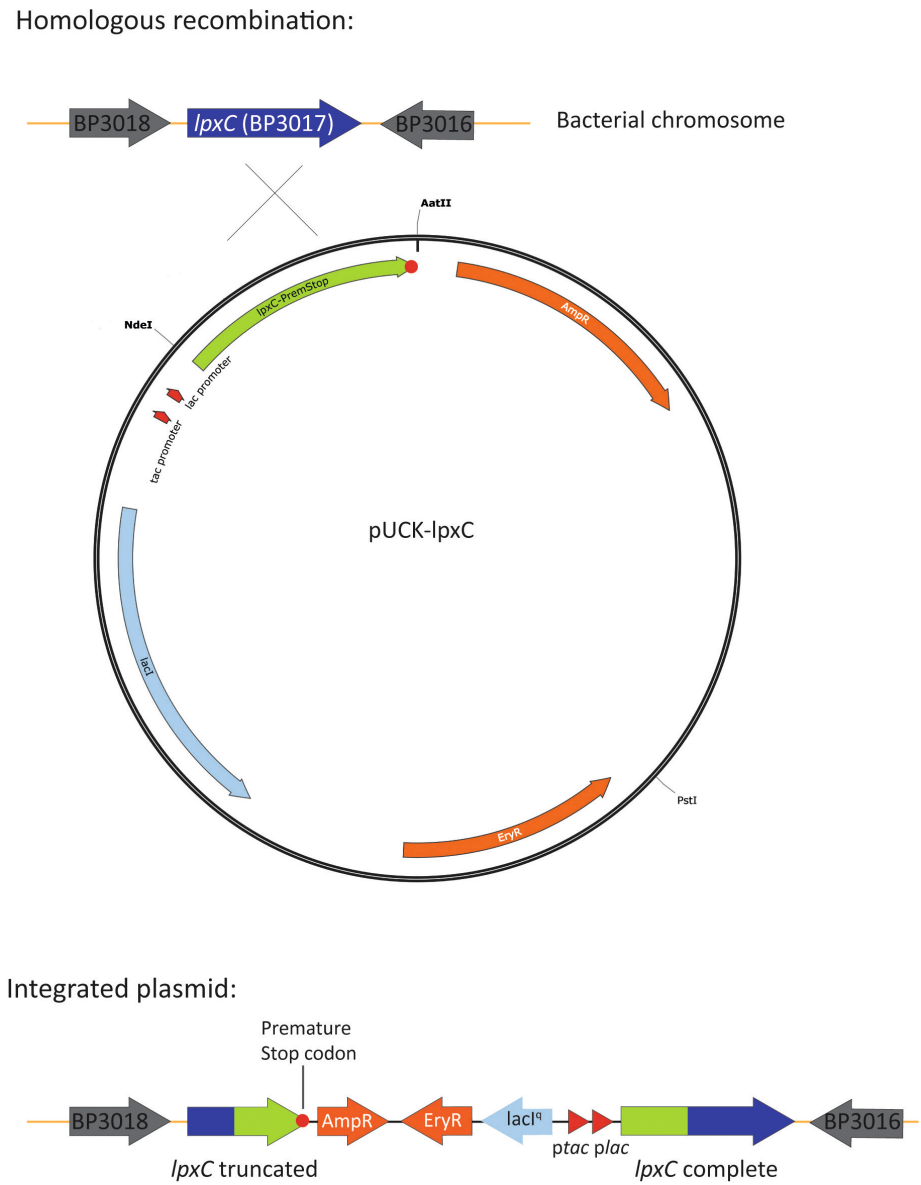

Figure S1. Schematic representation of the integration of the pUCK-lpxC construct into the *B. pertussis* chromosome. Integration of the plasmid is achieved by homologous recombination between a truncated *lpxC* gene (green) containing a premature stop codon (red circle) present on the plasmid and the intact chromosomal gene (dark blue). Chromosomal loci surrounding *lpxC* are depicted in grey. As a result of the integration, the intact *lpxC* copy with its own promoter is disrupted by the premature stop codon, whereas a new intact gene preceded by an IPTG-inducible double promoter (red arrowheads) is created. The presence of a *lacI*^{q} gene (light blue) allows for transcriptional repression in the absence of IPTG. The presence of antibiotic-resistance genes (orange) allows for selection of recombinants with the integrated plasmid. Restriction sites utilized during cloning are also depicted.

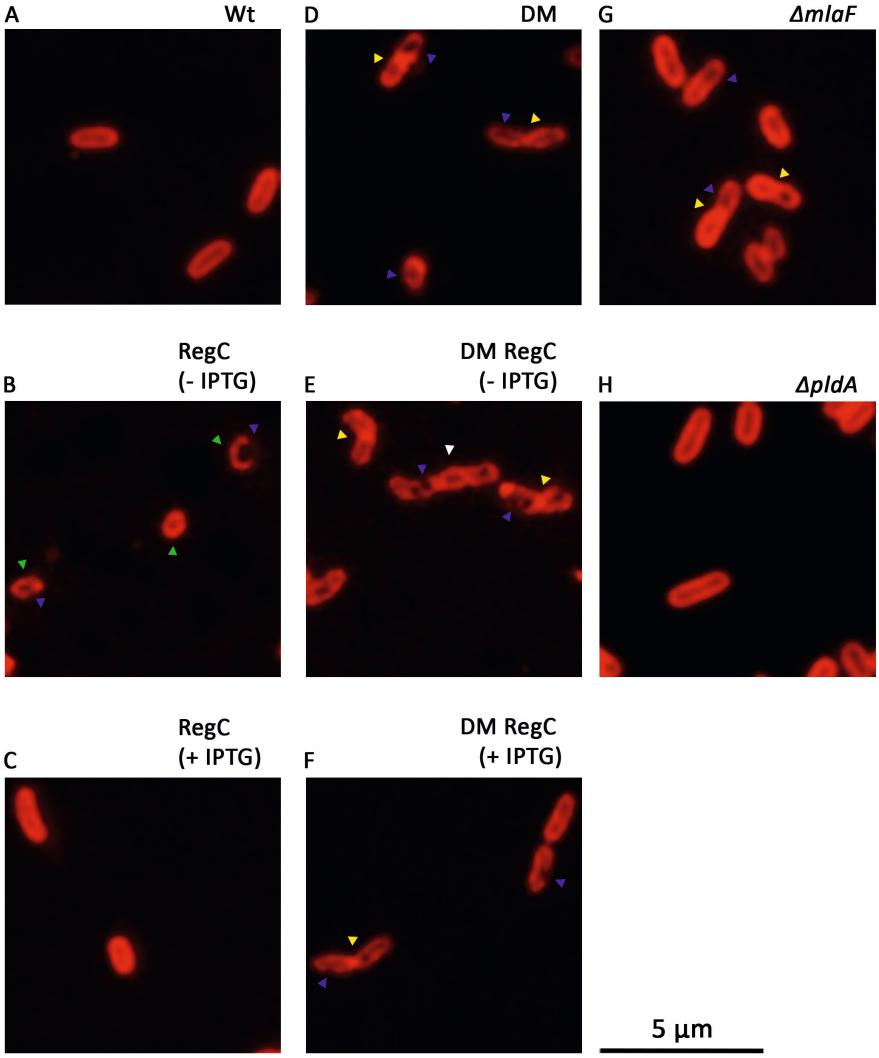

FIGURE S2. Morphology of *B. pertussis* cells visualized by fluorescence microscopy. This figure shows magnified captions from Figure 3. Scale bar represents 5 µm. Examples of cells showing shorter and more rounded shape than the wild-type cells (green), uneven dye distribution (blue), or appearing in pairs (yellow) or short chains (white) are indicated with colored arrowheads.

6

General and summarizing discussion

The cell envelope of Gram-negative bacteria consists of an inner membrane, a peptidoglycan layer, and an outer membrane (OM). The OM is characterized by its asymmetric lipid composition. It contains lipopolysaccharide (LPS, a.k.a. endotoxin) as the major component in its outer leaflet, while the inner leaflet is mainly formed of phospholipids (Raetz and Whitfield, 2002). LPS is responsible for the toxic immunological response upon infection with Gram-negative bacteria. This endotoxic reaction can also be a reason of reactogenicity of whole-cell vaccines against Gram-negative pathogens, as is the case for those developed against members of the genus *Bordetella*.

The strictly human pathogen *Bordetella pertussis* is responsible for the respiratory tract disease known as whooping cough or pertussis. It typically affects young children, and, hence, vaccination must be given shortly after birth. Initially, whole-cell pertussis (wP) vaccines were used worldwide to control the disease. However, to avoid the undesired endotoxicity, wP vaccines were later replaced in high-income countries by acellular pertussis (aP) alternatives, which comprise a limited number of purified antigens and are devoid of LPS (Esposito et al., 2019). Although aP vaccines proved to be safer and effective, resurgence of pertussis has occurred in the last decades in many countries, including in the Netherlands (Figure 1), one hypothesis explaining this resurgence being that these vaccines offer insufficient protection. It appears that the protection elicited by the aP vaccines wanes fast and fails to avoid colonization of the upper respiratory tract. Asymptomatic colonization of individuals vaccinated with aP was proved in baboons (Warfel et al., 2014) and, more recently, also in humans (de Graaf et al., 2020). Consequently, vaccinated individuals can function as carriers who transmit the disease to unvaccinated or incompletely vaccinated infants (Esposito et al., 2019). This seems to be the result of the stimulation of the wrong type of immunity. While natural infections and wP vaccines elicit a combined T-helper (Th)1 and Th17 immune response, aP vaccines induce mainly a Th2 response. The Th1 response seems necessary to avoid pertussis colonization. In addition, escape mutants evading the immune response are quickly spreading because of the limited number of antigens included in the aP vaccine (Esposito et al., 2019). The lower efficacy of the aP vaccine has forced the addition of booster doses and alternative strategies, such as vaccination during pregnancy, in the vaccination guidelines of the countries where aP is used.

Bordetella bronchiseptica is closely related to *B. pertussis* and is responsible for respiratory diseases in companion animals and livestock, such as tracheobronchitis in dogs and atrophic rhinitis in swine (Goodnow, 1980). In addition, it causes zoonotic infections in humans (Mattoo and Cherry, 2005). *B. bronchiseptica* LPS shows high endotoxicity and, thus, has become a concern for the wellbeing of animals vaccinated with whole-cell vaccines developed against this pathogen. Furthermore, whole-cell formulations commercially available against *B. bronchiseptica* require annual administration, which might be related to the use of suboptimal doses, necessary to avoid adverse reactions related to endotoxicity.

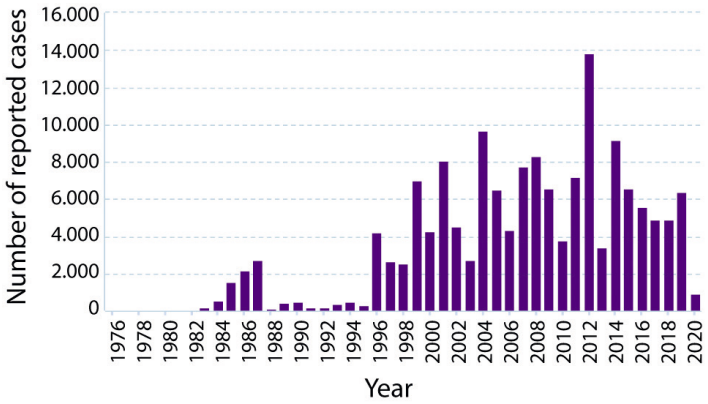

Figure 1. Pertussis incidence in the Netherlands. The numbers of pertussis cases annually reported in the Netherlands in the period 1976-2020 is depicted (Source: RIVM).

Development of next-generation vaccines against *B. pertussis* and *B. bronchiseptica* requires reliable alternatives that provide long-term protection in a safe manner. Whole-cell vaccines with weakened reactogenicity could be an option. Such vaccines would reduce the appearance of escape mutants because of the large number of antigens included, while they elicit the right type of immune response for a long-lasting protection against the disease and for avoidance of carriage. In addition, the reduced costs of whole-cell formulations would allow for the worldwide application of a safer pertussis vaccine. Moreover, wP vaccines could also protect against other pathogens, such as *Pseudomonas aeruginosa* (Blackwood et al., 2022), by virtue of the cross-reactivity amongst antigens in the vaccine and unrelated pathogens. A harmless whole-cell vaccine against *B. bronchiseptica* would improve animal welfare and reduce economic losses prompted by adverse reactions, while avoiding zoonosis. Alternatively, outer-membrane vesicles (OMVs) can potentially be used to generate effective vaccines against these pathogens. OMVs are non-replicative blebs of small diameter that are naturally released from the OM of Gram-negative bacteria (Figure 2). The composition of OMVs resembles that of the bacterial OM and, therefore, OMVs contain many of the OM components that can function as antigens to stimulate protective immunity (Schwechheimer and Kuehn, 2015). However, OMVs also contain LPS, which might dictate additional reduction of endotoxicity for the production of safe OMV-based vaccine formulations. Furthermore, spontaneous OMV release in *B. pertussis* and *B. bronchiseptica* is rather limited, which makes the production of OMV-based vaccines too costly.

In this thesis, we explored the possibilities of altering the LPS structure (**chapters 2** to **4**) or reducing LPS levels (**chapter 5**) in *Bordetella* spp. to diminish their endotoxicity and thus create novel tools for vaccine development. In addition, we studied the consequences of those alterations for the bacterial physiology and OM functioning.

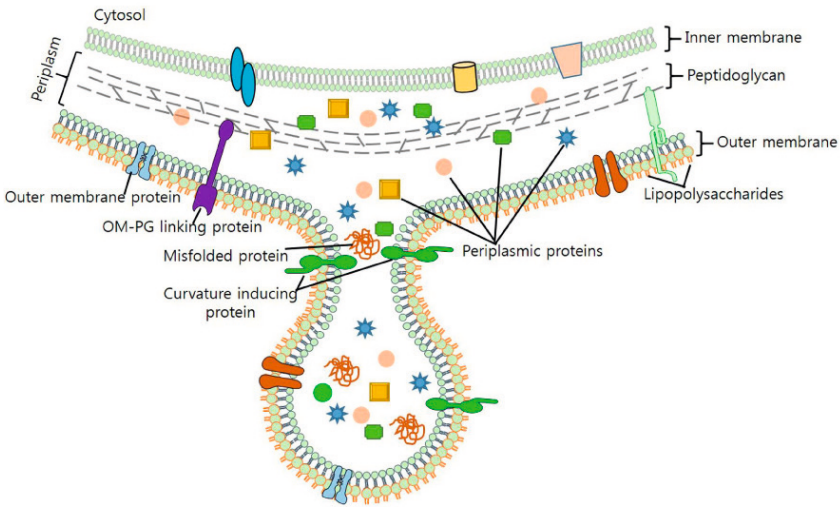

Figure 2. OMV biogenesis in Gram-negative bacteria. The formation of an OMV at the cell surface is illustrated. In addition to OM components, periplasmic proteins may appear as cargo in the lumen of the blebs. Reproduced from Jan (2017).

Bordetella LPS and Toll-like receptor 4 (TLR4)

LPS is recognized by a heterodimeric receptor consisting of TLR4 and myeloid differentiation factor 2 (MD-2) present at the surface of innate immune cells (see Figure 6 in **chapter 1**). Binding of LPS to the receptor is facilitated by the LPS-binding protein (LBP) and the co-receptor CD14 (Raetz and Whitfield, 2002; Zanoni and Granucci, 2013). Activation of the receptor results in the production of pro-inflammatory cytokines (e.g., TNFα and IL-1β), which are essentially responsible for the aforementioned endotoxicity of LPS. The classical three domains of LPS, i.e., lipid A, core oligosaccharide, and the O-antigen polysaccharide, are present in the LPS of *B. bronchiseptica* but not in that of *B. pertussis*, which lacks the O-antigen and, therefore, its LPS is also referred to as lipooligosaccharide (LOS) (see Figure 3 in **chapter 1**). The lipid As of *B. bronchiseptica* and *B. pertussis* display the archetypical lipid A structure, which consists of a glucosamine (GlcN) disaccharide substituted with phosphates at positions 1 and 4′ and usually acylated at positions 2, 2′, 3, and 3′ with β-hydroxylated fatty acids. These fatty acids can be substituted with secondary acyl chains by esterification at the hydroxyl groups (Raetz et al., 2007). However, the lipid A of *B. bronchiseptica* and that of *B. pertussis* still differ from each other in some of the substitutions displayed. The lipid A of *B. pertussis* is usually penta-acylated with four primary acyl chains and a single secondary myristate (C_{14}) connected to the primary chain at the 2′ position (see Figure 5A in **chapter 1**), which is added by the acylase LpxL2 (Geurtsen et al., 2007). On the other hand, *B. bronchiseptica* lipid A (see Figure 5B in **chapter 1**) usually lacks the primary 3-OH C_{10} at position 3 due to the activity of the OM-based deacylase PagL (MacArthur et al., 2011) and presents secondary fatty

acids attached to the three remaining primary chains. Besides the secondary myristate at the 2′ position, a secondary 2-hydroxy-laurate (2-OH C_{12}) is introduced at position 2 by the acylase LpxL1 (**chapter 2**), and the hydroxylation commonly present in this fatty acid is mediated by LpxO (MacArthur et al., 2011). In addition, a palmitate (C_{16}) can be attached to the primary chain at the 3′ position as a post-synthetic modification executed in the OM by acylase PagP (Preston et al., 2003). LgmB-mediated attachment of GlcN to the phosphate groups of the lipid A has been found in both *Bordetella* species (Marr et al., 2008), but *B. pertussis* lipid A barely presents those substitutions, while they seem to be a common feature in *B. bronchiseptica*.

Reduced acylation of lipid A

Modifications in the lipid A structure affect its recognition by the TLR4/MD-2 complex and, thereby, LPS endotoxicity. Variations in the number of acyl chains are commonly found as regulated post-synthesis modifications. These modifications are usually governed by two-component regulatory systems able to switch gene expression in response to changes in environmental conditions such as temperature, availability of divalent cations, and the presence of antimicrobial peptides (Simpson and Trent, 2019). In *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), the best studied two-component system is PhoP/Q. This system regulates the synthesis of two OM-based enzymes implicated in lipid A acylation level, i.e., the palmitoyltransferase PagP and the 3-*O*-deacylase PagL. Recombinant expression of *S*. Typhimurium PagL in *Escherichia coli* transformed its commonly hexa-acylated lipid A into penta-acylated lipid A. Purified lipid A with this modified structure showed reduced TLR4-stimulating capacity compared to the unmodified form (Kawasaki et al., 2004). In addition, the hepta-acylated form resulting from PagP activity also showed reduced endotoxicity. Interestingly, reduced endotoxicity was also observed for a hexa-acylated lipid A species obtained after the synthesis of both PagL and PagP combined, which suggests that not only the number of acyl chains but also their length and/or position are important for TLR4 activation (Kawasaki et al., 2004). PagL homologs have been identified in *B. bronchiseptica* and *B. pertussis*. In the latter, however, the *pagL* gene is disrupted by a frame shift (Geurtsen et al., 2005). The heterologous expression of *pagL* from *B. bronchiseptica* in *B. pertussis* resulted in deacylation of the 3 position and in a tetra-acylated lipid A. The resulting LPS showed reduced endotoxicity, but whole bacterial cells showed increased TLR4 activation, probably due to increased release of LPS from the bacterial surface (Geurtsen et al., 2006). This disfavors the use of PagL in the development of new wP vaccines with reduced endotoxicity. However, it may still be useful for OMV-based vaccines as nothing is known about the release of LPS from OMVs. PagP homologs were also identified in both *Bordetella* spp., but an insertion sequence in the promoter region of the *pagP* gene of *B. pertussis* prevents its expression. In *B. bronchiseptica*, PagP acylates the primary acyl chain at the 3′ position of the lipid A backbone (see Figure 5B in **chapter 1**), in contrast to *E. coli* and *S*. Typhimurium, where PagP attaches the secondary chain to position 2. The two-component system BvgA/S

is responsible for the regulation of the expression of *B. bronchiseptica pagP* (Preston et al., 2003), but apparently has no impact on PagL activity (MacArthur et al., 2011). Upon recombinant production of *B. bronchiseptica* PagP in *B. pertussis*, hexa-acylated LPS forms were produced which resulted in increased endotoxicity (Geurtsen et al., 2006). In **chapter 2**, we showed that purified LPS from wild-type *B. bronchiseptica* and from its *pagP* mutant derivative equally activated TLR4. The portion of palmitoylated LPS was rather low in the wild-type strain (~10%) and that could be the reason for the lack of an effect of *pagP* inactivation on TLR4 activation. However, heat-inactivated cells showed fully palmitoylated LPS, and a limited but statistically significant reduction in TLR4 activation was observed upon *pagP* inactivation when heat-inactivated cells were used in the TLR4-stimulation assays. These observations suggest a limited role of the secondary C_{16} at position 3′ of *B. bronchiseptica* lipid A in TLR4 activation, which contrasts with the effect on TLR4-stimulating capacity of this acylation in *B. pertussis* lipid A (Geurtsen et al., 2006). This could be related to the different organization of fatty acids in both lipid As.

Structural modifications can also be generated by manipulation of the lipid A biosynthesis pathway, a.k.a. Raetz pathway (see Figure 2 in **chapter 1**). Late acyltransferases add secondary acyl chains to the Kdo2-lipid IV_A precursor. In *E. coli*, LpxL (a.k.a. HtrB) transfers a C_{12} acyl chain onto the fatty acid at position 2′, while LpxM subsequently inserts a C_{14} residue to the acyl chain at the 3′ position (Raetz and Whitfield, 2002). In the *Bordetella* genome, two homologues of *lpxL* have been identified, i.e., *lpxL1* and *lpxL2*. Attempts to inactivate *lpxL2* have been futile in both *B. pertussis* (Geurtsen et al., 2007; Arenas et al., 2020) and *B. bronchiseptica* (**chapter 2**), which is consistent with the suggested essentiality of the gene in *B. pertussis* (Gonyar et al., 2019). The resulting tetra-acylated lipid A is probably a poor substrate for the MsbA flippase, which translocates the LPS precursor from the inner leaflet to the outer leaflet of the inner membrane. In *E. coli*, an *lpxL* mutant, which produces tetra-acylated LPS, is not viable but can be rescued by overproduction of MsbA or by specific *msbA* mutations that improve export of the poor substrate (Karow and Georgopoulos, 1993). It would be interesting to determine whether *lpxL2* can be knocked out in MsbA overproducers of the *Bordetella* species. On the other hand, *lpxL1* expression is usually absent in *B. pertussis* under laboratory conditions but its overexpression resulted in the addition of an extra acyl chain and, consequently, in a higher TLR4-stimulating capacity (Geurtsen et al., 2007). In *B. bronchiseptica*, LpxL1 stoichiometrically introduces a secondary 2-OH C_{12} at position 2 (see Figure 5B in **chapter 1**). Inactivation of *lpxL1* led to a complete loss of the secondary acyl chain but, unexpectedly, also of the GlcN decorations of the phosphate groups, generating mostly tetra-acylated and non-glycosylated lipid A (**chapter 2**). The absence of the secondary 2-OH C_{12} seems to impede the introduction of GlcN by the LgmB glycosyltransferase and might also be the reason why the GlcN substitutions are seldom detected in *B. pertussis* (Marr et al., 2008), inasmuch as LpxL1 activity is usually undetectable. Analogously, inactivation of *lpxM* in *E. coli* and *S.* Typhimurium results in the loss of the decorating group 4-amino-4-deoxy-L-arabinose (L-Ara4N) attached to the phosphates (Tran et al.,

2005). The modifications introduced into the *B. bronchiseptica* LPS upon inactivation of *lpxL1* resulted in a sharp reduction of TLR4 signaling in reporter cells and porcine macrophages (**chapter 2**). The reduced TLR4-stimulating capacity was observed for both purified LPS and whole cells, which contrasts with the aforementioned tetra-acylated lipid A of the *B. pertussis* derivative expressing *pagL*. This implies that the tetra-acylated LPS of the *B. bronchiseptica lpxL1* mutant remains firmly tied to the bacterial surface. In addition, the hydroxyl group present in the 2-OH C_{12} chain could play an anti-inflammatory role in the wild-type *B. bronchiseptica*, because hydroxylation of the acyl chains of lipid A can reduce the production of pro-inflammatory cytokines as has been demonstrated in other species such as *Klebsiella pneumoniae* (Llobet et al., 2015) and *Acinetobacter baumannii* (Bartholomew et al., 2019). However, although the *B. bronchiseptica* strains used in our lab presented fully hydroxylated lipid A (**chapter 2**), inactivation of the *lpxO* gene had no impact on the TLR4-activating capacity of their LPS (unpublished observations).

GlcN decoration of the phosphate groups of lipid A

The collateral loss of GlcN residues upon inactivation of *lpxL1* raises the question whether the reduced TLR4 activation and the other phenotypical alterations reported in **chapter 2** could be attributed to the loss of the secondary 2-OH C_{12} chain or of the GlcN residues. To elucidate the contribution of each modification to the different aspects studied in **chapter 2**, an *lgmB* mutant was also constructed, and its phenotypes were compared with those of an isogenic *lpxL1* mutant (**chapter 3**).

Previously, it has been reported that inactivation of *lgmB* in *B. bronchiseptica* did not affect TNFα production by murine macrophages (Rolin et al., 2014). In contrast, *lgmB* inactivation in *B. pertussis* and *Bordetella parapertussis* reduced the production of the pro-inflammatory cytokine IL-6 in human monocytes (Geurtsen et al., 2009). In **chapter 3**, we showed that *lgmB* deletion decreased the capacity of *B. bronchiseptica* to activate human (h-)TLR4 but had no effect on murine (m-)TLR4 stimulation, which is in line with a previous study, which showed that the inactivation of *lgmB* in *B. pertussis* affected its capacity to activate h-TLR4 but not m-TLR4 (Marr et al., 2010). Furthermore, the *lpxL1* mutation had a stronger effect on h-TLR4 activation than on m-TLR4 activation, which points to a combined effect of the loss of the secondary 2-OH C_{12} chain and the GlcN substitutions on h-TLR4 stimulation, while only the loss of the acyl chain affects m-TLR4 activation (**chapter 3**).

In addition to its effect on TLR4 stimulation, inactivation of *lpxL1* also affected the bacterial physiology in many other aspects. A reduced hydrophobicity of the cell surface observed in the mutant was initially related to an increased amount of LPS molecules containing O-antigen (**chapter 2**). However, this decreased hydrophobicity was also observed upon *lpxL1* inactivation in a different genetic background where no increment in O-antigen was noticed (**chapter 3**). Thus, the diminished surface hydrophobicity remains

unexplained. Probably as a result of the reduced surface hydrophobicity, the *lpxL1* mutant showed a reduced capability to form biofilms. Deletion of *lgmB* affected neither surface hydrophobicity nor biofilm production, which suggests that these changes are only related to the loss of the acyl chain.

Under-acylated LPS usually results in membrane instability. Yet, the *lpxL1* mutant showed reduced susceptibility to hydrophobic (rifampicin) and amphipathic (SDS) antimicrobials, indicating membrane reinforcement (**chapter 2**). The loss of the GlcN residues increases negative charges of LPS, which could stabilize the OM by increasing cross-links mediated by divalent cations (Zgurskaya et al., 2015). However, *lgmB* deletion barely altered the susceptibility to these antimicrobials (**chapter 3**). Overall, contrary to what would be expected, the loss of the secondary 2-OH C_{12} acyl chain seems to promote membrane stability, possibly because the loss of the hydroxylated fatty acyl chain improves the packing of the other fatty acids in the OM.

We demonstrated in **chapter 3** that glycosylation of lipid A does play a role in protection against the cationic antimicrobial peptide (CAMP) polymyxin B, possibly by allowing the binding of the cationic peptide to the negatively charged phosphates in lipid A. In addition, the loss of the GlcN residues hampered macrophage invasion. This could be due to increased sensitivity of the mutants to CAMPs produced by the macrophages, which can directly eradicate intracellular bacteria or avoid their replication (Rosenberger et al., 2004). The latter phenotype of the *lgmB* mutant qualifies a previous conclusion that relates the loss of the 2-OH C_{12} in a *B. pertussis lpxL1* mutant to impaired invasion of macrophages (Geurtsen et al., 2007). Most likely, LpxL1 activity and subsequent LgmB-mediated glycosylation are stimulated in *B. pertussis* during macrophage invasion. The inactivation of *lpxL1* then prevents the GlcN decoration, and the latter results in diminished survival in macrophages.

Additionally, we demonstrated the value of the *Galleria mellonella* infection model to study the effect of genetic modifications on *Bordetella* virulence in vivo (**chapter 3**). This insect presents an innate immune response similar to mammalian models and, therefore, minimizes the use of the latter models and their costs (Pereira and Rossi, 2020). In **chapter 3**, we showed that inactivation of *lpxL1* significantly reduces the virulence of *B. bronchiseptica*, while no effect was observed due to inactivation of *lgmB*. This indicates that the secondary 2-OH C_{12} chain in lipid A contributes to the virulence of the bacteria. Whether this is the result of an increased susceptibility of the *lpxL1* mutant to the *G. mellonella* defense mechanisms or of the reduced toxicity of its LPS should be further investigated.

Dephosphorylation of lipid A

The LPS of *B. pertussis* presents lower endotoxicity than that of *B. bronchiseptica* (Mann et al., 2005), but still, it's too reactogenic in whole-cell vaccines for human application. The lack of the secondary 2-OH C_{12} in the lipid A and the concomitant loss of the GlcN decorations are probably implicated in this lower endotoxicity. However, this means that strategies different to *lxpL1* inactivation as used for *B. bronchiseptica* are required to develop an improved wP vaccine with reduced reactogenicity for *B. pertussis*.

The phosphates at positions 1 and 4' of lipid A are very important for the binding of LPS to and the dimerization of the TLR4/MD-2 receptor complex and, consequently, decisive for endotoxicity (Raetz et al., 2007; Park et al., 2009). Some bacterial species produce inner-membrane-based phosphatases that remove these phosphate groups during LPS synthesis. These phosphatases, termed LpxE and LpxF, remove the phosphates from positions 1 and 4', respectively. In the *B. pertussis* genome, a putative *lpxE* homolog was identified (Geurtsen et al., 2006), but the encoded enzyme seems to act as pyrophosphatase instead (Zariri et al., 2016). Accordingly, when we expressed the corresponding gene from *B. bronchiseptica* in *B. pertussis*, no significant dephosphorylation of lipid A was detected (unpublished observation). In **chapter 4**, we aimed at dephosphorylating the lipid A of *B. pertussis* by the heterologous production of LpxE and LpxF from *Francisella novicida* ($LpxE_{Fn}$ and $LpxF_{Fn}$, respectively). Although $LpxF_{Fn}$ has been proven to dephosphorylate tetra- and penta-acylated lipid A molecules (Wang et al., 2006), no significant lipid A dephosphorylation was observed in our study. In contrast, the production of $LpxE_{Fn}$ did reduce phosphorylation but, unexpectedly, no effect on the TLR4-stimulating capacity was observed (**chapter 4**). This result contrasts with those in a previous study, in which it was shown that dephosphorylation achieved by $LpxE_{Fn}$ production in *S*. Typhimurium resulted in reduced endotoxicity (Kong et al., 2011). The absence of an effect on TLR4 signaling in *B. pertussis* could be related to the presence of negatively charged residues in the inner core of the LPS, which might be able to replace the lipid A 1-phosphate in the interaction with the TLR4/MD-2 complex as seems to be the case in *Capnocytophaga canimorsus* (Ittig et al., 2012). This bacterial species, whose lipid A is dephosphorylated at position 4' and presents a phosphoethanolamine (PEA) instead of phosphate at position 1, contains in the inner core of its LPS a single 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) substituted with PEA at position 4. Interestingly, upon removal of the core domain, the lipid A of *C. canimorsus* lost its capacity to activate TLR4 demonstrating that core residues in this case contribute to TLR4 activation (Ittig et al., 2012).

Modification of the inner core

In the inner core of *B. pertussis* LPS, the single Kdo present is decorated with a phosphate at position 4, which, in turn, is substituted with PEA (Caroff et al., 2000) (see Figure 3 in **chapter 1**). In the strains studied in this thesis, this PEA substitution is abundant but non-stoichiometrically present (**chapter 4**). To investigate whether this part of the *B. pertussis* LPS is implicated in TLR4 activation, we targeted genes involved in the inner core biosynthesis. Inactivation of the genes encoding the enzymes KdtA (WaaA) and KdkA, which introduce the Kdo and the phosphate substitution of the Kdo, respectively, was unsuccessful (**chapter 4**). Probably, these genes are essential for the viability of the bacteria as was suggested previously (Gonyar et al., 2019; Belcher et al., 2020). KdtA in *E. coli* ($KdtA_{Ec}$), which introduces two Kdo residues in the inner core, could only be replaced by a mono-functional homolog when the resulting single Kdo was additionally substituted with a phosphate by co-production of a KdkA (Brabetz et al., 2000). Similarly, inactivation of *kdtA* and *kdkA* was accomplished in *B. pertussis* when the bi-functional $KdtA_{Ec}$ was heterologously produced (**chapter 4**), indicating the need in the inner core of a Kdo substituted with either a phosphate or with a second Kdo for viability of both species. Nevertheless, the presence of a secondary Kdo in *B. pertussis* LPS resulted in growth reduction and in a defect in cell division, which could be related to additional alterations observed in the LPS structure upon incorporation of the secondary Kdo, i.e., the (partial) loss of the terminal trisaccharide in the outer core and of an acyl chain in lipid A. Most probably, the partial loss of the secondary C_{14} chain at the 2' position of lipid A, which is inserted by LpxL2, plays a role in this impairment. The presence of a secondary Kdo instead of a phosphate decoration was previously reported to reduce the activity of *B. pertussis* LpxL2 in vitro (Hankins and Trent, 2009). Since, as discussed above, the *lpxL2* gene is essential for viability, the reduced capability to insert the secondary C_{14} chain is expected to result in cellular stress. In addition, we found that the Bvg^+-phase is partially switched off in the mutants expressing the bifunctional KdtA. Whether the loss of the terminal trisaccharide in mutants presenting the second Kdo is the result of a physical impediment for the transferase that attaches the terminal trisaccharide *en bloc* to the LPS or due to changes in one of the bacterial regulatory systems, such as BvgA/S (Turcotte et al., 1997; Schaeffer et al., 2004) or the RpoE-dependent envelope stress response (Klein and Raina, 2015), is still unknown. In any case, the structural modifications in the LPS generated by $KdtA_{Ec}$ production resulted in increased stimulation of h-TLR4 and the loss of a relevant epitope for immunity induction, i.e., the terminal trisaccharide (Kubler-Kielb et al., 2011) (**chapter 4**), which exclude their application in the development of vaccines with reduced endotoxicity.

Additionally, we targeted the PEA decoration of the phosphorylated Kdo in *B. pertussis* (**chapter 4**). The gene with locus tag BP3136 was identified as the gene encoding the specific PEA transferase EptB. Inactivation of this gene resulted in the loss of PEA, and no other structural changes were observed in the LPS. The complete absence of PEA reduced

the TLR4-stimulating capacity of whole cells and indicates that PEA decoration of the phosphate on the Kdo in the inner core increases the endotoxicity of the *B. pertussis* LPS. This resembles the increased h-TLR4 stimulatory activity observed in *B. bronchiseptica* due to the decorations of the phosphates in lipid A (**chapter 3**) and suggests that shielding of any of the phosphates enhances h-TLR4 activation. This is rather surprising considering the role of the phosphates in TLR4/MD-2 binding and dimerization. Similarly, inactivation of *eptB* in the *B. pertussis* strain producing $LpxE_{Fn}$ resulted in reduced TLR4 activation (**chapter 4**). However, lipid A dephosphorylation by the phosphatase was disrupted in the *eptB* mutant. The loss of $LpxE_{Fn}$ activity was also observed when a secondary Kdo residue was introduced in the strain by producing $KdtA_{Ec}$, which suggests the need of the PEA decoration for the activity of the lipid A phosphatase (**chapter 4**). Regardless of this effect, *eptB* inactivation could allow for the development of whole-cell vaccines against *B. pertussis* with reduced endotoxicity. Further studies, such as reactogenicity and immunogenicity tests in vivo will be required to determine whether this modification is suitable for the production of safe and effective vaccines.

Regulation of LPS production

In this thesis, an alternative strategy to diminish the endotoxicity of *B. pertussis* whole cells was explored, i.e., the reduction of the total amount of LPS at the cell surface. The absence of LPS would result in bacterial cells devoid of endotoxicity. However, LPS is essential for the viability of numerous Gram-negative bacteria and, to our knowledge, only five species that normally produce LPS have proved to survive in the absence of LPS synthesis, i.e., *Neisseria meningitidis* (Steeghs et al., 1998), *Moraxella catarrhalis* (Peng et al., 2005), *A. baumannii* (Moffatt et al., 2010), *Yersinia ruckeri* (Altinok et al., 2016), and *Caulobacter crescentus* (Zik et al., 2022). For this reason, previously developed whole-cell vaccines with reduced LPS content entailed the use of organic solvents, which results in the loss of additional OM components and can therefore be detrimental for optimal immune responses.

The UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase LpxC (see Figure 2 in **chapter 1**) catalyzes the first committed reaction in the biosynthesis pathway of lipid A and is, thus, instrumental to the regulation of LPS production (Raetz and Whitfield, 2002). Accordingly, LPS production correlates with the LpxC levels in the cell (Guest et al., 2021). In the absence of *lpxC* expression, *B. pertussis* was not able to grow (**chapter 5**), proving the essentiality of LPS synthesis for viability in this species. In *A. baumannii* strains devoid of LPS, growth remarkably improved upon inactivation of the systems involved in the degradation or removal of phospholipids aberrantly located in the outer leaflet of the OM, i.e., the OM phospholipase A (OMPLA) and the maintenance-of-lipid-asymmetry (Mla) system, respectively (Powers and Trent, 2018; Nagy et al., 2019). In *B. pertussis*, inactivation of these systems resulted in accumulation of phospholipids in the outer leaflet of the OM (de Jonge et al., 2022b), which could potentially substitute for the

absence of LPS. However, the combined inactivation of these systems and *lpxC* did not preserve bacterial growth (**chapter 5**).

In any case, despite the toxic effects of LPS, its complete absence is not necessarily beneficial for development of new whole-cell vaccines because it induces the desirable cellular immune response required for protection, i.e., Th1 and Th17, which is commonly observed after immunization with wP vaccines (Higgins et al., 2006). In addition, LPS is considered a relevant antigen in *B. pertussis* to which an immune response is desirable (Trollfors et al., 2001). Controlled production of LpxC allowed us to maintain bacterial growth while reducing cellular LPS levels (**chapter 5**). Cells produced using this strategy presented lower levels of LPS and, consequently, reduced TLR4-stimulating capacity. Furthermore, the protein profile of isolated OMs from the LpxC-depleted cells was comparable to those of their parental strains, discarding drastic alterations in the antigenic content. In addition, increased release of OMVs was observed, which typically happens when the lipid asymmetry in the OM is disrupted (Roier et al., 2016; de Jonge et al., 2022b) and is probably a mechanism used by the bacterium to eliminate the accumulated phospholipids from the outer leaflet of the OM. Accordingly, the LPS content of OMVs shed from cells depleted of LpxC was even more reduced than that of the whole cells (**chapter 5**), making these OMVs an interesting alternative for development of vaccines with improved safety and cost efficiency. Furthermore, because of the possibility to regulate the *lpxC* expression, fine tuning can be applied to adjust the LPS content to the immunogenic and reactogenic requirements of the vaccine.

Future prospects

In this thesis, we studied the effect of several genetically engineered modifications in the *B. bronchiseptica* lipid A structure on TLR4 activation. The inactivation of *lpxL1* resulted in a drastic reduction of TLR4-stimulating activity (**chapter 2**), and the potential of this strain for use as a whole-cell vaccine in terms of safety and efficacy is currently being further evaluated in vivo. Additionally, combined inactivation of the *lpxL1* and *pagP* genes might be worthwhile to consider, since an increase in PagP-mediated palmitoylation was observed in the *lpxL1* mutant (**chapter 2**). This increment may be due to the capacity of PagP of *B. bronchiseptica* to attach a C_{16} also to the primary chain at the 2 position (Hittle et al., 2015). As this primary chain is not substituted in the *lpxL1* mutant (see Figure 5B in **chapter 1**), it might be a target for PagP-mediated palmitoylation. Because of its reduced TLR4-stimulating activity, higher doses of a whole-cell vaccine based on an *lpxL1* mutant could potentially be administered than of the currently available vaccines and, conceivably, this could result in better and longer protection. Co-infection with *B. bronchiseptica* and toxigenic *Pasteurella multocida* produces the progressive form of porcine atrophic rhinitis. To develop a multivalent vaccine that protects against such disease, an *lpxL1* mutant strain of *B. bronchiseptica* producing a detoxified version of the *P. multocida* toxin was also generated (unpublished data), and immunogenicity studies must

be performed with that strain as well. OMV-based vaccines could also be considered for use in animals, particularly in companion animals, where vaccine cost is less problematic than for livestock. Increased OMV release was observed in an *lpxL1* mutant (unpublished data), which would facilitate the cost-effective production of such formulations.

Until now, attempts to reduce the endotoxicity of *B. pertussis* LPS have exclusively focused on structural modifications of the lipid A (Geurtsen et al., 2006, 2007; Arenas et al., 2019, 2020). However, in some Gram-negative species, chemical groups of the inner core of the LPS play a role in the interaction with the TLR4 receptor and, thus, affect the response of the immune system. This seems to be the case also in *B. pertussis*, whose LPS presents a PEA decoration in the inner core that increases its TLR4-stimulating capacity (**chapter 4**). A strain lacking this decoration, generated by inactivation of the *eptB* gene, showed reduced activation of TLR4 and, therefore, could be employed for the development of whole-cell formulations with reduced endotoxicity. However, whether the endotoxicity is sufficiently reduced should be further evaluated in *in vivo* experiments. Additionally, we developed a strain with reduced levels of LPS by regulated expression of *lpxC* (**chapter 5**). Such a strain could be used as an alternative strategy but could also be applied in a combined strategy. Furthermore, this strain produced increased amounts of OMVs with greatly reduced LPS content, which could potentially be used to generate affordable and safe OMV-based vaccines. If favorable conclusions are reached with this strain in terms of immunogenicity and reactogenicity *in vivo*, further refinement will probably be required prior to commercial use. Adaptation of the protocol developed for LPS depletion will be required for growth in large-scale fermenters. In addition, as LPS depletion affects growth, the selection pressure for mutants with normal amounts of LPS is high and there is a risk of accumulating such mutants during growth, particularly with a repressor-based promoter because any mutation that inactivates the repressor will lead to constitutive LPS synthesis. Probably, it would be better to replace the *lac/tac* promoter used by an activator-controlled promoter or by a weak constitutive promoter. Selection of a *B. pertussis* isolate more representative of the currently circulating strains might be beneficial as well. Besides that, if the reduction of LPS epitopes in the LPS-depleted wP vaccine turns out to be problematic, supplementing the formulation with purified oligosaccharides could be used to enhance induction of immunity (Kubler-Kielb et al., 2011). Either way, we have provided proof of concept for the generation of novel vaccines with reduced reactogenicity based on genetic regulation of LPS levels in whole cells and OMVs. This novel approach is presumably more broadly applicable than the structural modification of lipid A usually adopted for Gram-negative bacteria inasmuch as those approaches are not always applicable to different species. For example, lipid A dephosphorylation had no effect on TLR4 activation in *B. pertussis* (**chapter 4**), despite being an effective strategy in many other bacteria. This seems to be related to the specific structure of the LPS and its interaction with TLR4. Similarly, the target animal to be vaccinated might be relevant to establish an effective LPS modification. For example, the *lgmB* mutation in *B. bronchiseptica* reduced activation of the human TLR4 while having

no effect on the murine variant (**chapter 3**). In addition, some structural modifications are simply not feasible in some species, as is the case for *lpxL2* inactivation in *B. pertussis* (Geurtsen et al., 2007; Arenas et al., 2020) and *B. bronchiseptica* (**chapter 2**), while in other species, such as *N. meningitidis* (van der Ley et al., 2001), this mutation could be made and resulted in reduced endotoxicity. If the OMV production is insufficient in the selected strains for production of affordable OMV-based vaccines, one could rely on alternative approaches to increase OMV release that have recently been developed for *B. pertussis* and *B. bronchiseptica* (de Jonge et al., 2021, 2022b, 2022a) and that could be combined with the detoxified strains developed here.

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APPENDICES

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Nederlandse samenvatting

Infectieziekten worden veroorzaakt door ziekteverwekkers die het weefsel van hun gastheer binnendringen en schadelijke stoffen produceren. *Bordetella pertussis* en *Bordetella bronchiseptica* zijn pathogene Gram-negatieve bacteriën, die luchtweginfecties veroorzaken bij zoogdieren, inclusief de mens. *B. pertussis* infecteert uitsluitend mensen en is de veroorzaker van een zeer besmettelijke luchtweginfectie die bekend staat als kinkhoest of pertussis. Deze ziekte treft voornamelijk jonge kinderen met de meest agressieve symptomen bij baby's jonger dan 1 jaar. Voordat vaccins tegen *B. pertussis* werden geïntroduceerd, was deze ziekteverwekker één van de belangrijkste oorzaken van ziekte en sterfte bij kinderen. *B. bronchiseptica* veroorzaakt ademhalingsziekten bij verschillende zoogdieren, zoals kennelhoest bij honden en snuffelziekte bij varkens. De bacterie kan ook mensen infecteren, meestal mensen met een verzwakt immuunsysteem, resulterend in zoönose.

In de jaren 40 van de vorige eeuw werden wereldwijd hele-cel vaccins tegen *B. pertussis* geïntroduceerd. Deze vaccins bestonden uit geïnactiveerde bacteriën en hun introductie leidde tot een snelle afname van de kinkhoestincidentie. Helaas resulteerde vaccinatie met deze formuleringen in ongewenste neveneffecten (reactogeniciteit), waaronder koorts, braken, anorexia en stuiptrekkingen. Om dit probleem te ondervangen werden deze vaccins in de jaren 90 in de geïndustrialiseerde landen vervangen door acellulaire formuleringen. Deze acellulaire vaccins bevatten slechts enkele gezuiverde eiwitten (antigenen) van de bacterie en bleken effectief in de bescherming tegen de ziekte, terwijl zij leidden tot minder ongewenste effecten. In de laatste decennia is er echter een toename van het aantal kinkhoestgevallen waargenomen in landen waar de acellulaire vaccins worden gebruikt. Dit lijkt het gevolg te zijn van verschillende factoren, waaronder (i) de mogelijkheid dat gevaccineerden weliswaar beschermd zijn, maar de pathogeen toch dragen in de bovenste luchtwegen en verspreiden naar andere mensen, (ii) de opkomst van bacteriële varianten die de opgewekte immuniteit kunnen ontwijken door structurele veranderingen in de antigenen die in het vaccin zijn opgenomen en (iii) de snelle afname van de immuniteit die wordt opgewekt door acellulaire vaccins.

Vaccins tegen *B. bronchiseptica* bevatten complete bacteriën en worden voornamelijk toegepast bij honden en varkens. Deze vaccins veroorzaken echter ook bijwerkingen, wat een negatief effect kan hebben op het welzijn van de dieren en op de voedselproductie.

De celenveloppe van Gram-negatieve bacteriën, zoals *B. pertussis* en *B. bronchiseptica*, bevat twee membranen, een binnenmembraan en een buitenmembraan (OM). Het OM is een bilaag die gekenmerkt wordt door zijn asymmetrische samenstelling, met lipopolysachariden (LPS) in de buitenste laag en fosfolipiden in the binnenste laag. LPS is verantwoordelijk voor de toxische immunologische respons (endotoxiciteit) die optreedt bij infectie met Gram-negatieve bacteriën en zijn aanwezigheid in hele-cel vaccins lijkt

de belangrijkste reden voor de ongewenste bijwerkingen van deze formuleringen. LPS wordt herkend door receptoren die aanwezig zijn op het oppervlak van cellen van het aangeboren immuunsysteem. De LPS receptor bestaat uit twee eiwitten, de *Toll-like receptor* 4 (TLR4) en *myeloid differentiation factor* 2 (MD-2), maar wordt hier verder kortweg aangeduid als TLR4. Binding van LPS aan de receptor activeert een signaaltransductieroute die resulteert in de secretie van ontsteking-bevorderende eiwitten (pro-inflammatoire cytokines). Bij lage concentraties activeren deze moleculen het immuunsysteem om de infectie op te ruimen. De overweldigende immuunrespons die optreedt bij hoge concentraties LPS resulteert echter in ernstige bijwerkingen die kunnen leiden tot septische shock en de dood.

LPS-moleculen bestaan uit drie onderdelen: het lipide A, een oligosacharide genaamd de *core* en een polysacharide genaamd het *O-antigen*. Het lipide A is verantwoordelijk voor de verankering van het LPS in het membraan en bestaat uit een disacharide van glucosamines (GlcN) waaraan vetzuren en fosfaten zijn bevestigd. De fosfaten kunnen op hun beurt worden gemodificeerd met andere groepen zoals GlcN. Lipide A wordt gemaakt in het cytoplasma en de binnenste laag van het binnenmembraan via de Raetz *pathway*, die negen stappen omvat, elk gekatalyseerd door een eigen specifiek enzym. Lipide A is verantwoordelijk voor de endotoxische activiteit van LPS. Het binnenste deel van de *core* bevat een 3-deoxy-D-manno-oct-2-ulosonzuur (Kdo) die de *core* aan het lipide A bindt. Het O-antigeen is het buitenste onderdeel van LPS en bestaat uit repeterende suikereenheden die sterk kunnen variëren in samenstelling afhankelijk van de bacteriële stam. De klassieke drie onderdelen zijn aanwezig in het LPS van *B. bronchiseptica* maar niet in dat van *B. pertussis*. Het LPS van *B. pertussis* mist het *O-antigen* en wordt daarom ook lipooligosacharide (LOS) genoemd.

Het lipide A van *B. pertussis* bevat vier primaire vetzuurketens (acylketens), die direct gekoppeld zijn aan het GlcN disacharide, en een enkel, indirect gekoppeld secundair vetzuur. Het *B. bronchiseptica* lipide A mist meestal een van de primaire vetzuren, terwijl de drie resterende primaire ketens elk gemodificeerd zijn met een secundaire keten, resulterend in totaal in zes acylketens. Structurele wijzigingen in lipide A kunnen de TLR4-stimulerende capaciteit en dus de endotoxiciteit ervan beïnvloeden. Door *bio-engineering* kan de LPS-structuur worden gemanipuleerd om de endotoxiciteit te verminderen. Dit biedt mogelijkheden tot de ontwikkeling van hele-cel vaccins met verminderde reactogeniciteit. Bijvoorbeeld, genen die coderen voor specifieke enzymen betrokken bij de biosynthese van LPS kunnen worden uitgeschakeld en LPS-biosynthese genen van bacteriën met een andere LPS structuur kunnen tot expressie worden gebracht in de doelbacterie (heterologe genexpressie). Deze strategieën zijn met succes toegepast bij verschillende bacteriesoorten, waaronder *B. pertussis*, om hun LPS-structuur te wijzigen.

Verlaging van het aantal acylketens in lipide A vermindert meestal de herkenning ervan door TLR4. Variaties in het aantal acylketens worden vaak aangebracht als post-

synthetische modificaties. Dergelijke modificaties worden meestal gereguleerd door systemen die in staat zijn om de genexpressie te veranderen in reactie op veranderingen in omgevingsomstandigheden, zoals temperatuur, beschikbaarheid van voedingsstoffen en de aanwezigheid van antimicrobiële peptiden. Een van deze modificaties wordt uitgevoerd door PagP, een enzym dat in het OM een secundair vetzuur hecht aan de primaire acylketen aanwezig op positie 3' van lipide A in *B. bronchiseptica*. In **hoofdstuk 2** hebben we aangetoond dat inactivatie van het *pagP* gen de aanhechting van het secundaire vetzuur weliswaar verhinderde maar slechts een beperkt effect had op TLR4-activatie. Dit resultaat staat in contrast met het effect van PagP synthese in *B. pertussis*. In *B. pertussis* komt het *pagP* gen normaal niet tot expressie. In een eerdere studie is echter aangetoond dat de artificiële productie van PagP in deze bacterie resulteert in de invoering van de extra acylketen in het lipide A, wat een toename van TLR4-activatie veroorzaakte. Een andere strategie om het aantal acylketens in *B. bronchiseptica* lipide A te verminderen was de inactivatie van het gen *lpxL1*. Dit gen codeert voor het enzym dat een secundaire acylketen toevoegt aan het primaire vetzuur op positie 2 van lipide A (**hoofdstuk 2**). Inactivatie van *lpxL1* leidde tot sterke vermindering van TLR4-activatie en van de productie van pro-inflammatoire cytokines. Structuuranalyse toonde echter aan dat de mutatie niet alleen leidde tot het verwachte verlies van de secundaire acylketen, maar ook van de GlcN-decoraties aan de fosfaatgroepen. Om de individuele bijdrage van het verlies van de acylketen en van GlcN te bepalen, hebben we ook het *lgmB* gen dat codeert voor het enzym dat GlcN aan de fosfaatgroepen van lipide A hecht, uitgeschakeld (**hoofdstuk 3**). Terwijl inactivatie van *lpxL1* resulteerde in sterk verminderde activatie van TLR4 van mens en muis (**hoofdstukken 2** en **3**), had inactivatie van *lgmB* geen effect op de activatie van muizen TLR4 en slechts een beperkt effect op de activatie van humaan TLR4 (**hoofdstuk 3**). We ontdekten ook dat de inactivatie van *lpxL1* een groot aantal fenotypische veranderingen in de bacterie teweegbracht, waaronder verhoogde membraanstabiliteit en resistentie tegen hydrofobe en amphipathische antimicrobiële middelen (**hoofdstuk 2**). We toonden ook aan dat het verlies van GlcN-residuen de gevoeligheid van de bacteriën voor kationische antimicrobiële peptiden verhoogt en de invasie van macrofagen belemmert (**hoofdstuk 3**). Deze bevindingen helpen ons het complexe samenspel tussen LPS-modificaties en bacteriële fysiologie en pathogenese te begrijpen.

In **hoofdstuk 4** onderzochten we de mogelijkheid om de endotoxiciteit van het LPS van *B. pertussis* te verminderen door lipide A en de *core* te modificeren. De fosfaatgroepen in lipide A dragen meestal bij aan de interactie met, en daarbij de stimulering van TLR4. We probeerden de fosfaatgroepen van lipide A van *B. pertussis* te verwijderen door heteroloog de genen voor de fosfatases LpxE en LpxF van *Francisella novicida* tot expressie te brengen. LpxE en LpxF verwijderen respectievelijk de fosfaatgroepen op posities 1 en 4' van lipide A van *F. novicida*. Alleen LpxE synthese leidde tot sterk verminderde fosforylering van *B. pertussis* lipide A. Deze modificatie had verrassenderwijs echter geen effect op TLR4-activering. We onderzochten vervolgens de mogelijkheid

dat verlies van het fosfaat in de interactie met TLR4 wellicht gecompenseerd wordt door de aanwezigheid van negatieve ladingen in het binnenste deel van de *core* van het LPS, zoals eerder is gerapporteerd bij een andere bacteriesoort, *Capnocytophaga canimorsus*. Hiervoor probeerden we de *core* van *B. pertussis* LPS te modificeren door verschillende genen die betrokken zijn bij de biosynthese van het binnenste deel van de *core* uit te schakelen (**hoofdstuk 4**). Maar het uitschakelen van de *kdtA* en *kdkA* genen, die coderen voor de enzymen die, respectievelijk, de enkelvoudige Kdo in de *core* introduceren en deze Kdo met fosfaat decoreren, bleek niet mogelijk. Waarschijnlijk zijn deze genen essentieel voor de levensvatbaarheid van de bacteriën. De inactivatie van de chromosomale *kdtA* en *kdkA* genen bleek wel mogelijk toen KdtA van een andere bacteriesoort, namelijk *Escherichia coli*, heteroloog vanaf een plasmide werd geproduceerd in *B. pertussis*. KdtA van *E. coli* introduceert twee Kdo-suikers in de *core*. Dit geeft aan dat LPS met één Kdo, die gedecoreerd is met ofwel een fosfaat of een tweede Kdo, nodig is voor de levensvatbaarheid van *B. pertussis*. De aanwezigheid van een secundair Kdo in *B. pertussis* LPS resulteerde in verhoogde TLR4-activatie evenals in een vermindering van bacteriële groei en een defect in celdeling, wat zou kunnen worden gerelateerd aan extra veranderingen die werden waargenomen in de LPS-structuur bij introductie van het secundaire Kdo.

In *B. pertussis* kan het fosfaat dat aan de Kdo-suiker is bevestigd, op zijn beurt worden gemodificeerd met een fosfo-ethanolamine (PEA). We hebben in **hoofdstuk 4** het *eptB* gen geïdentificeerd dat codeert voor het enzym dat PEA hecht aan het Kdo-gebonden fosfaat. De inactivatie van *eptB* resulteerde in het verlies van PEA zonder andere structurele veranderingen in de LPS. De afwezigheid van deze PEA verminderde de capaciteit van de cellen om TLR4 te stimuleren, wat aangeeft dat de PEA-groep de endotoxiciteit van *B. pertussis* LPS verhoogt. Inactivatie van *eptB* in de *B. pertussis* stam die LpxE van *F. novicida* produceert, resulteerde in een nog sterkere vermindering van TLR4-activatie, ook al werd de afsplitsing van fosfaat uit het lipide A grotendeels verhinderd door de afwezigheid van PEA. Het verlies van LpxE-activiteit werd ook waargenomen in de stam met een secundair Kdo-residu als gevolg van de productie van KdtA uit *E. coli*, wat suggereert dat de PEA-decoratie nodig is voor de activiteit van de lipide A-fosfatase. Inactivatie van *eptB*, mogelijk in combinatie met de heterologe productie van LpxE van *F. novicida*, zou de ontwikkeling van hele-cel vaccins tegen *B. pertussis* met verminderde endotoxiciteit mogelijk maken. Verder onderzoek, zoals reactogeniciteits- en immunogeniciteitstesten, moet bij dieren worden uitgevoerd om te bepalen of deze modificaties geschikt zijn voor de productie van veilige en effectieve vaccins tegen *B. pertussis*.

In **hoofdstuk 5** werd regulering van de productie van LPS onderzocht als alternatieve strategie om de endotoxiciteit van *B. pertussis* te verminderen. Terwijl de totale afwezigheid van LPS zou resulteren in cellen zonder endotoxiciteit, is dit in de praktijk moeilijk te realiseren omdat LPS essentieel is voor de levensvatbaarheid van veel Gram-negatieve bacteriën. Daarom was het doel om het LPS-gehalte te verminderen zonder

de groei van bacteriën aan te tasten. Het geselecteerde doelwit voor deze benadering was LpxC, het enzym dat de eerste toegewijde reactie in de biosyntheseweg van lipide A katalyseert. Dit enzym werd gekozen omdat zijn cellulaire niveaus in het algemeen correleren met LPS-productie in Gram-negatieve bacteriën. In *B. pertussis* bleek verhindering van *lpxC*-genexpressie letaal te zijn, wat aantoont dat ook in deze bacteriën LPS-synthese essentieel is voor de levensvatbaarheid (**hoofdstuk 5**). Eerdere studies toonden aan dat *Acinetobacter baumannii* wel levensvatbaar is in afwezigheid van LPS-synthese, maar dat de groei van LPS-loze mutanten aanzienlijk verbeterde na inactivatie van de moleculaire mechanismen die de fosfolipiden, die LPS in de buitenste laag van de OM van dergelijke mutanten vervangen, afbreken of verwijderen. Uitschakeling van deze mechanismen in *B. pertussis* herstelde echter niet de groei in afwezigheid van LpxC-synthese (**hoofdstuk 5**). Niettemin maakte gecontroleerde productie van LpxC het mogelijk de bacteriën te groeien tot hoge dichtheid, terwijl de LPS-niveaus in de cellen werden verlaagd. Deze cellen hadden daardoor een verminderde TLR4-stimulerende capaciteit. Het eiwitprofiel van geïsoleerde OMs van de LPS-arme cellen was vergelijkbaar met dat van hun ouderstammen, waardoor drastische veranderingen in het gehalte van eiwitantigenen in mogelijke vaccinformuleringen onwaarschijnlijk zijn.

Buitenmembraanblaasjes (*outer membrane vesicles*, OMVs) zijn kleine bolvormige structuren die worden gevormd door het uitstulpen van het OM van Gram-negatieve bacteriën. Zij worden spontaan geproduceerd als reactie op verschillende omgevingsstressoren of tijdens normale groei- en delingsprocessen. Ze bevatten in principe alle bacteriële oppervlakte-antigenen, waaronder LPS, wat ze geschikt maakt voor de ontwikkeling van nieuwe vaccins die een robuuste immuun bescherming bieden. OMVs zijn al effectief gebleken in de ontwikkeling van vaccins tegen *Neisseria meningitidis*. Spontane OMV-afgifte bij *B. pertussis* is echter beperkt, wat de productie van OMV-gebaseerde vaccins duur maakt. We hebben in de LPS-arme cellen een verhoogde afgifte van OMVs waargenomen (**hoofdstuk 5**), waarschijnlijk als een mechanisme dat de bacterie gebruikt om het overschot aan fosfolipiden dat ophoopt in het OM van deze bacteriën te verwijderen. In overeenstemming hiermee bleek het LPS-gehalte in deze OMVs nog meer verminderd dan dat van de hele cellen. Deze OMVs zijn daarom een interessant alternatief voor de ontwikkeling van kostenefficiënte vaccins met verbeterde veiligheid. Hoewel LPS toxisch is, is de volledige afwezigheid ervan niet noodzakelijkerwijs gunstig voor de ontwikkeling van nieuwe vaccins op basis van hele cellen. LPS is namelijk ook een sterk adjuvans dat de gewenste immuunrespons opwekt die nodig is voor de bescherming die vaak wordt waargenomen na immunisatie met klassieke pertussis-vaccins op basis van hele cellen. Gereguleerde *lpxC*-expressie biedt de mogelijkheid het LPS-gehalte zodanig fijn te stemmen dat een goede balans ontstaat tussen de verminderde reactogeniciteit en voldoende immunogeniciteit van het vaccin.

Samengevat hebben we de waarde aangetoond van genetische modificatie voor de ontwikkeling van nieuwe vaccins tegen *B. pertussis* en *B. bronchiseptica*. De inactivatie

van het *lpxL1*-gen in *B. bronchiseptica* resulteerde in een drastische vermindering van de TLR4-stimulerende capaciteit. Hele-cel vaccins gebaseerd op een *lpxL1*-mutant kunnen potentieel worden toegediend voor veiligere en langere bescherming van dieren, zoals honden en varkens. Hele-cel formuleringen met minder bijwerkingen zouden de vervanging van de huidige acellulaire vaccins tegen kinkhoest mogelijk kunnen maken. Dergelijke vaccins zouden vermoedelijk ook de bescherming tegen kinkhoest kunnen verhogen. Door het *eptB*-gen te inactiveren, hebben we een *B. pertussis* stam ontwikkeld met verminderde TLR4-stimulerende activiteit. Daarnaast werd een stam met verlaagde LPS-niveaus gegenereerd door de productie van LpxC te reguleren. Interessant genoeg produceerde deze stam verhoogde hoeveelheden OMVs met sterk verminderde LPS-inhoud. Deze stam zou dus ook potentieel gebruikt kunnen worden voor de ontwikkeling van betaalbare en veilige OMV-gebaseerde vaccins tegen kinkhoest. Voor de commerciële ontwikkeling van nieuwe vaccins voor mensen, zullen deze bacteriële kandidaten eerst verder getest moeten worden op immunogeniciteit en reactogeniciteit in dieren.

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Curriculum vitae

Jesús was born on April 20, 1984 in Málaga, Spain. After finishing high school in 2002 at Instituto Monterroso in Estepona (Spain), he studied Pharmacy at University of Granada (Spain). During his master's studies in Biological Analysis and Laboratory Diagnosis, he evaluated the interferon gamma release assay (IGRA) and the tuberculin skin test for the diagnosis of latent tuberculosis at the University Hospital Virgen de las Nieves (Granada, Spain) and collaborated in the development of tests based on nucleic acid amplification for diagnosis of human infectious diseases at the research and development department of the company Vircell S.L. (Granada, Spain). After the completion of that internship, Jesús was granted from the Ícaro program to continue his work at Vircell S.L. for a total of a year. In 2015, he was granted from the Erasmus + program to study the interbacterial interactions in meningococcal biofilms under the supervision of dr. Jesús Arenas at the Molecular Microbiology group of prof. dr. Jan Tommassen at Utrecht University (The Netherlands), where he continued studying the role of bacterial autotransporters in the interaction among *Neisseria* species as well as between bacteria and eukaryotic cells until 2017. In June 2017, Jesús started his PhD project at the Molecular Microbiology group of Utrecht University under the supervision of prof. dr. Jan Tommassen. His research focused on the reduction of bacterial endotoxicity in *Bordetella* species by lipopolysaccharide bioengineering for the development of vaccines with reduced reactogenicity, and the results of his work are described in this thesis. Since June 2022, he is working as a scientist at the company Human Health Vision Biotech, which aim is the development of novel compounds to combat biofilm-related diseases.

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