Improving pertussis vaccines by lipopolysaccharide engineering

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Puig de San Salvador (Mallorca)
Sven Bruijns

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Wilma Witkamp

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Improving pertussis vaccines by lipopolysaccharide engineering

Verbetering van kinkhoestvaccins door aanpassingen in lipopolysaccharide samenstelling

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 19 april 2007 des ochtends te 10.30 uur

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Jeroen Johannes Gerardus Geurtsen

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Promotor Prof.Dr. J.P.M. Tommassen

Co-promotor Dr. P.A. van der Ley

Voor jou

“Science may set limits to knowledge, but should not set limits to imagination”

Bertrand Russel (1872-1970)

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Chapter 1

General introduction

Pertussis disease

Pertussis or whooping cough is a severe respiratory tract disease that is characterised by paroxysmal coughing and a distinctive “whooping” sound when air is subsequently inhaled. The disease is highly contagious and most severe in neonates and children younger than one year. Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*, which was first isolated in 1906 by Bordet and Gengou (Bordet and Gengou, 1906). Pertussis disease spreads via aerosolised droplets produced during coughing and has an incubation period of 7 to 10 days. During this period, *B. pertussis* first colonises the trachea and nasopharynx by interacting with ciliated epithelial cells, after which replication is initiated and further colonisation of adjacent areas starts. Toxins produced by the bacterium damage the epithelial cell lining and thereby lead to the loss of ciliated epithelial cells. Although pertussis disease has very distinctive symptoms, the first medically documented disease case is from 1578 in Paris (Gerlach *et al.*, 2001). Since this is relatively recent, it is believed that pertussis disease has only recently emerged or increased in virulence. Obviously, good data on the total number of pertussis cases from these early times are not available. However, as of the beginning of the 20th century, people have started to systematically document pertussis incidence and reasonable estimations on the total number of pertussis cases can be made. An important note concerning the interpretation of the available data is that there is an important difference between the epidemiology of pertussis infection and the epidemiology of reported pertussis cases (Cherry, 2005). In the pre-vaccination era, pertussis was an endemic disease that was characterised by a cyclic pattern with incidence peaks every 2 to 5 years (Cherry, 2003). Reported pertussis cases occurred almost exclusively in children younger than the age of 10 (>93%) with a mean age of infection of 5 years (Cherry, 1984; Grenfell and Anderson, 1989). Before mass-vaccination started, the average yearly incidence of reported pertussis was 157 per 100,000 population in the United States and 230 per 100,000 population in England and Wales (Cherry, 1984). However, due to significant underreporting, these numbers are thought to be underestimates of the actual number of pertussis cases (Cherry, 1984).

Introduction of whole-cell pertussis (wP) vaccines in the 1940s and 1950s, and later of acellular pertussis (aP) vaccines in the 1980s and 1990s, led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. Despite high vaccination coverage, pertussis disease has remained endemic and kept showing a cyclic pattern with peaks in incidence every 2 to 5 years. During the last two decades, several countries, including the Netherlands, have experienced increases in numbers of reported pertussis cases (Fig. 1). Interestingly, in some areas, a shift in age

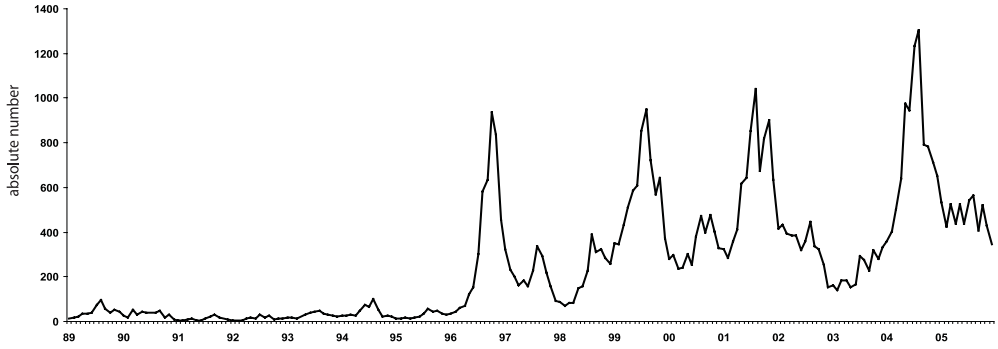

Fig.1 Reported pertussis per month in the Netherlands for the period 1989-2005. Notifications are corrected for the first day of illness (Source: National Institute of Public Health and the Environment).

distribution has also been observed. Whereas in the pre-vaccination and early vaccine era pertussis cases were predominantly reported in young children, adults, adolescents, and young infants have accounted for an increasing proportion of the cases in recent years. Several reasons for the re-emergence of reported pertussis have been proposed, including: (1) genetic changes in circulating *B. pertussis* strains that decrease vaccine efficacy, (2) reduced potency of pertussis vaccines, (3) waning immunity, (4) increased reporting of pertussis cases, and (5) the improved diagnosis of pertussis disease.

Thus, although pertussis vaccination has been proved very successful, the illness remains an important health issue. It is therefore of great importance that new and improved vaccines are being developed. The work described in this thesis mainly focuses on the engineering of *B. pertussis* strains that could form the basis for improved wP vaccines. A problem that should be dealt with is that such vaccines exhibit considerable reactogenicity, which is caused by the presence of a bioactive molecule, known as lipopolysaccharide (LPS). Reducing the reactogenicity of *B. pertussis*, and in particular of its LPS, currently forms the main bottleneck that prohibits the development of new wP vaccines. Furthermore, we investigate whether addition of non-toxic LPS derivatives may be a useful strategy for improving both wP and aP vaccines. In the remaining of this introduction, I will provide more insight into the challenges that the development of improved pertussis vaccines faces. First I will give an overview of *B. pertussis* and its virulence factors, then I will discuss how natural and acquired immunity against *B. pertussis* are obtained, and finally, I will introduce LPS and provide the aims and outline of the thesis.

Bordetella pertussis

The pathogenic *Bordetellae*

B. pertussis is a Gram-negative bacterium implicating that its cell envelope is composed of two membranes, an inner and an outer membrane, which are separated by the periplasmic space. The inner membrane is a symmetrical bilayer composed of phospholipids, whereas the outer membrane is asymmetric and consists of phospholipids in the inner leaflet and LPS in the outer leaflet. Although the genus *Bordetella* currently encompasses nine species, apart from *B. pertussis* only three additional members, i.e., *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella holmesii*, have been associated with respiratory infections in humans and other mammals. *B. bronchiseptica* has a broad host range and is capable of colonising rabbits, rats, guinea pigs, dogs, and piglets (Goodnow, 1980). Normally, *B. bronchiseptica* does not infect humans; however, some cases of infection in elderly and in immunocompromised individuals have been described (Amador *et al.*, 1991; Gueirard *et al.*, 1995). *B. parapertussis* was first isolated in the 1930s and causes a milder pertussis-like disease (Bradford and Slavin, 1937; Eldering and Kendrick, 1938). *B. parapertussis*, like *B. pertussis*, lacks an environmental reservoir. *B. holmesii*, the most recent of the *Bordetella* species associated with human respiratory tract infection, was first isolated from the blood of young adults suffering from septicaemia (Weynant *et al.*, 1995). Recently, the genomes of three *Bordetella* strains (*B. pertussis* strain Tohama I, *B. parapertussis* strain 12822, and *B. bronchiseptica* strain RB50) have been sequenced (Parkhill *et al.*, 2003). The genome of strain RB50 is 5.34 Mb, while those of strains Tohama I and 12822 are 4.09 and 4.77 Mb, respectively. The differences in genome sizes and sequence comparison of the three genomes support the hypothesis that *B. pertussis* and *B. parapertussis* recently and independently evolved from *B. bronchiseptica*-like ancestors. Interestingly, adaptation to the human host involved significant loss of DNA (Parkhill *et al.*, 2003).

Bordetella virulence factors

Bordetellae can express a wide variety of different virulence factors. The virulence factors that are relevant for this thesis will now be discussed in more detail. A complete overview of all currently identified *Bordetella* virulence factors is presented in Table 1.

Table 1 Expression and function information for various virulence determinants for *B. pertussis* and *B. bronchiseptica* (adapted from Mattoo and Cherry, 2005)

Virulence determinant	Description	Gene expression[a]		Protein expression[a]	
		B. pertussis	*B. bronchiseptica*	*B. pertussis*	*B. bronchiseptica*
Adhesins					
Filamentous hemagglutinin (FHA)	220-kDa surface-associated and secreted protein; dominant adhesin; required for tracheal colonisation; highly immunogenic; primary component of acellular pertussis vaccines	+	+	+	+
Fimbriae (FIM)	Filamentous cell surface structures; required for persistent tracheal colonisation; component of some acellular pertussis vaccines	+	+	+	+
Autotransporters					
Pertactin (PRN)	68–70-kDa surface protein; mediates eukaryotic cell binding *in vitro*; enhances protective immunity	+	+	+	+
Vag8	95-kDa outer membrane protein	+	+	+	+
BrkA	73-kDa surface-associated N-terminal passenger domain with 30-kDa outer membrane C-terminal protein; putative adhesin; confers serum resistance and protection against antimicrobial peptides in *B. pertussis*	+	+	+	+
SphB1	Subtilisin-like Ser protease /lipoprotein required for FHA maturation in *B. pertussis*	+	+	+	+
Tracheal colonisation factor (TcfA)	60-kDa secreted protein; role in tracheal colonisation in murine model	+	-	+	-
Toxins					
Pertussis toxin (PT)	A-B-toxin; ADP-ribosylates G proteins; responsible for pertussis-associated lymphocytosis; strong adjuvant and primary component of pertussis vaccines	+	-	+	-
Adenylate cyclase (CyaA)	Calmodulin-activated RTX family toxin with dual adenylate cyclase /hemolysin activity; acts as anti-inflammatory and antiphagocytic factor during infection	+	+	+	+
Type III secretion	Allows *Bordetella* to translocate effector proteins directly into host cells; required for persistent tracheal colonisation; inhibits host immune response; activates ERK1/2; mislocalises NF-κB; causes caspase-independent cell death	+	+	-	+
Dermonectrotic toxin (DNT)	160-kDa heat-labile secreted toxin; activates Rho; induces necrosis *in vitro*	+	+	+	+
Tracheal cytotoxin (TCT)	Disaccharide-tetrapeptide monomeric by-product of peptidoglycan synthesis; causes mitochondrial bloating, disruption of tight junctions, damage to cilia, IL-1α and $NO^{\cdot}$ production	+	+	+	+
LPS					
wlb locus	Consists of 12 genes required for LPS (band A) biosynthesis	+	+	+	+
wbm locus	Encodes O antigen; may be important for conferring serum resistance	-	+	-	+
PagP	Mediates palmitoylation modification of lipid A; may be important for persistence and resistance to serum killing	-	+	-	+
Additional loci					
Flagella	Peritrichous cell surface appendages required for motility; highly antigenic; ectopic expression of flagella in the Bvg^{+} phase is detrimental to the infection cycle	-	+	-	+
Type IV pili	Polar pili usually with an N-methylated phenylalanine as the N-terminal residue; possible functions include adherence, twitching motility, and DNA uptake	Δ	ND	NA	ND
Capsule	A type II polysaccharide coat predicted to be comprised of an *N*-acetylgalactosaminuronic acid Vi antigen-like polymer; possible role in protection against host defense mechanisms or survival in the environment	Δ	ND	NA	ND
Alcaligin	A siderophore for complexing iron, which is internalised through outer membrane receptors (*B. bronchiseptica* encodes 16 such receptors while *B. pertussis* encodes 12); iron uptake may be important for survival within mammalian hosts	+	+	+	+
Vrg loci	Several loci of uncharacterised function	+	-	+	-

[a] +, positive for expression; -, no expression; Δ, genome contains deletion mutations in these genes; ND, not determined; NA, not applicable.

Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) forms the critical determinant for adhesion of *Bordetellae* in animal model systems (Relman *et al.*, 1989). FHA is encoded by *fhaB* and is synthesised as a 367-kDa precursor, FhaB. After extensive N- and C-terminal processing of the precursor, i.e., first by leader peptidase and subsequently by the subtilisin-like autotransporter/protease SphB1, FHA is secreted into the external environment where it facilitates adherence to ciliated respiratory epithelial cells and, thereby, initiates the pathogenic lifecycle. FhaB is transported over the inner membrane by the general protein export apparatus, the Sec system. Its secretion over the outer membrane is dependent on the accessory protein FhaC, which forms a pore through which FhaB is secreted (Guedin *et al.*, 2000; Jacob-Dubuisson *et al.*, 1999). This type of secretion is known as two-partner secretion and is also found in many other Gram-negative bacteria (Jacob-Dubuisson *et al.*, 2004). *In vitro* studies indicate that FHA contains at least four separate binding domains. These domains mediate the interaction of FHA with several host structures, including integrins (via an RGD motif), sulphated sugars (heparin) found in the extracellular matrix and on epithelial cells, and specific carbohydrates found on ciliated epithelial cells (reviewed in Mattoo and Cherry, 2005 and Smith *et al.*, 2001).

Fimbriae

Fimbriae, also known as pili and agglutinogens, are long filamentous, polymeric protein structures that protrude from the bacterial cell surface and mediate the attachment to host cells. *Bordetella* fimbriae consist of major and minor subunits. The major subunits, encoded by the unlinked chromosomal loci *fim2* and *fim3*, form pentameric repeat units that together constitute the fimbrial strand (Livey *et al.*, 1987; Mooi *et al.*, 1987). At the tip of this strand, the minor subunits, encoded by *fimD*, are located which function as tip adhesins (Geuijen *et al.*, 1997). Besides Fim2 and Fim3, which form the predominant fimbrial serotypes, several other related major-subunit loci have been identified. The *fimX* locus encodes for a protein that is expressed at very low levels if at all (Riboli *et al.*, 1991). The *fimX* open reading frame (ORF) is intact in *B. pertussis* and *B. bronchiseptica*, but frame-shifted in *B. parapertussis* (Preston *et al*, 2004). Another related locus, the *fimA* locus, is only intact in *B. bronchiseptica* and *B. parapertussis*. It was found that *B. bronchiseptica* expresses a fully intact FimA protein (Boschwitz *et al.*, 1997b). A fifth fimbrial locus, *fimN*, was recently identified in *B. bronchiseptica*, but it was absent in *B. pertussis* (Kania *et al.*, 2000). The *fim* genes are subject to phase variation by slipped-strand mispairing within a stretch of

cytosine residues located in the promoter regions of *fim2*, *fim3*, *fimX*, and *fimN*. For this reason, bacteria may produce Fim2, Fim3, FimX, FimN, or any combination at any given time. The *fimD* gene, encoding the minor fimbrial subunit, is located within the fimbrial biogenesis operon, which, besides FimD, encodes two additional proteins, FimB and FimC. The FimB and FimC proteins are thought to function as a chaperone, facilitating transport of fimbrial subunits across the periplasm, and an usher, facilitating transport across the outer membrane, respectively. Interestingly, the *fimBCD* locus is located between *fhaB* and *fhaC*, the genes that encode FHA and its secretion partner.

Pertussis toxin

Pertussis toxin is an extensively studied 106-kDa ADP-ribosylating toxin and a member of the A-B bacterial toxin superfamily. It is composed of 5 different subunits, termed S1 to S5, which are encoded by the *ptxA-E* genes, respectively (Tamura *et al.*, 1982a). The S1 protein is the A subunit and possesses enzymatic activity, i.e., it catalyses the transfer of the ADP-ribose moiety of NAD^+ to the α subunit of guanine nucleotide-binding proteins (G-proteins). Substitution of G-proteins with ADP-ribose inactivates them and abrogates their function in host signalling pathways (Katada *et al.*, 1983). Biological effects of pertussis toxin include histamine sensitisation, leukocytosis, lymphocytosis, hypoglycaemia, neurological responses, and modulation of immune responses. The other subunits, S2 to S5, together constitute the toxin's B oligomer in the molar ratio 1:1:2:1 and facilitate the attachment to and subsequent entry of pertussis toxin into host cells (Tamura *et al.*, 1982a; Tamura *et al.*, 1982b). Secretion of pertussis toxin over the bacterial outer membrane is mediated by a specialised transport apparatus composed of nine pertussis toxin liberation (Ptl) proteins, which together constitute a type IV protein secretion apparatus (Farizo *et al.*, 1996; Weiss *et al.*, 1993). Although the *ptx* and *ptl* ORFs are intact in *B. bronchiseptica* and *B. parapertussis*, pertussis toxin is not produced in these organisms due to mutations in the promoter sequences.

Pertactin

Pertactin belongs to the family of autotransporters, which is a family of functionally diverse proteins, including toxins, adhesins, proteases, lipases, and invasins (Newman and Stathopoulos, 2004). Typically, autotransporters are produced as precursors with three domains, a signal sequence, a passenger domain, which exhibits the various effector functions, and a C-terminal translocator domain, which, directly or indirectly, mediates transport over the outer membrane. Pertactin is synthesised as a 93.5-kDa precursor, which, after transport over the inner membrane and removal of the signal peptide, is

transported over the outer membrane. It promotes the adherence to monocytes, but not to epithelial cells (Roberts *et al.*, 1991; van den Berg *et al.*, 1999; Hazenbos *et al.*, 1994). Like FHA, pertactin contains an RGD motif, which can interact with integrins and is proposed, thereby, to mediate the interaction with host cells. In addition, pertactin contains several proline-rich regions and leucine-rich repeats, motifs commonly found in molecules that form protein-protein interactions involved in eukaryotic cell binding (Emsley *et al.*, 1994). The exact *in vivo* roles and working mechanisms of pertactin remain unclear, as is its clinical relevance. For example, although pertactin-deficient mutants were found to adhere 30-40% less well to CHO and HeLa cells, consistent with a role for pertactin in bacterial adherence, such mutants were normally capable of colonising and multiplying in the murine respiratory tract (Leininger *et al.*, 1991; Roberts *et al.*, 1991).

The BvgAS virulence control system

B. pertussis, *B. parapertussis*, and *B. bronchiseptica* encode a nearly identical virulence control system, the BvgAS system. This two-component signal transduction system controls the expression of almost every virulence factor encoded by the various *Bordetellae*. BvgA is a 23-kDa DNA-binding response regulator, whereas BvgS is a 135-kDa transmembrane sensor kinase (Boucher and Stibitz, 1995; Stibitz and Yang, 1991). The BvgAS system responds to environmental changes. However, the exact environmental stimuli that control the system *in vivo* are yet to be determined.

Already long ago, it was recognised that *Bordetellae* could exist in three distinctive phenotypes. These phases are now known as the Bvg^+, Bvg^i, and Bvg^- phases. Bacteria in the Bvg^+ phase, i.e., the virulent phase, express several virulence factors, such as FHA, pertactin, and pertussis toxin. During the Bvg^- phase, i.e., the a-virulent phase, the majority of virulence factors is down regulated, whereas other genes, for example those that are required for motility, are up regulated (Akerley *et al.*, 1992). Some virulence genes, including those encoding FHA and fimbriae, are expressed also in a Bvg-intermediate phase, known as Bvg^i (Cotter and Miller, 1997). Comparison of the protein expression profiles indicate the presence of at least four different classes of BvgAS-controlled genes: (1) those that are expressed maximally only in the Bvg^+ phase, e.g., adenylate cyclase (*cyaA*), and are known as late Bvg-activated genes, (2) those that are expressed maximally in both the Bvg^+ and Bvg^i phase, e.g., *fhaB*, and are known as early Bvg-activated genes, (3) those that are expressed exclusively in the Bvg^i phase, e.g., *bipA*, and (4) those that are only expressed in the Bvg^- phase, e.g., *flaA* (Fig. 2) (Mattoo and Cherry, 2005). In the laboratory, *Bordetellae* can be forced

into a particular phenotypic phase by growing them under specific conditions. When grown at 37°C in the relative absence of $MgSO_4$ or nicotinic acid, expression of *bvgAS* is activated and the bacteria grow in the Bvg^+ phase (Melton and Weiss, 1989; Melton and Weiss, 1993). On the other hand, when the bacteria are grown either at 25°C or at 37°C in the presence of ≥ 40 mM $MgSO_4$ or ≥ 10 mM nicotinic acid (Melton and Weiss, 1993), or in the presence of nicotinic acid concentrations between 0.4 and 2 mM for *B. bronchiseptica*, *Bordetellae* display the Bvg^- or Bvg^i phenotype, respectively (Cotter and Miller, 1997). The role of these distinct phenotypic phases in the pathogenicity of the *Bordetellae* has been a subject of extensive research. Experiments with phase-locked and ectopic expression mutants have indicated that the Bvg^+ phase is essential for a successful colonisation of the host's respiratory tract by both *B. pertussis* and *B. bronchiseptica* (Cotter and Miller, 1994; Martinez de Tejada *et al.*, 1996). These experiments also demonstrated that the Bvg^- phase of *B. bronchiseptica* is not needed for respiratory tract colonisation, but rather increases the bacterium's survival under nutrient-limiting conditions (Cotter and Miller, 1994). By introducing a single amino acid substitution at position 733 of *bvgS* (Thr to Met), *Bordetellae* can be locked in the Bvg^i-phase. Bvg^i-phase-locked bacteria display an increased resistance to nutrient starvation and a decreased ability to colonise their host (Cotter and Miller, 1997). This observation, amongst others, led to the hypothesis that the Bvg^i phase may play a role in respiratory transmission (Fuchslocher *et al.*, 2003). In addition, the Bvg^i phase was recently also associated with the formation of biofilms by *B. bronchiseptica* (Irie *et al.*, 2004).

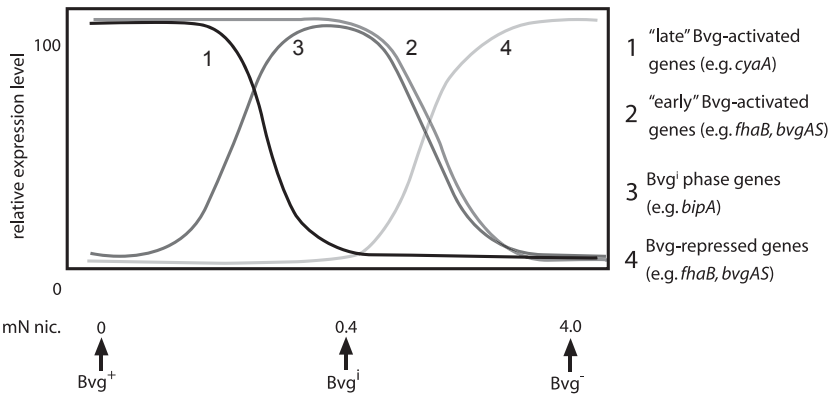

Fig. 2 Expression curves for the four classes of genes regulated by BvgAS. Genes expressed maximally in the Bvg^+ phase (such as *cyaA*) are referred to as "late" Bvg-activated genes and are represented by the black curve (curve 1). Genes that are expressed maximally under both Bvg^+ and Bvg^i phase conditions (such as *fhaB*) are referred to as "early" Bvg-activated genes and are represented by the green curve (curve 2). Genes expressed maximally only under Bvg^i phase conditions (such as *bipA*) are represented by the gold curve (curve 3). Finally, genes that are repressed by BvgAS and expressed maximally only under Bvg^- phase conditions are represented by the red curve (curve 4). Abbreviation: nic, nicotinic acid. Adapted from Mattoo and Cherry, 2005.

Immunity

The innate and adaptive immune system

The immune system has traditionally been divided into an innate and an adaptive component. Adaptive immunity is basically exerted by two classes of specialised cells, T- and B-lymphocytes. Each lymphocyte expresses a single, structurally unique receptor. Through usage and (re)combination of available gene segments, the complete repertoire of antigen receptors in the entire population of lymphocytes is enormous and extremely diverse, which increases the probability that each antigen will be recognised by an individual lymphocyte. This antigen-receptor interaction, together with co-stimulatory signals, then activates the lymphocyte and promotes proliferation and differentiation. This process, called clonal expansion, accounts for most of the basic properties of the adaptive immune system and is absolutely necessary for the generation of an efficient immune response. However, it usually takes at least three to five days before sufficient clones and effector cells have been produced to combat the pathogen. For most pathogens, this allows more than enough time to already damage the host. In contrast, the effector mechanisms of the innate immune system, which include phagocytes (e.g., macrophages and dendritic cells), natural killer cells, mast cells, granulocytes, antimicrobial peptides, and the complement system, are activated immediately upon infection and try to prevent replication and spread of the invading pathogen.

There are important differences between the innate and the adaptive immune systems in the mechanisms and receptors used for recognition of ligands. In the adaptive immune system, the T- and B-cell receptors are generated by somatic recombination in such a way that each lymphocyte has a structurally unique receptor. Since these receptors are not germ-line encoded, they are not designed to recognise a particular antigen. In contrast, recognition by the innate immune system is based upon germ-line encoded receptors, which means that receptor-specificity is genetically predetermined. The innate immune system has therefore not evolved to recognise every possible antigen, but rather focuses on a few, highly conserved structures that are unique to micro-organisms. These structures are referred to as pathogen-associated molecular patterns (PAMPs). Well-known PAMPs are LPS, peptidoglycan, lipoproteins, flagellin, mannans, bacterial DNA (CpG DNA), double-stranded RNA, and glucans. The receptors that recognise these PAMPs are called pattern-recognition receptors (PRRs). Functionally, PRRs can be divided into three classes: secreted, endocytic, and signalling PRRs. The best-known PRRs are probably the members of the Toll-like receptor (TLR) family, such as TLR-4, which recognises LPS (Fig. 3). Another important difference between the innate and the adaptive immune system is that the receptors of the innate immune

system, unlike the receptors of the adaptive immune system, are expressed by many different cells, including macrophages, dendritic cells, and B cells, i.e., the professional antigen-presenting cells. Responses induced by the innate immune system, including activation of NF-κB and the increased production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), and co-stimulatory cell-surface molecules, in turn, control the activation of adaptive immune responses; the adaptive immune system can only respond to a pathogen after its recognition by the innate immune system.

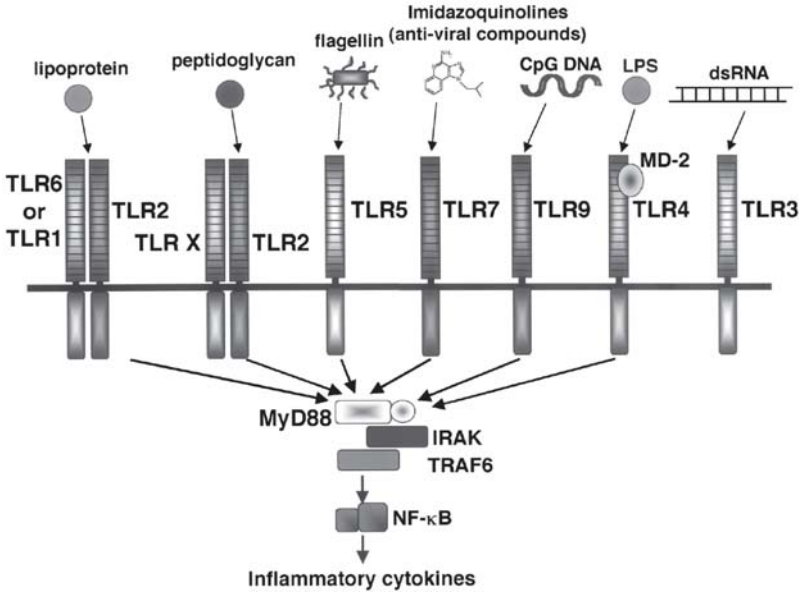

Fig. 3 Summary of ligands recognised by TLR family. The responses to all ligands, except for dsRNA, are dependent on MyD88, as assayed by inflammatory cytokine production. MyD88 does not appear to be involved in dsRNA-dependent TLR3 signalling. X, unknown. Adapted from Akira *et al.*, 2003.

Immunity against *B. pertussis*

Innate immunity

The contribution of early innate immune responses to inflammation, pathology, and the development of an adaptive immune response against *B. pertussis* is currently not well understood. Recently, it was shown that TLR4-deficient mice are highly susceptible to infection by *B. bronchiseptica* and rapidly develop bordetellosis and lethal pneumonia after inoculation with doses as low as 10^3 colony-forming units (Mann *et al.*, 2004b). It was also shown that the major reason for this increased susceptibility is the impairment of the transient early TNF-α response that is normally elicited by the cells of

the innate immune system (Mann *et al.*, 2004a). Thus, although the importance of the innate immune system in immunity to *Bordetella* is now clearly recognised, the exact mechanisms and factors involved have to be further elucidated.

Adaptive immunity

Adaptive immunity against *B. pertussis* develops after natural infection with *B. pertussis* or after immunisation with pertussis vaccines and provides a relatively long-lived protection against clinical pertussis disease (Mills, 2001). However, in contrast to the natural immunity developed after infection, vaccination-derived immunity provides little protection against subsequent sub-clinical infections and, in addition, does not cross-protect against infection with other *Bordetella* species (Mills, 2001; Watanabe and Nagai, 2001). Although extensive research has been performed, the exact immunological correlates of protection against *B. pertussis* remain to be elucidated. Unlike other diseases, such as tetanus or diphtheria, it is clear that immunity against *B. pertussis* is not simply mediated by antibodies against a single target antigen, but is much more complex and involves both the cell-mediated and humoral arms of the immune system (Leef *et al.*, 2000; Mills *et al.*, 1998). Indeed, although *B. pertussis* infection is mediated by virulence factors, such as toxins and adhesins, emphasising the importance of antibodies and thus the humoral response, it also comprises intracellular survival within macrophages and other cells, suggesting an important role for cell-mediated immunity. It has been shown that natural infection and vaccination with pertussis vaccines can induce both T- and B-cell responses against various known antigens, such as pertussis toxin, FHA, pertactin, fimbriae, and LPS, but also against several unidentified antigens (Mills, 2001). Experiments in mice demonstrated that wP vaccines confer immunity to *B. pertussis* in a similar way as natural infection (Mills, 2001). In contrast, aP vaccines were shown to give rise to a different response (Redhead *et al.*, 1993; Van den Berg *et al*., 2001). However, the observation that these vaccines can give comparable levels of immunity suggests the presence of redundancy in the immune protective mechanisms induced by the different vaccines.

Humoral immunity

The importance of antibodies in the protection against *B. pertussis* infection and disease has been subject of debate for already a long time. B cells, but also antibodies alone, have been shown to play an important role in protection against pertussis disease (Granstrom *et al.*, 1991; Bruss and Siber, 1999; Hellwig *at al*., 2003; Kirimanjeswara *et al*., 2003). Several studies, both in human and mice, have identified a significant

correlation between antibody titers against certain antigens and the protection against *B. pertussis* (Van den Berg *et al*., 2001; Cherry *et al*., 1998; Storsaeter *et al*., 1998). However, there are also reports that could not show such a correlation (Giuliano *et al*., 1998). Antibodies may function by either inhibiting the adherence of bacteria to the host cells, neutralising bacterial toxins, or promoting the uptake and destruction of bacteria by macrophages and neutrophils (opsonic antibodies).

Many studies in mice have shown that immunisation with various antigens, such as pertussis toxin, FHA, pertactin, and others, can confer protection against respiratory or inter cranial challenge with *B. pertussis* (Mills, 2001). Whereas most of these studies showed robust antibody responses upon immunisation, some of them demonstrated protection in the absence of detectable antibody titers (Leef *et al*., 2000; Mills *et al*., 1993), suggesting that besides antibody-mediated immunity, also other immune mechanisms can confer protection. Nevertheless, direct proof of the protective capacity of pertussis antibodies in humans came from a passive immunisation study, in which patients with pertussis disease that were treated with anti-sera were shown to have a reduced severity of disease (Granstrom *et al.*, 1991). Also in mice, passive immunisation experiments showed that antibodies possess protective capacity (Bruss and Siber, 1999). Further insight into the importance of antibody-mediated immunity to *B. pertussis* came from studies that used Ig-deficient ($Ig^{-/-}$) mice. Respiratory infection of these mice with *B. pertussis* led to the development chronic infections, whereas in the wild-type control group, infection was cleared after 8-10 weeks (Mahon *et al.*, 1997). However, a problem with $Ig^{-/-}$ mice is that they do not have mature B cells and display a defective T-cell response (Mahon *et al.*, 1997). It is indeed known that B cells not only function as memory or antibody-secreting cells, but also have an important function in generating a good, overall immune response, for instance by functioning as antigen-presenting and/or cytokine-secreting cells (Rodriguez-Pinto, 2005). Therefore, experiments using $Ig^{-/-}$ mice should be interpreted with care.

Cellular immunity

Already more than 35 years ago, it was described that *B. pertussis* can have a partially intracellular life cycle (Cheers and Gray, 1969). Later on, it was found that *B. pertussis* can invade and survive within several mammalian cells, including various epithelial cells and phagocytes, such as human macrophages (Ewanowich *et al.*, 1989; Friedman *et al.*, 1992; Guzman *et al.*, 1994). However, during its intracellular phase, *B. pertussis* hardly multiplies and survival appears to depend on the numbers of bacteria taken up (Friedman *et al.*, 1992). The significance of the intracellular state in pertussis

disease is not yet clear. It could for instance be important for the establishment of a chronic or quiescent stage, although there is no good evidence for this. The possibility of *B. pertussis* to survive within host cells suggests that cellular immunity may have an important role in the complete bacterial elimination from the respiratory tract.

The possible involvement of cellular immunity in protection to *B. pertussis* in humans was demonstrated in a study in which antigen-specific proliferation of peripheral blood mononuclear cells or purified T cells was found in persons who had been immunised for pertussis or had suffered from pertussis in their childhood (Redhead, 1995). Prior to this, when antigen-specific T cells and T-cell responses were studied, it had already become clear that in both humans and mice, natural infection and wP vaccination led to characteristic T-helper 1 (Th1) responses (Peppoloni *et al.*, 1991; Ryan *et al.*, 1997). In contrast, aP vaccines were found to evoke a clear T-helper 2 (Th2) response in mice and a more mixed Th1/Th2 response in humans (Redhead *et al.*, 1993; Ryan *et al.*, 1998; Assiello *et al.*, 1997). T-helper cells are specialised $CD4^+$ T-cells that provide help to other cells of the immune system by activation of cells and secretion of cytokines, thereby mounting a broad immune response. In mice, several distinct types of T-helper cells have been identified, i.e., Th1, Th2, Th3, and Tr1 cells. These cells exhibit different functions during an immune response (Mosmann and Coffman, 1989a; Mosmann and Coffman, 1989b). Th1 cells provide strong cell-mediated immunity, but only weak and transient antibody responses (Mosmann *et al.*, 1986; Cherwinsky *et al.*, 1987; Mosmann and Coffman, 1989a; Mosmann and Coffman, 1989b). In general, intracellular microbes, such as viruses, some mycobacteria, some yeasts, and some parasitic protozoans, stimulate Th1 responses. Th1 cells secrete various cytokines, such as IL-2, IFN-γ, IL-12, and TNF-β, collectively known as Th1 cytokines or Type 1 cytokines. Th2 cells evoke especially strong antibody responses but relatively weak cell-mediated immunity (Mosmann *et al.*, 1986; Cherwinsky *et al.*, 1987; Mosmann and Coffman, 1989a; Mosmann and Coffman, 1989b). Th2 responses are usually induced by extra-cellular bacteria, but also by some other parasites. Th2 cells secrete cytokines, known as Th2 cytokines or Type 2 cytokines, which include IL-4, IL-5, IL-6, IL-10, and IL-13. Also in humans, T-helper cells similar to those first identified in mice have been found. However, human T-helper cells display a less restricted cytokine profile in that the secretion of IL-2, IL-6, and IL-10 is not stringently restricted to one type of T-helper cells (Del-Prete *et al.*, 1991). In addition, T cells expressing both Th1 and Th2 cytokines have been identified during differentiation (Firestein *et al.*, 1989; Kamogawa *et al.*, 1993) and among terminally differentiated cells (Paliard *et al.*, 1988), indicating that expression of these distinct cytokines profiles is not mutually exclusive.

Th3 cells are regulatory $CD4^+$ T-cells, which are associated with immune mechanisms involving oral tolerance towards antigens (Groux *et al.*, 1997; Weiner *et al.*, 2001). Th3 cells are characterised by the secretion of TGF-β and/or IL-10 and have suppressive properties for both Th1 and Th2 cells. T-regulatory 1 cells (Tr1) are similar to Th3 cells. However, they secrete large amounts of IL10 and were first characterised on the basis of their role in preventing autoimmune colitis (Levings and Roncarolo, 2000; Groux *et al.*, 1997).

The first direct evidence that cell-mediated immunity is important for the clearance of *B. pertussis* came from experiments with athymic or severe combined immunodeficiency mice. It was shown that these mice, in contrast to control BALB/c mice, were unable to clear the bacteria or even became lethally infected (Mills *et al.*, 1993; Leef *et al.*, 2000). Transfer of $CD4^+$ T cells from infection-primed mice or of an FHA-specific T-cell line to athymic or sublethally radiated mice was shown to provide protection in the absence of a detectable antibody response, further confirming the protective role of T cells (Mills *et al.*, 1993). More evidence came from experiments in which it was shown that CD4 knock-out mice cannot be protected by intranasal immunisation and from experiments in which pre-immunised, $CD4^+$-depleted mice showed abrogated protection upon challenge (Leef *et al.*, 2000). Interestingly, the results described above could only be obtained by using $CD4^+$ T-cells and not by using $CD8^+$ T cells (Mills *et al.*, 1993; Leef *et al.*, 2000). This suggests that $CD4^+$ T-cells, but not $CD8^+$ T cells, mediate protection against *B. pertussis*. An overview of a current working model for innate and acquired immunity to *B. pertussis* is provided in Fig. 4.

Modulation of immune responses by *Bordetella* virulence factors

It is well established that *Bordetella* virulence factors display various immune modulating activities. An overview of immune effector functions exhibited by the virulence factors relevant for this thesis is provided below.

FHA

FHA exhibits several immune modulating functions. It has for instance been shown that *B. pertussis* can inhibit T-cell proliferation in an FHA-dependent manner (Boschwitz *et al.*, 1997a). Moreover, FHA can suppress LPS-induced IL-12 production by macrophages via an IL-10 dependent mechanism and thereby inhibit Th1 T-cell responses (McGuirk and Mills, 2000). This inhibition is not *B. pertussis* specific, as administration of FHA to mice infected with influenza virus was shown to also suppress

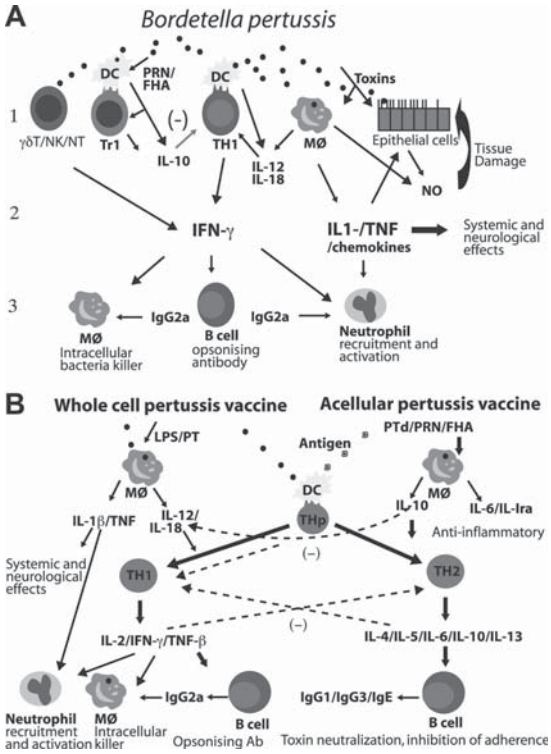

Fig. 4 (A) Immunity to *B. pertussis* in a naïve host: evidence from the murine respiratory challenge model. (1) Recognition of bacterial components by cells of the innate and acquired immune system, (2) Production of soluble mediators and (3) recruitment and activation of effector cells and molecules. When *B. pertussis* enters the respiratory tract, the bacteria bind to ciliated epithelial cells, but are also recognised and taken up by cells of the innate immune system, such as macrophages (Mϕ), dendritic cells (DCs), γδ T cells, natural killer (NK) or natural T cells (NT). DCs process and present bacterial antigens to T cells. The production of IL-12 and IL-18 by innate cells results in polarisation of the T-cell response to the Th1 subtype. However, early in infection the local Th1 response is suppressed due to the effects of IL-10 secreted by antigen-stimulated Tr1 cells or by FHA-stimulated macrophages and DCs. Nitric oxide (NO) and the pro-inflammatory cytokines, IL-1β and TNF-α induced by bacterial toxins, especially LPS, tracheal colonisation factor (TCT) and pertussis toxin (PT), as well as contributing to bacterial elimination, also mediate local lung pathology and are responsible for many of the systemic and neurological consequences of the infection. IFN-γ secreted early in infection by cells of the innate immune system, and later in infection by Th1 cells, stimulates recruitment and activation of macrophages and neutrophils and provides help for B cells to secrete opsonising and complement-fixing antibody (IgG2a in the mouse). Opsonised or non-opsonised bacteria are taken up by neutrophils and macrophages, which are killed by NO or reactive oxygen intermediates. (B) Distinct mechanisms of immunity induced with whole cell and acellular vaccines: a central role of Th1 and Th2 cells in regulation of the acquired immune response. wP include residual bacterial toxins, such as LPS, which activates IL-1β, TNF-α, IL-12 and IL-18 production by macrophages and other cells of the innate immune system. These pro-inflammatory cytokines regulate the selective induction of Th1 cells from the precursor T cell (Thp), but also contribute to the side effects of immunisation with wP. Cytokines secreted by Th1 cells, especially IFN-γ, provide help for opsonising antibody production and activate macrophages and neutrophils to take up and kill intracellular bacteria. In contrast, aP are devoid of bacterial toxins that stimulate IL-12 and IL-18, but include components such as FHA, which stimulate IL-10 production, and consequently have anti-inflammatory activity and preferentially induce Th2 cells. Th2 cells provide help for B cells to secrete IgE and murine IgG1, IgG3 antibodies, which neutralise toxins and prevent adherence of bacteria in the respiratory tract. Adapted from Mills *et al.*, 2001.

the virus-specific Th1 response (McGruik and Mills, 2000). Thus, whereas FHA primarily functions as an adhesin, it may also contribute significantly to suppression of the immune response, and in particular the Th1 response, following immunisation or primary infection with *Bordetellae* or other pathogens.

Fimbriae

Although there is no evidence that fimbriae can directly modulate immune responses, there are indications that they may facilitate the immune modulatory effects of FHA. For instance, it has been shown that FimD can function as a ligand that mediates the binding of *B. pertussis* to very late antigen-5 on the surface of monocytes. Ligation of FimD and very late antigen-5 activates complement receptor 3, which is an integrin that can interact with FHA, and thereby enhances the ability of FHA to modulate the protective immune response (Hazenbos et al., 1995a; Hazenbos et al., 1995b). In addition, fimbriae have been shown to induce nitric oxide production by murine macrophages (Xing *et al.*, 2000).

Pertussis toxin

Pertussis toxin exhibits several immune modulating functions. For instance, it has been shown that it can interfere with signals that promote B-cell survival *in vitro* (Lyons, 1997). Furthermore, pertussis toxin has been shown to inhibit macrophage chemotaxis *in vivo* and neutrophil and lymphocyte chemotaxis *in vitro* by changing the intracellular calcium levels (Meade *et al.*, 1985; Spangrude *et al.*, 1985). Pertussis toxin may also suppress immune responses to other antigens, as indicated by the observation that removal of pertussis toxin from *B. pertussis* resulted in an increased immune response to FHA in mice (Mielcarek *et al.*, 1998). Paradoxically, pertussis toxin has also been shown to function as a strong adjuvant in several immunological systems and in various animal and human models by boosting antibody and T-cell responses to unrelated antigens. One could argue that increasing the serum antibody responses may not be beneficial to the pathogen for survival in the host. However, pertussis toxin has also been implicated in shifting the immune response from protective Th1 responses to less protective Th2 responses (Samore and Siber, 1996; Mu and Sewell, 1993).

Pertactin

There is no clear evidence that pertactin can directly modulate immune responses. Nonetheless, it has been shown that pertactin can augment the suppressive effect of FHA on LPS-induced IL-12 production *in vitro* (McGuirk and Mills, 2000).

LPS

LPS is a major constituent of the Gram-negative outer membrane and is a potent immunostimulatory molecule. LPS exhibits both endotoxic and adjuvant properties and forms, together with other active toxins, the major determinant for the reactogenicity and adjuvant effect of wP vaccines (Mills, 2001). *Bordetella* LPS is pyrogenic, mitogenic, and toxic and induces the production and secretion of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-12, and IL-18 and the anti-inflammatory cytokine IL-10 both *in vivo*, upon natural infection with *B. pertussis*, and *in vitro*, by murine macrophages after stimulation with *B. pertussis* LPS (Ayme *et al.*, 1980; Loscher *et al.*, 2000; Mahon *et al.*, 1996). In addition, *B. pertussis* LPS functions as an adjuvant, as indicated by the enhanced antibody responses to tetanus and diphtheria toxoids when LPS was co-administered during vaccination (Nurminen and Olander, 1991). LPS induces the production of IL-12 (Mahon *et al.*, 1996) and thereby induces skewing towards a Th1 T-cell response (Redhead *et al.*, 1993; Mills *et al.*, 1993). The structure, biosynthesis, and endotoxic activity of LPS, and in particular of *Bordetella* LPS, will be discussed in more detail in the next paragraph.

Lipopolysaccharide

Chemical structure of LPS

LPS is an amphiphilic molecule that is located in the outer leaflet of the outer membrane of Gram-negative bacteria. LPS consists of three distinct structural domains: lipid A, the core, and the O-antigen (Fig. 5). Lipid A functions as a hydrophobic membrane anchor and forms the bioactive component of the molecule (Takada and Kotani, 1989). The structure of lipid A is reasonably conserved among different bacterial groups, indicating its importance for the correct functioning of the outer membrane. In general, lipid A consists of a β-1,6-linked D-glucosamine (GlcN) disaccharide carrying ester- and amide-linked 3-hydroxy fatty-acids at positions C-2, C-3, C-2', and C-3', and phosphate groups at positions C-1 and C-4' (Raetz and Whitfield, 2002). The core region consists of a complex oligosaccharide, which, as compared to the O-antigen, shows limited structural variability. In some bacteria, e.g., *Enterobacteriaceae*, the core domain can be divided into an inner core and an outer core. The outer core primarily consists of pyranosidic hexoses, e.g., D-glucose, D-galactose, and GlcN, whereas the inner core primarily consists of octulosonic acids and heptopyranoses. In the vast majority of Gram-negative bacteria, the core domain is connected to the lipid A domain by a specific carbohydrate, 2-keto-3-deoxyoctulosonic acid (Kdo) (Raetz and Whitfield,

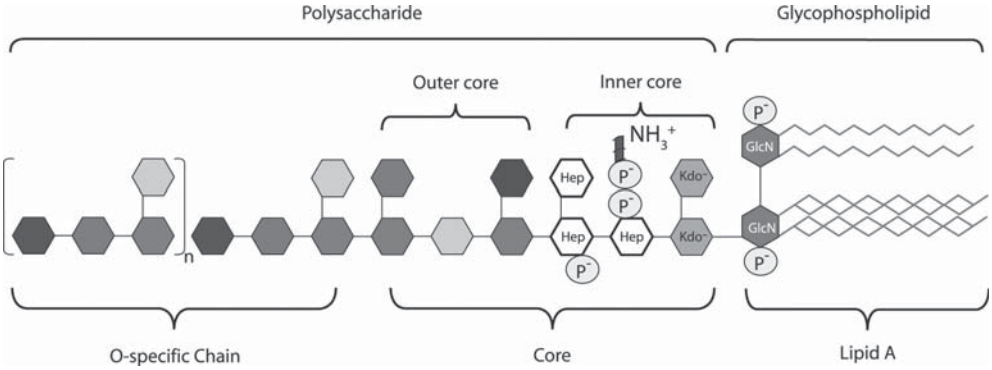

Fig. 5 Schematic representation of the chemical structure of enterobacterial LPS. Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; P, phosphate; EtN, ethanolamine; zigzag lines, fatty acids. Adapted from Caroff *et al.*, 2002.

2002). Usually, this connection embraces two Kdo sugars, but in some bacteria, e.g., the *Bordetellae*, there is only one (Caroff *et al.*, 2000). The O-antigen comprises the most variable part of the LPS and confers bacteria serotype specificity. It is composed of repeating sugar subunits of one to eight sugars. Each O-chain can contain up to 50 of these subunits. The presence of the O-antigen has been implicated in bacterial immune-escape, especially the escape from serum complement-mediated lysis (Raetz and Whitfield, 2002). Some Gram-negative bacteria, including *B. pertussis*, do not contain an O-antigen. For a long time, it was thought that the minimal structure of LPS needed for the growth of Gram-negative bacteria was a lipid A moiety glycosylated with two Kdo residues (Re LPS). However, recently, a viable, non-conditional *Escherichia coli* strain that produces lipid A without Kdo has been isolated (Meredith *et al.*, 2006). Furthermore, mutants of *Neisseria meningitidis* and *Moraxella catarrhalis* completely devoid of LPS have been isolated (Steeghs *et al.*, 1998; Peng *et al.*, 2005).

Structure of *B. pertussis* LPS

In contrast to the LPS of *B. bronchiseptica* and *B. parapertussis*, the LPS molecule of *B. pertussis* never contains an O-antigen domain (Peppler, 1984; Di Fabio *et al.*, 1992) Therefore, *B. pertussis* LPS is often referred to as lipooligosaccharide. *B. pertussis* produces two dominant LPS forms, band A and band B LPS (Peppler, 1984). Band B LPS is composed of lipid A and a core oligosaccharide consisting of 9 carbohydrates (Fig. 6) (Caroff *et al.*, 2000). Addition of a terminal tri-saccharide, consisting of *N*-acetyl glucosamine (GlcNAc), 2,3-diacetamido-2,3-dideoxy-mannuronic acid, and 2-acetamido-4-*N*-methyl-2,4-dideoxy-fucose, to band B LPS forms the LPS referred to as band A (Fig. 6) (Caroff *et al.*, 2000). In *B. pertussis*, lipid A is substituted

with *R*-3-hydroxytetradecanoic acid (3OH C14) residues at positions 2, 2', and 3' and with an *R*-3-hydroxydecanoic acid (3OH C10) residue at the 3 position via ester or amide linkage. A secondary myristoyl group (C14) substitutes the hydroxyl group of 3OH C14 at the 2' position (Fig. 6) (Caroff *et al.*, 1994).

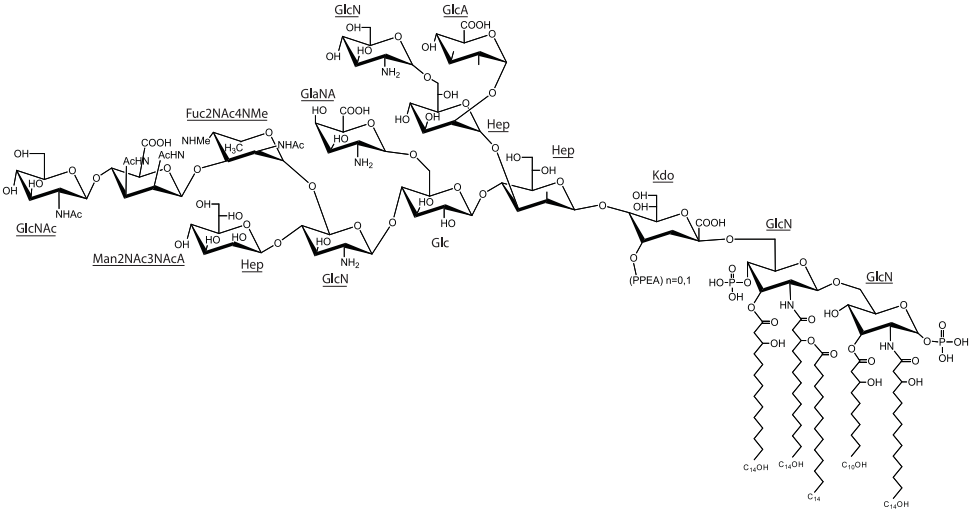

Fig. 6 Structure of the LPS of *B. pertussis* 1414. Abbreviations used: glucose (Glc), L-glycero-D-manno-heptose (Hep), 2-keto-3-deoxyoctulosonic acid (Kdo), glucosamine (GlcN), glucuronic acid (GlcA), *N*-acetyl glucosamine (GlcNAc), 2-acetamido-4-*N*-methyl-2,4-dideoxy-fucose (Fuc2NAc4NMe), 2,3-diacetamido-2,3-dideoxy-mannuronic acid (Man2NAc3NAcA), galactosaminuronic acid (GalNA), phosphoethanolamine (PPEA). Adapted from Caroff *et al.*, 2000.

For a long time, it was thought that species from the same genus would share almost identical lipid A structures. However, when the LPS compositions from several *Bordetella* species were compared, the structure of lipid A appeared to vary significantly (Fig. 7), while the composition and arrangement of the core was the same (Preston *et al.*, 2006; Caroff *et al.*, 2001). This variation is mainly observed in the fatty acids at the 3 and 3' positions. Interestingly, LPS heterogeneity was not only found to exist between the different *Bordetella* species, but also between different strains from a single species, e.g., *B. bronchiseptica* (Zarrouk *et al.*, 1994), or even within a single strain, e.g., *B. pertussis* strain 1414 (Caroff *et al.*, 1994).

LPS biosynthesis

Lipid A

Current knowledge about lipid A, core, and O-antigen biosynthesis mainly comes from studies in *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.*

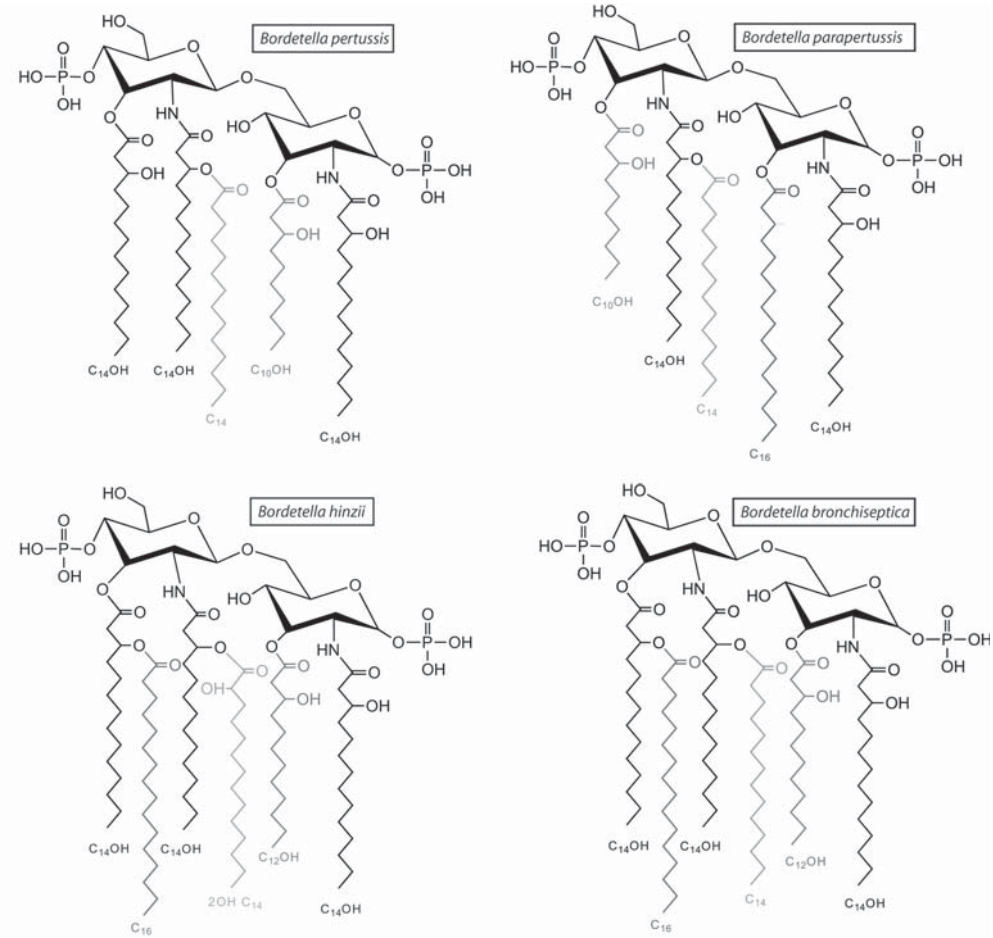

Fig. 7 Examples of lipid A structures from different *Bordetella* strains. Each colour indicates a specific fatty acid. Adapted from Caroff *et al.*, 2002.

Typhimurium). In *E.* coli, the biosynthetic pathway required for the synthesis of the Kdo_2-lipid A moiety of LPS has been completely elucidated. It consists of nine enzymes that work in a coordinate order (Fig. 8). In the first step, an acyl chain is transferred from the 3OH C14-acyl carrier protein ACP to the glucosamine 3 position of UDP-GlcNAc by the acyltransferase LpxA (Crowell *et al.*, 1986; Coleman and Raetz, 1988). In most bacteria, LpxA is highly specific with regard to its acyl-ACP substrate (Anderson and Raetz, 1987; Odegaard *et al.*, 1997; Sweet *et al.*, 2001), resulting in a symmetrical acyl-chain substitution at the 3 and 3' positions of mature lipid A. However, this is not the case for *Bordetellae*, where these positions never carry the same fatty acid (Fig. 7). A recent study showed that this asymmetry is due to a relaxed substrate specificity of the *Bordetella* LpxA enzyme (Sweet *et al.*, 2002). In the second step, the acylated

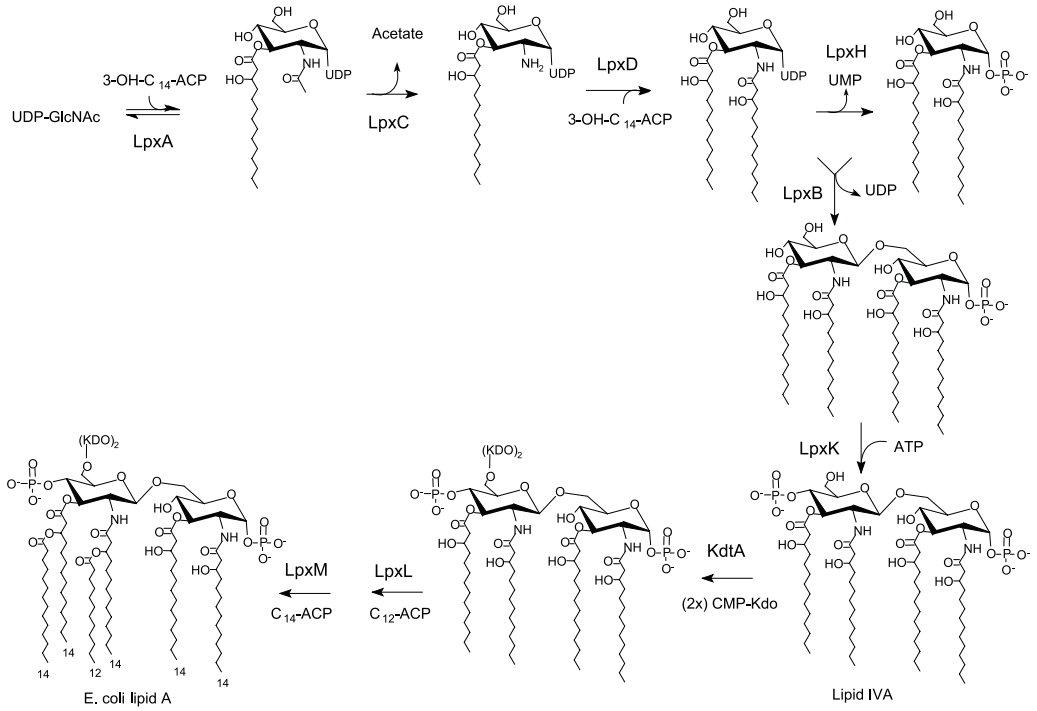

Fig. 8 Lipid A biosynthesis route of *E. coli*.

UDP-GlcNAc is de-acetylated by the LpxC enzyme (Young *et al.*, 1995), after which LpxD adds a 3-hydroxyl acyl chain at this position (Kelly *et al.*, 1993), resulting in a UDP-2,3-diacylglucosamine molecule. Next, UMP is removed from a proportion of the UDP-2,3-diacylglucosamine pool by LpxH (Babinski *et al.*, 2002), before a tetra-acylated glucosamine disaccharide is formed by LpxB (Crowell *et al.*, 1986). Then, after 4'-phosphorylation by LpxK, which forms a molecule known as lipid IV_A (Garrett *et al.*, 1997), two Kdo residues are added by KdtA (a.k.a. WaaA) (Clementz and Raetz, 1991), and finally the secondary acyl chains are added by the late acyltransferases LpxL (a.k.a. HtrB) and LpxM (a.k.a. MsbB) (Clementz *et al.*, 1996; Clementz *et al.*, 1997).

Core oligosaccharide

In *E. coli* and *S.* Typhimurium, the core oligosaccharide (core OS) biosynthesis gene cluster consists of three operons, the *gmhD*, the *waaQ*, and *WaaA* operons. The *gmhD* operon consists of four genes, *gmhD* and *waaFCL*, which are involved in the synthesis of the inner core (Schnaitman and Klena, 1993). The *gmhD*, *waaF*, and *waaC* genes encode proteins involved in the biosynthesis and transfer of Heptoses I and II to

Kdo_2-lipid A (Schnaitman and Klena, 1993), whereas the *waaL* gene product is a ligase that is involved in the attachment of the O-antigen (MacLachlan *et al.*, 1991). The *waaQ* operon is the largest of the three operons and encodes proteins that are involved in the biosynthesis of the outer core and for modification/decoration of the core OS. The number and types of genes present within in the *waaQ* operon differ per strain, which explains the strain-specific differences in core composition among *Enterobacteriaceae* (Heinrichs *et al.*, 1998). The *waaA* operon often encodes only one protein, KdtA. Only in *E. coli* K-12, an additional ORF is present (Raetz and Whitfield, 2002). The *kdtA* gene of *Enterobacteriaceae* encodes the bifunctional Kdo transferase that adds the two Kdo residues in the Kdo_2-lipid A biosynthesis (Clementz and Raetz, 1991) (Fig. 8).

Although the *Bordetella* and *E. coli* core OS show some resemblance, the exact composition and configuration of residues also display marked differences. For example, the *Bordetella* core OS contains only one Kdo residue, instead of the two or three residues that are found in most other Gram-negative bacteria (Fig. 6). Recently, this was shown to be due to the functioning of *Bordetella* KdtA as a monofunctional, rather than as a bifunctional, Kdo transferase (Isobe *et al.*, 1999). Like in *E. coli*, the *Bordetella* core OS starts with two heptose residues attached to Kdo (Fig. 6). The responsible glucosyltransferases were identified and shown, as expected, to be homologues of the WaaC and WaaF enzymes, respectively (Allen *et al.*, 1998a; Sisti *et al.*, 2002). Additionally, the *wlb* locus containing the genes responsible for the addition of the terminal trisaccharide in band A LPS has been identified and characterised (Allen and Maskell, 1996; Allen *et al.*, 1998b). The enzymes responsible for the synthesis of the remaining portion of the *Bordetella* core OS are currently unknown and await further identification.

O-antigen

The genes involved in O-antigen biosynthesis are usually found in a single cluster on the chromosome, and the structural variation of O-antigens is mirrored by the genetic variation in this cluster. O-antigen biosynthesis genes can roughly be divided into three major groups. One group comprises genes involved in the biosynthesis of nucleotide sugars. These genes are often clustered together and have a high level of identity. Many of such genes have been identified and they seem to be conserved in the different O-antigen clusters across a wide range of species. A second group comprises genes encoding glycosyltransferases. These genes are often scattered throughout the cluster and show a low level of sequence similarity. A third group are the O-antigen modifying genes (Samuel and Reeves, 2003).

In contrast to *B. pertussis*, B*. parapertussis* and *B. bronchiseptica* synthesise O-antigens. Initially, the O-antigens of these species were reported to be identical and composed of linear polymers of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-galacturonic acid (Di Fabio *et al.*, 1992), but, later on, differences between the terminal ends on *B. bronchiseptica* O-antigens were reported (Vinogradov *et al.*, 2000). The O-antigen is linked to the core via a five-sugar linker that is similar to the band A trisaccharide, but in which α-*N*-acetyl galactosamine replaces α-*N*-acetyl glucosamine, and extra β-2,3-acetamido-3-acetamido-2,3-dideoxy-glucoronic acid and β-β-2,3-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid residues are present (Preston *et al.*, 2006). Recently, the *Bordetella* O-antigen biosynthesis locus was identified (Preston *et al.*, 1999). This locus, designated the *wbm* locus, contains 24 genes and is well conserved among *B. parapertussis* and *B. bronchiseptica* strains, consistent with the fact that their O-chains comprise the same linker region and the same homopolymeric repeat. It is postulated that the genes from this locus are responsible for the synthesis of both the -α-GalNAc3NAcA- homopolymer and the five-sugar linker region (Preston *et al.*, 2006). The *wbm* locus is replaced by an insertion sequence element in *B. pertussis*, explaining the lack of O-chain biosynthesis in this species (Preston *et al.*, 1999).

LPS-modifying enzymes

In recent years, several LPS-modifying enzymes have been identified (Fig. 9). These enzymes are mainly capable of modifying the lipid A moiety. Substitution of the lipid A 1 and 4' phosphate groups with one or two 4-amino-4-deoxy-L-arabinose moieties in *S.* Typhimurium was found to be mediated by the enzyme ArnT (Trent *et al.*, 2001b). Recently, the PmrC protein was found to facilitate the addition of phosphoethanolamine to lipid A in *S.* Typhimurium (Lee *et al.*, 2004). Substitution of lipid A with phosphoethanolamine (pEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N) lowers its negative charge and thereby promotes resistance to cationic antimicrobial peptides, e.g., polymyxin (Zhou *et al.*, 2001). The enzyme LpxO catalyses the O_2-dependent hydroxylation of lipid A (Gibbons *et al.*, 2000), and recently lipid A 1- and 4'-phosphatases, i.e., LpxE and LpxF, respectively, were identified in *Rhizobium leguminosarum* and *Francisella novicida*, respectively (Karbarz *et al.*, 2003; Wang *et al.*, 2006). All these enzymes are thought to reside within the inner membrane or periplasmic space. Recently, a new class of outer membrane-localised lipid A-modifying enzymes was discovered. One of them is the lipid A-oxidase LpxQ, which generates a 2-amino-2-deoxy-gluconate unit from a glucosamine-containing precursor (Que-Gewirth *et al.*, 2003). Another one is the palmitoyl transferase PagP, which adds a secondary palmitoyl

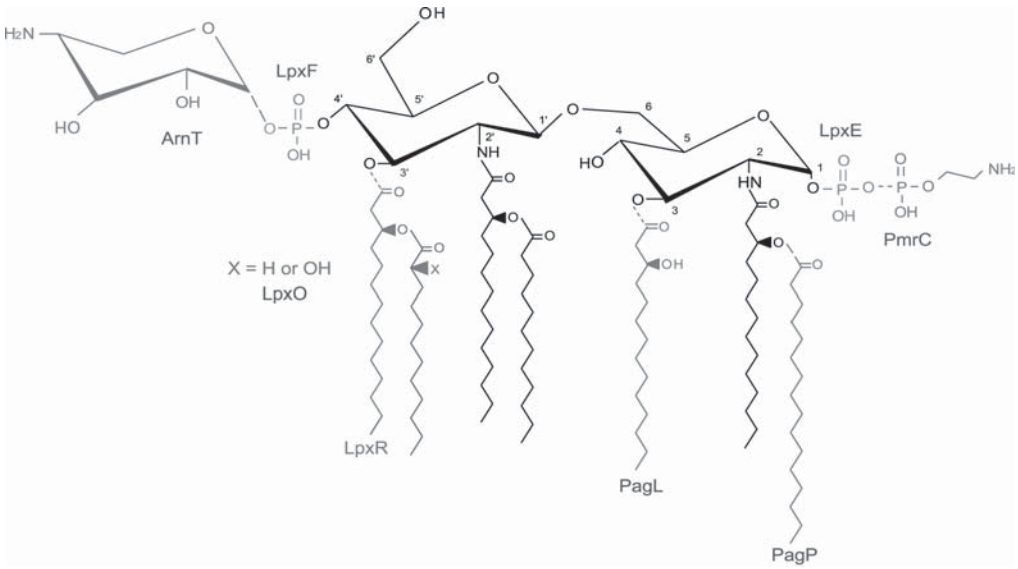

Fig. 9 Regulated modifications of lipid A. LpxE and LpxF function as 1- and 4'-phosphatases, respectively. Substitution of the phosphate moieties with L-Ara4N or pEtN is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary palmitoyl chain at the 2-position by PagP, and the removal of the acyl chain moieties at the 3- and 3' position by PagL and LpxR, respectively.

chain (C16) at the 2 position of lipid A (Bishop *et al.*, 2000). Palmitoylation of lipid A leads to an increased resistance of the bacterium to cationic anti-microbial peptides (Guo *et al.*, 1998). Furthermore, palmitoylated LPS is attenuated for signalling through TLR4/MD-2 (Janusch *et al.*, 2002). Homologs of PagP are found, amongst others, in *S.* Typhimurium, *Legionella pneumophila*, *E. coli*, and *Yersinia pestis* (Bishop *et al.*, 2000; Robey *et al.*, 2001). A PagP homolog, which, in contrast to *E. coli* and *Salmonella* PagP, adds a secondary C16 chain at the 3' position of lipid A, has been identified in *B. bronchiseptica* (Preston *et al.*, 2003). In *B. pertussis*, however, expression of PagP seems to be abolished through insertion of an IS element in the promoter region (Preston *et al.*, 2003). It was shown that PagP is required for resistance to antibody-mediated complement lysis during *B. bronchiseptica* respiratory infection (Pilione *et al.*, 2004). Another outer membrane-localised lipid A-modifying enzyme is the 3-*O*-deacylase PagL (Trent *et al.*, 2001a). This enzyme was discovered in *S.* Typhimurium and shown to hydrolyse the ester bond at the 3 position of lipid A, thereby releasing the primary 3-hydroxymyristoyl moiety (Trent *et al.*, 2001a). At that time, no obvious homologs of this protein could be found in the nonredundant or unfinished microbial databases, except in the closely related strains *S. enterica* serovars Typhi and Paratyphi (Trent *et al.*,

2001a). Nevertheless, some other Gram-negative bacteria, including *Pseudomonas aeruginosa* (Ernst *et al.*, 1999), *R. leguminosarum* (Bhat *et al.*, 1994), *Helicobacter pylori* (Moran *et al.*, 1997), and *Porphyromonas gingivalis* (Kumada *et al.* 1995) contain 3-*O*-deacylated lipid A species, suggesting that these organisms contain enzymes with a similar activity as PagL. Indeed, as shown in **chapter 2** of this thesis, *pagL* homologs are widely disseminated among Gram-negative bacteria. Recently, another lipid A-deacylase has been described. This enzyme, designated LpxR, is, like PagL, outer membrane localised and was shown to hydrolyse the ester bond at the 3' position of lipid A (Reynolds *et al.*, 2006).

LPS endotoxic activity: recognition and signalling by the LPS-receptor complex

LPS is also known as endotoxin, due to its endotoxic activity when administered to higher organisms. This endotoxic activity is based on the recognition of LPS by the host LPS-receptor complex, which leads to the activation of NF-κB and, consequently, to an increased production and secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α (Fig. 3). The first protein involved in LPS recognition is LPS-binding protein (LBP) (Schumann *et al.*, 1990). LBP is an acute-phase protein, produced in the liver, which recognises and binds the lipid A moiety of LPS, as free molecules, in aggregates, in membrane fragments, or still attached to the outer membrane of intact bacteria. The function of LBP appears to be that of a scaffolding protein, which recognises, binds and subsequently transfers LPS to the LPS-receptor complex present on host cells. After the primary interaction, the LBP-LPS complex first forms a ternary complex with the monocyte antigen CD14, after which LPS is transferred to the LPS receptor-complex consisting of TLR4 and the extracellular adaptor glycoprotein MD-2 (Hailman *et al.*, 1994; Tobias *et al.*, 1995). CD14 can be found in two forms. The first one, soluble CD14, resides in the plasma and allows LPS signalling in cells lacking membrane-bound CD14, e.g., endothelial and epithelial cells (Kitchens and Thompson, 2005). The other one, membrane-bound CD14, is linked to the surface of myeloid cells via a glycosylphosphatidylinositol tail (Simmons *et al.*, 1989). Currently, it is thought that the physiological role of CD14 is that of binding LPS and subsequently presenting it to the MD-2/TLR4 complex. However, despite all the evidence for a role of CD14 in LPS signalling, studies in CD14 knockout mice show a significant response of these mice to LPS, indicating that LPS can also be recognised independently of CD14 (Haziot *et al.*, 1996).

The secreted glycoprotein MD-2 acts as an extracellular adaptor protein and is essential for LPS signalling to occur (Schromm *et al.*, 2001; Visintin *et al.*, 2001).

MD-2 is thought to play a role in ligand recognition by TLR4. In immunoprecipitation studies, it was shown that LPS binds directly to the MD-2/TLR4 complex on the cell surface and that this interaction is dependent on CD14 (Akashi *et al.*, 2003). However, *in vitro*, CD14 is not needed for the interaction between LPS and soluble MD-2 to occur (Akashi *et al.*, 2003). A direct interaction of CD14 with the signalling complex could not be demonstrated, which is consistent with the notion that the main role of CD14 is the loading of LPS onto the MD-2/TLR4 complex (Akashi *et al.*, 2003).

TLR4 was the first TLR to be described and was originally designated human Toll (hToll) (Medzhitov *et al.*, 1997). It was shown that TLR4 is directly involved in innate immunity and that this could specifically be attributed to its intracellular Toll/IL-1R (TIR) domain (Medzhitov *et al.*, 1997). To date, 13 murine and 10 human TLRs have been described. The key features of these type 1 receptors are the extracellular leucine-rich repeats, a single transmembrane region, and the intracellular TIR domain. Small differences in the extra- and intracellular regions of TLRs give rise to distinct responses to specific microbial products. Recently, it was postulated that LPS with a conical shape, e.g., from *E. coli*, induces cytokine production through TLR4, whereas cylindrically shaped LPS, e.g., from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, as well as precursors and analogues of hexa-acylated lipid A, such as lipid IV_A and penta-acylated LPS, will lead to activation of TLR2 and may even act as antagonists to TLR4 (Netea *et al.*, 2002). In addition to causing MD-2/TLR4 to homodimerise, LPS also induces the association of several other combinations of signalling proteins with the receptor complex. In a recent study, it was shown that stimulation with hexa-acylated LPS and penta-acylated LPS induced different combinational associations of receptors (Triantafilou *et al.*, 2004). Thus, the physical form of the LPS determines the specific combinations and make-up of the TLR4/MD-2 receptor cluster and is thereby the critical determinant for the endotoxic properties of the LPS.

Upon clustering and activation of TLR4, several downstream signalling pathways become activated. This activation is dependent on a specialised subset of adapter proteins, including the myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR domain-containing adapter molecule (TRIF), and TRIF-related adapter molecule (TRAM). The TLR4-mediated response to LPS can be divided into two categories: an early MyD88-dependent response and a delayed MyD88-independent response (Fig. 10) (reviewed by Pålsson-McDermott and O'Neill, 2004).

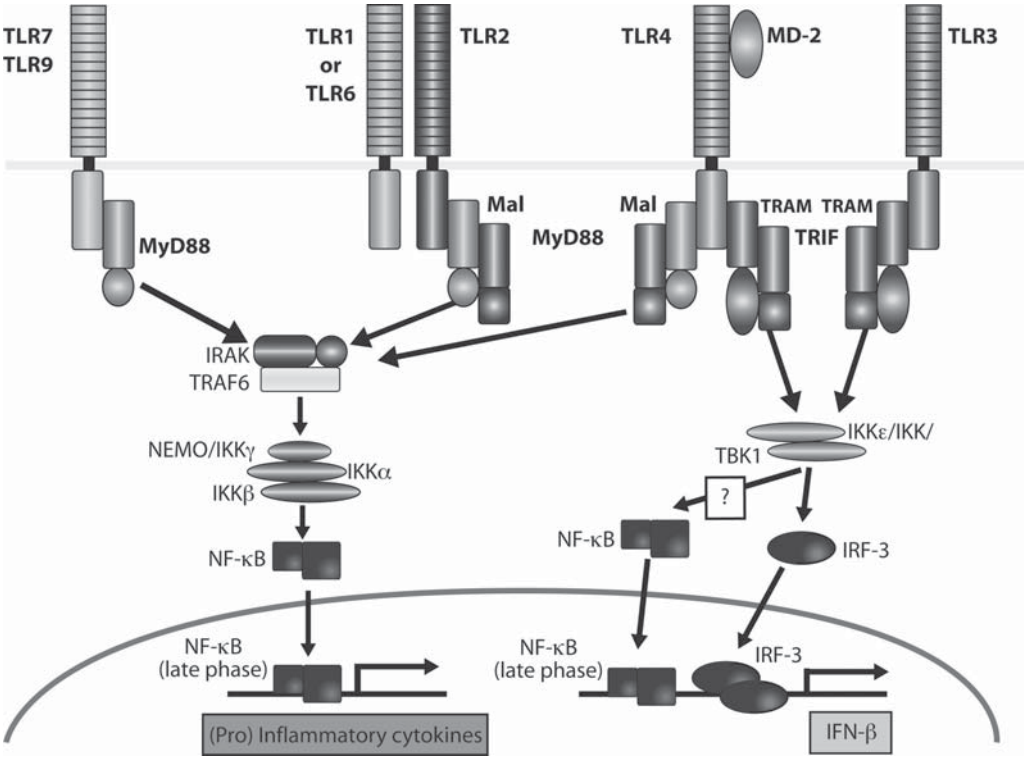

Fig. 10 TIR domain-containing adaptors and TLR signalling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via the majority of TLRs. Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In the TLR4- and TLR3-mediated signalling pathways, a MyD88-independent pathway exists that leads to activation of IRF-3 via TBK1 and IKKε/IKK*i*. The TIR domain-containing adaptor TRIF and the TRIF-related adapter molecule TRAM mediate this MyD88-independent pathway. Adapted from Takeda an Akira, 2004.

Endotoxic activity of *B. pertussis* LPS

Bordetella LPS is highly immunogenic, acts as an immunological adjuvant, and exhibits the properties that are expected of an endotoxin (Amano *et al.*, 1990; Chaby and Caroff, 1988; Watanabe *et al.*, 1990). It was previously shown that the strongest CD14-dependent LPS response requires the presence of an O-antigen, six acyl chains in lipid A, and two Kdo units (Gangloff *et al.*, 1999). With the knowledge that *B. pertussis* LPS has no O-antigen, only five acyl chains in lipid A, and only one Kdo residue (Fig. 6), it is not unexpected that this LPS was found to be far less active in endotoxic activity assays than for example *E. coli* and *Neisseria meningitidis* LPS (Laude-Sharp *et al.*, 1990). Moreover, *B. pertussis* lipid A was found to be a weak inducer of cytokines in humans (Caroff *et al.*, 1986). As indicated before, the composition of LPS from different

Bordetella species can vary significantly. In a recent study, in which the endotoxic activity of three different *Bordetella* species was compared, it was shown that *B. bronchiseptica* LPS induces a stronger TNF-α response in murine macrophages than both *B. pertussis* and *B. parapertussis* LPS. It was postulated that a reduction in LPS toxicity has been a strategy for adaptation to the human host (Mann *et al.*, 2005).

Modulation of LPS endotoxic activity

The endotoxic activity of LPS is mainly determined by the composition of its lipid A moiety (Homma *et al.*, 1985). Previous studies have shown that the phosphate groups as well as the number and length of the acyl chains are critical determinants of the endotoxic activity of lipid A and that changes in lipid A composition often modulate its biological activity (Loppnow *et al.*, 1989; Raetz and Whitfield, 2002; Steeghs *et al.*, 2002). Basically, there are two ways of obtaining bacteria that contain lipid A with altered physico/chemical properties. The first one is by making mutations or alterations in the lipid A biosynthesis route, the other one by making use of the newly identified LPS-modifying enzymes.

Proof of principal that alterations in the lipid A biosynthesis route can lead to LPS with an altered endotoxic activity has been provided in multiple studies (Somerville *et al.*, 1996; Low *et al.*, 1998). Recently, it was shown that *N. meningitidis* strains, deficient for the late acyltransferases LpxL1 or LpxL2, display a dramatically decreased endotoxic activity (van der Ley *et al.*, 2001). When these LPSs were tested for their adjuvant activity, tetra-acylated *lpxL2* mutant LPS proved to be poorly immunostimulatory, but the penta-acylated *LpxL1* mutant LPS was still capable of boosting the immune response up to wild-type levels (van der Ley *et al.*, 2001). Another example is a study, again in *N. meningitidis*, in which the endogenous *lpxA* gene was substituted by *lpxA* genes from different bacteria resulting in LPS with different fatty acyl chain lengths at the 3 and 3' positions (Steeghs *et al.*, 2002). These LPSs showed an adjuvant activity comparable to wild-type LPS, yet the endotoxic activity was reduced 10-fold (Steeghs *et al.*, 2002). These results indicate that manipulation of the lipid A biosynthesis route can lead to novel LPS species that are more suitable for inclusion in human vaccines. However, a problem with these genetic approaches is that mutations in the lipid A biosynthesis route often lead to severe phenotypes or sometimes even to non-viability. For example, the introduction of heterologous *lpxA* genes in *N. meningitidis* led to reduced amounts of LPS (Steeghs *et al.*, 2002) and an *E. coli lpxL* mutant shows a growth defect above 33^{o}C (Karow *et al.*, 1991). So the approach of reducing LPS endotoxic activity by genetically altering the lipid A biosynthesis route may not always be straightforward.

A second approach to alter the structure of LPS is by making use of enzymes that specifically modify the lipid A part of LPS. The main advantage of this approach is that expression of these enzymes can be easily controlled by making use of inducible promoters, and that at least some of these enzymes work on fully assembled lipid A, meaning that the biosynthesis route remains unaltered and that possible problems and feedback mechanisms as a consequence of alterations in the biosynthesis route are excluded. Also the comprehension that already about a dozen of these enzymes has been identified, all with their own unique activity, and that new enzymes are still regularly being discovered, emphasises that the opportunities of creating novel LPS forms using lipid A-modifying enzymes, or combinations of them, are wide-ranging.

Aims and outline of this thesis

LPS is most likely one of the major causes of the relatively high reactogenicity of wP vaccines. At present, the majority of developing countries uses wP vaccines because they are cheap, relatively effective, and easy to produce, whereas most industrialised countries have switched to less reactogenic, but more expensive aP vaccines. These latter vaccines do not contain LPS and were shown to be comparably effective. Yet, aP vaccines have, as compared to wP vaccines, some important drawbacks. These include high production costs, prohibiting administration in the developing world, the potential to rapidly select for escape mutants due to the small number of purified antigens on which aP vaccines are based, and the comprehension that aP vaccines evoke a different immunological response (Th2-type response) than do wP vaccines and natural infection, which elicit Th1-type immune responses. These notions, together with the problems described above concerning pertussis re-emergence and alterations in age distribution, emphasise the importance of the development of improved pertussis vaccines. In this thesis, we explore the possibility of improving pertussis vaccines by altering their LPS content either with the help of LPS-modifying enzymes (**chapters 2** to **5**), by adding non-toxic LPS derivatives (**chapters 6** and **7**), or by changing LPS biosynthesis (**chapters 8** and **9**). The work is mainly focused on the development of a less-reactogenic wP vaccine; however, as demonstrated in **chapter 7**, LPS (derivatives) may also be useful for the improvement of aP vaccines.

In **chapter 2**, we identified functional homologs of the LPS-modifying enzyme PagL in a large variety of Gram-negative bacteria, including *Bordetella spp.* and *P. aeruginosa*. We demonstrate that two completely conserved residues, i.e., a histidine and a serine, function as active-site residues, providing the first clue that PagL functions

as a serine hydrolase. In **chapter 3**, we describe the crystal structure of PagL from *P. aeruginosa* to 2.0-Å resolution. PagL was found to consist of an 8-stranded β-barrel. The analysis revealed that PagL contains an active site with a Ser-His-Glu catalytic triad and an oxyanion hole that comprises a conserved Asn. *In silico* modelling revealed that the 3-*O*-acyl chain is accommodated in a hydrophobic groove perpendicular to the membrane plane. In addition, we show that a particular Asp residue probably provides specificity of PagL towards lipid A. In **chapter 4**, we explored the consequences of PagL and PagP expression on the *in vitro* toxicity of *B. pertussis*. We show that expression of PagP and PagL in *B. pertussis* leads to an increased and decreased endotoxic activity of the LPS, respectively. As expected, PagP activity also results in an increased endotoxic activity of the whole bacterial cells. However, more unexpectedly, this was also the case for PagL. We show that this paradoxical result may be explained, in part, by an increased release of LPS from the PagL-expressing cells. In **chapter 5**, we assess the consequences of PagP and PagL expression on the efficacy and reactogenicity of wP vaccines. By evaluating vaccine efficacy, *B. pertussis*-specific antibody responses, and cytokine profiles, we show that expression of PagL, but not of PagP, significantly increases vaccine efficacy without altering vaccine reactogenicity. In **chapter 6**, we explore the consequences of supplementing wP vaccines with LPS analogs that are capable of modulating immune responses. We show that addition of monophosphoryl lipid A (MPL) increases vaccine efficacy without changing the reactogenicity. Moreover, we demonstrate that *N. meningitidis* LpxL2 LPS functions as an LPS antagonist and that supplementation of this LPS to wP vaccines not only decreases their reactogenicity, but also increases their efficacy. In **chapter 7**, we evaluated the effect of supplementing a combined diphtheria, tetanus, aP (DTaP) vaccine with various adjuvants. Three different adjuvants were studied: aluminum phosphate, MPL, and LpxL2 LPS. By determining vaccine efficacy, antibody responses, histological alterations, and cytokine (expression) profiles, we show that the DTaP vaccines supplemented with the LPS analogs exhibited an increased efficacy as compared to the vaccine supplemented with aluminium as an adjuvant. Interestingly, we found that supplementation of the vaccine with LPS analogs decreased type-1 hypersensitivity and re-directed the immune response towards a Th1-type response. Finally, in the last two experimental chapters, we altered LPS composition by manipulating the LPS biosynthesis route. In **chapter 8**, we identified two lipid A late acyltransferase homologues in the genome of *B. pertussis*. We show that one of them, LpxL2, is responsible for the addition of the secondary C14 group that is normally present at the 2' position of *B. pertussis* lipid A, whereas the other one, LpxL1, mediates the addition of a previously unrecognised secondary 2-OH C12. In addition,

we demonstrate that an *lpxL1*-deficient mutant of *B. pertussis* displays alterations in outer membrane integrity, reactogenicity, and haemolytic activity, as well as a defect in the infection of human macrophages. The goal of **chapter 9** was to identify and characterise new LPS glycosyltransferase homologs in the *B. pertussis* genome by using sequences of known LPS glycosyltransferases as leads. Via this approach, we identified a four-gene operon. By insertionally inactivating the genes and studying the resulting LPS structures, we show that at least two of the genes encode active LPS glycosyltransferases. In addition, we demonstrate that mutations in the operon differentially affect DC maturation and macrophage activation. Interestingly, during our analysis, we also found a previously unknown modification of lipid A with hexosamine. Finally, in **chapter 10**, we summarise and discuss the results obtained and present suggestions for the improvement of current pertussis vaccines.

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Chapter 2

Dissemination of lipid A deacylases (PagL) among Gram-negative bacteria

IDENTIFICATION OF ACTIVE-SITE HISTIDINE AND SERINE RESIDUES

Jeroen Geurtsen, Liana Steeghs, Jan ten Hove,
Peter van der Ley, and Jan Tommassen

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Abstract

Lipopolysaccharide (LPS) is one of the main constituents of the Gram-negative bacterial outer membrane. It usually consists of a highly variable O-antigen, a less variable core oligosaccharide, and a highly conserved lipid moiety, designated lipid A. Several bacteria are capable of modifying their lipid A architecture in response to external stimuli. The outer membrane-localised lipid A 3-*O*-deacylase, encoded by the *pagL* gene of *Salmonella enterica* serovar Typhimurium, removes the fatty acyl chain from the 3 position of lipid A. Although a similar activity was reported in some other Gram-negative bacteria, the corresponding genes could not be identified. Here, we describe the presence of *pagL* homologs in a variety of Gram-negative bacteria. Although the overall sequence similarity is rather low, a conserved domain could be distinguished in the C-terminal region. The activity of the *Pseudomonas aeruginosa* and *Bordetella bronchiseptica pagL* homologs was confirmed upon expression in *Escherichia coli*, which resulted in the removal of an *R*-3-hydroxymyristoyl group from lipid A. Upon deacylation by PagL, *E. coli* lipid A underwent another modification, which was the result of the activity of the endogenous palmitoyl transferase PagP. Furthermore, we identified a conserved histidine-serine couple as -residues, suggesting a catalytic mechanism similar to serine hydrolases. The biological function of PagL remains unclear. However, because PagL homologs were found in both pathogenic and non-pathogenic species, PagL-mediated deacylation of lipid A probably does not have a dedicated role in pathogenicity.

Introduction

Lipopolysaccharide (LPS), a major component of the Gram-negative bacterial outer membrane, is known to be important for the functioning of this membrane as a permeability barrier and for the resistance against complement-mediated cell lysis (for review, see Raetz and Whitfield, 2002). It consists of three covalently linked domains: lipid A, the core, and the O-antigen. Lipid A forms the hydrophobic membrane anchor and is responsible for the endotoxic activity of LPS. In *Escherichia coli*, it consists of a 1, 4'-bisphosphorylated β-1,6-linked glucosamine disaccharide, which is replaced by *R*-3-hydroxymyristic acid residues at positions 2, 3, 2', and 3' via ester or amide linkage. Secondary lauroyl and myristoyl groups replace the hydroxyl group of *R*-3-hydroxymyristoyl at the 2' and 3' positions, respectively (Fig. 1A). Previous studies have shown that the phosphate groups, the glucosamine disaccharide, and the correct number and length of the acyl chains are important for the biological activity of lipid A (Raetz and Whitfield, 2002; Loppnow *et al.*, 1989; Steeghs *et al.*, 2002). The basic structure of lipid A is reasonably well conserved among Gram-negative bacteria, although slight variations in the pattern of the substitutions of the two phosphates and the acyl chain number and length are observed (Nikaido and Vaara, 1987; Caroff *et al.*, 2002). Additional modifications of lipid A (Fig. 1B) are regulated in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) by the two-component regulatory system PhoP/PhoQ (Guo *et al.*, 1997; Guo *et al.*, 1998). In response to low Mg^{2+} levels, the sensor kinase PhoQ phosphorylates and thereby activates the transcriptional activator PhoP, which leads to the activation or repression of 40 different genes (Guo *et al.*, 1997; Gunn *et al.*, 1998a). A second regulatory system involved in lipid A modification is the PmrA/PmrB two-component system, which itself is PhoP/PhoQ-regulated (Gunn *et al.*, 1998b; Gunn *et al.*, 2000). Mutants with alterations in the PhoP/PhoQ system exhibit reduced virulence and an increased susceptibility to anti-microbial peptides (Miller *et al.*, 1989; Gunn and Miller, 1996). Homologs of the PhoP/PhoQ and PmrA/PmrB systems have been identified in other Gram-negative bacteria, including *E. coli, Yersinia pestis*, and *Pseudomonas aeruginosa* (Ernst *et al.*, 1999a; Ernst *et al.*, 1999b).

Until now, several lipid A-modifying enzymes have been identified (Fig. 1B). Substitution of the 1- and 4'-phosphate groups with one or two 4-amino-4-deoxy-L-arabinose (L-Ara4N) moieties in *S.* Typhimurium was found to be dependent on the enzyme ArnT (Trent *et al.*, 2001b). Recently, the PmrC protein was identified to mediate the addition of phosphoethanolamine to lipid A in *S. enterica* (Lee *et al.*, 2004). Another enzyme, designated LpxO, catalyses the O_2-dependent hydroxylation of lipid A (Gibbons *et al.*, 2000), and a lipid A 1-phosphatase was identified in *Rhizobium leguminosarum*

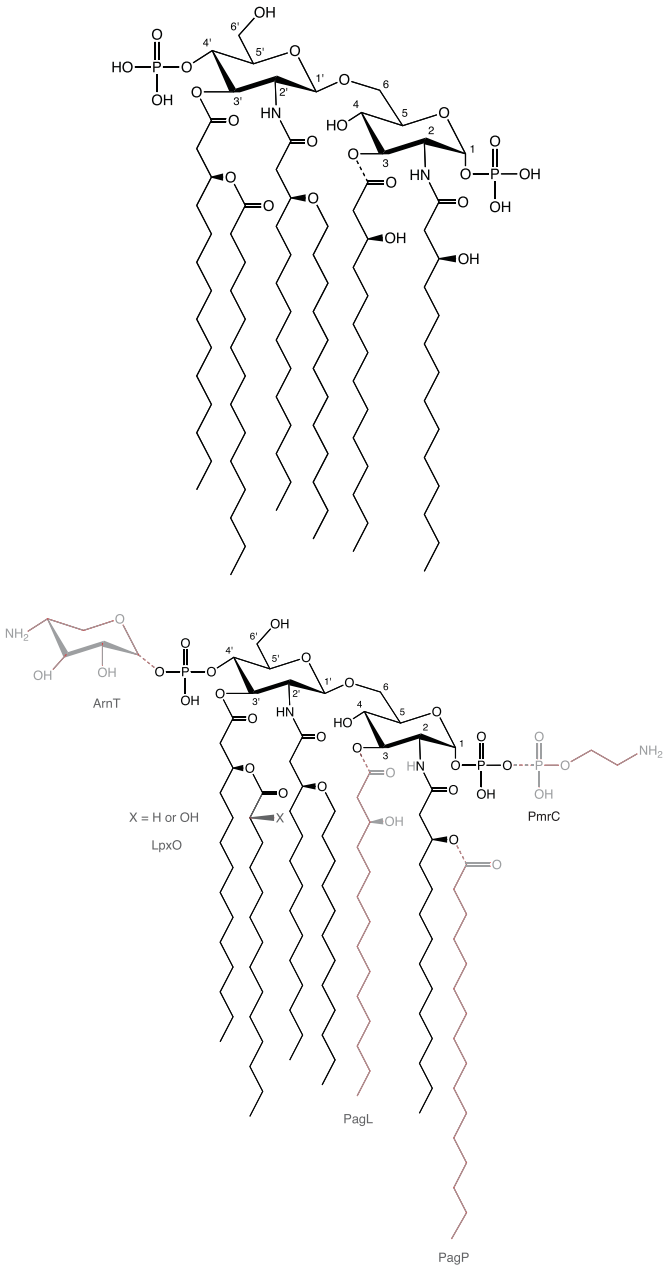

Fig. 1 Lipid A architecture. (A) *E. coli* lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with four *R*-3-hydroxymyristoyl moieties, of which the 2'- and 3'-fatty-acyl chains are esterified with laurate and myristate, respectively. (B) regulated modifications of *Salmonella* lipid A. Substitution of the phosphate moieties with L-Ara4N or phosphoethanolamine is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary palmitoyl chain at the 2 position by PagP, and the removal of the 3-hydroxymyristoyl moiety at the 3 position by PagL are shown.

(Karbarz *et al*, 2003). All these enzymes are thought to reside within the inner membrane or periplasmic space (Trent *et al.*, 2001b; Lee *et al.*, 2004; Gibbons *et al.*, 2000; Karbarz *et al.*, 2003). Recently, a new class of outer membrane-localised lipid A-modifying enzymes was discovered. One of them is the palmitoyl transferase PagP (Bishop *et al.*, 2000). Palmitoylation of lipid A leads to an increased resistance to cationic antimicrobial peptides (Guo *et al.*, 1998). Furthermore, palmitoylated lipid A antagonises LPS-induced activation of human cells (Tanamoto and Azumi, 2000). Homologs of PagP are found, among others, in *S.* Typhimurium, *Bordetella pertussis, Bordetella bronchiseptica, Bordetella parapertussis, Legionella pneumophila, E. coli*, and *Y. pestis* (Bishop *et al.*, 2000; Robey *et al.*, 2001). Another outer membrane-localised lipid A-modifying enzyme is the 3-*O*-deacylase PagL (Trent *et al.*, 2001a). This enzyme was discovered in *S.* Typhimurium and shown to hydrolyse the ester bond at the 3 position of lipid A, thereby releasing the primary 3-hydroxymyristoyl moiety (Trent *et al.*, 2001a). At that time, no obvious homologs of this protein could be found in the nonredundant or unfinished microbial databases, except in the closely related strains *S. enterica* serovars Typhi and Paratyphi (Trent *et al.*, 2001a). Nevertheless, some other Gram-negative bacteria, including *P. aeruginosa* (Ernst *et al.*, 1999b), *R. leguminosarum* (Bhat *et al.*, 1994), *Helicobacter pylori* (Moran *et al.*, 1997), and *Porhyromonas gingivalis* (Kumada *et al.*, 1995) contain 3-*O*-deacylated lipid A species, suggesting that these organisms contain enzymes with an activity similar to that of PagL. We report now the identification of PagL homologs in a variety of Gram-negative bacteria. The limited sequence similarity among the various proteins was used to identify active-site residues.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are described in Table 1. Unless otherwise notified, the *E. coli* and *P. aeruginosa* strains were grown at 37°C and 30°C, respectively, in a modified Luria-Bertani broth, designated LB (Tommassen *et al.*, 1983), supplemented with 0.2% glucose, or in minimal medium (SV) (Winkler and de Haan, 1949) supplemented with 0.5% glucose, while shaking at 200 rpm. To induce expression of the *pagL* genes cloned behind the T7 promoter, the bacteria were grown in LB supplemented with glucose until an absorbance at 600 nm (A_{600}) of 0.4–0.6 was reached. Expression of the *pagL* genes was then induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and incubation at 37°C was continued. When appropriate, bacteria were grown in the presence of 100 μg/ml ampicillin, 50 μg/ml kanamycin, 10 μg/ml tetracycline, or

100 μg/ml streptomycin, for plasmid maintenance. *S.* Typhimurium SR11 was grown on LB agar plates at 37°C. *B. bronchiseptica* and *B. pertussis* strains were grown at 35°C on Borduet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading). Stain JG101, carrying a tetracycline-resistance transposon insertion in *pagP*, was obtained by P1 transduction by using *E. coli* BL21 Star™ (DE3) and *E. coli* SK2257 (Table 1) as the acceptor and donor, respectively.

TABLE 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
B. bronchiseptica		
B505	Wild-type strain	N.V.I.[a]
B. pertussis		
BP509	Dutch vaccine strain	N.V.I.[a]
BP134	Dutch vaccine strain	N.V.I.[a]
P. aeruginosa		
PAO1	Wild-type strain	Jacobs *et al.*, 2003
PAO25	PAO1 *leu arg*	Haas and Holloway, 1976
#32751	PA4661 (*pagL*) mutant-derivative of PAO1	Jacobs *et al.*, 2003
S. Typhimurium		
SR11	Wild-type strain	Pace *et al.*, 1993
E. coli		
TOP10F'	*F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	*F$^-$ Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	Hanahan, 1983
BL21 Star™ (DE3)	*F$^-$ ompT hsdS B (rB$^-$ mB$^-$) gal dcm rne131 (DE3)*	Invitrogen
SK2257	*F$^-$ crcA280::Tn10^c thyA6 rpsL120(Str^R) deoC1*	CGSC[b]
JG101	BL21 Star™ (DE3) *crcA280::Tn10^c*	This study
Plasmids		
pCRII-TOPO	*E. coli* cloning vector Amp^R Kan^R	Invitrogen
pET-11a	*E. coli* high-copy expression vector, Amp^R, T7 promotor	Novagen
pPagL$_{(Pa)}$	pET-11a derivative harboring *P. aeruginosa pagL*	This study
pPagL$_{(Bb)}$	pET-11a derivative harboring *B. bronchiseptica pagL*	This study
pPagL$_{(St)}$	pET-11a derivative harboring *S.* Typhimurium *pagL*	This study
pPagL$_{(Pa)}$(-)	pET-11a derivative encoding *P. aeruginosa* PagL without signal sequence	This study
pPagL$_{(Pa)\ (H81A)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with H81A substitution	This study
pPagL$_{(Pa)\ (H81N)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with H81N substitution	This study
pPagL$_{(Pa)\ (S84A)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S84A substitutiion	This study
pPagL$_{(Pa)\ (S84C)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S84C substitution	This study
pPagL$_{(Pa)\ (H149A)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with H149A substitution	This study
pPagL$_{(Pa)\ (H149N)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with H149N substitution	This study
pPagL$_{(Pa)\ (S151A)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S151A substitution	This study
pPagL$_{(Pa)\ (S151C)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S151C substitution	This study

[a] Netherlands Vaccine Institute, Bilthoven, The Netherlands

[b] *E. coli* genetic stock center, Yale university, New Haven (CT)

[c] *pagP* is also known as *crcA*

Recombinant DNA techniques

The plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega Wizard® *Plus* SV Minipreps system. Calf intestine alkaline phosphatase and restriction endonucleases were used according to the instructions of the manufacturer (Fermentas). DNA fragments were isolated from agarose gels using the Qiagen quick gel extraction kit. Ligations were performed by using the rapid DNA ligation kit (Roche Applied Science).

The *pagL* genes from *S.* Typhimurium SR11 ($pagL_{(St)}$), *B. bronchiseptica* B505 ($pagL_{(Bb)}$), and *P. aeruginosa* PAO25 ($pagL_{(Pa)}$) were cloned into pET-11a (Novagen) behind the T7 promoter. The genes were amplified by PCR using chromosomal DNA as template. Template DNA was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x *g*, after which the supernatant was used as template DNA. The sequences of the forward primers, which contained an NdeI site (underlined), including an ATG start codon, were 5'-AACATATGAAGAGAATATTTATATATC-3' ($pagL_{(St)}$), 5'-AACATATGAAGAAACTACTTCCGCTGG-3' ($pagL_{(Pa)}$), and 5'-AACATATGCAATTTCTCAAGAAAAACA-3' ($pagL_{(Bb)}$). The sequences of the reverse primers, which contained a BamHI site (underlined) and included a stop codon, were 5'-AAGGATCCTCAGAAATTATAACTAATT-3' ($pagL_{(St)}$), 5'-AAGGATCCCTAGATCGGGATCTTGTAG-3' ($pagL_{(Pa)}$), and 5'-AAGGATCCTCAGAACTGGTACGTATA-G-3' ($pagL_{(Bb)}$). The PCRs were done under the following conditions: 50-μl total reaction volume, 25 pmol of each primer, 0.2 mM dNTPs, 3 μl of template DNA solution, 1.5% dimethyl sulfoxide, 1.75 units of Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche Applied Science). The temperature program was as follows: 95°C for 3 min, a cycle of 1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C repeated 30 times, followed by 10 min at 72°C and subsequent cooling to 4°C. The PCR products were purified from agarose gel and subsequently cloned into pCRII-TOPO. Plasmid DNA from correct clones was digested with NdeI and BamHI, and the PagL-encoding fragments were ligated into NdeI/BamHI-digested pET-11a. The ligation mixture was used to transform *E. coli* DH5α using the $CaCl_2$ method (Sambrook *et al.*, 1989). Plasmid DNA from transformants was checked for presence of the correct PagL-encoding insert by digestion with NdeI and BamHI. Plasmids that gave a correct digestion profile were designated $pPagL_{(Pa)}$, $pPagL_{(Bb)}$, and $pPagL_{(St)}$ (Table 1). The correct coding sequences of the cloned *pagL* genes were confirmed by nucleotide sequencing in both directions. Mutations were introduced in *pagL* by using the QuikChange site-directed mutagenesis kit (Stratagene)

and the primers listed in Table 2. Plasmid $pPagL_{(Pa)}$ was used as the template in which the mutations were created. The presence of the correct mutations was confirmed by nucleotide sequencing in both directions.

TABLE 2
Primers used for site-directed mutagenesis

Name[a]	Sequence (5'-3')[b]
H81A_FW	GAAGGCGCCGGCAAGGCGTCGCTGTCGTTCGCT
H81A_REV	AGCGAACGACAGCGACGCCTTGCCGGCGCCTTC
H81N_FW	GAAGGCGCCGGCAAGAACTCGCTGTCGTTCGCT
H81N_REV	AGCGAACGACAGCGAGTTCTTGCCGGCGCCTTC
S84A_FW	GGCAAGCATTCGCTGGCGTTCGCTCCGGTATTC
S84A_REV	GAATACCGGAGCGAACGCCAGCGAATGCTTGCC
S84C_FW	GGCAAGCATTCGCTGTGCTTCGCTCCGGTATTC
S84C_REV	GAATACCGGAGCGAAGCACAGCGAATGCTTGCC
H149A_FW	GGCGTTCGGGCGATCGCGTATTCCAACGCCGGC
H149A_REV	GCCGGCGTTGGAATACGCGATCGCCCGAACGCC
H149N_FW	GGCGTTCGGGCGATCAACTATTCCAACGCCGGC
H149N_REV	GCCGGCGTTGGAATAGTTGATCGCCCGAACGCC
S151A_FW	CGGGCGATCCACTATGCGAACGCCGGCCTGAAA
S151A_REV	TTTCAGGCCGGCGTTCGCATAGTGGATCGCCCG
S151C_FW	CGGGCGATCCACTATTGCAACGCCGGCCTGAAA
S151C_REV	TTTCAGGCCGGCGTTGCAATAGTGGATCGCCCG

[a] The primer name gives the amino acid substitution, e.g. H81A_FW indicates that the oligonucleotide shown was used as the forward primer in a site-directed mutagenesis procedure to substitute the histidine at position 81 of the precursor $PagL_{(Pa)}$ by an alanine.
[b] Introduced mutations are underlined.

Isolation of cell envelopes

Cells were harvested by centrifugation for 10 min at 1,500 x *g* and washed once in 50 ml of cold 0.9% sodium chloride solution. The cell pellets were frozen for at least 15 min at -80°C and then suspended in 20 ml of 3 mM EDTA, 10 mM Tris-HCl (pH 8.0) containing Complete protease inhibitor mixture (Roche Applied Science). The cells were disrupted by sonication, after which unbroken cells were removed by centrifugation for 10 min at 1,500 x *g*. The cell envelopes were pelleted from the supernatant by centrifugation for 1.5 h at 150,000 x *g* and resuspended in 2 mM Tris-HCl (pH 7.4). The cell envelopes were stored at -80°C in aliquots.

SDS-PAGE and immunoblotting

Proteins were analysed by SDS-PAGE (Laemmli, 1970) with 0.2% SDS in the running gel using the Bio-Rad Mini-PROTEAN®3 apparatus. Samples were applied to a 13% polyacrylamide gel with a 4% stacking gel and subjected to electrophoresis at 150 V. Proteins were stained with Coomassie Brilliant Blue. Prestained or unstained Precision

Plus Protein™ Standard from Bio-Rad was used to determine the relative molecular mass. For Western blotting, proteins were transferred from SDS-polyacrylamide gels onto nitrocellulose membranes. The membranes were blocked overnight in phosphate-buffered saline (pH 7.6), 0.5% nonfat dried milk, 0.1% Tween 20 and incubated with guinea pig antibodies directed against $PagL_{(Pa)}$ in blocking buffer followed by an incubation with horseradish peroxidase-conjugated rabbit anti-guinea pig IgG antibodies (Sigma) in blocking buffer. Blots were developed using SuperSignal® WestPico Chemiluminescent Substrate (Pierce).

Polyclonal antibodies

For antibody production, the *pagL* gene from *P. aeruginosa* PAO25 without the signal sequence-encoding part was PCR amplified by using the forward primer (5'-AACATATGGCGGACGTCTCGGCCGCCG-3'), which contained an NdeI site (underlined), including an ATG start codon, and the reverse primer (5'-AAGGATCCCTAGATCGGGATCTTGTAG-3'), which contained an BamHI site (underlined) and included a stop codon. The PCR product was cloned into pET-11a, and the resulting plasmid, $pPagL_{(Pa)(-)}$, was used to transform *E. coli* BL21 Star™ (DE3) to allow for expression of the truncated *pagL* gene. The $PagL_{(Pa)}$ protein, accumulating in inclusion bodies, was isolated (Dekker *et al.*, 1995), purified from a preparative SDS-polyacrylamide gel, and used for immunisation of guinea pigs at Eurogentec.

Microsequencing

Proteins were transferred from SDS-polyacrylamide gels to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) in 192 mM glycine, 25 mM Tris (pH 8.3), 10% methanol (v/v) at 100 V for 1 h using the Bio-Rad Mini-PROTEAN®2 blotting apparatus. After transfer, the membrane was washed three times for 15 min with distilled water. Transferred proteins were stained with Coomassie Brilliant Blue. The membrane was dried in the air, and the putative PagL bands were excised and subjected to microsequencing at the Sequencing Center Facility, Utrecht University, The Netherlands.

LPS analysis by Tricine-SDS-PAGE

Approximately 10^9 bacteria were suspended in 50 μl of sample buffer (Laemmli, 1970), and 0.5 mg/ml proteinase K (end concentration) was added. The samples were incubated for 60 min at 55°C followed by 10 min at 95°C to inactivate proteinase K. The samples were then diluted 10-fold by adding sample buffer, after which 2 μl of

each sample was applied to a Tricine-SDS-polyacrylamide gel (Lesse *et al.*, 1990). The bromphenol blue was allowed to run into the separating gel at 35 V, after which the voltage was increased to 105 V. After the front reached the bottom of the gel, the samples were left running for another 45 min. The gels were fixed overnight in water/ethanol/acetic acid 11:8:1 (v/v/v) and subsequently stained with silver as described previously (Tsai and Frasch, 1982).

Gas chromatography-mass spectrometry (GC/MS) and electrospray ionisation-mass spectrometry (ESI/MS)

LPS was isolated using the hot phenol/water extraction method (Westphal and Jann, 1965). For fatty acid analysis by GC/MS, a 5-fold volume excess of acetone was added to an aliquot of the isolated LPS (0.2 mg/ml), after which the solution was dried at 60°C under a nitrogen flow. Subsequently, 10 μg of 2OH C12 (1 mg/ml in ethanol) was added as an internal standard, as well as 100 μl of acetylchloride/ethanol 1:9 (v/v), after which the samples were derivatised for 1 h at 90°C. After cooling, the reaction was stopped by adding 200 μl of 1 M K_2HPO_4 (pH 8.0), followed by extraction of the acylethyl esters with 200 μl of ethyl acetate. A 1-μl volume of the upper phase was used for analysis by GC/MS on a Finnigan MAT SSQ in the electron-impact mode. For ESI/MS, an aliquot of isolated LPS was freeze-dried and taken up in 1.8 ml of 12.5 mM sodium acetate (pH 4.5) containing 1% SDS. The mixture was boiled for 30 min to hydrolyse the LPS and release the lipid A moiety, after which the mixture was cooled to room temperature and converted into a two-phase Bligh and Dyer mixture by adding 2 ml of methanol and 2 ml of chloroform. Phases were separated by centrifugation, after which the lower phase was collected and washed twice with the upper phase of a fresh two-phase Bligh and Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). Structural analysis of purified lipid A was performed by nanoelectrospray tandem MS on a Finnigan LCQ in the negative ion mode (Wilm and Mann, 1996).

Results

Identification of PagL homologs in various Gram-negative bacteria

The 187-amino acid sequence of the *S.* Typhimurium PagL precursor protein (GenBank accession no. AAL21147) was used as a lead to identify putative PagL homologs in other Gram-negative bacteria, by searching all completed and unfinished genomes of Gram-negative bacteria present in the NCBI data base (www.ncbi.nlm.nih.gov/sutils/ genom_table.cgi). BLAST search (Altschul *et al.*, 1990) revealed the

presence of putative homologs in the *Bordetella* spp. *B. pertussis, B. bronchiseptica*, and *B. parapertussis* (Fig. 2). The PagL homologs of *B. bronchiseptica* and *B. parapertussis* are two mutually identical 178-amino acid polypeptides (Fig. 2) with, as predicted by the SignalP server (Nielsen *et al.*, 1999), a 25-amino acid N-terminal signal peptide. A gene for a PagL homolog was also found in the genome of the *B. pertussis* Tohama I strain (Parkhill *et al.*, 1999), but this open reading frame (ORF) was disrupted by a frame shift. Nucleotide sequencing of the PagL ORFs from *B. pertussis* strains BP509 and BP134 also showed the presence of the same frameshift,[1] which indicates that disruption of the PagL ORF might be a common feature in *B. pertussis* strains. By using the newly identified *B. bronchiseptica pagL* homolog as a probe for further BLAST analysis, additional putative *pagL* homologs could be identified in the genomes of *P. aeruginosa, Pseudomonas fluorescens, Pseudomonas syringae, Pseudomonas putida, Ralstonia metallidurans, Ralstonia solanacearum, Burkholderia mallei, Burkholderia pseudomallei, Burkholderia fungorum*, and *Azotobacter vinelandii* (Fig. 2). Together, all PagL homologs exhibited a low overall mutual sequence identity but contained a clear homologous domain near the C terminus.

Cloning of *pagL* and heterologous expression in *E. coli*

To verify their putative lipid A deacylase activity, we cloned the *pagL* homologs of *P. aeruginosa* ($pagL_{(Pa)}$) and *B. bronchiseptica* ($pagL_{(Bb)}$). We included in these studies *pagL* from *S.* Typhimurium ($pagL_{(St)}$) as a reference. These *pagL* genes were amplified from the chromosomes by PCR and eventually cloned into pET-11a under the control of the T7 promoter, resulting in plasmids, $pPagL_{(Pa)}$, $pPagL_{(Bb)}$, and $pPagL_{(St)}$. To investigate expression and membrane localisation of PagL in *E. coli, E. coli* BL21 Star™ (DE3) containing the empty vector pET-11a or the pPagL plasmids was grown overnight in LB, after which cell envelopes were isolated. Analysis by SDS-PAGE revealed the presence of prominent additional bands with molecular masses of 15,000–18,000 in the cell envelopes of the cells expressing PagL (Fig. 3). This was consistent with the expected molecular masses of the mature PagL proteins, i.e., $PagL_{(Pa)}$ 16.1 kDa, $PagL_{(Bb)}$ 17.2 kDa, and $PagL_{(St)}$ 18.2 kDa. To identify the additional protein bands, they were subjected to microsequencing. The sequences of the first 5 amino acid residues of $PagL_{(Pa)}$, $PagL_{(Bb)}$, and $PagL_{(St)}$ were ADVSA, QPTQG, and NDNVF, respectively, indicating that cleavage of the signal peptide by leader peptidase occurs between amino acid residues 23 and 24 (AQA and ADV), 25 and 26 (AQA and QPT), and between 20 and 21 (CSA and NDN), respectively. Particularly in the case of expression of $PagL_{(Bb)}$, an additional band with a higher molecular mass was visible on the gel (Fig. 3).The N-terminal sequence of this band, MQFLK, corresponded with that of the precursor of $PagL_{(Bb)}$.

[1] H. J. Hamstra and P. van der Ley, unpublished observation.

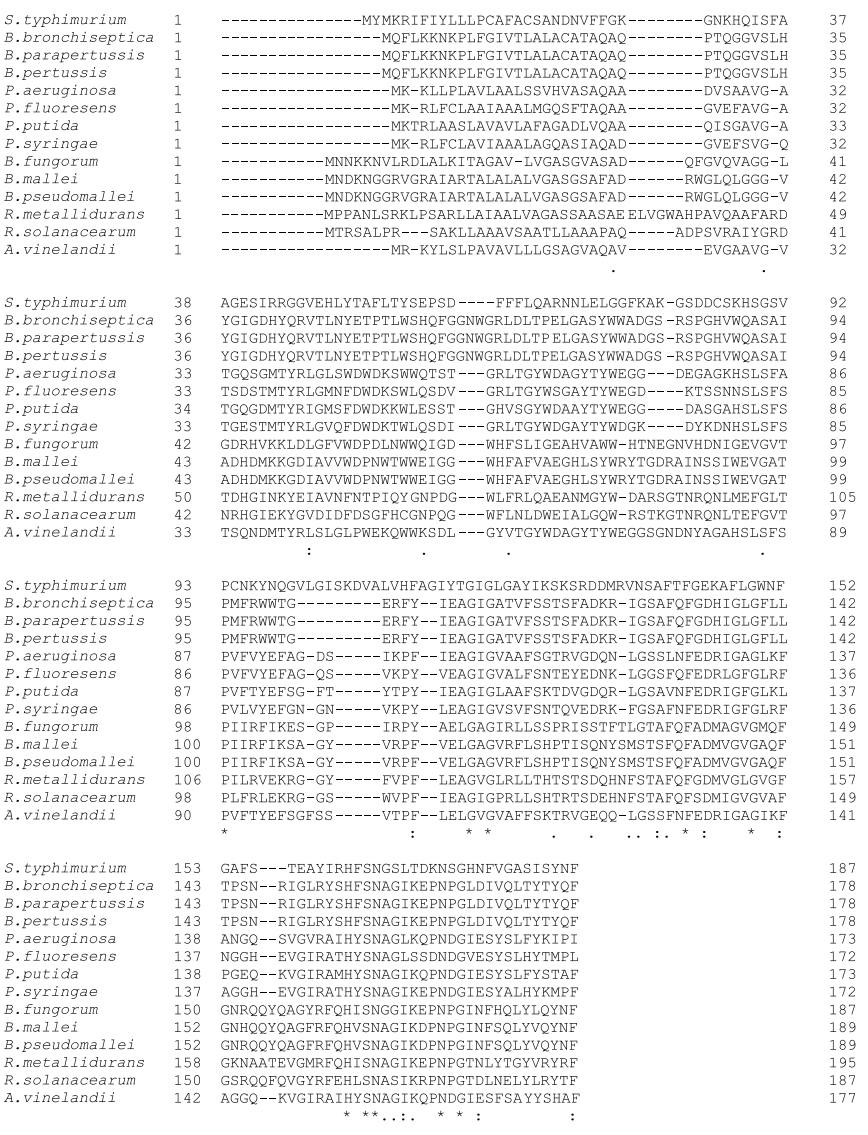

Fig. 2 Multiple sequence alignment of the PagL proteins. Sequences were aligned using ClustalW (www.ch.embnet.org/software/ClustalW.html). Hyphens indicate gaps introduced for optimal alignment. Absolutely conserved residues are marked with asterisks. Colons and dots indicate strongly and weakly conserved residues, respectively. The *pagL* ORF in *B. pertussis* is disrupted by a frameshift, which was restored for this alignment by adding two nucleotides in codon 33. The GenBank™ protein accession nos. for the PagL homologs are: *S.* Typhimurium, AAL21147; *B. bronchiseptica*, NP_890306; *B. parapertussis*, NP_885487; *B. pertussis*, BX470248§; *P. aeruginosa*, NP_253350; *P. fluorescens*, NZ_AAAT03000006§; *P. putida*, NC_002947§; *P. syringae*, ZP_00125465; *B. fungorum*, NZ_AAAJ03000003§; *B. mallei*, NC_002970§; *B. pseudomallei*, NC_002930§; *R. metallidurans*, ZP_00274744; *R. solanacearum*, NP_522762; and *A. vinelandii*, ZP_00089534. The symbol § indicates GenBank™ accession numbers of whole (unfinished) genomes, in which the PagL homologs were identified manually.

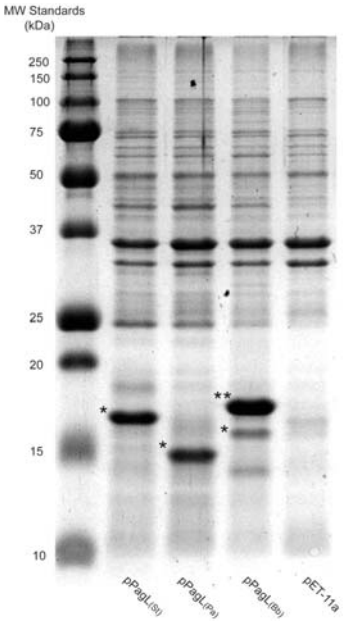

Fig. 3 Expression and membrane localisation of PagL in *E. coli* BL21 Star™ (DE3). Membranes from *E. coli* BL21 Star™ (DE3) containing empty vector pET-11a or the pPagL plasmids were isolated and analysed by SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue. *Asterisks* indicate the bands that were subjected to microsequencing and were found to correspond to the mature PagL proteins. The band indicated by the double asterisk corresponds to the $PagL_{(Bb)}$ precursor protein. Molecular mass standard proteins are present on the left side.

In vivo modification of *E. coli* LPS by PagL

To study whether the cloned PagL homologs were active on *E. coli* LPS, IPTG was added to exponentially growing *E. coli* BL21 Star™ (DE3) cells containing the empty vector pET-11a or the pPagL plasmids, and after various incubation periods, samples equivalent to one A_{600} unit were collected, and their LPS content was analysed by Tricine-SDS-PAGE. In accordance with the expected hydrolysis of the *R*-3-hydroxymyristate at the 3 position of lipid A, expression of any of the three *pagL* homologs converted the LPS into a form with a higher electrophoretic mobility (Fig. 4). The conversion was almost complete within 75 min after $PagL_{(Pa)}$ or $PagL_{(Bb)}$ was induced but took somewhat longer in the case of $PagL_{(St)}$. To test whether the efficiency of *in vivo* modification in *E. coli* was dependent on growth temperature (30, 37, or 42°C), the presence of magnesium chloride (10 mM), time of PagL induction during the growth phase (early log phase, mid log phase, or stationary phase), or nutrient availability (rich medium (LB), or minimal medium (SV)), $PagL_{(Pa)}$ was expressed in *E. coli* under various conditions, after which the LPS profile was analysed by Tricine-SDS-PAGE. Under all conditions tested, no obvious changes in deacylation efficiency could be observed (data not shown).

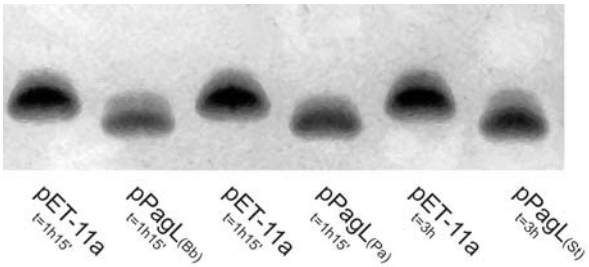

Fig. 4 Analysis by Tricine-SDS-PAGE of LPS modification *in vivo*. Exponentially growing *E. coli* BL21 Star™ (DE3) cells containing pET-11a or the pPagL constructs were induced with IPTG for the indicated time, after which 1 A_{600} unit culture samples were collected and analysed by Tricine-SDS-PAGE.

Structural analysis of PagL-modified LPS

To determine its fatty acid content, LPS was isolated from bacteria that were grown in the presence of 10 mM $MgCl_2$ to suppress PhoP/PhoQ-regulated modifications of lipid A and analysed by GC/MS. The C14/3OH C14 ratio in the PagL-modified LPS samples was increased compared with that in the wild-type LPS (Fig. 5), consistent with the expected removal of a 3OH C14 from lipid A. To confirm these data, the lipid A moieties were isolated and analysed by ESI/MS in the positive ion mode, which revealed the presence of four major lipid A species in wild-type LPS (Fig. 6A). The peak at *m*/*z* 1797 represents the characteristic hexaacylated *bis*-phosphate species that is typically found in *E. coli*, whereas the peak at *m*/*z* 1928 corresponds to a hexaacylated *bis*-phosphate species replaced with an L-Ara4N moiety. The two remaining peaks at *m*/*z* 1716 and *m*/*z* 1847 most likely represent fragment ions of the two former species missing a phosphate group. Upon expression of $PagL_{(St)}$ (Fig. 6B), $PagL_{(Pa)}$ (Fig. 6C), or $PagL_{(Bb)}$ (Fig. 6D), the major lipid A species were present at *m*/*z* 1622 and *m*/*z* 1490, which correspond to the loss of one β-hydroxymyristate residue and one phosphate group from the major species at *m*/*z* 1928 and *m*/*z* 1797 present in the empty vector control, respectively. Also here, the loss of the phosphate group is probably an artifact of the ionisation procedure. Based upon the GC/MS and ESI/MS data, it can be concluded that the identified PagL homologs of *P. aeruginosa* and *B. bronchiseptica*, like that of *S.* Typhimurium, are active lipid A deacylases. Furthermore, the data suggest that the deacylation is not dependent upon the absence or presence of an L-Ara4N moiety because both species were deacylated efficiently.

Subsequent *in vivo* modification of PagL-deacylated LPS

In the course of these experiments, it was observed that after prolonged PagL expression, PagL-modified LPS was no longer detectable on Tricine-SDS-PAGE and that the LPS migrated again at the position of wild-type LPS, as illustrated for the strain

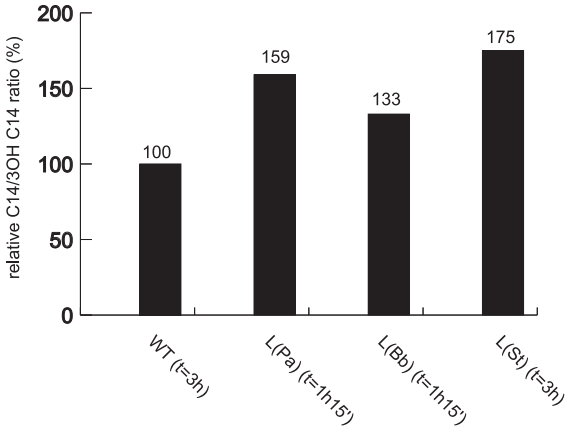

Fig. 5 GC/MS analysis of wild-type and PagL-modified *E. coli* BL21 Star™ (DE3) LPS. GC/MS analysis of purified *E. coli* BL21 Star™ (DE3) wild-type LPS (*WT*), $PagL_{(St)}$-modified LPS (*L*(*St*)), $PagL_{(Bb)}$-modified LPS (*L*(*Bb*)), and $PagL_{(Pa)}$-modified LPS (*L*(*Pa*)) (*t* = time after induction). Indicated are the normalised C14/30A C14 ratios with wild-type LPS set at 100 (values shown above bars).

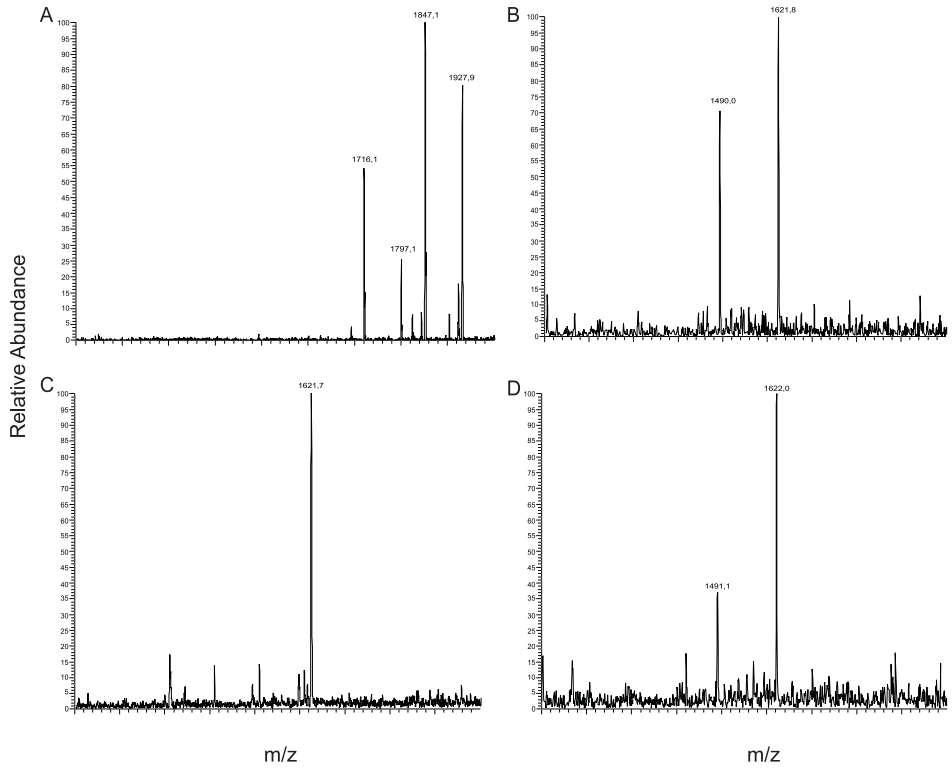

Fig. 6 Structural analysis by ESI/MS of wild-type and PagL-modified *E. coli* BL21 Star™ (DE3) LPS. Lipid A species from wild-type *E. coli* BL21 Star™ (DE3) containing empty vector pET-11a (A) and lipid A species modified by $PagL_{(St)}$ (B), $PagL_{(Pa)}$ (C), and $PagL_{(Bb)}$ (D) were analysed by ESI/MS. Major peaks at *m*/*z* 1797, 1928, 1622, and 1490 were interpreted as the characteristic hexaacylated *bis*-phosphate species that is typically found in *E. coli*, a hexaacylated *bis*-phosphate species substituted with an L-Ara4N moiety, a 3-*O*-deacylated *mono*-phosphate species substituted with an L-Ara4N moiety, and a 3-*O*-deacylated *mono*-phosphate species, respectively. The major peaks at *m*/*z* 1716 and 1847 probably represent fragment ions of the species at *m*/*z* 1797 and 1928.

expressing $PagL_{(Bb)}$ (Fig. 7A). The PagL protein was still abundantly present at this time point, as revealed on SDS-PAGE (data not shown). Furthermore, analysis by GC/MS revealed that the C14/3OH C14 ratio was not decreased again for the LPS isolated after a 5-h induction of $PagL_{(Bb)}$ (Fig. 7B). Thus, the secondary modification observed on the Tricine-SDS-polyacrylamide gel (Fig. 7A) was not the consequence of restoration of the PagL modification, but the result of (an) additional modification(s) that restored the electrophoretic mobility to that of wild-type LPS. Therefore, other fatty acid ratios were compared. A striking increase in the C16/C14 ratio was found in the LPS of cells induced 5 h for PagL production (Fig. 7C), suggesting that the PagL-deacylated LPS was subsequently palmitoylated.

A protein that adds palmitate to lipid A is the outer membrane protein PagP (Bishop *et al.*, 2000) (Fig. 1). Therefore, we hypothesised that the secondary modification of PagL-modified LPS might have been the result of endogenous PagP activity. To investigate this possibility, we transformed wild-type *E. coli* BL21 Star™ (DE3) and its *pagP* mutant derivative JG101 with the $pPagL_{(Pa)}$ plasmid. The secondary modification of PagL-modified LPS was again observed in the case of the wild-type strain, but not in that of the mutant strain (Fig. 7D). This result strongly suggests that the secondary modification of PagL-modified LPS (Fig. 7A) was indeed the consequence of endogenous PagP activity.

Identification of PagL active-site residues

The mutual sequence identity between the identified PagL homologs is very low (Fig. 2). Among the few totally conserved residues are a histidine and a serine, which, we hypothesise, might be part of a “classical” Asp/Glu-His-Ser catalytic triad of serine hydrolases. These putative active-site residues are located at the lipid-exposed side near the top of a β-strand in a topology model we propose (Fig. 8). Interestingly, in the outer membrane phospholipase A (OMPLA), the active-site His and Ser are located in a similar position (Snijder *et al.*, 1999). To test whether these residues, located at positions 149 and 151 of the $PagL_{(Pa)}$ precursor protein, respectively, are indeed important for catalytic activity, they were replaced by alanine or asparagine, and by alanine or cysteine, respectively. As a control, the same substitutions were made for a non-conserved histidine and serine residue, located at positions 81 and 84 of the $PagL_{(Pa)}$ precursor, respectively. The protein and LPS profiles of *E. coli* BL21 Star™ (DE3) cells carrying the relevant plasmids and induced for 75 min with IPTG were analysed by immunoblotting (Fig. 9A) and Tricine-SDS-PAGE (Fig. 9B), respectively. Whereas substitution of the non-conserved His-81 and Ser-84 did not affect LPS deacylation, deacylation of LPS

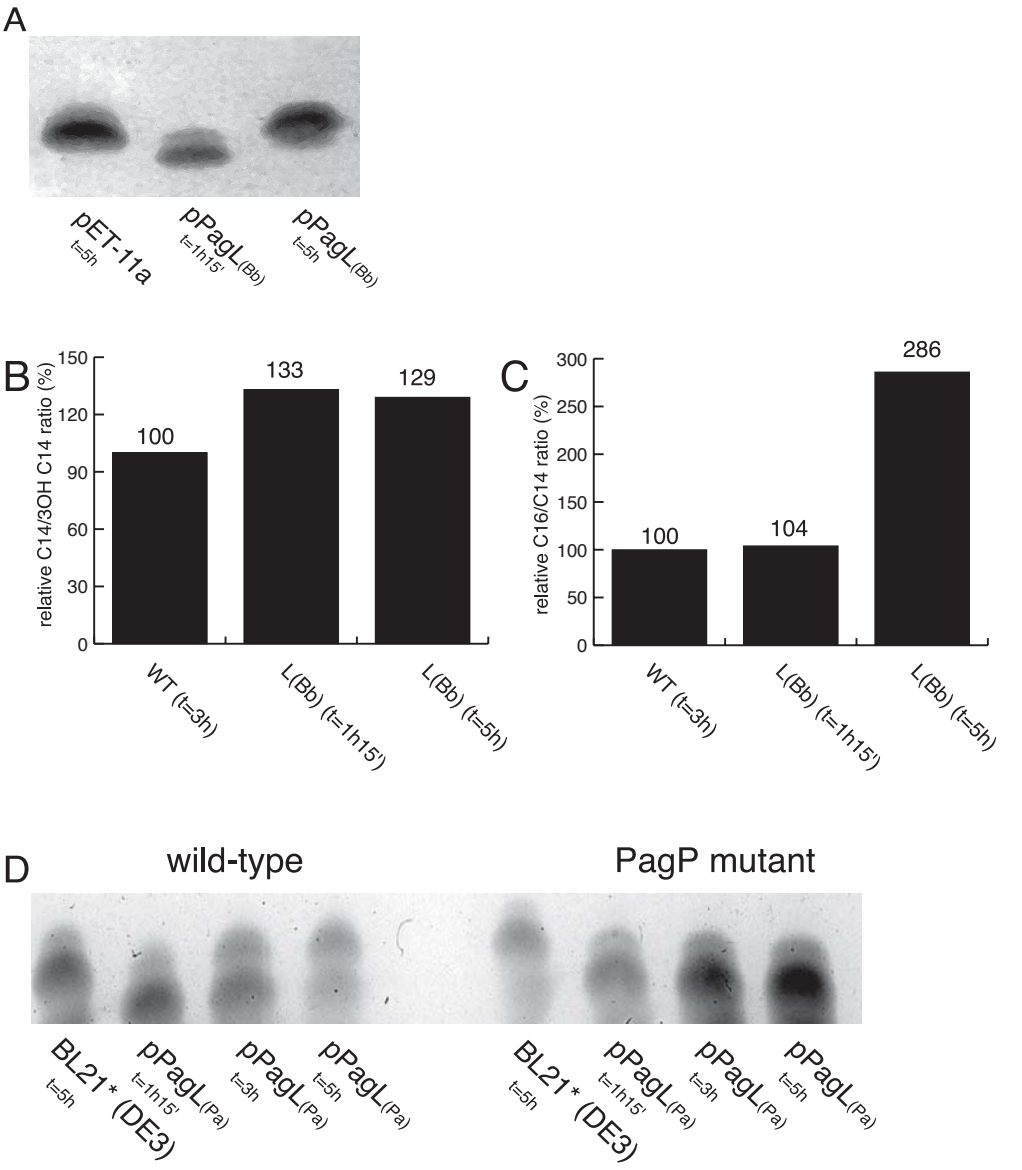

Fig. 7 *In vivo* remodification of deacylated LPS and the role of endogenous PagP. (A), exponentially growing *E. coli* BL21 Star™ (DE3) cells containing the empty pET-11a vector or the $pPagL_{(Bb)}$ plasmid were induced with IPTG for the indicated time period. Samples corresponding to 1 A_{600} unit were collected and analysed by Tricine-SDS-PAGE. (B) and (C), the fatty acid content of purified *E. coli* BL21 Star™ (DE3) wild-type LPS (*WT*) and $PagL_{(Bb)}$-modified LPS (*L*(*Bb*)), isolated at the indicated time after induction of *pagL* expression, was analysed by GC/MS. Indicated are the normalised C14 30H C14 (*B*) and C16/C14 (C) ratios with wild-type LPS set at 100 (*values* shown above *bars*). (D), exponentially growing wild-type *E. coli* BL21 Star™ (DE3) or *E. coli* BL21 Star™ (DE3) and its *pagP* mutant derivative JG101, containing $pPagL_{(Pa)}$, were induced with IPTG for the indicated time period, after which 1 A_{600} unit culture samples were collected and analysed on Tricine-SDS-PAGE gel.

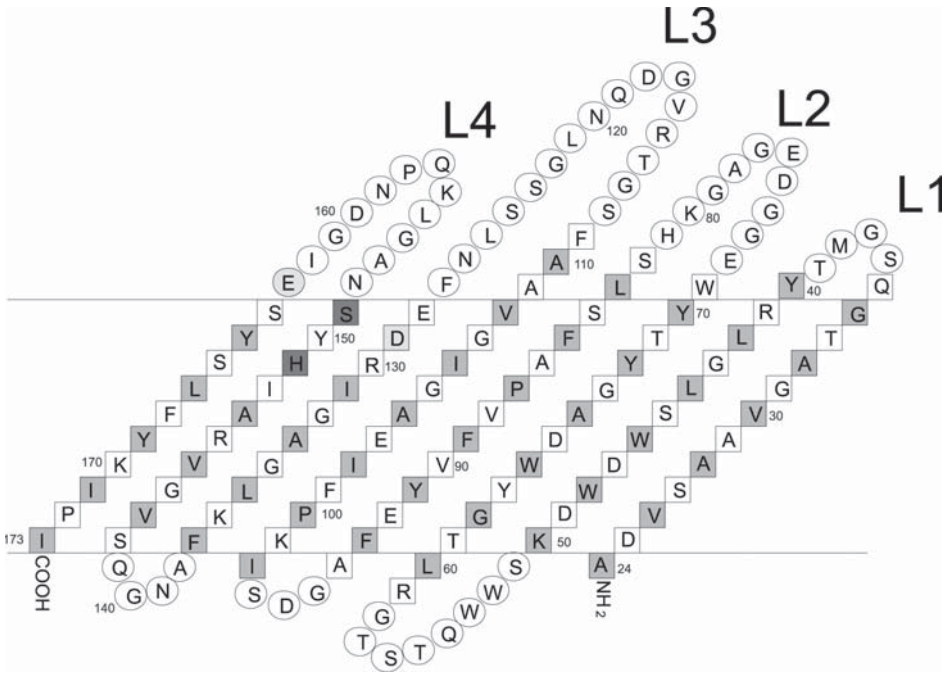

Fig. 8 Topology model for PagL from *P. aeruginosa*. A model for the topology of $PagL_{(Pa)}$ was constructed using the general rules of outer membrane protein architecture as described by Jacobs *et al.* (2003). The proposed model consists of an eight-stranded β-barrel with four loops (L1–4) extending into the external environment. Residues in the postulated β-strands are shown in diamonds, which are shaded for residues that are exposed to the lipid bilayers. His-149 and Ser-151 (marked in red; position in the $PagL_{(Pa)}$ precursor) are absolutely conserved (Fig. 2) and are suggested to be part of a classical catalytic triad of a serine hydrolase. Potential candidates for the acidic residue of the catalytic triad are indicated in yellow. Numbers refer to the positions of the residues in the precursor sequence.

was no longer observed when the conserved His-149 and Ser-151 were replaced (Fig. 9B), even though the expression of these mutant proteins was not affected (Fig. 9A). These results strongly support the hypothesis that the conserved histidine at position 149 and serine at position 151 of the precursor $PagL_{(Pa)}$ protein are active-site residues and that PagL mechanistically functions as a serine hydrolase.

PagL expression in *P. aeruginosa* and phenotypic characterisation of a *pagL* mutant

Because *pagL* homologs were identified in many Gram-negative bacteria, including non-pathogenic soil bacteria (Fig. 2), a primary role for the enzyme in pathogenesis appears unlikely. To gain insight in the possible function of PagL in *P.*

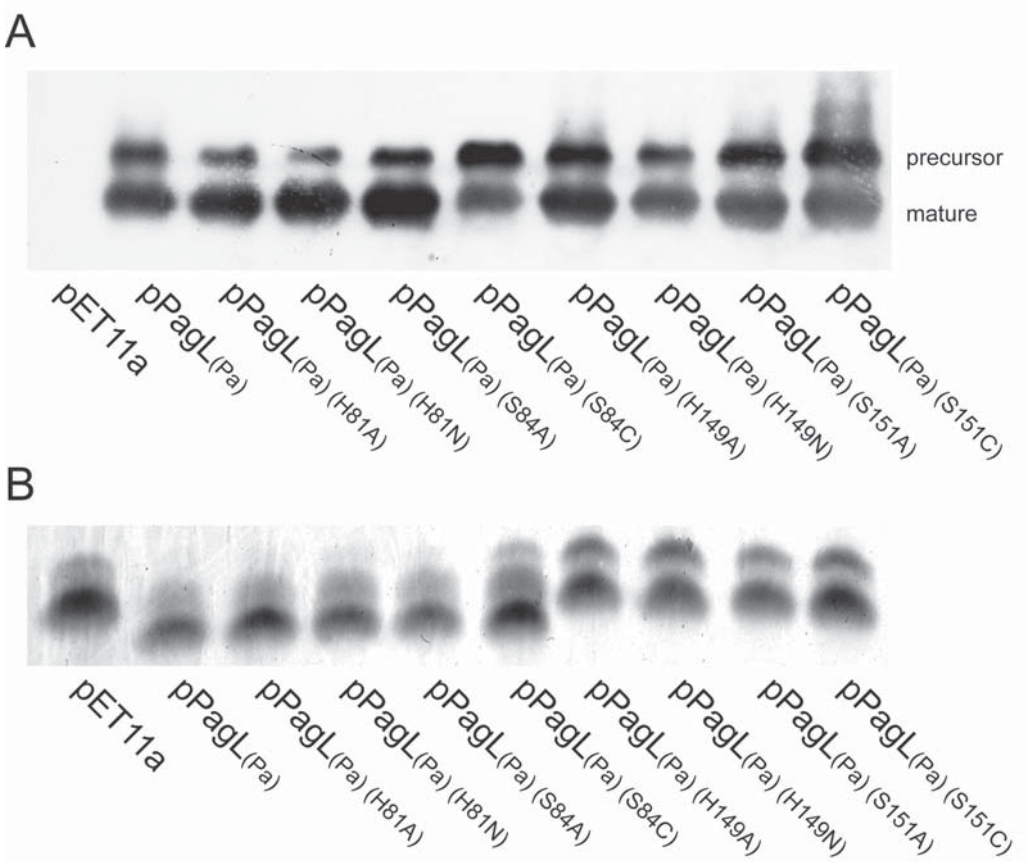

Fig. 9 Identification of $PagL_{(Pa)}$ active-site residues by amino acid substitution. Exponentially growing *E. coli* BL21 Star™ (DE3) cells containing the empty pET-11a vector, the $pPagL_{(Pa)}$ plasmid, or the mutant $pPagL_{(Pa)}$ plasmids were induced with IPTG for 75 min, after which 1 A_{600} unit culture samples were collected and analysed by SDS-PAGE followed by immunoblotting with primary antibodies against $PagL_{(Pa)}$ (A) and by Tricine-SDS-PAGE to visualise LPS (B).

aeruginosa, we tested whether endogenous *pagL* expression levels in *P. aeruginosa* can be influenced by the growth conditions. Therefore, wild-type *P. aeruginosa* PAO1 was grown in LB supplemented or not with 0–5% ethanol, 10 mM magnesium chloride, or 5 μg/ml EDDHA (to create iron-limiting conditions), or in minimal medium, or on solidified media (LB agar, and SV agar). Endogenous PagL levels were analysed by immunoblotting. The result showed that under all conditions tested, PagL was expressed, and the expression levels were similar (data not shown). Furthermore, we compared the growth characteristics of a *P. aeruginosa pagL* transposon-insertion mutant (32751) and its parental strain (PAO1). Both strains were tested for their ability to grow in LB medium at different temperatures (25, 30, or 37°C) or in LB medium supplemented with 0–4 M

sodium chloride, 0–1,500 μg/ml chloramphenicol, 0–5% ethanol, or 0–1% chloroform. The results showed that both strains had similar growth characteristics under most circumstances. Only when chloramphenicol was present in the medium, a difference in the ability to grow was observed. This difference was most pronounced at a concentration of 650 μg/ml chloramphenicol, where the absorbance of the wild-type culture after overnight growth was 1.7-fold higher than that of the PagL mutant (data not shown). This suggests that PagL probably does not function in the adaptation to different growth temperatures or in the resistance against osmotic stress or organic solvents but that its activity affects the permeability of the outer membrane for hydrophobic compounds, such as chloramphenicol.

Discussion

A lipid A 3-*O*-deacylase, PagL, was originally identified in *S.* Typhimurium (Trent *et al.*, 2001a). Although similar activity was detected in some other bacteria, no homologs of *pagL* were identified (Trent *et al.*, 2001a). Now, we identified *pagL* homologs in a range of Gram-negative bacteria, and we showed by cloning and expression of the corresponding genes in *E. coli* that at least two of them, those of *P. aeruginosa* and *B. bronchiseptica*, are functional lipid A deacylases. The newly identified PagL homologs are from organisms with genomes with a high GC content, and no other homologs were identified in Enterobacteriaceae, which suggested that *Salmonellae* might have acquired the *pagL* gene by horizontal gene transfer from an organism with a high GC content. However, although all newly identified *pagL* genes have a relatively high GC content (55–66%), *pagL* of *S.* Typhimurium has a GC content of only 39.5%, which is considerably lower than the *S.* Typhimurium average chromosomal GC content of 53% (McClelland *et al.*, 2001). This observation suggests that *S.* Typhimurium has indeed acquired the *pagL* gene by horizontal gene transfer but not from an organism with a high GC content.

In previous work, it was demonstrated that outer membranes prepared from *P. aeruginosa* PAO1 harboured lipid A 3-*O*-deacylase activity (Basu *et al.*, 1999). Furthermore, it is known that *P. aeruginosa* contains partially 3-*O*-deacylated lipid A species (Kulshin *et al.*, 1991). The *P. aeruginosa* PagL homolog identified here is most likely responsible for the 3-*O*-deacylase activity found previously in *P. aeruginosa* membranes. However, we did not find an intact PagL homolog in the *R. leguminosarum* genome sequence, although 3-*O*-deacylase activity in its membranes has been described (Basu *et al.*, 1999). BLAST searches with the unfinished *R. leguminosarum*

genome sequence did show the presence of two *pagL* homologs[2]; however, the ORFs encoding the homologs were found to be disturbed by premature stop codons. Possibly, the available sequence still contains some errors. Also in other Gram-negative bacteria that are known to contain partially 3-*O*-deacylated lipid A, such as *H. pylori* (Bhat *et al.*, 1994), no *pagL* homologs could be found. It is possible that the PagL homologs in these bacteria show only very low sequence similarity to the PagL family described in this paper, or 3-*O*-deacylation in these bacteria is mediated by entirely different proteins. On the other hand, we have also identified PagL homologs in bacteria that have not previously been described to contain 3-*O*-deacylated lipid A, for example, in the genomes of *Bordetella* spp. Strikingly, the *pagL* ORF in the *B. pertussis* strains appears to be disrupted by a frame-shift mutation, whereas the closely related species *B. parapertussis* and *B. bronchiseptica* have an intact *pagL* gene. Perhaps inactivation of *pagL* in *B. pertussis* is an example of host-specific adaptation. Large scale analysis of the capacity of *Bordetella* strains of expressing PagL will probably give insight in the relevance of the absence of intact *pagL* in *B. pertussis*.

We observed that *E. coli* lipid A upon deacylation by PagL was subsequently modified by the addition of palmitate, which was the consequence of endogenous PagP activity. An interesting question is whether the additional modification by PagP is actively triggered by lipid A deacylation or whether it is the consequence of a changed physiological state of the outer membrane, e.g., by the accumulation of fatty acids resulting from PagL activity. In the recently described NMR structure of *E. coli* PagP, the active-site was found to be located at the outer surface of the outer membrane, where phospholipids are normally not present (Hwang *et al.*, 2002). The authors suggested that PagP activity might depend on the aberrant migration of phospholipids into the outer leaflet of the outer membrane. Thus, the observed secondary modification might be a consequence of the PagL activity, resulting in the migration of phospholipids from the inner leaflet to the outer leaflet of the outer membrane, where they can become a substrate for PagP. Interestingly, it was reported recently that some membrane-spanning proteins passively induce the flip-flop of phospholipids across lipid bilayers (Kol *et al.*, 2003). Thus, the mere overproduction of PagL in our studies may already be sufficient to induce phospholipids transport. This notion is consistent with the appearance of a slower migrating LPS form, even when inactive PagL proteins were expressed (Fig. 9B).

The high expression of the PagL homologs in *E. coli* allowed the determination of the processing sites by N-terminal sequence analysis. The identified leader peptidase I cleavage sites for $PagL_{(Pa)}$ and $PagL_{(Bb)}$ correspond to the location predicted by the SignalP server (Nielsen *et al.*, 1999). However, for $PagL_{(St)}$, the cleavage site did

[2] J. Geurtsen and J. Tommassen, unpublished observation.

corresponded neither to the SignalP predicted site (between amino acids 17 and 18 (AFA and CSA)) (Nielsen *et al.*, 1999) nor to the position that was identified in earlier work, in which the cleavage position was determined to be between residues 22 and 23 (AND and NVF) (Trent *et al.*, 2001a). Because the -1 residue of the latter cleavage site does not conform to the consensus sequence S_nXA (S_n stands for an amino acid with a small neutral side chain), which is necessary for recognition by leader peptidase (von Heijne, 1983), this cleavage site is unlikely to be correct. The SignalP predicted site does match the consensus sequence for the -3 and -1 position, but the C domain, comprising the last six residues of the signal sequence does not include polar residues as one would expect it to do (von Heijne, 1983). The cleavage position identified here between residues 20 and 21 (CSA and NDN) does match both criteria, with a cysteine at the -3 (S_n) position and a serine at -2, increasing the polarity of the C domain.

The poor conservation of the PagL sequence allowed us to speculate about the catalytic mechanism. Among the few totally conserved residues were a histidine and a serine, which are located in our topology model in a position similar to that of the active-site residues in OMPLA (Snijder *et al.*, 1999), i.e., at the lipid-exposed side of a β-strand, close to the cell surface. By means of amino acid substitutions, we demonstrated that these conserved residues are indeed essential for catalytic activity. The position of the active-site residues at the lipid-exposed side of the β-barrel suggests that PagL, like OMPLA, may be active as a dimer to be able to form a substrate binding pocket. Good candidates for the acidic component of the catalytic triad are the highly conserved aspartate and glutamate at positions 129 and 163 of the $PagL_{(Pa)}$ precursor sequence, respectively, which, in our model, are located in the β-strands flanking the one containing the active-site His and Ser residues (Fig. 8). The Asp-129 is conserved in all homologs, except in *S.* Typhimurium, where it is replaced by glutamate. The Glu-163 varies more, with possible substitutions by an aspartate, or an asparagine. Although rare, asparagine can substitute for the acidic residue in serine hydrolases, as it does, for instance, in *E. coli* OMPLA (Snijder *et al.*, 1999).

The physiological role of PagL-mediated deacylation of lipid A remains to be elucidated. The identification of PagL homologs in a variety of Gram-negative bacteria, including non-pathogenic ones, such as *P. putida* and *R. metallidurans*, suggests that lipid A deacylation does not have a dedicated function in pathogenicity. To gain insight in its function, we tested whether *pagL* expression in *P. aeruginosa* can be influenced by the growth conditions. Supplementation of the growth medium with Mg^{2+} ions did not affect *pagL* expression, consistent with the observation that 3-*O*-deacylase activity in *P. aeruginosa* membranes is not affected by the Mg^{2+} concentration in the medium nor

by a *phoP* mutation (Trent *et al.*, 2001a). Also, other growth conditions tested did not appear to affect *pagL* expression. Furthermore, we compared the growth of wild-type *P. aeruginosa* and a *pagL* mutant derivative under different conditions. The results showed a decreased resistance of the *pagL* mutant to chloramphenicol, suggesting that PagL activity, at least in *P. aeruginosa*, affects the permeability barrier of the outer membrane to hydrophobic compounds. The PagL family described here probably includes many more proteins because new BLAST searches just before submission of this paper (December 2004) using the newly identified homologs as leads revealed several additional PagL homologs in the genomes of, for example, *Agrobacterium tumefaciens* (GenBank™ accession no. AAK87616), *Methylobacillus flagellatus* (GenBank™ accession no. ZP_ 00173991), and *Geobacter sulfurreducens* (GenBank™ accession no. AAR36806). Again, these proteins have low overall sequence similarity but a clearly conserved C-terminal region, including the histidine-serine couple that was identified here as part of the active-site.

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Chapter 3

Crystal structure and catalytic mechanism of the LPS 3-O-deacylase PagL from *Pseudomonas aeruginosa*

Lucy Rutten*, Jeroen Geurtsen*, Wietske Lambert, Jeroen Smolenaers, Alexandre Bonvin, Alex de Haan, Peter van der Ley, Maarten Egmond, Piet Gros, and Jan Tommassen

***** L.R. and J.G. contributed equally to this work

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Abstract

Pathogenic Gram-negative bacteria can modify the lipid A portion of their lipopolysaccharide in response to environmental stimuli. 3-*O*-deacylation of lipid A by the outer membrane enzyme PagL modulates signalling through Toll-like receptor 4, leading to a reduced host immune response. We found that PagL is widely disseminated among Gram-negative bacteria. Only four residues are conserved: a Ser, His, Phe, and Asn residue. Here, we describe the crystal structure of PagL from *Pseudomonas aeruginosa* to 2.0-Å resolution. It consists of an eight-stranded β-barrel with the axis tilted by ~30° with respect to the lipid bilayer. The structure reveals that PagL contains an active-site with a Ser-His-Glu catalytic triad and an oxyanion hole that comprises the conserved Asn. The importance of active-site residues was confirmed in mutagenesis studies. Although PagL is most likely active as a monomer, its active-site architecture shows high resemblance to that of the dimeric 12-stranded outer membrane phospholipase A. Modelling of the substrate lipid X onto the active-site reveals that the 3-*O*-acyl chain is accommodated in a hydrophobic groove perpendicular to the membrane plane. In addition, an aspartate makes a hydrogen bond with the hydroxyl group of the 3-*O*-acyl chain, probably providing specificity of PagL toward lipid A.

Introduction

The outer membrane of Gram-negative bacteria functions as a permeability barrier that protects the bacteria against harmful compounds from the environment. It is an asymmetric bilayer with phospholipids and lipopolysaccharide (LPS) in the inner and outer leaflet, respectively. LPS contains three covalently linked domains: lipid A, the core, and the *O*-antigen (Raetz and Whitfield, 2002). Lipid A forms the hydrophobic membrane anchor and is responsible for the endotoxic activity of LPS. In *Escherichia coli*, it consists of a 1,4'-bisphosphorylated β-1,6-linked glucosamine disaccharide, which is substituted with *R*-3-hydroxymyristic acid (3OH C14) residues at positions 2, 3, 2', and 3' via ester or amide linkage. Secondary lauroyl and myristoyl groups substitute the hydroxyl group of 3OH C14 at the 2' and 3' positions, respectively. The underlying mechanism for LPS toxicity is its recognition by the host Toll-like receptor 4–MD2 complex, resulting in a signalling cascade within the host cell. Signalling eventually leads to activation of the transcription factor NF-κB, which regulates the production of pro-inflammatory cytokines (reviewed in Miyake, 2004).

Several Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), covalently modify their LPS in response to environmental stimuli. These modifications usually require the two-component regulatory system PhoP/PhoQ, which can be activated by antimicrobial peptides or repressed by high concentrations of divalent cations (Bader *et al.*, 2005). The PhoP/PhoQ system activates or represses the expression of >40 genes, some of which are involved in antimicrobial peptide resistance. Two integral outer membrane enzymes, the PhoP/PhoQ-activated gene products PagP and PagL, can alter the number of acyl chains in lipid A (reviewed in Trent, 2004). PagP is a palmitoyl transferase that transfers a palmitoyl moiety from a phospholipid to the *R*-3-hydroxyacyl chain at the 2 position of the glucosamine disaccharide of lipid A (Bishop *et al.*, 2000). PagL hydrolyses the ester bond at the 3 position of lipid A, thereby releasing the primary 3OH C14 moiety (Trent *et al.*, 2001). Both lipid A modifications reduce Toll-like receptor 4 signalling, which could help bacteria to evade the host immune system (Kawasaki *et al.*, 2004).

PagL was originally identified in *S.* typhimurium (Trent *et al.*, 2001). Recently, we identified PagL homologs in numerous other Gram-negative bacteria (Geurtsen *et al.*, 2005). Although the overall sequence conservation was rather low, the enzymatic activity of two of these homologs, including that of *Pseudomonas aeruginosa*, was confirmed because they deacylated *E. coli* LPS. Because *P. aeruginosa* lipid A carries 3OH C10, rather than 3OH C14 at the 3 position, these results also demonstrate that the enzyme lacks fatty acyl chain-length specificity. Furthermore, PagL was predicted to

form an eight-stranded β-barrel, and Ser-128 and His-126 of *P. aeruginosa* PagL were shown to be essential for activity (Geurtsen *et al.*, 2005). Here, we present the crystal structure of PagL from *P. aeruginosa* at 2.0-Å resolution and provide insights into the catalytic mechanism and substrate binding of the enzyme.

Materials and Methods

Site-directed mutagenesis

Mutations were introduced in *pagL* by using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and the primers listed in Table 1. Plasmid $pPagL_{(Pa)}$ encoding wild-type PagL, including the signal sequence (Geurtsen *et al.*, 2005), was used as the template in which the mutations were created. The presence of the correct mutations was confirmed by nucleotide sequencing in both directions.

Construction of an *E. coli metE* mutant

The *metE* gene from *E. coli* strain AM1095 (Hoekstra *et al.*, 1976) was amplified by PCR. Chromosomal template DNA was prepared by resuspending ~10^9 bacteria in 50 ml of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x *g*, and the supernatant was used as template DNA. The sequence of the forward primer, including an ATG start codon, was 5'-AAATGACAATATTGAATCA-3'. The sequence of the reverse primer, which included a stop codon, was 5'-TTTTACCCCCGACGCAAGT-3'. The PCR was done under the following conditions: 50 ml total reaction volume, 25 pmol of each primer,

TABLE 1

Primers used for site-directed mutagenesis

Name[a]	Sequence (5'-3')[b]
D106A_FW	TCCCTGAACTTCGAAGCGCGCATCGGCGCCGGC
D106A_REV	GCCGGCGCCGATGCGCGCTTCGAAGTTCAGGGA
N129A_FW	GCGATCCACTATTCCGCGGCCGGCCTGAAACAG
N129A_REV	CTGTTTCAGGCCGGCCGCGGAATATGTGATCGC
N136A_FW	GGCCTGAAGTTCGCCGCGGGCCAGTCGGTCGGC
N136A_REV	GCCGACCGACTGGCCCGCGGCGAACTTCAGGCC
E140A_FW	CCGAACGACGGTATCGCGTCCTACAGCCTGTTC
E140A_REV	GAACAGGCTGTAGGACGCGATACCGTCGTTCGG

[a] The primer name gives the amino acid substitution, e.g., N136A_FW indicates that the oligonucleotide shown was used as the forward primer in a site-directed mutagenesis procedure to substitute the Asn at position 136 PagL by an alanine.
[b] Introduced mutations are underlined.

0.2 mM dNTPs, 3 nl of template DNA solution, 1.5% dimethyl sulfoxide, 1.75 units of Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche). The temperature program was as follows: 95°C for 3 min, 30 cycles of 1 min at 95°C, 1 min at 60°C, and 3 min at 72°C, followed by 10 min at 72°C and subsequent cooling to 4°C. The PCR product was purified from agarose gel and subsequently cloned into pCRII-TOPO. The resulting plasmid, designated pMetE, was partially digested with MluI and then ligated with the 801-bp MluI fragment of pBSL141 (Alexeyev and Shokolenko, 1995) harbouring a gentamicin (Gm)-resistance cassette. The ligation-mixture was used to transform *E. coli* DH5α using the $CaCl_2$ method (Sambrook *et al.*, 1989). A plasmid with the correct restriction digestion profile was designated pMetE::Gm^R, and harboured the *metE* gene with a inserted Gm-resistance gene in the same orientation as the *metE* gene itself. A methionine-auxotrophic derivative of AM1095 was constructed by marker exchange. Strain AM1095 was transformed with 6 µg of the 3-kb EcoRI fragment, containing the *metE* gene with the inserted Gm^R cassette from pMetE::Gm^R. Colonies were selected on LB agar plates containing 1 µg/ml Gm. Gm^R transformants were tested for methionine auxotrophy by growing on minimal medium plates in the absence or presence of 20 µg/ml methionine, and a methionine-auxotroph was designated JG201. Strain JG301, a methionine-auxotrophic derivative *E. coli* BL21 Star (DE3) (Invitrogen), was subsequently obtained by P1 transduction, using strain JG201 as the donor.

Production of PagL

To induce expression of PagL without its signal sequence, *E. coli* BL21 Star (DE3) (Invitrogen) containing pPagL(-) (Geurtsen *et al.*, 2005) was grown in LB broth until mid-exponential phase. Expression of the *pagL* gene was then induced by adding 1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG), after which incubation at 37°C was continued. To obtain PagL containing selenomethionine (Se-Met), *E. coli* strain JG301 (see above) transformed with pPagL(-) was grown in minimal medium (Winkler and de Haan, 1948) supplemented with 0.5% glucose and 50 µg/ml Se-Met. Inclusion bodies, containing PagL, were isolated as described (Dekker *et al.*, 1995), with some adaptations, i.e., centrifugation steps to collect the inclusion bodies were prolonged to 2 h. Inclusion bodies were solubilised in 8 M urea/10 mM glycine (pH 8.0) to a protein concentration of ~10 mg/ml. The urea-solubilised PagL was centrifuged at 150,000 ×*g* for 1 h to remove insoluble material. To remove residual non-protein contaminants, four volumes of 20% (wt/vol) trichloroacetic acid were added. Precipitated proteins were collected by centrifugation and washed with water in three rounds of resuspending and centrifuging. The protein pellet was then solubilised in 8 M urea/10 mM glycine (pH 8.0).

In vitro folding and purification of PagL

PagL was folded *in vitro* by 2-fold dilution from a stock solution in 8 M urea into 10% (wt/vol) lauryldimethylamine-oxide, followed by 10 min of sonication using a Branson 1210 Sonifier. Refolded PagL was diluted 3- to 4-fold with buffer A [20 mM Tris·HCl, pH 8.5/0.08% (wt/vol) pentaethyleneglycol monodecyl ether ($C_{10}E_5$)] and purified by fast-protein liquid chromatography (FPLC) using a 1-ml monoQ ion-exchange column (Amersham Pharmacia) that was pre-equilibrated with buffer A. PagL was eluted with a linear gradient of 0–1 M NaCl in buffer A. Fractions containing PagL were pooled and concentrated to ~10 mg/ml by using Centricon concentrators with a molecular mass cut-off of 3 kDa (Amicon). The protein was then dialysed three times overnight against 10 ml of 2 mM Tris·HCl, pH 8.5/0.06% $C_{10}E_5$ (vol/vol) using a membrane with a molecular mass cut-off of 3.5 kDa.

Crystallisation and structure determination

PagL was crystallised by using the hanging-drop vapour diffusion method. Crystals grew in the C2 space group, using a condition containing 3% PEG3000/25% glycerol/10 mM calcium acetate/0.1 M Tris-HCl, pH 8.5 at 20°C. Se-Met PagL crystals were grown in the same condition, with 1 mM DTT added to the protein solution. Crystals were harvested from the drops with a cryo-loop and directly cooled into liquid nitrogen. Data sets were obtained at 100 K on a charge-coupled device detector at beam line ID14-EH4 at the European Synchrotron Radiation Facility. Native data were collected to 2.0-Å resolution. Data of the Se-Met PagL crystal was collected at λ_{peak} = 0.9795 to 2.8-Å resolution. All data were indexed and processed by using DENZO and SCALEPACK (Otwinowski and Minor, 1997). Table 2 summarises data collection information. The structure of PagL was solved by using a combination of Se-Met single-wavelength anomalous dispersion (SAD) and molecular replacement (MR). From the Se-Met SAD data set, the two selenium atoms in the asymmetric unit (one per PagL molecule) were located by using standard programs (Storoni *et al.*, 2004; Weeks and Miller, 1999; Schneider and Sheldrick, 2002). Unfortunately, the anomalous signal was too low to obtain an interpretable density-modified electron-density map. Therefore, we attempted MR. Whereas all commonly used MR programs failed, the program PHASER (Storoni *et al.*, 2004) provided an MR solution using a polyalanine β-barrel model of the NspA structure, lacking the loops. The poor phases obtained from the incomplete model were sharpened by using the prime-and-switch algorithm in the program SOLVE (Storoni *et al.*, 2004). The positions of the seleniums indicated the locations of the methionines, which enabled us to correctly build a few β-strands by using the program

O (Jones *et al.*, 1991). Subsequently, the complete model could be built automatically by using ARP/WARP (Perrakis *et al.*, 2001), which was not the case directly after MR. The structure was refined by using REFMAC 5.0 (Winn *et al.*, 2001), using TLS groups for the separate molecules in the asymmetric unit. Refinement statistics are summarised in Table 2, and the electron density map is shown in Fig. 1.

TABLE 2

Data collection and refinement statistics

	Native	Se-Met
Data collection		
Space group	C2	C2
Cell dimensions		
a(Å)	92.26	93.21
b(Å)	48.99	47.66
c(Å)	105.03	103.45
β(°)	115.46	113.39
Resolution , Å (outer shell)	2.00(2.05)	2.80(2.87)
R_{sym} or R_{merge}	0.051(0.380)	0.098(0.515)
$I / \sigma I$	20.9(3.0)	19.3(3.4)
Completeness (%)	90.3(52.2)	99.8(97.6)
Redundancy	3.1(2.7)	6.8(6.9)
Refinement		
Resolution (Å)	2.00	
No. of reflections	26283	
R_{work} / R_{free}	0.198 / 0.233	
No. of atoms	2518	
Protein	2	
Ligand/ion	12 ($C_{10}E_5$ parts) / 1 (Ca^{2+})	
Water	121	
B-factors		
Protein	29.40	
ligand/ion	50.22 / 22.41	
Water	39.97	
rms deviations		
Bond lengths (Å)	0.017	
Bond angles (°)	1.657	

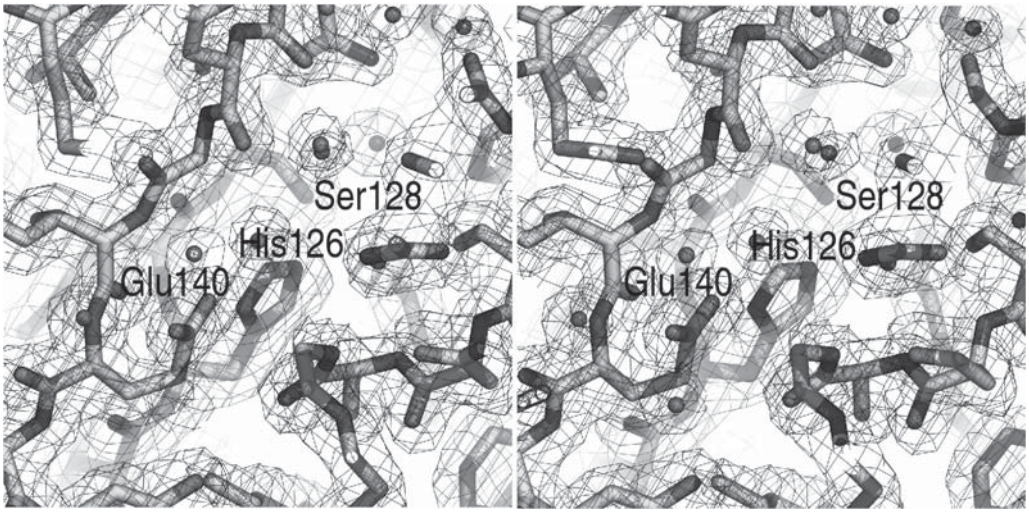

Fig. 1. Stereoview of the catalytic site with the final $2F_o$-F_c electron density map, at 2.0 Å resolution and contoured at 1σ, shown as chicken wire. Protein is shown as sticks. Carbon atoms are shown in yellow, oxygen in red and nitrogen in blue. Catalytic triad residues are labelled.

Modelling of lipid X onto the active-site of PagL

Models of lipid X docked onto PagL were created by using the program HADDOCK (Dominguez *et al.*, 2003). The coordinates of PagL were taken from chain A of the structure and the lipid X coordinates were based on those of the LPS molecule that was co-crystallised with FhuA (Ferguson *et al.*, 1998). Ambiguous interaction restraints (AIRs) were defined, based on the information on the reaction mechanism: 3-Å-distance restraints were defined between the Ser-128 oxygen of PagL and the carboxyl carbon of lipid X and between the His-126 nitrogen (Nε2) and the ester oxygen of lipid X. Additional AIRs were imposed merely to ensure that the lipid X acyl chains were positioned at least as close to the PagL molecule as they could be in the outer leaflet of the outer membrane. First, an ensemble of 20 conformations of lipid X was generated by simulated annealing and molecular dynamics refinement in DMSO, which was subsequently used to generate 1,000 rigid body docking solutions. The best 200 solutions based on their intermolecular energy (sum of van der Waals, electrostatic, and AIR energies) were then submitted to a semiflexible refinement, during which lipid X was treated as fully flexible, whereas PagL could only move at defined flexible regions (side chains first, subsequently both side chains and backbone). All 200 models were finally refined in explicit solvent (DMSO was used to mimic the hydrophobic environment imposed by the membrane) and clustered based on root mean square deviation criteria.

The lowest energy structure from the lowest energy cluster was taken as best solution.

PagL assays

PagL activity was measured *in vivo* and *in vitro*. In the *in vivo* assay, exponentially growing cultures of *E. coli* BL21 Star (DE3) containing $pPagL_{(Pa)}$ or its mutant derivatives were induced by adding 1 mM IPTG (end concentration). After 75-min further incubation at 37°C, deacylation of the endogenous LPS was monitored by the analysis of whole-cell samples in Tricine-SDS/PAGE (Lesse *et al.*, 1990) and staining of the LPS with silver (Tsai and Frasch, 1982). For the quantitative *in vitro* assay, cell envelopes of the *E. coli* strains expressing wild-type or mutant PagL proteins, induced as described above, were isolated as described (Geurtsen *et al.*, 2005). The protein content of the cell envelopes was determined by using the Pierce BCA protein assay after boiling the samples in 0.1% SDS. The amount of native PagL in the cell envelopes was estimated by comparison of the band intensities with a dilution series of purified refolded PagL on a blot of an SDS/PAGE gel, which was stained with Ponceau. For all PagL variants, the concentration was between 70 and 90 mg per mg total cell envelope protein. PagL activity was determined by incubating cell envelopes (444 total protein content per ml) with *Neisseria meningitidis* L3 LPS as described (Bos *et al.*, 2004). The amount of 3OH C12 released in time was determined by gas chromatography using 2OH C12 as an internal standard as described (Patterson *et al.*, 1999). The *in vitro* assay was also used to determine the activity of *in vitro*-folded PagL. Apart from the release of 3OH C12 in time, the deacylation of the LPS was also verified by Tricine-SDS/PAGE and ESI/MS (Geurtsen *et al.*, 2005).

Results

Production, folding, and activity of recombinant PagL

PagL from *P. aeruginosa* was refolded from inclusion bodies. Folding could be monitored by SDS/PAGE analysis. Like many outer membrane proteins (OMPs), PagL showed heat modifiability (Fig. 2A). Usually, the folded form of an OMP has a higher electrophoretic mobility than its denatured form. In the case of PagL, however, the folded protein has a lower electrophoretic mobility as has been observed also for a few other small OMPs, i.e., OmpA171t, OmpX, and NspA (Pautsch *et al.*, 1999; Vandeputte-Rutten *et al.*, 2003). The same mobility shift was observed on Western blot for wild-type PagL folded *in vivo* (data not shown). Folded PagL was insensitive to denaturation by SDS, as it remained folded even in the presence of 2% SDS when not heated (Fig. 2A).

To verify that PagL was correctly folded *in vitro*, it was incubated with purified LPS of *N. meningitidis*. The LPS was converted into a form with a higher electrophoretic mobility (Fig. 2B), in agreement with the expected hydrolysis of the primary acyl chain at the 3 position of lipid A, which is 3OH C12 in the case of *N. meningitidis*. Deacylation of the LPS was confirmed by electrospray ionisation mass spectrometry (Fig. 3). The reaction was independent of the presence of divalent cations, as deacylation of LPS was still observed in the presence of 5 mM EDTA (Fig. 2B). Quantification of the amount of 3OH C12 released in time revealed a specific activity of 0.40 nmol 3OH C12 released per min per nmol of *in vitro* folded PagL. *E. coli* membranes containing overexpressed *in vivo* folded wild-type PagL displayed a lipid A-deacylase activity of ~0.22 nmol/min per nmol of PagL, which is comparable to the specific activity of *in vitro* folded PagL. We concluded that PagL was correctly folded *in vitro* into its active conformation and, thus, suitable for structure determination.

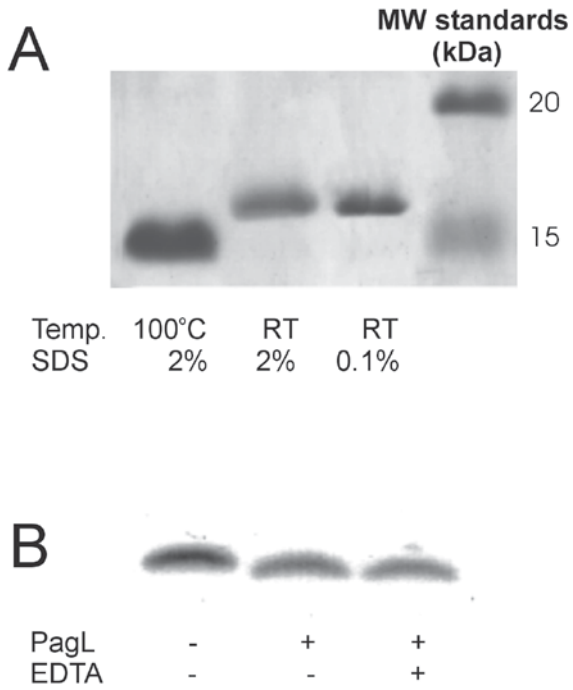

Fig. 2. Folding and *in vitro* activity of recombinant PagL. (A) Coomassie-stained SDS/PAGE gel showing the heat modifiability of purified, refolded PagL. Samples were treated in sample buffer containing either 2% or 0.1% SDS and at room temperature (RT) or 100°C before electrophoresis. The positions of molecular mass standard proteins are shown at the right. (B) Purified *N. meningitidis* LPS was incubated in a detergent-containing buffer with or without refolded PagL and analysed by Tricine-SDS/PAGE and staining with silver.

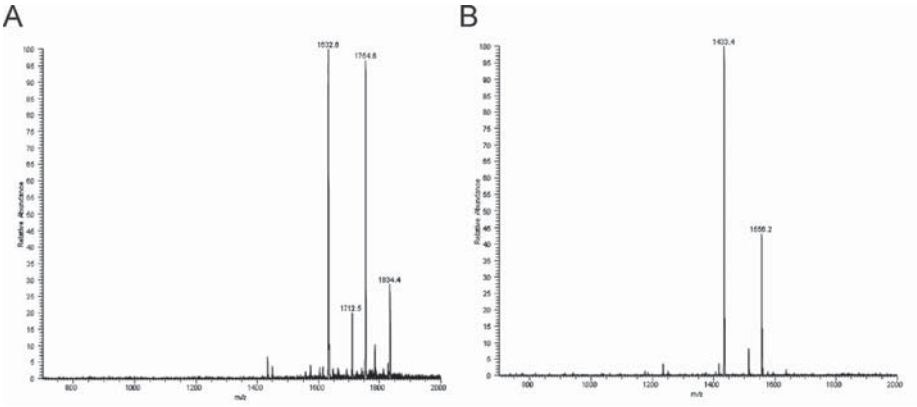

Fig. 3. Structural analysis by ESI/MS of wild-type and *in vitro* PagL-modified *N. meningitidis* L3 LPS. Lipid A moieties of wild-type (A) and *in vitro* PagL-modified (B) *N. meningitidis* L3 LPS were analysed by ESI/MS as described (Geurtsen *et al.*, 2005). Major peaks at *m*/*z* 1713, 1834, 1556, and 1433 were interpreted as the characteristic hexaacylated *bis*-phosphate species that is typically found in *N. meningitidis*, a hexaacylated *bis*-phosphate species substituted with a phosphoethanolamine, a 3-*O*-deacylated *mono*-phosphate species substituted with a phosphoethanolamine, and a 3-*O*-deacylated *mono*-phosphate species, respectively. The major peaks at *m*/*z* 1632 and 1754 probably represent fragment ions of the species at *m*/*z* 1713 and 1834.

Structure determination of PagL

PagL was crystallised in the C2 space group, containing two molecules in the asymmetric unit (Fig. 4). The structure of PagL was solved with a combination of MR using the program PHASER (Storoni *et al.*, 2004), together with single-wavelength anomalous dispersion. MR was successfully performed by using a polyalanine model of the β-stranded part of NspA. The calculated $2F_o - F_c$ maps (even after prime-and-switch in RESOLVE (Terwilliger, 2003) did not allow for initial model tracing by hand or by automated model building with ARP/wARP (Perrakis *et al.*, 1997). Determination of the position of the single methionine present in PagL, using the anomalous signal of the selenium from a crystal of Se-Met-substituted protein, enabled limited model tracing and subsequent automated model building. The structure was refined to 2.0-Å resolution.

The overall structure of PagL consists of an eight-stranded anti-parallel β-barrel, which is consistent with our earlier prediction of its topology (Geurtsen *et al.*, 2005). So far, the N and C termini of the β-barrel part in all OMP structures solved are facing the periplasm (reviewed in Schulz, 2000). Therefore, we propose that the same holds for PagL. In this orientation, the previously identified active-site residues Ser-128 and His-126 (Geurtsen *et al.*, 2005) are located near the hydrophilic/hydrophobic boundary of the outer leaflet of the outer membrane (Fig. 5A). This position would be consistent with the location of the scissile bond of the substrate LPS. Like other OMPs, PagL has long extracellular loops (L) and short periplasmic turns (T), except for T1, which is exceptionally long (Fig. 5A).

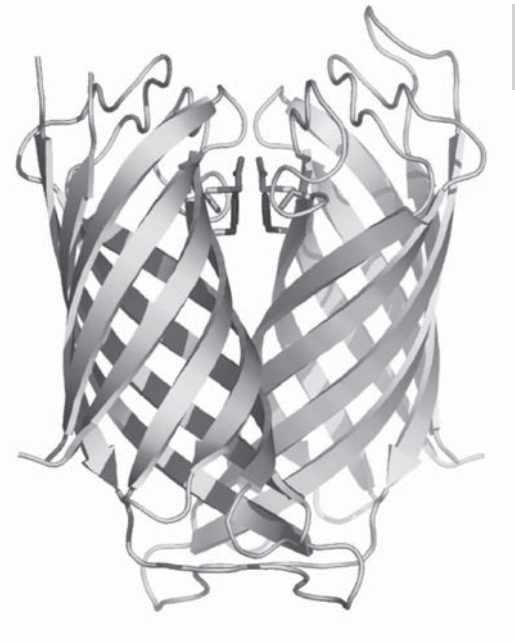

Fig. 4. PagL dimer in the crystal structure. The active-site residues Ser-128, His-126, and Glu-140 are coloured pink, blue, and red, respectively.

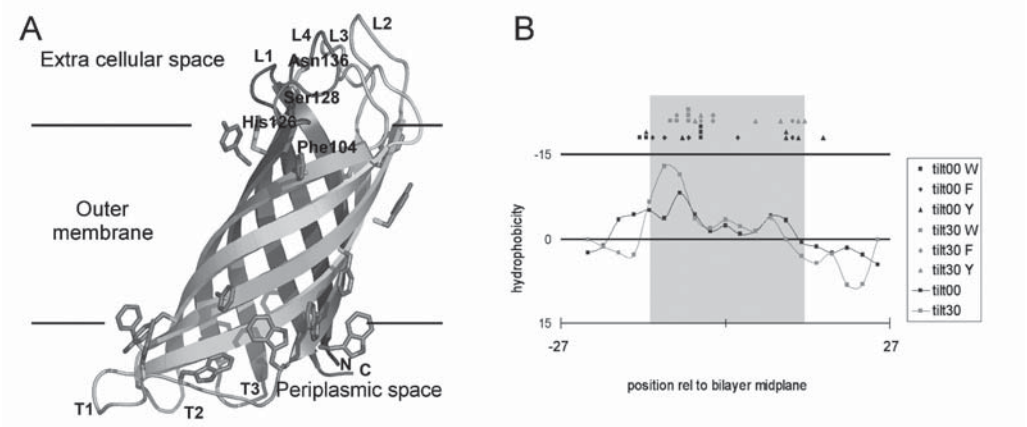

Fig. 5. PagL structure and membrane orientation. (A) Ribbon representation of PagL. The N and C termini are labelled and coloured blue and red, respectively, with gradient colours in between. The four extracellular loops are labelled L1–L4, and the three periplasmic turns are labelled T1–T3. Aromatic residues located at the presumed membrane boundaries are shown in gray, with nitrogen and oxygen atoms shown in blue and red, respectively. The only four completely conserved residues among PagL homologs are labelled. (B) Hydrophobicity profiles for the outward-facing PagL residues as a function of membrane position (periplasmic side left, extracellular side right) are shown as solid lines. Negative $\Sigma(\Delta G)$ values indicate regions that are more hydrophobic. The blue line and symbols present results for the positions with the β-barrel axis aligned along the membrane normal, whereas the magenta line and symbols are for the protein tilted by 30°. The symbols represent the $C\gamma$ positions of Trp (squares), Tyr (circles), and Phe (triangles) residues that form the inner and outer aromatic girdles. The image shown in (A) was prepared with PYMOL (www.pymol.org).

Orientation of PagL in the membrane

Like most other OMPs, PagL contains two girdles of aromatic residues, which are located at the hydrophobic/hydrophilic boundaries of the membrane and are assumed to stabilise the position of the protein in the membrane. Because these girdles are not perpendicular to the β-barrel axis of PagL, as they usually are in OMPs, the orientation of PagL in the membrane may be tilted (Fig. 5A). Therefore, we calculated the net hydrophobicity at the β-barrel surface as a function of the height in the membrane as described (Wimley, 2002); this was done for two orientations, i.e., with the β-barrel axis parallel or with a 30° tilt angle relative to the membrane normal (Fig. 5B). In the tilted orientation, the graph resembles the average graph calculated for all OMP β-barrel structures, in which two hydrophobic peaks represent the aromatic girdles (Wimley, 2002). Remarkably, the hydrophobic peak at the extracellular side is much lower than that at the periplasmic side, which can easily be explained by the presence of a highly hydrophilic active-site at that height in the barrel (see below). Also, in the parallel orientation, two hydrophobic peaks can be discriminated. However, the area of hydrophobicity of the intracellular peak extends considerably into the periplasm, suggesting that a parallel orientation is energetically highly unfavourable. Therefore, we propose that PagL has a tilted orientation in the membrane.

PagL catalytic-mechanism

Previously, we identified PagL homologs in the genomes of a wide variety of Gram-negative bacteria (Geurtsen *et al.*, 2005). These homologs exhibited a low overall mutual sequence identity, with only 10 conserved residues among 14 different homologs. Here, we performed a more extensive search, which revealed additional PagL homologs in *Caulobacter crescentus*, *Collimonas fungivorans*, *Chlorobium tepidum*, *Rhodospirillum rubrum*, *Sinorhizobium meliloti*, and *Ralstonia eutropha*. An alignment of all currently identified PagL homologs is presented in Fig. 6. Among all PagL homologs identified so far, only four amino acid residues are fully conserved, i.e., Phe-104, His-126, Ser-128, and Asn-136 of the *P. aeruginosa* PagL sequence.

Active sites of hydrolases comprise three important regions: the catalytic site, a substrate-binding site, and an oxyanion hole. Classical serine esterases contain a catalytic site consisting of a Ser-His-Asp/Glu triad. The histidine abstracts a proton from the serine residue, after which the serine performs a nucleophilic attack on the carbonyl carbon of the scissile bond. This attack can only be performed efficiently if the Nε2 of the histidine is deprotonated. Deprotonation of the histidine is achieved by stabilisation of the proton on the Nδ1 of the histidine via a hydrogen bond with an acidic residue.

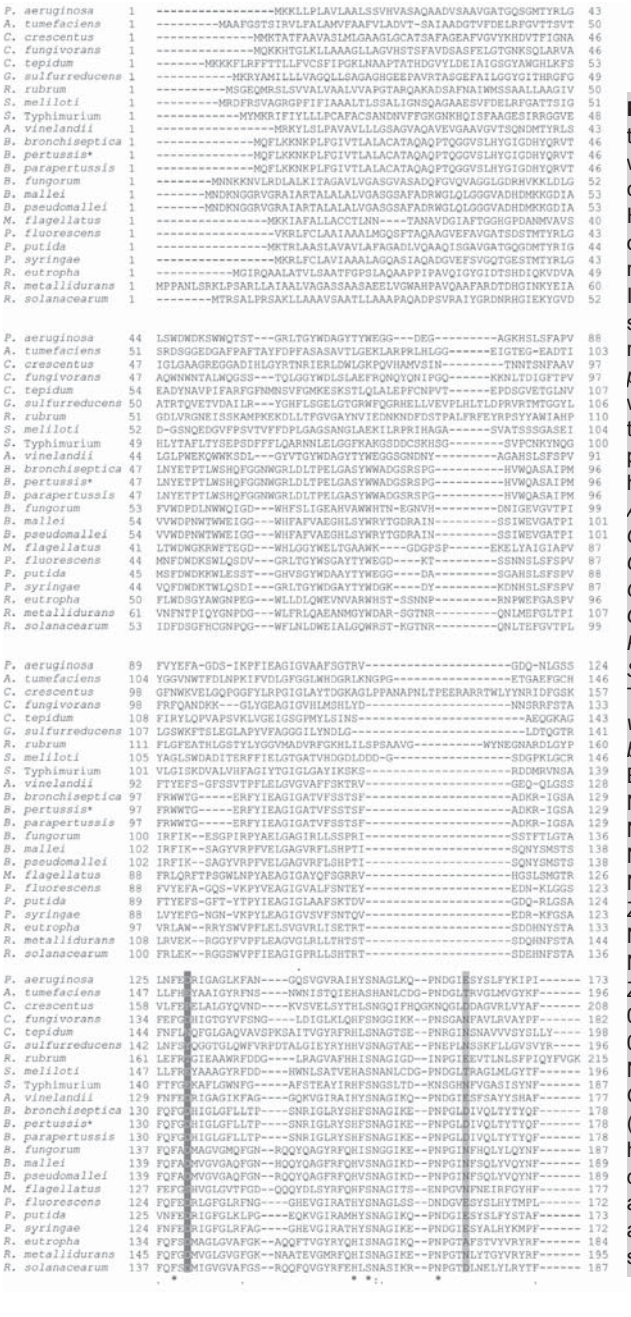

Fig. 6. Multiple sequence alignment of the precursor PagL proteins. Sequences were aligned using CLUSTALW (www.ch.embnet.org/software/ClustalW.html). Hyphens indicate gaps introduced for optimal alignment. Absolutely conserved residues are marked with asterisks. Indicated by colons and dots are strongly and weakly conserved residues, respectively. The *pagL* ORF in *Bordetella pertussis* is disrupted by a frame shift, which was restored for this alignment by adding two nucleotides in codon 33. The GenBank protein accession numbers for the PagL homologs are: *P. aeruginosa* NP_253350, *Agrobacterium tumefaciens* AAK87616, *Caulobacter crescentus* AAK25066, *Collimonas fungivorans* AAT42436, *Chlorobium tepidum* NP_663142, *Geobacter sulfurreducens* AE017180§, *Rhodospirillum rubrum* ZP_00270083, *Sinorhizobium meliloti* CAC46357, *S.* Typhimurium AAL21147, *Azotobacter vinelandii* ZP_00089534, *Bordetella bronchiseptica* NP_890306, *B. pertussis* BX470248§, *Bordetella parapertussis* NP_885487, *Burkholderia fungorum* NZ_AAAJ03000003§, *Burkholderia mallei* NC_002970§, *Burkholderia pseudomallei* NC_002930§, *Methylobacillus flagellatus* ZP_00564368, *Pseudomonas fluorescens* NZ_AAAT03000006§,*Pseudomonasputida* NC_002947§, *Pseudomonas syringae* ZP_00125465, *Ralstonia eutropha* ZP_00168928, *Ralstonia metallidurans* ZP_00274744, and *Ralstonia solanacearum* NP_522762. The symbol § indicates GenBank™ accession numbers of whole (unfinished) genomes, in which the PagL homologs were manually identified. The dark and light gray boxes indicate residues at the equivalent positions of Asp-106 and Glu-140 of the *P. aeruginosa* PagL sequence, respectively.

Usually, this acidic residue is an aspartate or a glutamate. However, in a few exceptions, such as in outer membrane phospholipase A (OMPLA) of *E. coli*, an asparagine residue is also able to stabilise the deprotonated state of the Nε2 of the histidine (Snijder *et al.*, 1999).

Site-directed mutagenesis studies have shown that His-126 and Ser-128 are important for catalytic activity and constitute part of the active-site of PagL (Geurtsen *et al.*, 2005). In the crystal structure, two acidic residues, Glu-140 and Asp-106, are located in close proximity to His-126. A hydrogen bond is present between the carboxylate of Glu-140 and the Nδ1 hydrogen of His-126, which implies that Glu-140 is the acidic component of the catalytic triad. This notion is supported by the observation that a Glu140Ala substitution reduced the activity of PagL *in vivo* in *E. coli* membranes (Fig. 7). LPS was almost completely converted into its 3-*O*-deacylated form 75 min after induction of expression of wild-type *pagL*, whereas ~50% remained in the acylated form when the mutant PagL was produced. In the quantitative assay, in which the release of 3OH C12 from exogenously added Neisserial LPS is measured, the mutated protein appeared 142-fold less active than the wild type.

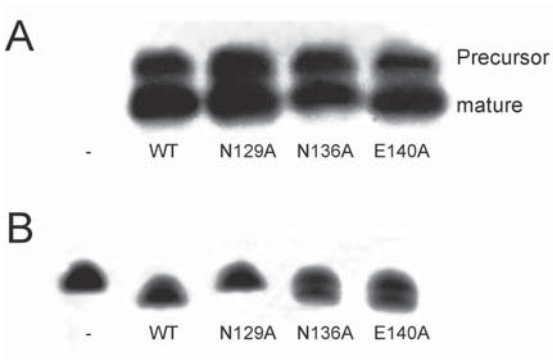

Fig. 7. Identification of residues important for PagL activity. Cells of *E. coli* BL21 Star (DE3) containing the empty pET11a vector, the $pPagL_{(Pa)}$ plasmid, or the mutant $pPagL_{(Pa)}$ plasmids, exponentially growing in LB, were induced with isopropyl-β-D-thiogalactoside for 75 min, after which 1-A_{600}-unit culture samples were collected and analysed by SDS/PAGE followed by immunoblotting with primary antibodies against PagL (A) and by Tricine-SDS/PAGE to visualise LPS (B). In *A*, the positions of mature PagL and of the precursor form, which accumulated because of overexpression, are indicated.

Another acidic residue, Asp-106, is present on the opposite side of His-126. In the crystal structure, Asp-106 coordinates a Ca^{2+} ion. Because PagL activity is not influenced by EDTA, this Ca^{2+} ion is likely not relevant for activity and may just be a crystallisation artefact. Because Asp-106 points toward the hydrophobic region of the membrane, which is not a favourable position for a charged residue, it seems plausible that it has an important function in the catalytic mechanism. To test this possibility, we substituted Asp-106 by Ala. Unfortunately, the mutant protein was poorly expressed (data not shown), indicating that the substitution caused folding and/or stability problems of the protein.

To gain further insight into the catalytic mechanism, we modelled lipid X, the smallest known substrate of PagL *in vitro* (Trent *et al.*, 2001) onto the active-site of PagL by using the program HADDOCK (Dominguez *et al.*, 2003). Several restraints were used. The distances between the hydroxyl oxygen of Ser-128 and the carbonyl carbon of the scissile bond and between the Nε2 of His-126 and the main-chain oxygen of the ester bond were both restrained to 3 Å. Furthermore, the acyl chains of lipid X were restrained to point towards the periplasm. In the resulting model (Fig. 8), the residual acyl chain (i.e., the chain to be cleaved off from the substrate) of lipid X is bound in a well defined hydrophobic groove, whereas the acyl chain of the leaving group is loosely bound into a second hydrophobic groove. The conserved residue Phe-104, which makes a hydrophobic interaction with the residual acyl chain, is likely to be a key residue for positioning the substrate into the correct orientation. Furthermore, the hydroxyl group of the residual acyl chain makes a hydrogen bond to Asp-106, suggesting a possible role for this residue in substrate specificity.

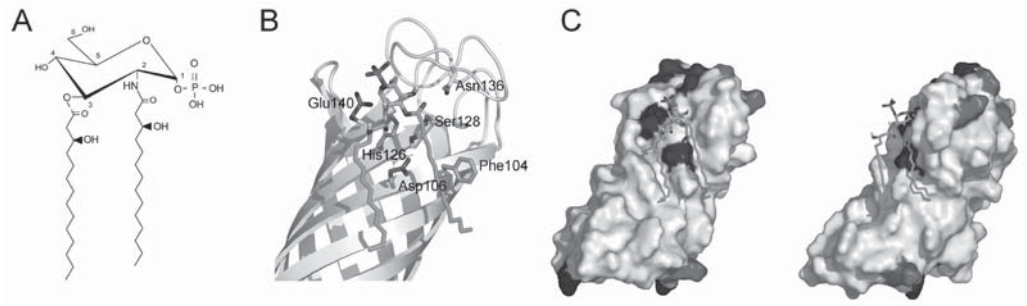

Fig. 8. Modelling of lipid X onto the active site of PagL. (A) Schematic representation of lipid X. (B) Lipid X modelled onto the active site of PagL. PagL is represented as a gray ribbon diagram. Lipid X is shown as green sticks with oxygen atoms in red and a phosphate atom in magenta. The hydrogen atoms from hydroxyl groups are shown in gray. Some amino acid residues important for PagL activity are shown as sticks and are labelled. (C) Two views (~90° rotated) of the electrostatic surface potential of PagL with lipid X. Positively and negatively charged residues are coloured blue and red, respectively. Lipid X is shown as green sticks. The images in *B* and *C* were prepared with PYMOL (www.pymol.org).

The structure of one other outer membrane esterase has been solved, i.e., that of OMPLA, which forms a 12-stranded β-barrel with a catalytic site composed of a Ser-His-Asn catalytic triad (Dominguez *et al.*, 2003). To compare the catalytic sites of OMPLA and PagL, we superposed Ser-128 and His-126 of PagL onto Ser-144 and His-142 of OMPLA (Fig. 9). In both PagL and OMPLA, the active sites are located at comparable heights in the barrel relative to the membrane. Furthermore, Glu-140 of PagL occupies the structurally equivalent position of Asn-156, the acidic residue in the catalytic triad of OMPLA, consistent with Glu-140 being the acidic residue of the PagL catalytic triad. In

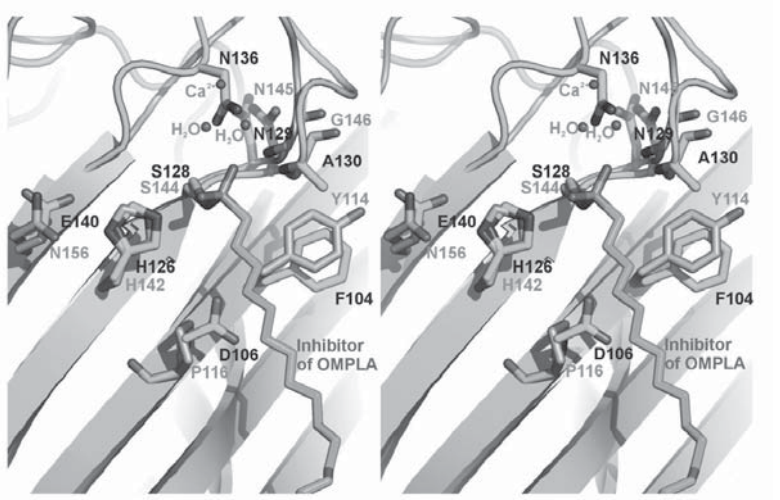

Fig. 9. Stereo diagram of the active site Ser-128 and His-126 of PagL superposed on the active site Ser-144 and His-142 of OMPLA. PagL is shown in cyan, whereas OMPLA is represented in orange. Residues and atoms that may have an important role for activity are shown as sticks and are labelled with cyan and orange text for PagL and OMPLA, respectively. The hexadecanesulfonyl moiety of an OMPLA inhibitor is covalently attached to Ser-144 of OMPLA and coloured green. The image was prepared by using PYMOL (www.pymol.org).

many serine hydrolases, the oxyanion hole consist of two backbone amide groups that form a slightly positively charged centre that stabilises the transient negative charge on the carbonyl oxygen of the substrate during the reaction. The oxyanion hole of OMPLA is formed by two backbone nitrogens from two glycine residues and two water molecules, coordinated by a calcium ion (Snijder *et al.*, 1999). For PagL, the oxyanion hole could well be formed by the two backbone nitrogens of the highly conserved Ala-130 and Gly-131 residues, which are located at the equivalent positions of the oxyanion-hole glycines in OMPLA. Comparison of the OMPLA and PagL active sites further showed that the side-chain nitrogen of Asn-136, the fourth completely conserved residue among the PagL homologs, occupies the position of one of the oxyanion-hole waters in OMPLA (Fig. 9). Therefore, we speculate that the side-chain amide group of Asn-136 is part of the oxyanion hole of PagL. This possibility is supported by the observation that an Asn136Ala mutant derivative of PagL was less active *in vivo* than wild-type PagL (Fig. 7). In the quantitative assay, the activity of this mutant protein was reduced 304-fold. In addition to the three highly or even completely conserved residues Ala-130, Gly-131, and Asn-136, which we postulate to form the oxyanion hole of PagL, one other residue, Asn-129, shows a high conservation among the PagL homologs (Fig. 6). Furthermore, the same residue is present at the equivalent position in OMPLA (Fig. 9). We propose that Asn-129 of PagL might play a crucial role in stabilising the conformation of loop 4

and thereby that of the oxyanion hole and the active-site. The importance of Asn-129 for the enzymatic activity is supported by the observation that an Asn129Ala mutant derivative of PagL showed no activity *in vivo* (Fig. 7), nor in the quantitative assay even after 27 h incubation with the substrate.

Discussion

The lipid A deacylase PagL was initially discovered in *S.* Typhimurium, and, recently, PagL homologs were identified in a wide variety of Gram-negative bacteria, including *P. aeruginosa*. By using these PagL homologs as leads, we were able to identify additional homologs, which now brings their total number to 23. Remarkably, besides the previously identified active-site His and Ser residues, only a Phe and an Asn residue are completely conserved among all homologs identified so far. Modelling with HADDOCK suggested that the phenylalanine is important for positioning the substrate correctly into a hydrophobic groove present on PagL, thereby bringing the scissile bond into close proximity to the active-site serine. This hypothesis is sustained by the presence of another aromatic residue, Tyr-114, at the equivalent position in OMPLA, which was shown to have similar active-site architecture. This tyrosine was shown to make a hydrophobic interaction in the crystal structure of OMPLA with the substrate analog hexadecanesulfonylfluoride. The close resemblance of the PagL active-site to that of OMPLA also allowed us to speculate about the location of the oxyanion hole and the possible role of the completely conserved asparagine (Asn-136) therein.

The catalytic triad of PagL is formed by His-126, Ser-128, and, most likely, Glu-140. The hypothesis that Glu-140, rather than Asp-106, is the third member of the catalytic triad is supported by several lines of evidence. First, Glu-140 is located at the structurally equivalent position of Asn-156, the postulated acidic residue in the catalytic triad of OMPLA. Second, Glu-140 forms a hydrogen bond with His-126. Third, mutagenesis showed that Glu-140 is important for PagL activity, although not essential, because residual activity was detected after its substitution by Ala. In OMPLA, Asn-156 is not essential either, because Asn156Ala substitution reduced OMPLA activity ~20-fold (Kingma *et al.*, 2000). A possible explanation is that OMPLA, and probably also PagL, has a highly functional oxyanion hole that can partly compensate for the loss of the acidic residue. The acidic residue of the catalytic triad of serine hydrolases can be formed by three different amino acids, i.e., Asp, Glu, and, more rarely, Asn. An alignment of all identified PagL homologs (Fig. 6) shows that always one of these residues is present at the position of Glu-140 in *P. aeruginosa* PagL, except in three cases, i.e., the PagL

homologs of *Agrobacterium tumefaciens*, *S. meliloti*, and *R. eutropha*, where a threonine, a threonine, and an alanine, respectively, are present. Theoretically, a threonine could take over the role of the acidic residue in forming a hydrogen bond with the active-site histidine, and it will be interesting to determine by site-directed mutagenesis and x-ray crystallography whether the threonine indeed plays such a role in the *A. tumefaciens* and *S. meliloti* enzymes. The *R. eutropha* enzyme may be less active, like the Glu140Ala-mutant derivative of *P. aeruginosa* PagL, and it will be interesting to determine whether its activity can be enhanced by substituting an acidic residue for the alanine. In the modelled PagL/lipid X structure, the hydroxyl group of the residual acyl chain makes a hydrogen bond to Asp-106. In OMPLA, a proline (Pro-116) is present at the equivalent position. It is tempting to speculate that Asp-106 determines the specificity of PagL for substrates with a hydroxyl group present at the correct position, i.e., for LPS. It will be interesting to determine the consequences of Asp106Pro and Pro116Asp substitutions in PagL and OMPLA, respectively, for the substrate specificity of the enzymes. It should be noted, however, that Asp-106 is not completely conserved among all PagL homologs, and one enzyme, i.e., that of *C. crescentus*, contains a proline at the equivalent position (Fig. 6).

Taken together, monomeric PagL has all features needed for activity, i.e., an active-site, an oxyanion hole, and a substrate-binding pocket within a single molecule. In contrast, OMPLA activity is activated *in vitro* by dimerisation (Snijder *et al.*, 1999). This raises the question in what way enzymatic activity of PagL is controlled. In *S.* Typhimurium, expression of *pagL* is under the control of the PhoP/PhoQ two-component regulatory system. However, silencing of PagL activity, when it is no longer beneficial to the bacterium, is not explained. A recent observation may provide an answer: PagL of *S.* Typhimurium is inhibited by modification of LPS with aminoarabinose (Kawasaki *et al.*, 2004). It was suggested that such modification might affect the membrane localisation or conformation of PagL. However, in *P. aeruginosa*, modification of LPS with aminoarabinose is found in combination with 3-*O*-deacylation of lipid A, suggesting that PagL activity is not regulated in this way in *P. aeruginosa* (Ernst *et al.*, 1999). In contrast to the activation caused by dimerisation of OMPLA, dimerisation of PagL could inhibit PagL activity. The active-sites of the two PagL molecules in the asymmetric unit are in close proximity (Fig. 4). It is tempting to speculate that this structure represents a dimeric state of PagL. After substitution of the active-site serine by a cysteine, we detected dimers of the protein when the membranes were analysed by SDS/PAGE in the absence of a reducing agent (data not shown), indicating that the active sites of the two PagL molecules could be very close *in vivo* as well. If indeed such a dimer is formed,

the active-site of one monomer is shielded for substrate binding by the other monomer. Therefore, dimerisation may be an inactivation mechanism for PagL, whereas it is an activation mechanism in the case of OMPLA.

Both the length of turn T1 and the position of the aromatic residues raised the idea that the PagL β-barrel is tilted relative to the membrane plane. Interestingly, such an assumption has been made for only one other OMP, i.e., the lipid A-modifying enzyme PagP (Ahn *et al.*, 2004). Analysis of the hydrophobic surface of the β-barrel indeed showed that a tilted orientation of PagL is more consistent with the average hydrophobicity profile of other β-barrels of known structure. The tilted orientation also explains why the periplasmic turn (T1) between strands 2 and 3 is asymmetrical and contains many aromatic residues. Considering the hydrophobicity profiles of possible orientations tested, a 30°-tilted orientation is expected to be energetically most favourable. However, in a biological membrane, there are many more forces and factors that determine the position of a protein. The shape of the LPS and phospholipids and the lateral pressure exerted by these will form a counterbalancing force against tilting of the barrel. Therefore, it cannot be concluded that PagL has a 30° tilt *in vivo*, although such an angle might be considered as the maximal possible tilt. Furthermore, control of the barrel tilt angle and, thereby, of the position of the active-site relative to the membrane might form another mechanism to control the enzymatic activity of PagL *in vivo*.

LPS is known for its endotoxic activity when administered to higher organisms. This endotoxic activity is responsible for the side effects that are often seen when vaccines that contain LPS are administered. LPS also has a powerful adjuvant activity. Unfortunately, a broad-scale use of this adjuvant quality is not possible because of the endotoxic activity. Recently, it has been shown that changing the physico-chemical properties of LPS influences its endotoxic activity (Raetz and Whitfield, 2002; Loppnow *et al.*, 1989; Steeghs *et al.*, 2002). Thus, LPS-modifying enzymes may be valuable tools for detoxification of LPS. We showed here that purified and *in vitro* refolded PagL is active against externally added LPS. Thus, *in vitro* refolded PagL might be used for the detoxification of LPS *in vitro* and thereby be useful for the development of new vaccines or adjuvants. Furthermore, the structure of PagL may form, especially because the active-site is on the outside of the barrel, a basis for the design of inhibitors with possible therapeutic value.

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Chapter 4

Expression of the lipopolysaccharide-modifying enzymes PagP and PagL modulates the endotoxic activity of *Bordetella pertussis*

Jeroen Geurtsen, Liana Steeghs, Hendrik-Jan Hamstra, Jan ten Hove, Alex de Haan, Betsy Kuipers, Jan Tommassen, and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial cell envelope. Its endotoxic activity causes the relatively high reactogenicity of whole-cell vaccines. Several bacteria harbour LPS-modifying enzymes that modulate the endotoxic activity of the LPS. Here we evaluated whether two such enzymes, i.e., PagP and PagL, could be useful tools for the development of an improved and less reactogenic whole-cell pertussis vaccine. We showed that expression of PagP and PagL in *Bordetella pertussis* leads to increased and decreased endotoxic activity of the LPS, respectively. As expected, PagP activity also resulted in increased endotoxic activity of whole bacterial cells. However, more unexpectedly, this was also the case for PagL. This paradoxical result may be explained, in part, by an increased release of LPS, which we observed in the PagL-expressing cells.

Introduction

The genus *Bordetella* includes several well-known pathogens that are capable of colonising the upper respiratory tracts of mammals. *Bordetella pertussis* and the majority of *Bordetella parapertussis* strains are obligate human pathogens that usually cause acute respiratory tract diseases. *Bordetella bronchiseptica* has a much broader host range and is capable of colonising rabbits, rats, guinea pigs, dogs, and piglets (Goodnow, 1980). Normally, *B. bronchiseptica* does not infect humans; however, some cases of infection in elderly or immunocompromised individuals have been described (Amador *et al.*, 1991; Gueirard *et al.*, 1995; Reina *et al.*, 1991).

The majority of virulence factors in the Bordetellae are regulated by the two-component system BvgAS (Mattoo and Cherry, 2005; Smith *et al.*, 2001). In response to environmental stimuli, the BvgAS system regulates the transcription of several target genes (Cotter and Jones, 2003). Bacteria in the Bvg^+ phase express virulence factors, such as filamentous hemagglutinin, pertactin, and pertussis toxin. During the Bvg^- phase, the majority of virulence factors are down regulated, whereas other genes (for example, those that are required for motility) are up regulated (Akerley *et al.*, 1992). Some virulence genes, including those encoding filamentous hemagglutinin and fimbriae, are expressed also in a Bvg-intermediate phase, known as Bvg^i (Cotter and Miller, 1997).

Lipopolysaccharide (LPS) is one of the major components of the Gram-negative bacterial outer membrane. It is usually composed of a highly variable O antigen, a less variable core oligosaccharide, and a highly conserved lipid moiety designated lipid A. The structure of lipid A is well conserved among Gram-negative bacteria and consists of a glucosamine disaccharide substituted with one or two phosphate groups and a variable number of acyl chains (Fig. 1). LPS is also known as endotoxin, due to its endotoxic activity when administered to higher organisms. This endotoxic activity is based on recognition of the LPS by the innate immune system. LPS is first bound by the LPS-binding protein (LBP), after which it is transferred, with the help of CD14, to the Toll-like receptor 4 (TLR4)/MD-2 complex. Activation of the TLR4/MD-2 complex leads to a downstream signalling cascade and eventually to increased production of proinflammatory cytokines, such as interleukin-6 (IL-6) (reviewed in Pålsson-McDermott and O'Neill, 2004). The endotoxic activity of LPS is determined entirely by the composition of the lipid A moiety (Homma *et al.*, 1985). Previous studies have shown that the phosphate groups as well as the number and length of the acyl chains are critical determinants of the endotoxic activity of lipid A (Loppnow *et al.*, 1989; Raetz and Whitfield, 2002; Steeghs *et al.*, 2002). Besides its endotoxic activity, LPS also has a powerful adjuvant activity and in that respect is potentially an interesting vaccine component.

The role of LPS in *Bordetella* pathogenesis and immunity is unclear. It has been shown that *Bordetella* LPS is highly immunogenic, acts as an immunological adjuvant, and exhibits the properties that are expected of an endotoxin (Amano *et al.*, 1990; Chaby and Caroff, 1988; Watanabe *et al.*, 1990). Furthermore, *Bordetella* LPS and its recognition by the TLR4/MD-2 complex have been shown to be important determinants for the course of infection in mice (Banus *et al.*, 2006; Mann *et al.*, 2004a; Mann *et al.*, 2004b). *B. pertussis* can express two forms of LPS, i.e., band A and band B (Peppler, 1984). Band B LPS is composed of lipid A and a core oligosaccharide. Addition of a terminal trisaccharide to band B LPS forms the LPS referred to as band A. In contrast to *B. parapertussis* and some strains of *B. bronchiseptica*, *B. pertussis* lacks a repetitive O-antigen structure. In *B. pertussis*, lipid A is substituted via ester or amide linkage with *R*-3-hydroxytetradecanoic acid (3OH C14) residues at positions 2, 2', and 3' and with an *R*-3-hydroxydecanoic acid (3OH C10) residue at the 3 position via ester linkage. A secondary myristoyl group substitutes the hydroxyl group of 3OH C14 at the 2' position (Fig.1) (Caroff *et al.*, 1994). Limited information on the genetics of *B. pertussis* LPS biosynthesis is currently available. Detailed studies have been performed only on the *wlb* locus, which is required for addition of the band A trisaccharide (Allen and Maskell, 1996).

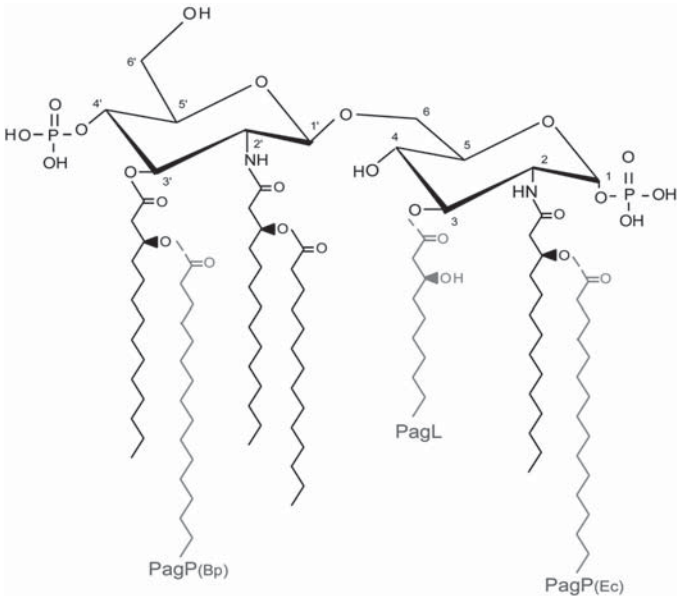

Fig. 1. *B. pertussis* lipid A architecture and modification by PagP and PagL. *B. pertussis* lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2' fatty acyl chain is esterified with myristate. *B. pertussis* PagP ($PagP_{(Bp)}$) and *E. coli* PagP ($PagP_{(Ec)}$) add a secondary palmitoyl chain at the 3' and 2 positions, respectively. Removal of the 3-hydroxydecanoic acid moiety at the 3 position is mediated by PagL.

In recent years, several enzymes capable of modifying mature lipid A in Gram-negative bacteria have been identified. One of them is the palmitoyl transferase PagP, which adds a secondary palmitoyl chain (C16) at the 2 position of lipid A (Bishop *et al.*, 2000). It was recently shown that treating *Escherichia coli* with EDTA leads to activation of PagP via a membrane perturbation mechanism (Jia *et al.*, 2004). Palmitoylation of lipid A leads to an increased resistance of the bacterium to cationic antimicrobial peptides (Guo *et al.*, 1998). Furthermore, palmitoylated LPS is attenuated for signalling through TLR4/MD-2 (Janusch *et al.*, 2002). A PagP homolog, which, in contrast to *E. coli* and *Salmonella* PagP, adds a secondary palmitoyl chain at the 3' position of lipid A (Fig. 1), has been identified in *B. bronchiseptica* (Preston *et al.*, 2003). In *B. pertussis*, however, expression of PagP seems to be abolished through insertion of an insertion sequence element in the promoter region (Preston *et al.*, 2003). Another lipid A-modifying enzyme, PagL, was discovered in *Salmonella enterica* serovar Typhimurium and shown to hydrolyse the ester bond at the 3 position of lipid A, thereby releasing the primary 3-hydroxymyristoyl moiety (Trent *et al.*, 2001). In *S.* Typhimurium, PagL activity can be inhibited by aminoarabinose modification of lipid A (Kawasaki *et al.*, 2005). Deacylation by PagL modulates the recognition of lipid A by the TLR4/MD-2 complex (Kawasaki *et al.*, 2004). We recently reported the identification of PagL homologs in a wide range of Gram-negative bacteria, including *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (Geurtsen *et al.*, 2005). Interestingly, in *B. pertussis*, the *pagL* open reading frame was disrupted by a frame shift, whereas in *B. bronchiseptica* and *B. parapertussis* the open reading frame was intact.

Until the second half of the previous century, pertussis was one of the major causes of early childhood mortality and morbidity. The introduction of whole-cell pertussis vaccines in the 1950s and 60s changed this and led to a dramatic decrease in the number of pertussis cases. However, a problem was that the whole-cell pertussis vaccines contained several toxins, such as LPS and pertussis toxin, and therefore displayed considerable reactogenicity. This led to the development of several acellular pertussis vaccines in the 1980s and 1990s. These vaccines are clearly less reactogenic and have an efficacy of between 58% and 97%, which is comparable to that of whole-cell pertussis vaccines (Schmitt, 1996). However, causes of concern are that these acellular vaccines are rather expensive, can be expected to select (potentially) for escape mutants more rapidly, and were shown to skew towards a Th2 immune response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997). The development of a new but less reactogenic whole-cell pertussis vaccine seems to be a feasible solution.

Here, we studied the consequence of PagP and PagL expression for the endotoxic activity of *B. pertussis* LPS and whole bacterial cells. The goal of this study was

to investigate whether the PagP and PagL enzymes might be useful tools for decreasing the LPS-mediated reactogenicity of whole-cell pertussis vaccines. In addition, this study provides the first description of *B. pertussis* mutants with altered lipid A.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used are described in Table 1. Typically, the *E. coli* strains were grown at 37°C in a modified Luria-Bertani broth, designated LB (Tommassen *et al.*, 1983), with shaking at 200 rpm. The medium was supplemented with 0.2% glucose. When appropriate, bacteria were grown in the presence of 100 μg/ml ampicillin, 50 μg/ml kanamycin, 10 μg/ml chloramphenicol, 50 μg/ml nalidixic acid, or 300 μg/ml streptomycin, for plasmid maintenance or strain selection. *B. pertussis* was grown at 35°C on Bordet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading). To induce the expression of the plasmid-carried *pagL* and *pagP* genes in *B. pertussis*, the bacteria were grown in synthetic THIJS medium (Thalen *et al.*, 1999) supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 35°C with shaking (175 rpm). To grow *B. pertussis* in the Bvg^- phase, 50 mM magnesium sulphate (final concentration) was added to the growth medium. A Bvg^+ phase-locked mutant of *B. pertussis* strain Tohama was created by using suicide plasmid pJM503 (Table 1) for allelic exchange mutagenesis. The presence of the correct mutation in the *bvgS* gene on the chromosome was confirmed by nucleotide sequencing in both directions after obtaining the gene by PCR. The correct phenotype was confirmed by testing colonies for haemolytic activity on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood and 50 mM $MgSO_4$. *Neisseria meningitidis* was grown at 37°C on GC agar plates (Oxoid) supplemented with Vitox (Oxoid). To induce the expression of *pagL* in *N. meningitidis*, bacteria containing plasmid pEN11-$PagL_{(Bb)}$ were grown on GC agar plates supplemented with Vitox and 1 mM IPTG at 37°C.

Recombinant DNA techniques

All plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega WizardPlus SV Minipreps system. Calf intestine alkaline phosphatase and restriction endonucleases were used according to the instructions of the manufacturer (Fermentas). DNA fragments were isolated from agarose gels by using the QIAGEN quick gel extraction kit. Ligations were performed using the rapid DNA ligation kit (Roche).

TABLE 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
B. pertussis		
Tohama	Wild-type strain	N.V.I.[a]
Tohama Bvg$^+$-phase lock	Bvg$^+$-phase locked mutant of *B. pertussis* Tohama strain	This study
E. coli		
TOP10F'	*F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	*F$^-$ Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	Hanahan, 1983
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir* R6K Kan^r	N.V.I.[a]
N. meningitidis		
H44/67	Wild-type strain	N.V.I.[a]
H44/67-*lpxA*	LPS-deficient derivative of H44/76	Steeghs *et al.*, 1998
Plasmids		
pCRII-TOPO	*E. coli* cloning vector Amp^R Kan^R	Invitrogen
pET-11a	*E. coli* high-copy expression vector, Amp^R, T7 promotor	Novagen
pMMB67EH	Broad-host-range expression vector	Fürste *et al.*, 1986
pPagL$_{(Pa)}$	pET-11a derivative harboring *P. aeruginosa pagL*	Geurtsen *et al.*, 2005
pPagL$_{(Bb)}$	pET-11a derivative harboring *B. bronchiseptica pagL*	Geurtsen *et al.*, 2005
pPagL$_{(Pa)\ (S151A)}$	pPagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S151A substitution	Geurtsen *et al.*, 2005
pPagP$_{(Bp)}$	pET-11a derivative harboring *B. pertussis pagP*	This study
pMMB67EH- PagL$_{(Pa)}$	pMMB67-EH derivative harboring *P. aeruginosa pagL*	This study
pMMB67EH- PagL$_{(Bb)}$	pMMB67-EH derivative harboring *B. bronchiseptica pagL*	This study
pMMB67EH- PagL$_{(Pa)\ (S151A)}$	pMMB67EH- PagL$_{(Pa)}$ encoding PagL$_{(Pa)}$ with S151A substitution	This study
pMMB67EH- PagP$_{(Bp)}$	pMMB67-EH derivative harboring *B. pertussis pagP*	This study
pMMB67EH- PagP$_{(Ec)}$	pMMB67-EH derivative harboring *E. coli pagP*	Tefsen *et al.*, 2005
pEN11-PagL	pEN11 derivative harboring *B. bronchiseptica pagL*	Bos *et al.*, 2004
pJM503	Suicide-vector harboring *bvgS* region with a G2527A substitution	Miller *et al.*, 1992

[a] Netherlands Vaccine Institute, Bilthoven, The Netherlands

The *pagP* gene from *B. pertussis* strain Tohama (*pagP*$_{(Bp)}$) was obtained by PCR. The chromosomal template DNA was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x *g*, after which the supernatant was used as template DNA. The sequence of the forward primer, which contained an NdeI site (underlined) and included an ATG start codon, was 5'-AACATATGACCCAGTATTTCCGGTCCC-3'. The sequence of the reverse primer, which contained a BamHI site (underlined) and included a stop codon, was 5'-AAGGATCCTTAGAACTCCCAGCGGCCA-3'. The PCR was done under the following condition: 50-μl total reaction volume, 25 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 3 μl of template DNA solution, 1.5% dimethyl sulfoxide, and 1.75 units of Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche). The temperature program was as follows: 95°C for 3 min; a cycle of 1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C repeated 30 times; followed by 10 min at 72°C and subsequent cooling to 4°C. The PCR product was purified from agarose gel and subsequently cloned into pCRII-TOPO. Plasmid DNA from a correct clone was digested with NdeI and BamHI, and the PagP-encoding fragment was ligated into NdeI- and

BamHI-digested pET-11a. The ligation mixture was used to transform *E. coli* DH5α by the $CaCl_2$ method (Sambrook *et al.*, 1989). A plasmid containing the correct insert was designated $pPagP_{(Bp)}$. The nucleotide sequence of the cloned *pagP* gene was confirmed by nucleotide sequencing in both directions.

To allow for expression in *B. pertussis*, $pagP_{(Bp)}$ and the *pagL* genes of *B. bronchiseptica* ($pagL_{(Bb)}$) and *Pseudomonas aeruginosa* ($pagL_{(Pa)}$) were subcloned into the broad-host-range, low-copy vector pMMB67EH. $pPagP_{(Bp)}$, $pPagL_{(Bb)}$, $pPagL_{(Pa)}$, and $pPagL_{(Pa)(S151A)}$ plasmid DNAs (Table 1) were digested with XbaI and HindIII, and the relevant fragments were ligated into XbaI- and HindIII-digested pMMB67EH. The ligation mixture was used to transform *E. coli* DH5α. Plasmids with the correct inserts were designated $pMMB67EH\text{-}PagP_{(Bp)}$, $pMMB67EH\text{-}PagL_{(Bb)}$, $pMMB67EH\text{-}PagL_{(Pa)}$, and $pMMB67EH\text{-}PagL_{(Pa)(S151A)}$, respectively (Table 1). The pMMB67EH-based plasmids were used to transform *E. coli* SM10(λpir), which allowed for subsequent transfer of the plasmids to *B. pertussis* by conjugation.

Isolation and analysis of LPS

LPS was isolated using the hot-phenol/water extraction method (Westphal and Jann, 1965) with slight modifications. In short, bacteria were grown in THIJS medium supplemented with 1 mM IPTG to stationary phase. Cells were harvested by centrifugation and resuspended in 40 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA. The cells were treated overnight with lysozyme at 4°C, after which an equal volume of phenol was added. The suspension was heated to 70°C, incubated for 30 min with shaking, and subsequently cooled to 10°C, after which phases were separated by centrifugation for 10 min at 8,000 x *g*. The upper phase was collected, and the extraction was repeated after addition of an equal volume of distilled water to the lower phase. The two upper phases were combined, dialysed against tap water until the phenol odour disappeared, freeze-dried, and subsequently taken up in distilled water. The LPS was pelleted by centrifugation for 3 h at 150,000 x *g* and dissolved in distilled water, after which the LPS concentration was determined by analysing the 3OH C14 content, using an Agilent 6890 gas chromatograph, as described previously (Welch, 1991). Approximately 250 pmol purified LPS was applied to a Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Lesse *et al.*, 1990). The electrophoresis was run for 17 h at a constant current of 20 mA, after which the gel was fixed with water-ethanol-acetic acid (11:8:1, vol/vol/vol) and subsequently stained with silver as described previously (Tsai and Frasch, 1982). For electrospray ionisation-mass spectrometry (ESI-MS), a 200-μl aliquot of isolated LPS (50 nmol/ml) was freeze-dried

and taken up in 0.1 ml of 2% acetic acid (pH 2.8). The mixture was heated for 2 h at 95°C to hydrolyse the LPS and release the lipid A moiety. Subsequently, the mixture was cooled to room temperature and centrifuged for 10 min at 16,100 x *g*. The pellet was washed twice in 0.1 ml of double-distilled water and taken up in 0.1 ml of double-distilled water, and 0.3 ml of chloroform-methanol (2:1, vol/vol) was added. After vigorous vortexing, phases were separated by centrifugation for 10 min at 16,100 x *g*. The upper phase was then used for structural analysis of the lipid A by nanoelectrospray tandem MS on a Finnigan LCQ instrument in the negative-ion mode (Wilm and Mann, 1996).

Determination of LPS release by *B. pertussis* cells.

To determine the total LPS content, stationary-phase cultures grown in THIJS medium in the presence of 1 mM IPTG were heat inactivated (1 h at 56°C), freeze-dried, and stored at 4°C. To determine the amount of LPS released into the culture medium, the cells were removed from cultures by centrifugation (10 min at 16,100 x *g*), after which remaining cells were removed from the supernatant by filtration through a 0.22-μm filter (Millipore). The filtrate was subsequently freeze-dried and also stored at 4°C. Whole-cell suspensions, used for endotoxic activity assays (see below), were prepared by collecting the cells from cultures by centrifugation (for *B. pertussis*) or from GC agar plates (for *N. meningitidis*). The cells were washed once with phosphate-buffered saline (PBS) and then resuspended in PBS at an A_{590} of 1.0, heat inactivated, and stored at 4°C. To determine whether LPS was released from the *B. pertussis* cells during storage, whole-cell *B. pertussis* suspensions that had been stored at 4°C for 48 h were centrifuged and remaining cells were removed from the supernatant by filtration. The filtrate was freeze-dried and stored at 4°C. The LPS content of the cells and cell-free supernatants was determined by measuring the 3OH C14 content in the different preparations. The freeze-dried materials were resuspended in PBS, after which the samples were analysed using an Agilent 6890 gas chromatograph as described previously (Welch, 1991). The results shown represent the data from four individual experiments and were statistically analysed using Student's *t* test (two-tailed, two-sample unequal variance). *P* values lower than 0.05 were considered to be statistically significant.

Endotoxic activity assays

Stimulation of IL-6 production was tested with the human macrophage cell line MM6 (Ziegler-Heitbrock *et al.*, 1998), the TLR4-deficient mouse macrophage cell line CTC3H/HeJ, or CTC3H/HeJ cells expressing human TLR4 (hTLR4) (Poltorak *et al.*, 2000). The macrophages were seeded in 24-wells plates (2.5 x 10^5 cells/well) in 400

μl of IMDM medium (Gibco BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (Gibco BRL), and 10% fetal calf serum (Gibco BRL). The cells were stimulated with 100 μl of serial dilutions of whole-cell suspensions. (prepared as described above, starting at an A_{590} of 0.2 in the total stimulation volume), cell-free supernatants of whole-cell suspensions (starting with 0.1 ml supernatant in the total stimulation volume), or purified *B. pertussis* LPS (starting at 10 nmol/ml in the total stimulation volume). The stimulation was for 16 to 18 h at 37°C in a humid atmosphere containing 5% or 10% CO_2 for the MM6 cells and CTC3H/HeJ cells, respectively. For the determination of the effect of EDTA on the endotoxic activity of a whole-cell suspension, MM6 cells were stimulated with a *B. pertussis* whole-cell suspension, at an A_{590} of 0.01 in the total stimulation volume, in the presence of serial dilutions of EDTA, starting at 10 mM. As an internal standard for the stimulation potency of the cells, CTC3H/HeJ cells were stimulated with 100 μl of serial dilutions of a whole-cell suspension of an LPS-deficient *N. meningitidis* strain (Steeghs *et al.*, 1998) or the TLR2/6 agonist lipoprotein FSL (InvivoGen) (Okusawa *et al.*, 2004). Following stimulation, IL-6 concentrations in the culture supernatants were quantified with an enzyme-linked immunosorbent assay (ELISA) against mouse or human IL-6 according to the manufacturers' instructions (eBioscience and PeliKine Compact, respectively).

Results

Contribution of LPS to the total endotoxic activity of whole *B. pertussis* cells

Since our goal was to investigate whether the PagP and PagL enzymes might be useful tools for the development of a less reactogenic whole-cell pertussis vaccine, we first investigated the contribution of LPS to the total endotoxic activity of whole *B. pertussis* cells. Serial dilutions of a whole-cell suspension of *B. pertussis* strain Tohama were used to stimulate the production of the proinflammatory cytokine IL-6 by TLR4-deficient C3H/HeJ cells or by C3H/HeJ cells expressing hTLR4. The hTLR4-expressing cells were stimulated much more strongly (Fig. 2A), indicating a major contribution of the LPS to the total endotoxic activity of the *B. pertussis* cells. However, we also tested whether the stimulation potencies of the two cell lines were comparable. Therefore, the macrophages were stimulated with a whole-cell suspension of an LPS-deficient *N. meningitidis* strain and with the TLR2/6 agonist lipoprotein FSL. In both cases, the TLR4-deficient C3H/HeJ cells exhibited a clearly reduced potency to be stimulated (Fig. 2A and 2B). These results demonstrate that the TLR4-transfected C3H/HeJ cells are much more sensitive to stimulation by non-TLR4 agonists than the parent cells, emphasising

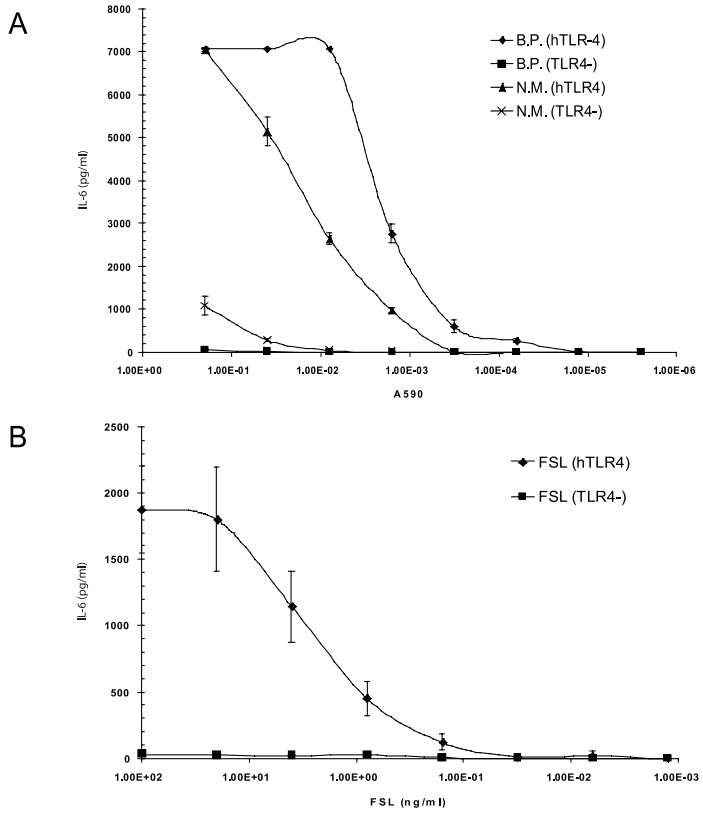

Fig. 2. Contribution of LPS to the endotoxic activity of whole *B. pertussis* cells. Stimulation of IL-6 production by (A) wild-type, whole *B. pertussis* cells (B.P.) or LPS-deficient *N. meningitidis* cells (N.M.) or by (B) the TLR2/6 ligand FSL was tested with the TLR4-deficient mouse macrophage cell line C3H/HeJ (TLR4–) and C3H/HeJ cells expressing hTLR4. C3H/HeJ cells were seeded in microtiter plates and stimulated with serial dilutions of a whole-cell suspension. The IL-6 concentration in the culture supernatant was quantified with an ELISA against mouse IL-6. The data represent the averages and standard deviations from three individual experiments.

the importance of proper controls when using these cells in determining the relative contributions of various TLR agonists to cytokine induction. Nevertheless, since the macrophages expressing hTLR4 produced more IL-6 after stimulation with *B. pertussis* cells than after stimulation with LPS-deficient *N. meningitidis*, whereas this was just the opposite for the TLR4-deficient C3H/HeJ cells, *B. pertussis* LPS still seems to contribute to the endotoxic activity of whole *B. pertussis* cells. Thus, reducing the endotoxic activity of *B. pertussis* LPS seems to be a valid strategy for obtaining a strain that can be used as the basis for a less reactogenic whole-cell pertussis vaccine.

Expression of PagP and PagL in *B. pertussis*

To produce PagP and PagL in *B. pertussis* strain Tohama, the *pagL* gene of *B. bronchiseptica* ($pagL_{(Bb)}$) and the *pagP* genes of *B. pertussis* ($pagP_{(Bp)}$) and of *E. coli* ($pagP_{(Ec)}$) were expressed from the broad-host-range, low-copy-number vector pMMB67EH. LPS isolated from the $PagL_{(Bb)}$-expressing strain appeared to be unaffected upon Tricine-SDS-PAGE analysis, whereas that from the PagP-expressing strains appeared to be modified, since a band with a lower electrophoretic mobility than that of the wild-type LPS was detected in case of the PagP-expressing strains (Fig. 3). The modification efficiency appeared to be higher in the $PagP_{(Ec)}$-expressing strain than in the $PagP_{(Bp)}$-expressing strain. To evaluate the possible LPS modifications in further detail, the lipid A moieties of the strains were analysed by ESI-MS in the negative-ion mode. This analysis revealed the presence of four major lipid A species in wild-type LPS (Fig. 4A). The peak at *m/z* 1557 represents the characteristic penta-acylated bisphosphate species that is typically found in *B. pertussis* (Caroff *et al.*, 1994), whereas the peak at *m/z* 1477 corresponds to a penta-acylated monophosphate species. The two remaining peaks at *m/z* 1307 and 1251 represent deacylated lipid A species of the molecular ion at *m/z* 1477, which lack the primary 3OH C10 residue at the 3 position and a primary 3OH C14 residue (at either the 2 or the 3' position), respectively. Interestingly, calculation of the relative amounts of the individual lipid A species from the corresponding peaks revealed that in wild-type *B. pertussis* LPS, a large quantity of lipid A species (~50%) consists of tetra-acylated forms. Furthermore, the large majority (~80%) of lipid A species are monophosphate forms. These results indicate a high heterogeneity among the lipid A species in wild-type *B. pertussis*, which was apparently not resolved in the gel analysis (Fig. 3). This observed heterogeneity could be either the consequence of fragment ionisation during mass spectrometry analysis, although this is an unlikely option with respect to the heterogeneity in acyl chain substitution, or the consequence of active modification by lipid A-modifying enzymes. However, to exclude the possibility that the observed heterogeneity was a consequence of overhydrolysis prior to the analysis, we tested whether shorter or longer periods of hydrolysis (varying between 1 and 4 h) influenced the ratio of the various lipid A species, and this was not the case (data not shown).

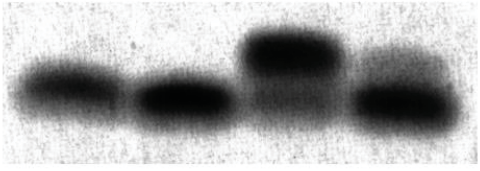

Fig. 3. Analysis by Tricine-SDS-PAGE of *in vivo* LPS modification. LPS was isolated from wild-type (WT) and PagP- or PagL-expressing *B. pertussis* strain Tohama and analysed by Tricine-SDS-PAGE.

Upon expression of $PagL_{(Bb)}$ (Fig. 4B), three lipid A species, at *m/z* 1081, 1307, and 1387, were detected. The major peak at *m/z* 1307 corresponds to the monophosphate deacylated form missing the 3OH C10 residue at the 3 position, whereas the peak at *m/z* 1387 corresponds to the bisphosphorylated form of the molecular ion at *m/z* 1307. The peak at *m/z* 1081 corresponds to a monophosphate form missing both the 3OH C10 residue and a 3OH-C14 residue. The relative content of lipid A species that lack the 3OH C10 residue at the 3 position was increased from ~37% in wild-type *B. pertussis* LPS to more than 92% in the strain expressing $PagL_{(Bb)}$. Thus, even though the electrophoretic mobility of the LPS was not affected (Fig. 3, lane 2), the *pagL*$_{(Bb)}$-encoded lipid A 3-*O*-deacylase was active in *B. pertussis*.

Upon expression of $PagP_{(Ec)}$ (Fig. 4C) and $PagP_{(Bp)}$ (Fig.4D), several new lipid A species were detected. The peaks at *m/z* 1320, 1490, 1545, 1625, 1715, and 1796 correspond to the expected PagP-mediated palmitoylation of the molecular ions present at *m/z* 1081, 1251, 1307, 1387, 1477, and 1557, respectively. The difference in modification efficiency between *E. coli* PagP and *B. pertussis* PagP, which was seen after analysis by Tricine-SDS-PAGE (Fig. 3), was also revealed in the mass spectrometry analysis. In the strain expressing *E. coli* PagP, approximately 47% of the total lipid A pool was palmitoylated, in contrast to only ~9% in the strain expressing $PagP_{(Bp)}$. Remarkably, palmitoylated lipid A species missing a 3OH C14 residue were found only in the strain expressing $PagP_{(Ec)}$ and not in that expressing $PagP_{(Bp)}$. *E. coli* PagP and *B. pertussis* PagP were previously shown to have different specificities (Bishop *et al.*, 2000; Preston *et al.*, 2003). Whereas *E. coli* PagP adds a secondary C16 to the primary acyl chain at the 2 position of lipid A, *B. pertussis* PagP adds the C16 to the acyl chain at the 3' position (Fig.1). Thus, if the *B. pertussis* lipid A species lacking a 3OH C14 residue (Fig.4A) lack it specifically at their 3' positions, the different modification efficiencies of the two PagP enzymes could be explained, as the substrate pool for *B. pertussis* PagP would then be smaller than that for *E. coli* PagP. Such an explanation would also be consistent with the presence of hypoacylated lipid A species *in vivo*. An overview of the relative abundances of all lipid A molecular ions as determined by ESI-MS is presented in Table 2.

Endotoxic activity of LPS

To assess the endotoxic activity of wild-type and PagP- or PagL-modified *B. pertussis* LPSs, their potency to stimulate the production of IL-6 by the human macrophage cell line MM6 was tested. Compared with wild-type LPS, the purified LPS from the strains expressing $PagP_{(Ec)}$ or $PagP_{(Bp)}$ had a strongly increased ability to stimulate the macrophages (Fig. 5A). In contrast, modification by $PagL_{(Bb)}$ reduced the

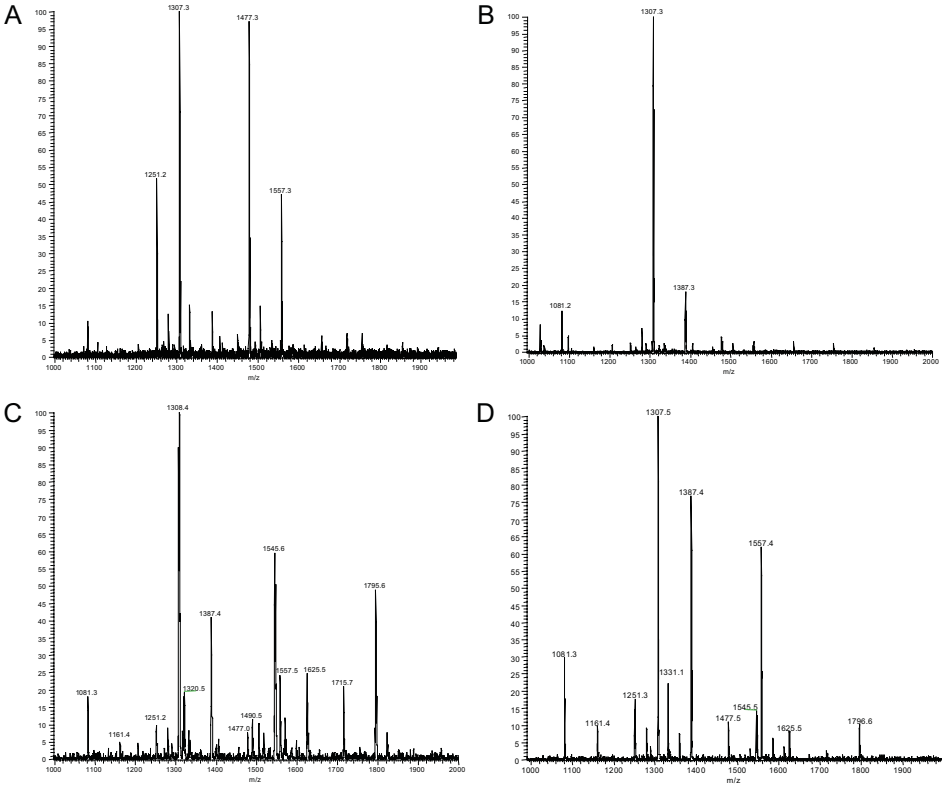

Fig. 4. Structural analysis by ESI-MS of purified *B. pertussis* LPS. Lipid A species from wild-type *B. pertussis* strain Tohama (A) and its derivatives expressing $PagL_{(Bb)}$ (B), $PagP_{(Ec)}$ (C), and $PagP_{(Bp)}$ (D) were analyzed by ESI-MS. Major peaks at *m/z* 1557, 1477, 1387, 1307, 1251, and 1081 were interpreted as the characteristic penta-acylated bisphosphate species that is typically found in *B. pertussis*, the corresponding penta-acylated monophosphate species, the deacylated lipid A species of the molecular ion at *m/z* 1557 missing the primary 3OH C10 residue at the 3 position, the deacylated lipid A species of the molecular ion at *m/z* 1477 missing the primary 3OH C10 residue at the 3 position, the deacylated lipid A species of the molecular ion at *m/z* 1477 missing a primary 3OH C14 residue, and the deacylated lipid A species of the molecular ion at *m/z* 1477 missing both the primary 3OH C10 residue at the 3 position and a primary 3OH C14 residue, respectively. The peaks at *m/z* 1320, 1490, 1545, 1625, 1715, and 1796 correspond to the PagP-mediated palmitoylation of the molecular ions present at *m/z* 1081, 1251, 1307, 1387, 1477, and 1557, respectively.

TABLE 2

Relative abundance of lipid A molecular ions as determined by ESI-MS

	1081 **-C14-3OH -C10-3OH -PO4**	**1161** **-C14-3OH -C10-3OH**	**1251** **-C14-3OH -PO4**	**1307** **-C10-3OH -PO4**	**1320** **-C14-3OH -C10-3OH -PO4 +C16**	**1331** **-C14-3OH**	**1387** **-C10-3OH**	**1477** **-PO4**	**1490** **-C14-3OH -PO4 +C16**	**1545** **-C10-3OH -PO4 +C16**	**1557**	**1625** **-C10-3OH +C16**	**1715** **-PO4 +C16**	**1796** **+C16**
Wild-type	3.0	0.0	15.6	29.9	0.0	4.5	3.9	29.0	0.0	0.0	14.1	0.0	0.0	0.0
$PagL_{(Bb)}$	8.5	0.0	2.1	70.9	0.0	0.0	12.8	3.5	0.0	0.0	2.1	0.0	0.0	0.0
$PagP_{(Ec)}$	4.5	1.3	2.3	25.0	5.0	2.3	10.3	2.0	3.0	14.8	6.0	6.3	5.3	12.3
$PagP_{(Bp)}$	8.3	2.5	5.0	27.6	0.0	6.1	21.3	3.0	0.0	3.9	17.1	2.5	0.0	2.9

ability to stimulate IL-6 production. Thus, it can be concluded that *in vivo* palmitoylation results in an increase in endotoxic activity of *B. pertussis* LPS, whereas $PagL_{(Bb)}$-mediated deacylation results in LPS that is, in comparison to wild-type *B. pertussis* LPS, biologically less active. Comparable results were also obtained for other cytokines, such as IL-10 and IL-8 (data not shown).

Endotoxic activity of whole *B. pertussis* cells

To determine whether expression of PagP and PagL affects the endotoxic activity of whole bacterial cells similarly to that of purified LPS, serial dilutions of whole-cell suspensions were used to stimulate IL-6 production by the macrophage cell line MM6. As expected, *B. pertussis* cells expressing $PagP_{(Ec)}$ or $PagP_{(Bp)}$ showed, compared to *B. pertussis* cells containing the empty vector, an increased potency to stimulate the macrophages (Fig. 5B). However, unexpectedly, the cells expressing $PagL_{(Bb)}$ also showed this effect (Fig. 5B), while the purified LPS of this strain was biologically less active (Fig. 5A). This paradoxical result suggests that some other mechanism counterbalances the reduced endotoxic activity of $PagL_{(Bb)}$-modified LPS in the whole cells. The majority of virulence factors in the Bordetellae are regulated by the two-component system BvgAS (Mattoo and Cherry, 2005). It is known that some of these virulence factors, e.g., adenylate cyclase, are capable of modulating the immune response and suppress the production of proinflammatory cytokines by macrophages (Boyd *et al.*, 2005; Ross *et al.*, 2004). Therefore, we hypothesised that the increased endotoxic activity of the whole cells expressing $PagL_{(Bb)}$ might be a consequence of an altered expression of virulence factors through an effect on the Bvg system. To test this hypothesis, we expressed $PagL_{(Bb)}$ in a Bvg^+ phase-locked *B. pertussis* strain and compared the whole-cell endotoxic activity of this strain with that of the same strain containing the empty vector. Expression of $PagL_{(Bb)}$ in the Bvg^+ phase-locked strain resulted in an increase in endotoxic activity comparable to that in the wild-type strain, indicating that *bvg*-mediated alterations in the expression levels of virulence factors are not responsible for the increased endotoxic activity of whole PagL-expressing *B. pertussis* cells (data not shown). In addition, we also observed a similar increase in IL-6 induction with PagL-expressing cells grown under conditions known to induce the Bvg^- phase (results not shown). To investigate whether the enzymatic activity as opposed to merely the expression of PagL is responsible for the observed increase in endotoxic activity, we constructed two other *B. pertussis* strains, one expressing *P. aeruginosa* PagL ($PagL_{(Pa)}$) and the other expressing an inactive variant thereof ($PagL_{(Pa)(S151A)}$) in which one of the catalytic-site residues, Ser151, has been replaced by alanine (Geurtsen *et al.*, 2005).

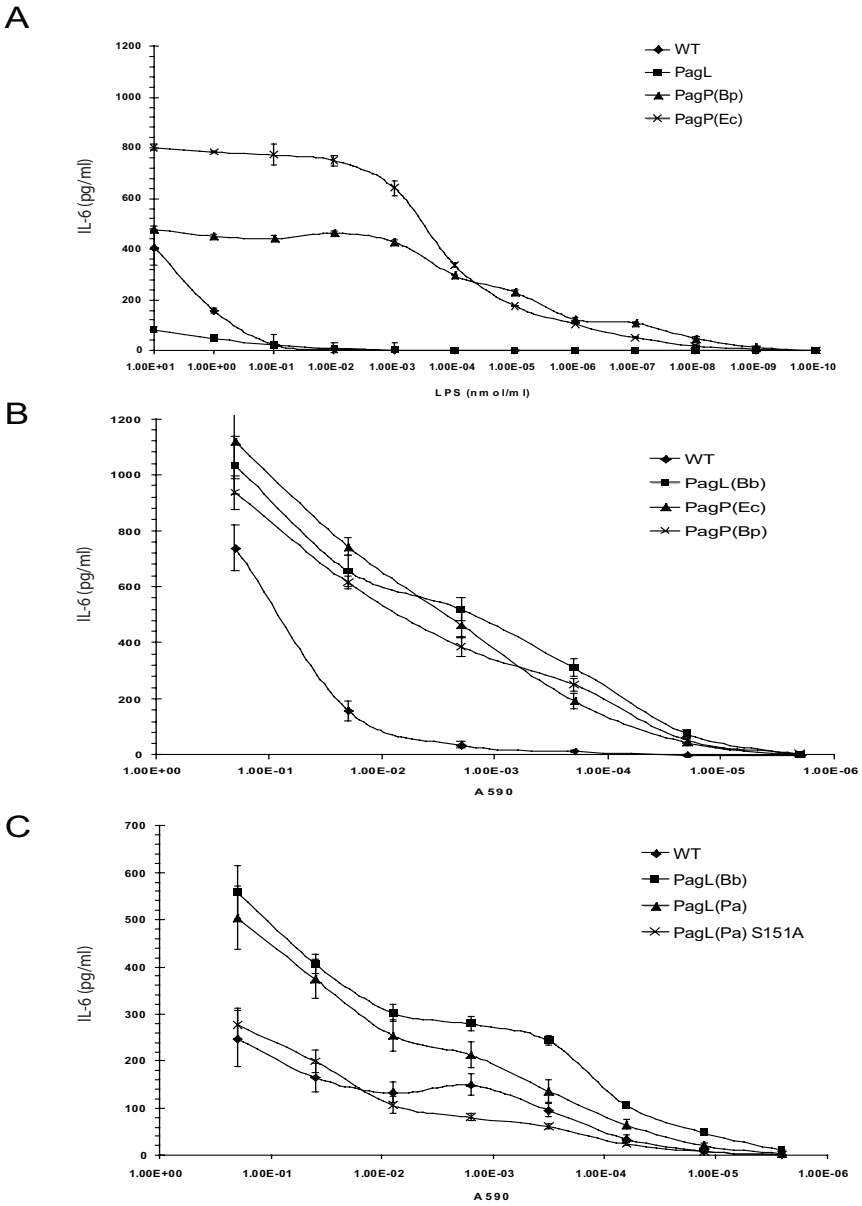

Fig. 5. IL-6 induction by purified *B. pertussis* LPS and whole cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of purified wild-type (WT) or PagP- or PagL-modified LPS from *B. pertussis* strain Tohama, (B) a whole-cell suspension of *B. pertussis* cells expressing PagP or PagL or containing the empty vector pMMB67EH (WT), or (C) a whole-cell suspension of whole *B. pertussis* cells containing the empty pMMB67EH vector (WT) or expressing $PagL_{(Bb)}$, wild-type *P. aeruginosa* PagL ($PagL_{(Pa)}$), or an active-site mutant derivative of $PagL_{(Pa)}$ ($PagL_{(Pa)(s151A)}$). The IL-6 concentration in the culture supernatant was quantified in an ELISA against human IL-6. The data represent the averages and standard deviations from three individual experiments.

Like that of *B. bronchiseptica* PagL, expression of *P. aeruginosa* PagL led to increased endotoxic activity of *B. pertussis* whole cells (Fig. 5C). Furthermore, enzymatic activity of PagL was needed for the increased endotoxic activity, since stimulation of the MM6 cells with bacteria expressing the mutant PagL did not lead to increased IL-6 production (Fig. 5C).

Deacylation of LPS influences its release from *B. pertussis* membranes

LPS is normally anchored into the outer membranes of Gram-negative bacteria via its lipid A part. However, by disturbing LPS-LPS interactions, e.g., by EDTA treatment (Leive, 1965), LPS can be released from the outer membrane into the external environment. In previous studies, it was shown that membrane-bound LPS has a lower proinflammatory cytokine-inducing activity than soluble LPS aggregates (Mirlashari and Lyberg, 2003; Post *et al.*, 2005), probably due to the better accessibility of LPS in soluble aggregates for extraction and subsequent transfer by LBP/CD14 to the TLR4/MD-2 complex. Deacylation of LPS could potentially weaken the LPS-LPS interactions, leading to increased release from the cells and counterbalancing the effect of the reduced endotoxic activity of the purified, deacylated LPS.

To test the hypothesis that deacylated *B. pertussis* LPS is more readily released, we compared the amounts of released LPS in whole-cell suspensions of wild-type and $PagL_{(Bb)}$-expressing cells. The fraction of released LPS was assessed by determining the total 3OH C14 content in the whole-cell suspensions and cell-free supernatants obtained from the suspensions. The proportion of LPS released in the whole-cell suspensions of the $PagL_{(Bb)}$-expressing strain was significantly higher than that from the wild-type strain, i.e., 17% (±1.8%) versus 11% (±0.7%), showing that deacylation indeed led to an increased release of LPS. For reasons of safety, *B. pertussis* cells were incubated for 1 h at 56°C during the preparation of whole-cell suspensions. To test whether the higher LPS release from the $PagL_{(Bb)}$-expressing cells was due to this heat inactivation step, we also determined the fraction of LPS released during growth into the culture medium. A significantly larger proportion of LPS was detected in the cell-free culture medium from the PagL-expressing strains than in that of the wild-type strain, i.e., 12% (±4.6%) for the wild-type strain and 20% (±1.6%) for the $PagL_{(Bb)}$-expressing strain. Interestingly, this analysis also revealed that the total amount of LPS produced, corrected for optical density, by wild-type cells was significantly higher, i.e., 21% (±8.3%), than that produced by the bacteria expressing $PagL_{(Bb)}$. Overall, these data indicate that deacylation of *B. pertussis* LPS indeed leads to an increased LPS release from the cells into the external environment and that this is independent of heat inactivation at 56°C. However, we

cannot exclude the possibility that this increased release is an indirect consequence of the removal of the acyl chain from the 3 position. For instance, PagL-mediated deacylation of lipid A could induce secondary changes in the LPS core region, which, in turn, could be primarily responsible for the observed release. Yet, the observation that deacylated LPS migrates at the same position as wild-type LPS on a Tricine-SDS-PAGE gel (Fig. 3, lanes 1 and 2) indicates that major changes in LPS composition, other than deacylation at the 3 position, are absent. Hence, the increased release of LPS after modification by PagL is probably directly related to the removal of the acyl chain from the 3 position.

EDTA treatment increases the endotoxic activity of a *B. pertussis* whole-cell suspension

To test the hypothesis that increased LPS release enhances endotoxic activity, we stimulated MM6 cells with a whole-cell suspension of wild-type *B. pertussis* cells in the presence of various amounts of EDTA (Fig. 6). The addition of EDTA indeed led to a concentration-dependent increase in the endotoxic activity of the whole-cell suspension, which is consistent with the hypothesis that destabilisation of the outer membrane and increased LPS release could potentially counterbalance the reduced endotoxic activity of deacylated LPS.

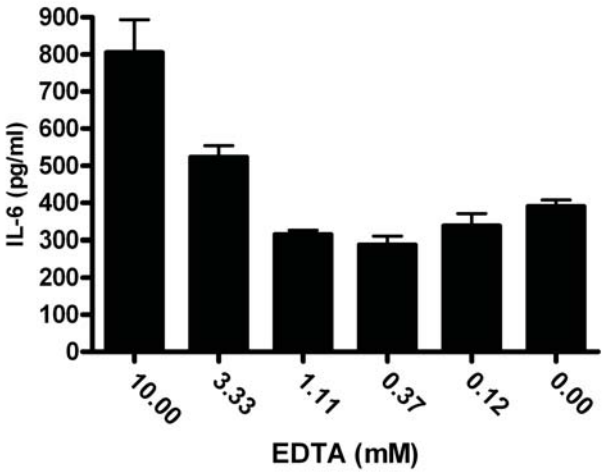

Fig. 6. Stimulation of IL-6 production by a *B. pertussis* whole-cell suspension in the presence of EDTA. The production of IL-6 by the human macrophage cell line MM6 was stimulated with a whole-cell suspension in the presence of various amounts of EDTA. The IL-6 concentration in the culture supernatant was quantified in an ELISA against human IL-6. The data represent the averages and standard deviations from three individual experiments.

Released LPS is the main determinant of whole-cell suspension endotoxicity

It was shown above that LPS release increases the endotoxic properties of a whole-cell suspension. To determine the contribution of released LPS to the endotoxic activity of whole-cell suspensions, MM6 cells were stimulated with either a whole-cell suspension of wild-type *B. pertussis* or with the cell-free supernatant of such a suspension. As shown in Fig. 7, ~90% of the endotoxic activity appeared to be present within the supernatant. Since only ~11% of the total LPS was released from the cells (see above), these data suggest that the majority of LPS, which remained associated with the bacterial cells, only marginally contributed to the endotoxic activity of the suspensions

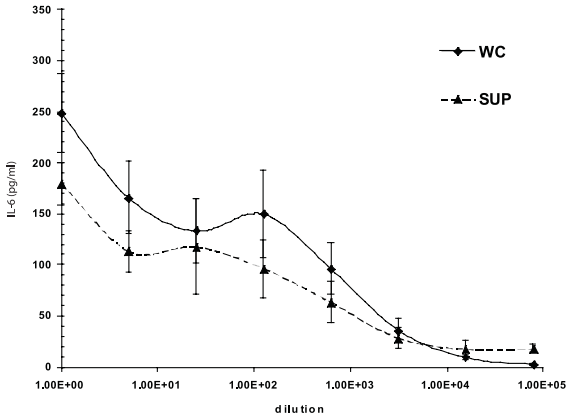

Fig. 7. Released LPS determines endotoxicity of *B. pertussis* whole-cell suspensions. Production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type *B. pertussis* (WC) or with the cell-free supernatant of such a suspension (SUP). The data represent the averages and standard deviations from three individual experiments.

PagL expression lowers the endotoxic activity of *N. meningitidis* cells.

To test whether PagL expression would also lead to an increased endotoxic activity in another Gram-negative bacterium, we stimulated MM6 cells with serial dilutions of a whole-cell suspension of wild-type or $PagL_{(Bb)}$-expressing *N. meningitidis* cells. Previously, we have shown that PagL(Bb) expression in this host leads to efficient 3-O deacylation of the LPS (Bos *et al.*, 2004). As a control, the macrophages were also stimulated with LPS-deficient *N. meningitidis* cells. Compared to the wild-type cells, both the $PagL_{(Bb)}$-expressing cells and the LPS-deficient cells displayed a reduced endotoxic activity (Fig. 8). This reduction was most pronounced for the LPS-deficient cells, implying that the LPS in the PagL-expressing strain still had residual endotoxic activity. Most

importantly, this result shows that, in contrast to *B. pertussis* cells, *N. meningitidis* cells expressing PagL do show a lowered biological activity compared to the wild-type cells. Thus, the paradoxical results obtained with PagL-expressing *B. pertussis* cells cannot directly be extrapolated to other Gram-negative bacteria.

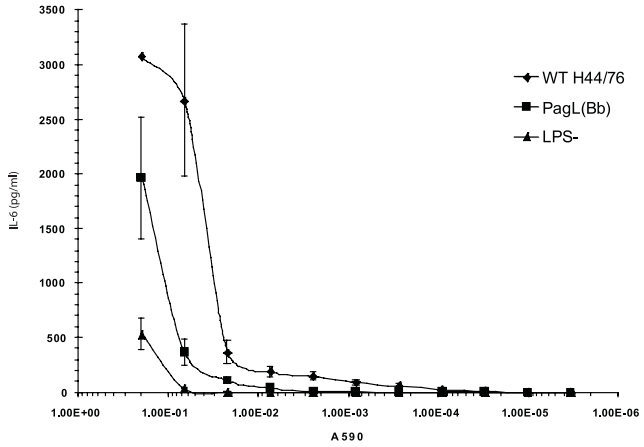

Fig. 8. PagL modification reduces endotoxic activity of *N. meningitidis* cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type *N. meningitidis* cells (WT H44/76), *N. meningitidis* cells expressing $PagL_{(Bb)}$ (PagL(Bb)), or LPS-deficient *N. meningitidis* cells (LPS–). The IL-6 concentration in the culture supernatant was quantified in an ELISA against human IL-6. The data represent the averages and standard deviations from three individual experiments.

Discussion

The lipid A part is mainly responsible for the endotoxic activity of LPS, and it is known that changes in its physicochemical properties influence its biological activity. In recent years, several enzymes that are capable of modifying lipid A have been discovered in various Gram-negative bacteria. Two of these enzymes, PagP and PagL, are located in the outer membrane and function as a lipid A acylase and deacylase, respectively. Although genes for these enzymes have been identified in *B. pertussis*, they are actually pseudogenes (Geurtsen *et al.*, 2005; Preston *et al.*, 2003). Here, we investigated the consequence of PagP and PagL expression in *B. pertussis* on the endotoxic activity of the LPS and whole bacterial cells in order to determine whether the PagP and PagL enzymes might be useful tools for the development of a less reactogenic whole-cell pertussis vaccine.

In initial experiments, we wanted to determine the contribution of the LPS to the endotoxic activity of whole *B. pertussis* cells. Importantly, we observed that TLR4-deficient C3H/HeJ macrophages, compared to C3H/HeJ macrophages expressing hTLR4, exhibit a strongly reduced potency to be stimulated by LPS-deficient *N. meningitidis* cells as well as by the TLR2/6 agonist lipoprotein FSL. These observations suggest possible cross talk between different TLRs or, alternatively, a reduced sensitivity of the C3H/HeJ macrophage cell line to TLR agonists in general. Therefore, the use of these cell lines to identify LPS responses is less straightforward than anticipated. Nevertheless, the observation that the *B. pertussis* cells stimulated the TLR4-expressing cells more strongly than did the LPS-deficient *N. meningitidis* cells, whereas the opposite was the case for the TLR4-deficient macrophages, suggests that *B. pertussis* LPS, in spite of its relatively low biological activity, might still contribute significantly to the endotoxic activity of the whole bacterial cells.

Interestingly, we found that *B. pertussis* LPS shows a high heterogeneity. This observation is consistent with the high LPS heterogeneity that was previously reported for other *Bordetella* strains (Caroff *et al.*, 1994; Zarrouk *et al.*, 1997) and other Gram-negative bacteria (reviewed in Caroff *et al.*, 2002). Since the enzymes that cooperate in the lipid A biosynthesis route are thought to be interdependent and to work in a preferred order (reviewed in Trent, 2004), it is hard to imagine how significant lipid A heterogeneity could arise during biosynthesis. It is therefore more likely that the heterogeneity observed is the consequence of active modification by lipid A-modifying enzymes. Mass spectrometry analysis showed that ~37% of the total LPS in the wild-type strain consists of tetra-acylated forms that miss the 3OH C10 residue at the 3 position, i.e., the position where PagL removes the acyl chain. Since *B. pertussis pagL* is a pseudogene, it cannot be responsible for removal of this fatty acyl chain. This finding thus suggests the presence of another enzyme in *B. pertussis*, with an activity comparable to that of PagL. However, no other PagL homolog could be identified in the genome sequence (data not shown). We recently solved the crystal structure of the *P. aeruginosa* PagL homolog (Rutten *et al.*, 2006). That study revealed a close resemblance of the active site of PagL to that of the outer membrane phospholipase A. It is therefore tempting to speculate that *B. pertussis* outer membrane phospholipase A has relaxed substrate specificity and might be responsible for removal of the 3OH C10 residue at the 3 position. Furthermore, the data suggest the existence of at least one additional lipid A deacylase with a specificity different from that of PagL, i.e., one that removes the 3OH C14 acyl chain from either the 2 or the 3' position. Recently, an outer membrane-located lipid A deacylase, i.e., LpxR, with specificity for the 3' position of lipid A has been identified in *S. enterica* serovar

Typhimurium (Reynolds *et al.*, 2006). During the analysis, we also observed a high abundance of monophosphate lipid A species. Although this hypophosphorylation could well be the consequence of fragment ionisation during mass spectrometry analysis as observed previously (Geurtsen *et al.*, 2005), it should be noted also that a lipid A-1-phosphatase, i.e., LpxE, was recently identified in *Rhizobium leguminosarum* (Karbarz *et al.*, 2003). Hence, we screened the *B. pertussis* genome sequence for possible homologs of the LpxE enzyme. BLAST searches resulted in the identification of open reading frame BP0835 (GenBank accession number CAE41138), which encodes a protein with a size comparable to that of LpxE and which also shares the conserved tripartite active-site motif of the lipid phosphatase family that was identified in LpxE (Karbarz *et al.*, 2003).

Previous studies have shown that both the phosphate groups and the number and length of the acyl chains are important determinants of the endotoxic properties of lipid A (Loppnow *et al.*, 1989; Raetz and Whitfield, 2002; Steeghs *et al.*, 2002). Recognition of LPS is based upon its binding to LBP and subsequent transfer to CD14, followed by interaction with the TLR4/MD2 complex. Earlier studies with purified LPS have shown that the strongest CD14-dependent response requires lipid A to be substituted with six acyl chains and two phosphates and the presence of an O antigen (Gangloff *et al.*, 1999). Moreover, it was shown that wild-type *B. pertussis* LPS already is a poor cytokine inducer in human monocytes, as can be expected from its penta-acylated structure (Haeffner-Cavaillon *et al.*, 1989). Indeed, in our IL-6 induction assay, wild-type *B. pertussis* LPS was found to be at least 1,000-fold less active, on a weight basis, than hexa-acylated *N. meningitidis* LPS (data not shown). Here we showed that further deacylation by PagL reduces the endotoxic activity of *B. pertussis* LPS even more, thus indicating that the number of acyl chains significantly contributes to the overall endotoxic activity of *B. pertussis* LPS. This notion is supported by the observation that the PagP-mediated addition of an extra acyl chain to *B. pertussis* LPS led to increased endotoxic activity. Notably, only a small increase in palmitoylated species, i.e., 9%, in the $PagP_{(Bp)}$-expressing strain led to a strongly increased potency to stimulate macrophages. This result can be explained by the notion that hexa-acylated LPS, compared to penta-acylated LPS, is biologically far more active (Raetz and Whitfield, 2002). Hence, small increases in hexa-acylated species content will give large effects on the displayed endotoxic activity. Although modification of hexa-acylated *Salmonella* LPS by PagP was earlier reported to result in LPS with reduced endotoxic activity (Janusch *et al.*, 2002; Kawasaki *et al.*, 2004), the increase in endotoxic activity of *B. pertussis* LPS after modification by PagP is not surprising, since *B. pertussis* LPS is a mixture of tetra- and

penta-acylated forms, which, after modification by PagP, will be converted into penta- and hexa-acylated forms.

Whereas purified PagL-modified LPS showed reduced endotoxic activity, we unexpectedly found that whole bacterial cells expressing PagL from either *B. bronchiseptica* or *P. aeruginosa* had increased potency to induce the production of IL-6. This increase in stimulatory potency was not caused by an altered expression of Bvg^--regulated virulence factors and was shown to be dependent on the enzymatic activity of PagL. It is well established that purified LPS, on a molar basis, is biologically more active than LPS incorporated into membrane structures such as in bacterial cells, outer membrane vesicles, or liposomes (Mirlashari and Lyberg, 2003; Post *et al.*, 2005). This can probably be explained by an increased accessibility of LPS in soluble aggregates for extraction and subsequent transfer by LBP/CD14 to the TLR4/MD-2 complex. As deacylation of LPS may diminish LPS-LPS interactions, we hypothesised that the increased endotoxic activity of the PagL-expressing cells could be due to an increased accessibility of the deacylated LPS to LBP/CD14. Such a mechanism could then counterbalance the effect of the reduced endotoxic activity of purified, deacylated LPS. Two observations support this interpretation. First, we found that PagL expression leads to an increased release of LPS from the bacterial cells into the medium. Second, a treatment known to result in LPS release, i.e., incubation in the presence of EDTA, results in an increased IL-6 induction by a *B. pertussis* cell suspension (Fig. 6). Therefore, increased release of deacylated LPS could eventually lead to a net increase in endotoxic activity of PagL-expressing cells. However, we cannot exclude the possibility that other factors also contribute to the observed increase in endotoxic activity of *B. pertussis* cells expressing PagL, since it is conceivable that after deacylation of LPS, other factors also can be more easily released from the *B. pertussis* cells.

It is tempting to speculate about a possible physiological significance of LPS release after PagL-mediated deacylation. By regulating PagL expression and/or activity, bacteria could, for instance, control the amount of LPS present within the outer membrane and thereby the membrane fluidity. Furthermore, released LPS could potentially function as a decoy and prevent recognition of the bacterium by the immune system. Interestingly, and consistent with this speculation, *S. enterica* serovar Typhimurium, one of the Gram-negative bacteria that does contain a PagL enzyme, was previously shown, in contrast to other Gram-negative bacteria, to be capable of releasing LPS, more or less selectively, from its outer membrane (Munford *et al.*, 1982). In addition to the increased LPS release, we also observed that the cells expressing PagL produced ~21% less

LPS than the control strain. The reason for this reduced LPS content remains elusive, but it suggests the presence of a feedback mechanism on LPS biogenesis, following deacylation of the LPS by PagL.

The endotoxic activity of LPS forms a serious problem for the development of new whole-cell vaccines. We studied the consequences of the expression of two LPS-modifying enzymes, PagP and PagL, for the endotoxic activity of *B. pertussis* LPS and whole bacterial cells to see whether they might be useful tools for decreasing the LPS-mediated reactogenicity of whole-cell pertussis vaccines. We showed that these enzymes indeed modulate the endotoxic activity of *B. pertussis* LPS. Our results demonstrate how LPS modification influences biological activity at different levels, i.e., directly through its effect on LPS per se and indirectly through its effect on LPS accessibility. In the case of PagL, these effects work in opposite directions. Determination of whether the PagP and/or PagL modifications can be used in improved pertussis vaccines will require further *in vivo* studies on immunogenicity and reactogenicity. We did, however, show that PagL could readily be used for lowering the endotoxic activity of *N. meningitidis* cells. We think that the discrepancy between the effects on *B. pertussis* and *N. meningitidis* cells can be explained by differences in the physical properties of their LPS species, i.e., a mixture of tetra- and penta-acylated species versus primarily hexa-acylated species. Nevertheless, our data show that PagP, PagL, and probably also other lipid A-modifying enzymes, such as LpxE and LpxR, can be used for altering the endotoxic activity of Gram-negative bacterial cells and that they thus potentially form useful tools for the development of future vaccines.

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Chapter 5

Consequences of the expression of lipopolysaccharide-modifying enzymes for the efficacy and reactogenicity of whole-cell pertussis vaccines

Jeroen Geurtsen, Rob J. Vandebriel, Eric R. Gremmer, Betsy Kuipers, Jan Tommassen, and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial outer membrane and is, due to its endotoxic activity, responsible for the relatively high reactogenicity of whole-cell vaccines. In addition, LPS has strong immune stimulating properties, which makes it, potentially, an interesting vaccine component. In a previous study, we have shown that expression of two LPS-modifying enzymes, i.e., PagP and PagL, modulates the endotoxic activity of the Gram-negative bacterium *Bordetella pertussis*, the causative agent of whooping cough. To assess the consequences of PagP and PagL expression on the efficacy and reactogenicity of whole-cell pertussis (wP) vaccines, we have immunised mice and challenged them intranasally with wild-type *B. pertussis*. Vaccine efficacy, *B. pertussis*-specific antibody responses, and cytokine profiles were evaluated. The results show that expression of PagL, but not of PagP, significantly increases vaccine efficacy without altering vaccine reactogenicity. Therefore, PagL-expressing *B. pertussis* strains may form a basis for the development of a new and safer wP vaccine, as higher vaccine efficacies may allow a reduced vaccine dosage. These data show, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

Introduction

Pertussis or whooping cough is a severe acute respiratory illness that is characterised by paroxysmal coughing and a distinctive “whooping” sound when air is subsequently inhaled. The disease is highly contagious and most severe in neonates and children younger than one year. Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*, which was first isolated in 1906 by Bordet and Gengou (Bordet and Gengou, 1906).

Introduction of whole-cell pertussis (wP) vaccines in the 1940s and 1950s led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after their introduction, it became clear that wP vaccines exhibited a considerable reactogenicity, which was caused by the presence of strong immunogens, such as lipopolysaccharide (LPS) and pertussis toxin. Therefore, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the 1980s and 1990s. Today, aP vaccines are broadly used in industrialised countries and have been shown to be highly effective. Nevertheless, aP vaccines have, as compared to wP vaccines, some important disadvantages. These include an increased cost/benefit ratio, which prohibits their use in the developing world, the loss of the adjuvant effect of the wP vaccines, and the fact that aP vaccination skews towards a Th2 immune response, whereas wP vaccination and natural infection have been shown to evoke more of a Th1 immune response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998; van den Berg *et al.*, 2001). For these reasons, the development of new and safer wP vaccines remains an important issue.

An important factor that contributes to the relatively high reactogenicity of wP vaccines is the presence of LPS. LPS is one of the major constituents of the Gram-negative bacterial outer membrane, where it is important for maintaining the membrane barrier function. LPS is an amphiphatic molecule that consists of three domains, i.e., the O-antigen, the core, and lipid A (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also possesses a powerful adjuvant activity. Both these properties are based upon the recognition of the LPS by the host TLR4/MD-2 receptor complex and the subsequent activation of NF-κB (reviewed in O’Neill, 2006; Pålsson-McDermott and O’Neill, 2004).

The endotoxic activity of LPS is largely determined by the composition of its lipid A moiety (Homma *et al.*, 1985). In general, lipid A consists of a β-1,6-linked D-glucosamine disaccharide carrying phosphate groups at positions C-1 and C-4’ and ester- and amide-linked 3-hydroxy fatty-acids (Raetz and Whitfield, 2002). Previous

studies have shown that the phosphate groups, as well as the number and the length of the acyl chains are critical determinants of the endotoxic activity of lipid A and that changes in lipid A composition often modulate its biological activity (Geurtsen *et al.*, 2006; Loppnow *et al.*, 1989; Steeghs *et al.*, 2002; van der Ley *et al.*, 2001).

After injection into mice, LPS rapidly induces increased serum pro-inflammatory cytokine levels (Durand *et al.*, 2004). Pro-inflammatory cytokines have been shown to play a central role in the systemic inflammatory effects elicited by a variety of Gram-negative bacteria, including *B. pertussis* (Kutukculer *et al.*, 1997; Loscher *et al.*, 2000; Sawa *et al.*, 1997). Furthermore, pro-inflammatory cytokines have been associated with the relatively high reactogenicity of wP vaccines (Armstrong *et al.*, 2003; Loscher *et al.*, 1998). Hence, a straightforward approach to reduce wP reactogenicity would be the generation of *B. pertussis* strains that contain LPS with reduced endotoxic activity. Such strains could then function as a basis for safer wP vaccines. Of course this approach is only feasible when the second important property of the LPS, i.e., its adjuvant activity, is retained.

In a previous study, we have shown that expression of two lipid A-modifying enzymes, i.e., PagP and PagL, modulates the endotoxic activity of *B. pertussis* (Geurtsen *et al.*, 2006). Expression of PagP, a lipid A acylase, increased the endotoxic activity of both LPS and whole bacterial cells, whereas expression of PagL, a lipid A 3-*O*-deacylase, decreased the endotoxic activity of the purified LPS, but increased the toxicity of the whole bacterial cells. This latter observation was rather unexpected and might be related to an increased release of the deacylated LPS from the bacterial membranes (Geurtsen *et al.*, 2006). In the study presented here, we evaluated the consequences of LPS acylation or deacylation on the vaccine potential of wP vaccines. We immunised mice with wP vaccines based upon wild-type *B. pertussis* or *B. pertussis* expressing either PagP or PagL and subsequently analysed vaccine efficacy, *B. pertussis*-specific antibody responses, and cytokine profiles.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise notified, *B. pertussis* strain B213, a streptomycin resistant derivative of *B. pertussis* strain Tohama (Kasuga *et al.*, 1953), and its plasmid-containing derivatives were grown at 35°C on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood (Tritium, Veldhoven, the Netherlands). When appropriate, 100 μg/ml ampicillin was added to the medium for plasmid maintenance.

Vaccine preparation

Vaccines were prepared from wild-type *B. pertussis* strain B213 harbouring the empty pMMB67EH vector, plasmid pMMB67EH-PagP$_{(Ec)}$, containing the *Escherichia coli pagP* gene (Tefsen *et al.*, 2005), or pMMB67EH-PagL$_{(Bb)}$, containing the *Bordetella bronchiseptica pagL* gene (Geurtsen *et al.*, 2006). The bacteria were grown in synthetic THIJS medium (Thalen *et al.*, 1999) supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 68 h at 35°C while shaking (175 rpm). The bacterial cell suspensions were heat inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100 x *g* and resuspended in phosphate-buffered saline (PBS) to an A_{590} of 2.5, i.e., 50 international opacity units (IOU). The suspensions were stored at 4°C. Prior to immunisation, the suspensions were further diluted in PBS to final concentrations of 3.2 IOU/ml or 16 IOU/ml (depending on the experiment), after which 0.3 mg/ml or 1.5 mg/ml aluminum phosphate (final concentration), respectively, was added as an adjuvant. The resulting vaccine concentrations corresponded to 1/10 and 1/2 human dose per 0.5 ml, respectively. Furthermore, Western blot analysis showed that the vaccines contained similar amounts of the virulence factors pertactin, fimbriae, pertussis toxin, and filamentous haemagglutinin (data not shown).

Immunisation and intranasal challenge

All animal experiments were performed at the Netherlands Vaccine Institute in accordance with the Dutch national guidelines for animal experimentation. Groups of nine 4- to 5-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml vaccine (prepared as described above), or with 0.5 ml PBS/$AlPO_4$ as a control, at days 0 and 14. At day 28, the mice were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *B. pertussis* strain B213 in 40 μl medium.

Autopsy

The left lung lobes were collected in 1 ml Verwey medium and homogenised using a tissue homogeniser (Pro-200, ProScientific, Monroe, CT, USA) at maximum speed for 10 s. The homogenates were diluted 10- and 100-fold for the immunised mice and 1000-fold for the control mice, and 100-μl aliquots of the dilutions were plated on BG plates supplemented with 30 μg/ml streptomycin and incubated at 35°C for 5 days. Results are reported as log protection (LOG_{Prot}) values, which allows for comparison of vaccine efficacies. The LOG_{Prot} was calculated using the following equation: LOG_{Prot} =

^{10}log (mean CFU of PBS/AlPO$_4$-treated mice) - ^{10}log (CFU of each individual immunised mouse). Blood was collected 4 h post immunisation at days 0 and 14 for cytokine analysis and at day 28 for the assessment of antibody titers.

Combined mouse toxicity test

The combined mouse toxicity test was performed as described (van Straaten *et al.*, 2002). In short, groups of 10 male N:NIH/RIVM outbred mice (14-17 g) were immunised intraperitoneally with 0.5 ml wP vaccine (1/2 human dose), or with 0.5 ml PBS/AlPO$_4$ as a control. Before immunisation, the mice were distributed randomly in groups of five per cage and weighed individually. Sixteen h after immunisation, the mice were weighed again. On day 7 after immunisation, a 10 μl blood sample was taken from the tail vein, using an EDTA-impregnated capillary, diluted in 1.6 ml Isoton (Coulter), after which the number of leukocytes was determined using a Coulter Counter.

Antibody titer determination

Total IgG, IgG1, and IgG2a antibody titers against whole cells of *B. pertussis* strain B213 were determined in an enzyme-linked immunosorbent assay (ELISA) as described (Abdillahi and Poolman, 1987). In short, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100-μl samples of a whole-cell suspension (A_{590} = 0.05 in PBS). Antibody titers were measured for individual sera using HRP-labelled goat anti-mouse secondary antibodies (SouthernBiotech). A four-parameter curve fit was made for optical density values of 3-fold serial dilutions, and the antibody titers were calculated in reciprocal dilutions that gave 50% of the maximum absorbance.

Cytokine determination

Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) concentrations in the sera were quantified with an ELISA against mouse IL-6 or TNFα according to the manufacturers' instructions (eBioscience). For determination of cytokine concentrations in the supernatant of lung homogenates, an 11-plex panel containing beads for mouse IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-α, TNFα, and GM-CSF (Bio-Rad, Hercules, CA) was used. 96-wells filter bottom plates (Bio-Rad) were pre-wet with 100 μl of Bio-Plex assay buffer (Bio-Rad). After each step, buffer was removed by vacuum. Beads were diluted in assay buffer, and 50 μl of the solution was added per well. The plates were washed twice with 100 μl of Bio-Plex wash buffer (Bio-Rad). The cytokine standards were diluted in Verwey medium to a range of 32,000 to 0.18 pg/ml. Of

standards and samples, 50 μl/well were added. The plates were incubated for 30 min; each incubation step consisted of vortexing the plates at 1100 rpm for 30 s, followed by incubation with shaking at 300 rpm. The plates were washed 3 times with 100 μl of wash buffer. Detection antibody was diluted in detection antibody diluent (Bio-Rad), and 25 μl/well was added. The plates were incubated for 30 min, and washed 3 times with 100 μl of assay buffer. Streptavidin-phycoerythrin was diluted in assay buffer, and 50 μl/well was added. The plates were incubated for 10 min, and washed 3 times with 100 μl of wash buffer. The beads were resuspended in 125 μl of assay buffer and analysed on a Bio-Plex (Bio-Rad). The results shown were obtained at low photo multiplier tube settings. All steps were performed at room temperature.

Statistical analysis

Data were statistically analysed using one-way analysis of variation (ANOVA) followed by Bonferroni's multiple comparison test. Differences were considered to be significant when $p < 0.05$.

Results

Protection against *B. pertussis* challenge after subcutaneous immunisation with whole-cell pertussis vaccines

To evaluate the protection offered by wP vaccines based upon wild-type *B. pertussis* strain B213 or its derivatives expressing *E. coli* PagP or *B. bronchiseptica* PagL, 4/5-wk-old BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a human dose of the vaccines and subsequently challenged with wild-type *B. pertussis* strain B213. All three vaccines conferred significant protection against colonisation by the wild-type strain. When mutually compared, immunisation with the PagL-expressing strain (LOG_{Prot}~2.8) provided significantly better protection against a challenge than did immunisation with the wild-type strain (LOG_{Prot}~1.5) (Fig. 1). Also immunisation with the PagP-expressing strain provided a better protection (LOG_{Prot}~2.2), but in this case the difference was not significant (Fig. 1). Thus, the results indicate that the vaccine consisting of the PagL-expressing strain had a significantly higher efficacy. Of note, the decision to use *E. coli* PagP in stead of *Bordetella* PagP for our study was based upon our previous finding that a *B. pertussis* strain expressing the latter enzyme showed only poor modification efficiency (Geurtsen *et al.*, 2006).

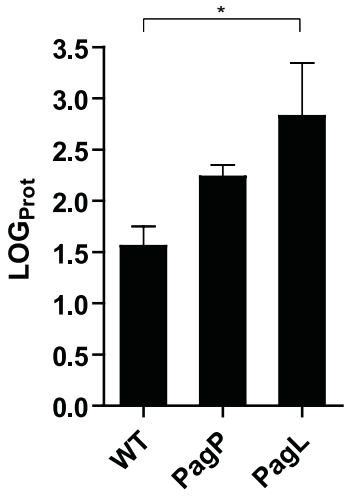

Fig. 1. Efficacy of whole cell pertussis vaccines in a mouse model. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT) or *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL) and challenged with wild-type *B. pertussis* at day 28. At day 5 after challenge, lung CFU numbers were determined. Log protection values were calculated by subtracting the Log10 CFU of individual mice from the mean Log10 CFU of the PBS/$AlPO_4$-treated mice. Results are expressed as mean log protection values (± SEM) from nine mice per group and are representative of three separate experiments. A single asterisk marks significant ($p<0.05$) differences.

B. pertussis specific antibody responses

The *B. pertussis*-specific total IgG, IgG1, and IgG2a serum antibody titers at day 28 after primary immunisation were determined in whole-cell ELISAs. High *B. pertussis*-specific serum IgG titers were elicited with all three vaccines (Fig. 2A). As compared to the mice immunised with the wild-type strain, the mice immunised with the PagP- and PagL-expressing strains showed similar IgG titers (Fig. 2A). Subsequent analysis of the IgG1/IgG2a subclass distribution showed similar IgG1 titers in all groups, although the titers in the group immunised with the PagL-expressing strain were slightly higher. The PagP- and PagL-expressing strains both elicited a higher IgG2a response than did the wild-type strain (Fig. 2B). A decreased IgG1/IgG2a ratio in the groups immunised with the PagP- and PagL-expressing strains suggests that these animals generally exhibited stronger Th1-type responses than the mice immunised with the wild-type strain.

Cytokine profiles in the lung

The supernatants of lung homogenates prepared at day 5 after challenge were assessed for their cytokine concentrations (Fig. 3). As compared to the PBS/$AlPO_4$ control group, the IL-1α level was significantly higher in the lungs of the mice immunised with the PagL-expressing strain, but not in those of the mice immunised with the wild-type or the PagP-expressing strain (Fig. 3A). In addition, the lungs of the mice immunised with the PagL-expressing strain showed a significantly higher IFN-γ level, as compared to all other groups (Fig. 3B). Analysis of the other cytokines, i.e., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNFα, and GM-CSF, did not reveal significant differences compared to the control group.

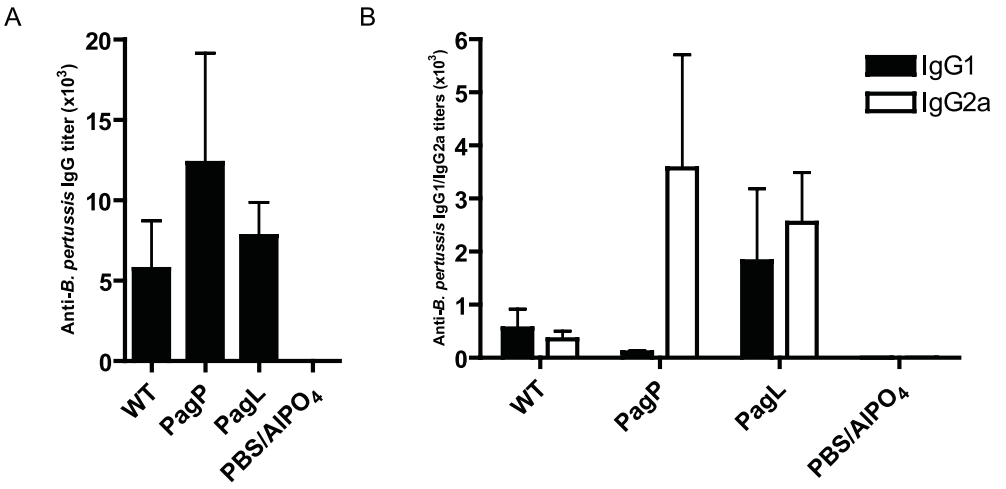

Fig. 2. *B. pertussis*-specific antibody responses. Anti-*B. pertussis* IgG (A) and IgG1/IgG2a (B) titers at day 28 post primary immunisation were measured in mice immunised with wP vaccines based upon *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control using a whole-cell ELISA. Results are expressed as mean antibody titers (± SEM) from nine mice per group and are representative of three separate experiments.

Pro-inflammatory cytokines in serum

To address the *in vivo* reactogenicity of the vaccines, we analysed the concentration of two pro-inflammatory cytokines, i.e., TNFα and IL-6, in serum samples taken 4 h after the primary or booster immunisation (Fig. 4). As shown in Fig. 4A, serum TNFα levels were similar in all groups and no significant differences were found. On the other hand, all three wP immunisations elicited significantly higher serum IL-6 levels than did the PBS/$AlPO_4$ control (Fig. 4B). Comparison of the IL-6 levels between the wP vaccinated groups showed no significant differences, indicating that the reactogenicity of the three wP vaccines, as assessed by this assay, was comparable.

Vaccine toxicity test

To obtain more insight into the overall toxicity displayed by the vaccines, a mouse weight gain test in combination with a leukocytosis promotion test was performed. The first test assesses the weight gain of individual mice at 16 h post immunisation and provides a general measure for vaccine toxicity, whereas the leukocytosis promotion test, in which

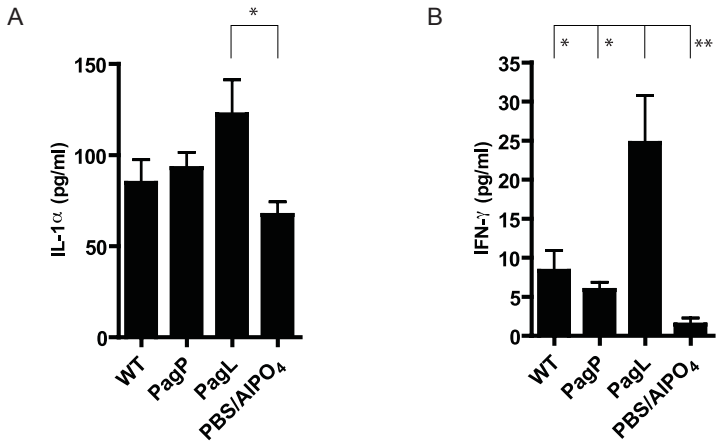

Fig. 3. Lung cytokine concentrations at day 5 post challenge. Mice were immunised with wP vaccines based upon *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control and challenged with wild-type *B. pertussis*. At day 5 after challenge, IL-1α (A) and IFN-γ (B) concentrations in the lung-homogenate supernatant were measured. Results are expressed as mean cytokine concentrations (± SEM) from six mice per group and are representative of two separate experiments. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

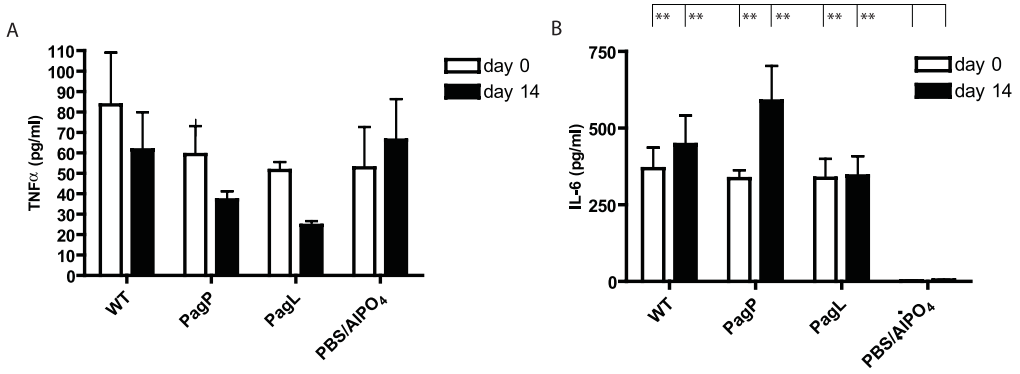

Fig. 4. Serum pro-inflammatory cytokine concentrations 4 h post immunisation. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control. Serum TNFα (A) and IL-6 (B) concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from nine mice per group and are representative of three separate experiments. Double asterisks mark highly significant ($p<0.001$) differences.

the numbers of leukocytes in the blood are determined at day 7 after immunisation, specifically assesses pertussis toxin-mediated leukocytosis (van Straaten *et al.*, 2002). As shown in Fig. 5A, immunisation with the wP vaccines resulted in a substantial loss of weight 16 h post immunisation, which is consistent with the relatively high toxicity of wP vaccines. When the wP vaccines were mutually compared, no significant differences in vaccine toxicity were found. However, as compared to the other vaccines, the mice immunised with the PagP-expressing strain exhibited significantly increased leukocyte counts in their blood (Fig. 5B), which indicates that in this particular vaccine, the activity of pertussis toxin may be enhanced.

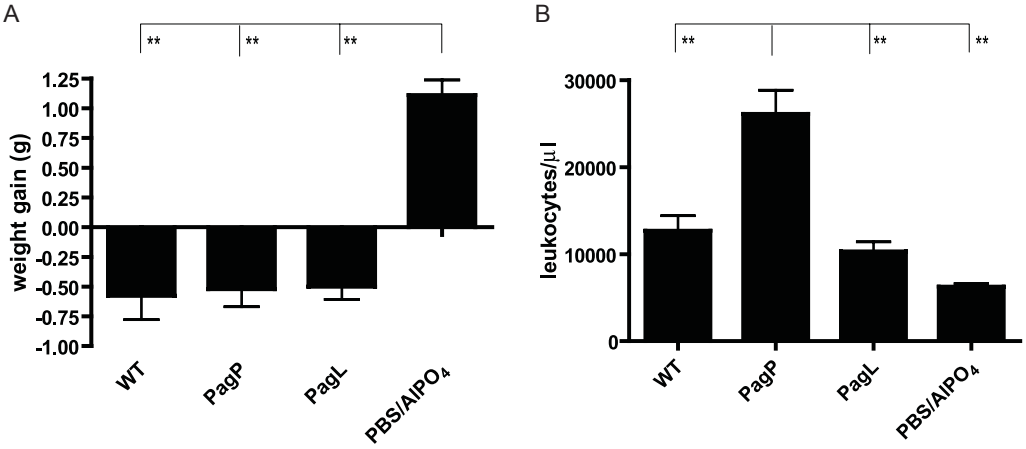

Fig. 5. Assessment of vaccine reactogenicity in a vaccine toxicity test. Mice were immunised (at day 0 and at day 14) with wP vaccines based upon wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 expressing *E. coli* PagP (PagP) or *B. bronchiseptica* PagL (PagL), or with PBS/$AlPO_4$ as a control and the weight gain 16 h post immunisation (A) or the number of leukocytes in the blood at day 7 (B) were determined. Results are expressed as mean weight gain or mean leukocyte number per µl blood (± SEM) from nine mice per group. Double asterisks mark highly significant ($p<0.001$) differences.

Discussion

The goal of this study was to evaluate the effects of the expression of two LPS-modifying enzymes, i.e., *E. coli* PagP and *B. bronchiseptica* PagL, on the efficacy and reactogenicity of wP vaccines in a mouse model. In a previous study, we have shown that expression of these enzymes in *B. pertussis* modulates the toxicity of both LPS and whole bacterial cells (Geurtsen *et al.*, 2006). Here, we showed that immunisation with either wild-type *B. pertussis* or *B. pertussis* expressing PagP or PagL conferred significant protection against a subsequent challenge with the wild-type *B. pertussis*

strain. We found that mice immunised with the PagL-expressing strain, as compared to the mice immunised with the wild-type strain, had significantly lower lung CFU numbers at day 5 after the challenge, indicating that the vaccine based upon the PagL-expressing strain was more efficacious. This result shows, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

To identify immunological determinants that may be responsible for the increased vaccine potential of the PagL-expressing strain, we first analysed the *B. pertussis*-specific total serum IgG levels. In spite of the different vaccine efficacies observed, either of the wP vaccines induced comparable serum IgG titers. Protective mechanisms against *B. pertussis* have complementary roles for both cellular and humoral immunity (Mills *et al.*, 1998). With regard to the cellular immunity, protection against *B. pertussis* is correlated with Th1 responses, whereas with humoral immunity protection against *B. pertussis* is correlated with the production of IgG2a antibodies (Mills, 2001). As compared to the wild-type strain, immunisation with the both the PagP- and PagL-expressing strain induced higher *B. pertussis*-specific IgG2a titers. However, since the CFU numbers in the mice vaccinated with the PagP-expressing were not significantly lower than those in mice vaccinated with the wild-type strain, it cannot be concluded that the increased vaccine potential of the PagL-expressing strain is attributed to its potential to induce higher IgG2a antibody titers. Possibly, changes in antibody levels against a particular antigen play a role. Nevertheless, these results show that LPS composition influences the *B. pertussis*-specific IgG1/IgG2a ratio and thus plays a role in the Th1/Th2 balance after immunisation.

To gain more insight into potential differences in immune effector mechanisms induced by the different vaccines, we went further to analyse the post-challenge cytokine profile in the lung. For the majority of the eleven cytokines that we assayed for, we found no significant differences between the wP-vaccinated groups and the PBS/$AlPO_4$-treated control group. However, in the lungs of the mice immunised with the PagL-expressing strain, both the IL-1α and the IFN-γ levels were significantly higher. For IL-1α, this increase was only significant as compared to the PBS/$AlPO_4$-treated control group, but, for IFN-γ, it was significant as compared to all other groups. A role for IL-1α in the response to *B. pertussis* infection is not known, but its increased level suggests a stronger inflammatory response in the lungs after vaccination with the PagL-expressing strain. Studies in IFN-γ receptor-disrupted and IFN-γ knock-out mice have demonstrated that IFN-γ plays a key role in the protection against *B. pertussis* infection (Barbic *et al.*, 1997; Mahon *et al.*, 1997; Mills, 2001). Thus, the observation that immunisation with the PagL-expressing strain induced the highest levels of IFN-

γ in the lungs provides a possible explanation for the higher vaccine potential of this strain. Besides measuring the cytokine profile in the lungs, we have also measured the production of the same set of cytokines by splenocytes and cells from the bronchial lymph nodes after their stimulation *in vitro* with a *B. pertussis* whole-cell suspension or Concanavalin A, respectively. However, significant differences were not observed (data not shown).

To assess vaccine reactogenicity *in vivo*, we used two different approaches. In the first approach, which was aimed at determining transient effects on cytokine profiles directly after immunisation, we measured the concentration of two pro-inflammatory cytokines in serum samples taken 4 h after the primary and the booster immunisation. Consistent with the relatively high reactogenicity of wP vaccines, mice immunised with the wP vaccines exhibited high serum IL-6 levels as compared to the control group. However, the serum TNF-α levels in the wP-immunised groups were similar to those in the control group. This observation was unexpected since it has previously been reported that injection of LPS transiently increases both IL-6 and TNF-α levels in serum (Durand *et al.*, 2004). Possible explanations for this discrepancy are the nature of the LPS, i.e., *E. coli* LPS vs. *B. pertussis* LPS, the amount of LPS administered, the different genetic backgrounds, i.e., C57/BL6 mice vs. BALB/c mice, and the different route of administration, i.e., intramuscular vs. subcutaneous. Furthermore, Durand *et al.* (2004) showed that TNF-α levels, after reaching a peak concentration, decreased more rapidly than the serum IL-6 levels. Thus, possibly, the TNF-α levels had already returned to background levels 4 h after immunisation in our experiments. Nonetheless, since all three wP vaccines tested here induced similar increases in IL-6 levels, these vaccines appear to exhibit similar reactogenicity. In the second approach, we immunised mice with the different vaccines and measured their weight gain after 16 h. Consistent with the conclusion from the previous experiment, the results indicated that the reactogenicity exhibited by the wP vaccines was similar. However, 7 days after immunisation, mice immunised with the PagP-expressing strain exhibited significantly increased leukocyte numbers in their blood, which might indicate that the vaccine based upon the PagP-expressing strain contains a higher amount of active pertussis toxin than the other vaccines. Alternatively, because of its increased biological activity the PagP-modified LPS (Geurtsen *et al.*, 2006) might also contribute directly to leukocytosis by acting as B-cell mitogen.

The results presented here show that the expression of the LPS deacylase PagL increases vaccine efficacy without altering vaccine reactogenicity. Interestingly, this conclusion deviates from that of our previous study in which we showed that PagL-

expressing *B. pertussis* cells exhibit an increased toxicity *in vitro* (Geurtsen *et al.*, 2006). Thus, apparently, *in vitro* toxicity data cannot predict the outcomes of *in vivo* experiments, emphasising the importance of both *in vitro* and *in vivo* analyses. Since increased vaccine potency probably allows for a reduction of the vaccine dosage, the data presented here suggest that PagL-expressing *B. pertussis* strains may form a basis for the development of less reactogenic wP vaccines, as lower vaccine dosages will probably reduce vaccine side-effects. However, more detailed studies using different animal models, different *B. pertussis* strains, such as the vaccine strains, and vaccine dose-response studies should be performed. In conclusion, here we have provided the proof of principle that LPS-modifying enzymes are indeed promising tools for the development of safer wP vaccines. Obviously, this observation is not only significant in the field of pertussis vaccinology, but may also be applicable for the development of vaccines against other Gram-negative bacteria.

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Chapter 6

Supplemention of whole-cell pertussis vaccines with lipopolysaccharide analogs: a novel strategy for modulating vaccine efficacy and reactogenicity

Jeroen Geurtsen, Floris Fransen, Rob J. Vandebriel, Eric R. Gremmer, Liset J. J. de la Fonteyne-Blankestijn, Betsy Kuipers, Jan Tommassen and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS) is one of the main constituents of the Gram-negative bacterial outer membrane. Besides being an endotoxin, LPS also possesses a powerful adjuvant activity. Previously, it has been shown that changes in the chemical composition of the lipid A domain of LPS modulate its biological activity. An example of this is monophosphoryl lipid A (MPL), an LPS analog that has been shown to be a non-toxic immunostimulatory compound. Moreover, several LPS analogs have been shown to function as LPS antagonists. In the present study, we show that supplementation of whole-cell pertussis (wP) vaccines with LPS analogs modulates their efficacy and reactogenicity. We show that addition of MPL to a wP vaccine increases vaccine efficacy without changing vaccine reactogenicity. Furthermore, we show that *Neisseria meningitidis* LpxL2 LPS, an LPS species derived from *N. meningitidis* by insertionally inactivating the *lpxL2* gene, functions as an LPS antagonist and that addition of this LPS not only decreases the *in vivo* reactogenicity of the wP vaccine, but also increases vaccine efficacy.

Introduction

Whooping cough, or pertussis, is a severe, highly-contagious respiratory tract disease that is caused by the Gram-negative bacterium *Bordetella pertussis*. Introduction of whole-cell pertussis (wP) vaccines in the second half of the 20th century led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after its introduction, it became clear that wP vaccines displayed a considerable reactogenicity, which was caused by the presence of potent immunomodulatory agents, such as lipopolysaccharide (LPS) and pertussis toxin. Hence, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the late 1980s and early 1990s. Nowadays, aP vaccines are broadly used in the industrialised countries and have been shown to be highly effective. Nevertheless, vaccination with aP vaccines has, compared to vaccination with wP vaccines, some important drawbacks, including high production costs, which prohibits their use in developing countries, the potential to select for escape mutants more rapidly, and the fact that aP vaccination evokes a Th2 immune response, whereas wP vaccination and natural infection induce a Th1 immune response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998; van den Berg *et al.*, 2001). Therefore, the development of safer wP vaccines remains an issue of importance.

An important factor contributing to the reactogenicity of wP vaccines is the presence of LPS or endotoxin. LPS is a large glycolipid that forms one of the major components of the Gram-negative bacterial outer membrane. It is usually composed of a highly variable O-antigen, a less variable core oligosaccharide, and a highly conserved lipid moiety, designated lipid A. The structure of lipid A consists of a phosphorylated glucosamine disaccharide substituted with a variable number of acyl chains (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also possesses a powerful adjuvant activity. Both these properties are based upon the recognition of the LPS by the host TLR4/MD-2 receptor complex and the subsequent activation of NF-κB (reviewed in Pålsson-McDermott and O'Neill, 2004; O'Neill, 2006). Due to its adjuvant activity LPS potentially forms a valuable vaccine component. However, the pyrogenicity and toxicity of LPS have precluded its development as a therapeutic compound in humans.

The endotoxic activity of LPS is dependent on the composition of the lipid A moiety (Homma *et al.*, 1985). Previous studies have indicated that the phosphate groups, as well as the number and length of the acyl chains are critical determinants of the endotoxic activity (Loppnow *et al.*, 1989; Raetz and Whitfield, 2002; Steeghs *et al.*, 2002; Geurtsen *et al.*, 2006). These studies have stimulated the development

of LPS derivatives with potentially useful properties. Some of these derivatives have been shown to exert a strongly reduced endotoxic activity, whereas their adjuvant and immunostimulatory properties were retained. One of the best known examples is monophosphoryl lipid A (MPL). MPL has been developed both as an adjuvant for application in human vaccines and as a prophylactic drug against septic shock (Rudbach *et al.*, 1994; Baldridge and Crane, 1999). The reduced endotoxic activity of MPL has been attributed to a reduced capacity to induce the secretion of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNFα (Gustafson and Rhodes, 1994; Henricson *et al.*, 1993; Okemoto et al, 2006). In addition, MPL has been shown to increase the secretion of the anti-inflammatory cytokine IL-10 by murine macrophages (Salkowski *et al.*, 1997). Another example is the LPS from *Porphyromonas gingivalis*. This LPS has been shown to be non-toxic and to block *in vitro* secretion of TNFα by both human and murine macrophage cell lines stimulated with *Escherichia coli* LPS (Henricson et al., 1992; Golenbock *et al.*, 1991; Takayama *et al.*, 1989). Furthermore, it has been shown to act *in vivo* as an LPS antagonist (Qureshi *et al.*, 1991).

To create novel LPS species with potentially useful properties the possibilities of manipulating the LPS biosynthesis route have been explored. Recently, it has been shown that *Neisseria meningitidis* strains deficient in the late acyltransferases LpxL1 or LpxL2 display a dramatically decreased endotoxic activity when tested for their capability to stimulate human macrophages (van der Ley *et al.*, 2001). However, when the LPSs from these strains were tested for their adjuvant activity in a mouse model, the mainly tetra-acylated *lpxL2* mutant LPS proved to be only moderately immunogenic, whereas the penta-acylated *lpxL1* mutant LPS was still capable of boosting the immune response up to wild-type levels (van der Ley *et al.*, 2001).

In the present study, we investigated whether supplementation with either a non-toxic, non-antagonistic LPS-analog or a non-toxic, antagonistic LPS-analog beneficially influences the efficacy and/or reactogenicity of wP vaccines and thus facilitates the development of safer wP vaccines.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise notified, *B. pertussis* strain B213, a streptomycin resistant derivative of *B. pertussis* strain Tohama (Kasuga *et al.*, 1953) was grown at 35°C on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood (Tritium, Veldhoven, the Netherlands).

In vitro endotoxic activity assay

Stimulation of IL-6 production was tested with the human macrophage cell line MM6 (Ziegler-Heitbrock *et al.*, 1998). The macrophages were seeded in 24-wells plates ($1x10^6$ cells/well) in 200 µl of IMDM medium (Gibco BRL), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 300 µg/ml L-Glutamine (Gibco BRL), and 10% heat-inactivated fetal calf serum (Gibco BRL). The cells were stimulated with 100 µl of serial dilutions of a whole-cell suspension of wild-type *B. pertussis* strain B213 or purified *B. pertussis* LPS in the absence or presence of 1 µg/ml *N. meningitidis* LpxL2 LPS (van der Ley *et al.*, 2001). The whole-cell suspension and *B. pertussis* LPS was prepared as described (Geurtsen *et al.*, 2006) After stimulation for 16-18 h at 37°C in a humidified atmosphere containing 5% CO_2, IL-6 concentrations in the culture supernatants were quantified with an ELISA for human IL-6 according to the manufacturers' instructions (PeliKine Compact™).

Vaccine preparation

Vaccines were prepared from 68-h cultures of *B. pertussis* strain B213. The bacterial cell suspensions were heat-inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100 x *g* and resuspended in PBS to an A_{590} of 2.5, i.e., 50 international opacity units (IOU). The suspensions were stored at 4°C. Prior to immunisation, the suspensions were further diluted in PBS to a final concentration of 3.2 IOU/ml, after which 0.3 mg/ml aluminum phosphate (final concentration), alone or in combination with 40 µg/ml MPL (Sigma; prepared from *Salmonella enterica* serotype Minnesota Re 595) or 40 µg/ml LpxL2 LPS, was added as an adjuvant. The resulting vaccine concentration corresponded to 1/10 human dose per 0.5 ml.

Immunisation and intranasal challenge

All animal experiments were performed at the Netherlands Vaccine Institute in accordance with the Dutch national guidelines for animal experimentation. Groups of nine 4- to 5-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml of the vaccine (prepared as described above), or with 0.5 ml of PBS/$AlPO_4$ as a control, at days 0 and 14. At day 28, the mice were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *B. pertussis* strain B213 in 40 µl of Verwey medium (Willems *et al.*, 1998).

Autopsy, analysis of bronchoalveolar lavage fluid cells, and CFU counting

To obtain bronchoalveolar lavage fluid (BALF) from the lungs, a cannula was placed intratracheally and fixed using a suture. 0.5 ml of PBS was brought into the right lung lobes and sucked up. This was repeated twice. BALF cells were pelleted by centrifugation, resuspended in PBS, counted using a Coulter Counter Z2 (Beckman Coulter B.V), and cytospin preparations were made using a cytospin centrifuge (Shandon) and cells were visually differentiated after staining according to May-Grunewald and Giemsa. The left lung lobes were collected in 1 ml Verwey medium (Tritium) and homogenised using a tissue homogeniser (Pro-200, ProScientific, Monroe, CT, USA) at maximum speed for 10 s. The homogenates were diluted 10- or 100-fold for the immunised mice or 1000-fold for the PBS/$AlPO_4$ treated mice, and 100-μl aliquots of the dilutions were plated on BG plates (Tritium) supplemented with streptomycin and incubated at 35°C for 5 days. Results are reported as log protection (LOG_{Prot}) values, which allows for comparison of vaccine efficacies. The LOG_{Prot} was calculated using the following equation: LOG_{Prot} = log10(mean CFU of PBS/$AlPO_4$ treated mice) – log10(CFU of each individual immunised mouse). Blood was collected 4 h post immunisation at days 0 and 14 for cytokine analysis and at day 28 for the assessment of antibody titers.

Antibody titer determination

Total IgG, IgG1, and IgG2a antibody titers against whole cells of *B. pertussis* strain B213 were determined in an ELISA as previously described (Abdillahi and Poolman, 1987). In short, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100-μl sample of a whole-cell suspension (A_{590} = 0.05 in PBS). Antibody titers were measured for individual sera. A four-parameter curve fit was made for optical density values of 3-fold serial dilutions, and the antibody titers were calculated in reciprocal dilutions that gave 50% of the maximum absorbance.

Cytokine determination

IL-6 and TNFα concentrations in the sera were quantified in an ELISA for mouse IL-6 or TNFα according to the manufacturers' instructions (eBioscience). For determination of cytokine concentrations in the supernatant of lung homogenates, an 11-plex panel containing beads for mouse IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNFα, and GM-CSF (Bio-Rad, Hercules, CA) was used. Ninety-six-wells filter bottom plates (Bio-Rad) were pre-wet with 100 μl of Bio-Plex assay buffer (Bio-Rad). After each step, buffer was removed by vacuum. Beads were diluted in assay

buffer, and 50 μl of the solution was added per well. The plates were washed twice with 100 μl of Bio-Plex wash buffer (Bio-Rad). The cytokine standards were diluted in Verwey medium to a range of 32,000 to 0.18 pg/ml. Of standards and samples, 50 μl/well were added. The plates were incubated for 30 min: each incubation step consisted of vortexing the plates at 1100 rpm for 30 s, followed by incubation with shaking at 300 rpm. The plates were washed 3 times with 100 μl of wash buffer. Detection antibody was diluted in detection antibody diluent (Bio-Rad), and 25 μl of the solution was added per well. The plates were incubated for 30 min, and washed 3 times with 100 μl of assay buffer. Streptavidin-phycoerythrin was diluted in assay buffer, and 50 μl/well was added. The plates were incubated for 10 min, and washed 3 times with 100 μl of wash buffer. The beads were resuspended in 125 μl of assay buffer and analysed on a Bio-Plex (Bio-Rad). The results shown were obtained at low photo multiplier tube settings. All steps were performed at room temperature.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (Graphpad). Alternatively, a Student's *t* test (two-tailed, two-sample unequal variance) was used. Differences were considered to be significant when $p < 0.05$.

Results

N. meningitidis LpxL2 LPS functions as an LPS antagonist *in vitro*

To test whether tetra-acylated *N. meningitidis* LpxL2 LPS functions as an antagonist for stimulation of macrophages, we stimulated the human macrophage cell line MM6 with serial dilutions of purified *B. pertussis* LPS (Fig. 1A) or a *B. pertussis* whole-cell suspension (Fig. 1B) in the absence or presence of 1 μg/ml of LpxL2 LPS. In the absence of LpxL2 LPS, *B. pertussis* LPS and whole-cell suspensions induced high levels of IL-6 in the culture supernatant. However, in the presence of LpxL2 LPS, IL-6 production was considerably lower (Fig. 1), indicating that LpxL2 LPS indeed functions as an antagonist for the stimulation of macrophages in these assays.

Protection against *B. pertussis* challenge

To evaluate whether supplementation of a wP vaccine with MPL or *N. meningitidis* LpxL2 LPS influences its efficacy, 4/5-wk-old BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a human dose of a standard wP vaccine

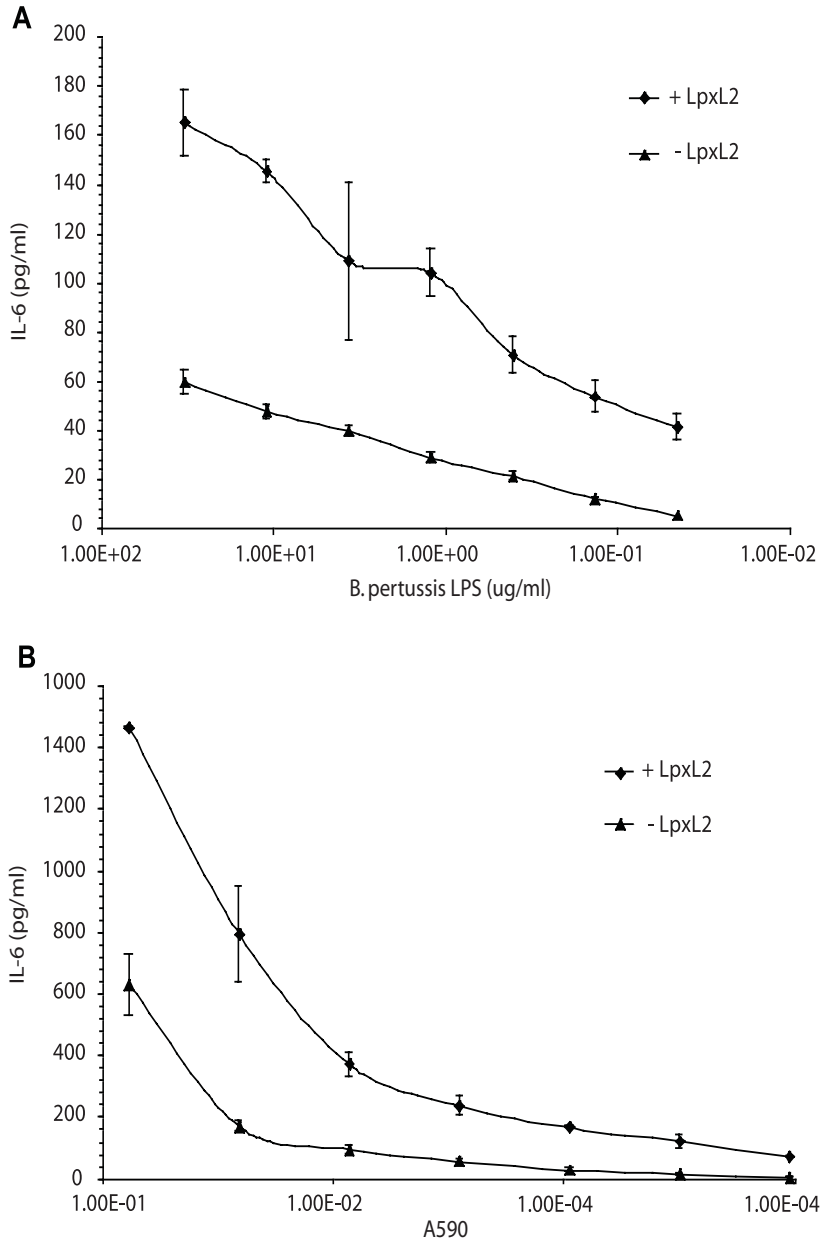

Fig. 1. IL-6 induction by purified *B. pertussis* LPS and whole cells in the absence or presence of *N. meningitidis* LpxL2 LPS. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of purified LPS from *B. pertussis*, (B) a whole-cell suspension of *B. pertussis*, both in the absence (-LpxL2) or presence (+LpxL2) of 1 μg/ml LpxL2 LPS. The IL-6 concentration in the culture supernatant was quantified using an ELISA for human IL-6. The data represent the averages of three individual experiments.

either supplemented or not with one of the LPS-analogs and subsequently challenged with *B. pertussis*. All three wP vaccines conferred significant protection against colonisation by the *B. pertussis* strain (Fig. 2). However, the MPL- and LpxL2 LPS-supplemented wP vaccines provided better protection (LOG_{Prot} ~2.5) than did the standard vaccine (LOG_{Prot}~1.5), indicating that both supplements increased the efficacy of the wP vaccine.

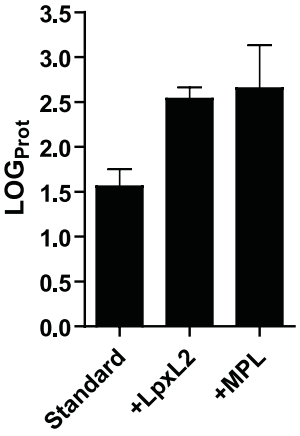

Fig. 2. Efficacy of whole cell pertussis vaccines in a mouse model. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL and challenged with *B. pertussis* at day 28. At day 5 after challenge, lungs were removed aseptically and CFU numbers were determined. Results are expressed as mean log protection values (± SEM) from nine mice per group and are representative of three separate experiments.

B. pertussis-specific antibody responses

B. pertussis-specific IgG serum antibody titers at day 28 after immunisation were determined in a whole-cell ELISA. *B. pertussis*-specific total serum IgG titers were similar between the different groups (Fig. 3A). Also the analysis of the IgG1/IgG2a subclass distribution did not reveal differences between the groups (Fig. 3B). Thus, supplementation of the wP vaccine with MPL or LpxL2 LPS did not alter the level of anti-*B. pertussis* serum IgG antibodies nor the IgG1/IgG2a subclass distribution.

Lung cytokine concentrations and cell-type distribution in BALF

Cytokine concentrations in the lung homogenate supernatant of mice sacrificed 5 days post-challenge were assessed in an 11-plex Bio-plex assay. Compared to the PBS/$AlPO_4$-treated control group, all groups of wP-immunised mice displayed increased levels of IFN-γ (Fig. 4A). This difference was significant for the group treated with the LpxL2-supplemented vaccine, but not for the groups treated with the standard or MPL-supplemented vaccine. Analysis of the other cytokines tested, i.e., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNFα, and GM-CSF did not reveal further differences.

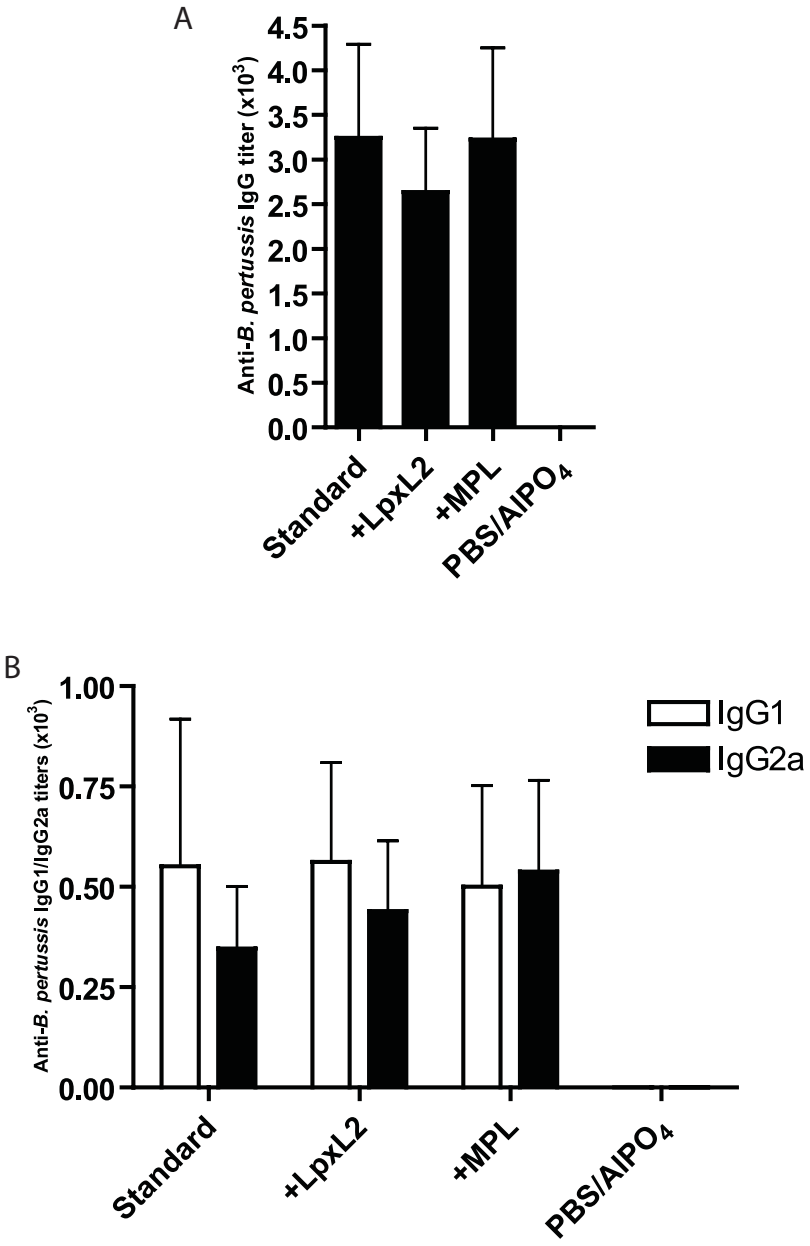

Fig. 3. *B. pertussis*-specific antibody responses. Anti-*B. pertussis* IgG (A) and IgG1/IgG2a (B) titers at day 28 post primary immunisation were measured in mice immunised with a standard wP vaccine, or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL using a whole-cell ELISA. Results are expressed as mean antibody titers (± SEM) from nine mice per group and are representative of three separate experiments.

To obtain more insight into possible variations in immune effector mechanisms within the lungs, we also analysed the cell-type distribution in BALF samples. As shown in Fig. 4B, the wP-immunised mice showed, as compared to the PBS/$AlPO_4$-treated control group, an increased neutrophil fraction, whereas the abundance of other cell types, such as macrophages, eosinophils, lymphocytes, and monocytes was similar (data not shown). This difference was significant for the group treated with the LpxL2-supplemented vaccine, but not for the other wP immunised groups. Overall, these data indicate that at day five after challenge the differences between the control group and the wP-immunised groups in the lungs are limited, with only higher IFN-γ and neutrophil levels found. Furthermore, these data show that the supplementation of the wP vaccine with LPS analogs did not markedly affect the lung cytokine concentrations or cell-type distribution.

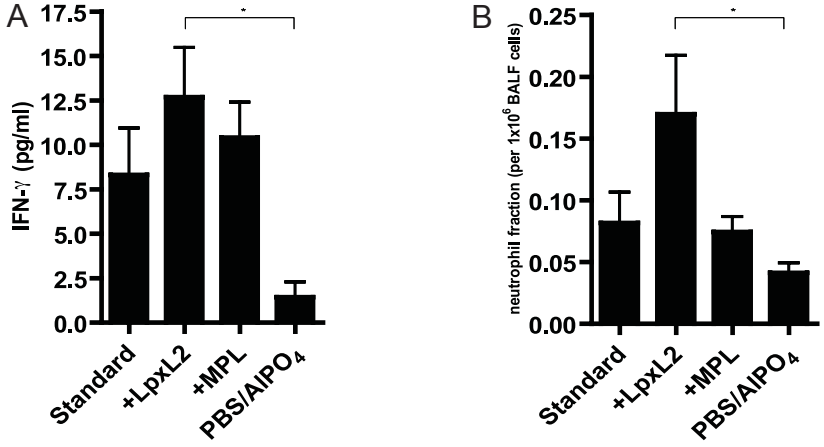

Fig. 4. Lung IFN-γ concentration and neutrophil fractions at day 5 post challenge. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL, or with PBS/$AlPO_4$ as a control and challenged with *B. pertussis*. At day 5 after challenge, IFN-γ concentration (A) and neutrophil fraction (B) in the lungs were determined. Results are expressed as mean cytokine concentrations and the fraction of neutrophils per $1x10^6$ BALF cells (± SEM), respectively, from six mice per group and are representative of two separate experiments. A single asterisk marks significant ($p<0.05$) differences.

Serum pro-inflammatory cytokine concentrations

Previously, it has been shown that an increased pro-inflammatory cytokine production is an important determinant for the reactogenicity of wP vaccines (Armstrong *et al.*, 2003; Loscher *et al.*, 1998). Hence, to determine whether the *in vivo* reactogenicity of the wP vaccines can be reduced by the addition of LPS analogs, we analysed the concentration of two pro-inflammatory cytokines, i.e., TNFα and IL-6, in serum samples

taken 4 h after immunisation. As shown in Fig. 5A, serum TNFα levels were similar in all groups. In contrast, compared to treatment with PBS/$AlPO_4$, wP immunisation elicited high serum IL-6 levels (Fig. 4B). Interestingly, the IL-6 levels in the group immunised with the LpxL2 LPS-supplemented vaccine were significantly lower than in the groups immunised with the standard or the MPL-supplemented vaccine. These results indicate that supplementation of the vaccine with LpxL2 LPS significantly decreased its reactogenicity, which shows that LpxL2 LPS works also *in vivo* as an LPS antagonist.

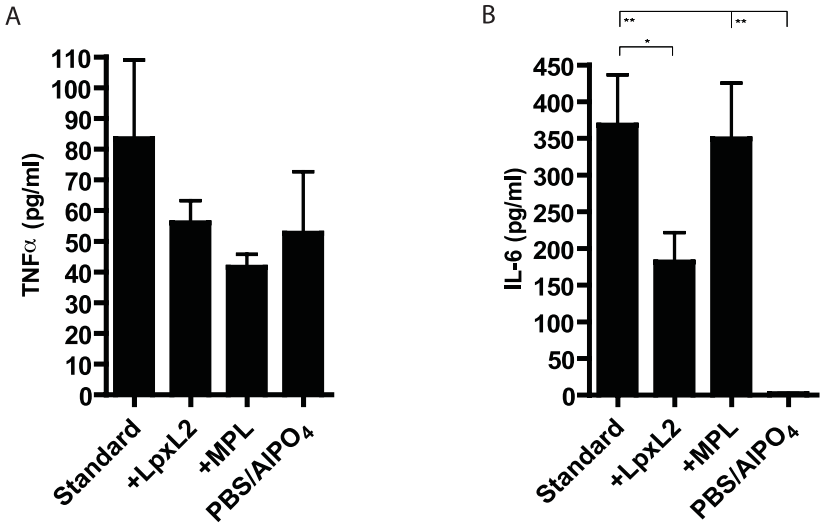

Fig. 5. Serum pro-inflammatory cytokine concentrations 4 h post immunisation. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL, or with PBS/$AlPO_4$ as a control. Serum TNFα (A) and IL-6 (B) concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from nine mice per group and are representative of three separate experiments. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Discussion

This study was aimed at determining whether the addition of non-toxic LPS derivatives may be a useful strategy to improve the efficacy and reduce the reactogenicity of wP vaccines. One of the LPS analogs studied, MPL, has been developed as an adjuvant for human vaccines. As is clear from data presented here, addition of MPL indeed increased the efficacy of a wP vaccine. Moreover, we showed that MPL did not lead to major differences in immunological responses, neither with respect to the antibody response nor to the cellular responses in the lung. Furthermore, addition of MPL did not increase vaccine reactogenicity, as assessed by measuring the levels of pro-inflammatory cytokines in serum after immunisation. Therefore, addition of MPL,

or compounds with similar properties, to wP vaccines seems to be a useful strategy by which the potency of wP vaccines can be improved. Such increased potency will possibly allow for the use of lower vaccine dosages and thereby reduce reactogenicity.

It has been shown previously that *P. gingivalis* LPS can act *in vivo* as an LPS antagonist in mice (Qureshi *et al.*, 1991). Such LPS-types are non-toxic and can antagonise the LPS-induced production of pro-inflammatory cytokines. We reasoned that addition of such a compound to a wP vaccine may reduce its *in vivo* reactogenicity. Here, we showed first that the tetra-acylated LpxL2 LPS of *N. meningitidis*, which was developed by making alterations in the lipid A biosynthetic route, also exhibits antagonistic properties. In the presence of LpxL2 LPS, the macrophages produced less IL-6 when stimulated with either a *B. pertussis* cell suspension or purified *B. pertussis* LPS. Interestingly, LpxL2 LPS did not completely inhibit stimulation of IL-6, suggesting that besides LPS, also other bacterial components induce the production of pro-inflammatory cytokines. This result is consistent with a previous study in which it was shown that LPS-deficient *N. meningitidis* cells still induce a substantial production of pro-inflammatory cytokines (Sprong *et al.*, 2001).

The addition of LpxL2 LPS to the wP vaccine resulted in a higher efficacy. This result was unexpected, since it has previously been shown that LpxL2 LPS has poor adjuvant properties (van der Ley *et al.*, 2001). Possibly, the different approaches by which this activity was measured form the reason for this discrepancy: whereas in the previous study adjuvant activity was correlated with the level of serum (bactericidal) antibody titers, in our study, adjuvant activity was assessed by determining lung CFU counts. Indeed, when we compared the antibody titers at day 28 after primary immunisation, we found that the LpxL2 LPS supplement, did neither induce higher *B. pertussis*-specific antibody titers in the serum, nor changes in the IgG subclass distribution. Apparently, the increased vaccine efficacy of the supplemented vaccines was not dependent on the induction of higher antibody titers, although it cannot be excluded that alterations in antibody levels against specific antigens play a role. This conclusion is consistent with a previous study in which we explored the effects of LPS modifications on the efficacy and reactogenicity of wP vaccines. Also in that study, a higher efficacy did not significantly correlate with higher antibody titers or changes in IgG subclass distributions (**chapter 5**). Furthermore, it is well-known that besides the antibody-mediated response, cellular immune responses play an important role in the protection against *B. pertussis* infection (Mills, 2001).

To gain more insight in the factors that may be responsible for the increased efficacy of the vaccines supplemented with LPS analogs, we analysed the levels of eleven

different cytokines in the lungs at day 5 after challenge. In most cases, no differences between the various wP-immunised and PBS/$AlPO_4$-treated control group were found. Only the levels of IFN-γ were 5- to 10-fold higher in the lungs of the three wP-immunised groups. The importance of IFN-γ for the protection against *Bordetella* infection has been shown in many experiments (Mills *et al.*, 1998; Mahon *et al.*, 1997; Barbic *et al.*, 1997; Pilione and Harvill, 2006; **chapter 5**. IFN-γ has been shown to induce the transcription of several activation markers, including CD14, proinflammatory cytokines, various cell surface receptors, chemokine receptors, and co-stimulatory receptors involved in antigen presentation, i.e., MHC-II (Dalton *et al.*, 1993; Ellis and Beaman, 2004; Janeway, 2001). Furthermore, IFN-γ has been shown to activate and recruit neutrophils (Ellis and Beaman, 2004; Burch *et al.*, 2006). Consistently, we observed that the wP-immunised mice exhibited increased lung neutrophil-counts. Neutrophils have been shown to play an important role in *Bordetella* clearance (Kirimanjeswara *et al.*, 2003; Kirimanjeswara *et al.*, 2005; Pishko *et al.*, 2004) Overall, our data suggest a central role for both IFN-γ and neutrophils in the clearance of *B. pertussis* from the lungs.

Analysis of pro-inflammatory cytokine levels in serum samples taken 4 h after primary immunisation showed that supplementation of LpxL2 LPS significantly reduced the induction of serum IL-6 levels, and, thus, probably decreased vaccine reactogenicity. Together with increased vaccine efficacy, this result suggests that addition of LpxL2 LPS may be a useful way to develop safer and improved wP vaccines.

Overall, this study shows that addition of LPS analogs may be a useful strategy to develop improved and safer wP vaccines. Moreover, such a strategy may also be applicable to other vaccines, including aP vaccines. In contrast to wP vaccines and natural *B. pertussis* infection, which evoke a Th1 response, these latter vaccines have been shown to skew towards a Th2 immune response (Ausiello *et al.*, 1997; Mills, 2001). Since LPS is potent Th1-skewing compound (Levy, 2005), addition of non-toxic LPS analogs may be a useful strategy to re-direct aP-mediated immune responses towards Th1 without increasing vaccine reactogenicity.

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Chapter 7

Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccines and reduce type-I hypersensitivity

Jeroen Geurtsen, Sander A. Banus, Eric R. Gremmer, Henke Ferguson, Jolanda P. Vermeulen, Jan A. M. A. Dormans, Jan Tommassen, Peter van der Ley, Frits R. Mooi and Rob J. Vandebriel

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Abstract

Pertussis is an infectious disease caused by the Gram-negative bacterium *Bordetella pertussis*. Although mass vaccination has drastically reduced pertussis incidence, the disease has remained often the least well-controlled disease in childhood vaccination programs. Therefore, improved pertussis vaccines need to be developed. In this study, we compared three different adjuvants with respect to their potential to improve the efficacy and the immunogenicity of a combined Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccine. The adjuvants tested were aluminum, which is used in current aP vaccines, and two lipopolysaccharide (LPS) analogs, i.e., monophosphoryl lipid A and *Neisseria meningitidis* LpxL2 LPS. Mice were immunised, challenged intranasally with *B. pertussis*, and vaccine efficacy, antibody responses, histological alterations, and cytokine expression profiles were evaluated. The results showed that the LPS analogs were superior to aluminium when vaccine efficacy was evaluated. Interestingly, parameters for type-I hypersensitivity and T-helper responses indicated that the LPS analogs re-directed the immune response towards a Th1-type response and decreased type-I hypersensitivity. Combined with the observation that the LPS analogs, as compared to aluminum, only slightly increased vaccine reactogenicity, these results suggests that the use of LPS analogs as adjuvant forms a promising strategy for improving aP vaccines.

Introduction

Pertussis is a highly contagious disease of the respiratory tract that is caused by the Gram-negative bacterium *Bordetella pertussis*. Introduction of whole-cell pertussis (wP) vaccines in the 1940s and 1950s led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after their introduction, it became clear that wP vaccines exhibited a relatively high reactogenicity, which was caused by the presence of strong immunogens, such as lipopolysaccharide (LPS) and pertussis toxin (PT). Therefore, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the 1980s and 1990s. Although pertussis mass vaccination has drastically reduced pertussis incidence, the disease remains often the least well-controlled disease in childhood vaccination programs, emphasising the importance of improving pertussis vaccines.

LPS or endotoxin is one of the major constituents of the Gram-negative bacterial outer membrane, where it is important for maintaining the membrane barrier function. LPS is an amphiphatic molecule that consists of three domains, i.e., the O-antigen, the core, and lipid A (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also has a powerful immune-stimulating potency. By engaging Toll-like receptor 4 (TLR4), LPS induces Th1 adaptive immunity (Dabbagh and Lewis, 2003; Dillon *et al.*, 2004; Kapsenberg, 2003; Medzhitov, 2001; Saito *et al.*, 2003). Since aP vaccines are devoid of LPS, concerns regarding their immunogenicity have been raised. In fact, this apprehension has been substantiated by an increase in invasive *Haemophilus influenzae* type B disease incidence in the UK that coincided with the distribution of combination vaccines that contain aP instead of wP (McVernon *et al.*, 2003). Furthermore, aP vaccination has been associated with a strong increase in parameters of type-I hypersensitivity, being total serum IgE, lung eosinophilia, and Th2 cytokine production by cells obtained from the lung-draining lymph nodes (LN) (Vandebriel *et al.*, 2007).

The non-toxic LPS derivative monophosphoryl lipid A (MPL) engages TLR4 (Evans *et al.*, 2003; Persing *et al.*, 2002), inducing Th1 adaptive immunity and deviating Th2-directed to Th1-directed responses (Baldridge *et al.*, 2000; Puggioni *et al.*, 2005; Reed *et al.*, 2003; Zhang *et al.*, 2005). MPL combined with aluminum (denoted AS04) is registered for clinical use as adjuvant in viral vaccines (hepatitis B virus (Boland *et al.*, 2004) and human papillomavirus (Giannini *et al.*, 2006)), while MPL combined with L-tyrosine is registered for clinical use as adjuvant in allergy therapy (Baldrick *et al.*, 2004; McCormack and Wagstaff, 2006). In mice, genetic mapping and functional studies have implicated a critical role for TLR4 in pertussis clearance and ensuing adaptive immunity

(Banus *et al.*, 2006). So, at least in mice, MPL engages a receptor critical for clearance of *B. pertussis* and immunity against this pathogen.

Besides naturally existing LPS forms, the possibilities of actively altering the LPS biosynthesis route has been explored in order to create novel LPS species with potentially useful properties. Recently, it was shown that a *Neisseria meningitidis* strain deficient for the late acyltransferase LpxL2 displays a dramatically decreased endotoxic activity when tested for its capability to stimulate human macrophages (van der Ley *et al.*, 2001). In addition, analysis of the adjuvant activity of the LPS from this strain showed that the mutant LPS exhibited only poor immune-stimulating activity (van der Ley *et al.*, 2001).

Here, we compared three different adjuvants with respect to their potential to improve the efficacy and the immunogenicity of a combined Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccine. We first immunised mice with DTaP vaccines, adjuvated either with aluminum, MPL, or *N. meningitidis* LpxL2 LPS, and then, after challenge, analysed vaccine efficacy, antibody responses, histological alterations, and cytokine secretion profiles.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise notified, *B. pertussis* strain B213, a streptomycin-resistant derivative of strain Tohama (Kasuga *et al.*, 1953), was grown at 35°C on Bordet-Gengou (BG) agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading).

Vaccines

The acellular vaccine was a combined diphtheria, tetanus, acellular pertussis vaccine composed of > 30 International Units (IU) diphtheria toxoid, > 40 IU tetanus toxoid, 25 μg formaldehyde- and glutaraldehyde-detoxified pertussis toxin, 25 μg filamentous hemagglutinin, and 8 μg pertactin in 0.5 ml saline (Infanrix, GlaxoSmithKline). The vaccine contained aluminum hydroxide as an adjuvant and is equivalent to 1 human dose (HD) per 0.5 ml.

The whole-cell pertussis (wP) vaccine was prepared from *B. pertussis* strain B213 as described (chapter 5). In short, the bacteria were grown in synthetic THIJS medium (Thalen *et al*., 1999) for 68 h at 35°C while shaking (175 rpm). The bacterial cell suspensions were heat inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100

x *g* and resuspended in phosphate-buffered saline (PBS) to an A_{590} of 2.5, i.e., 50 international opacity units per ml (~1.6 HD/ml). The suspensions were stored at 4°C.

Prior to immunisation, the DTaP and wP vaccines were further diluted in PBS to final concentrations of 1/5 or 1/10 of a HD per 0.5 ml (depending on the experiment), after which either 3 mg/ml aluminum phosphate (Brenntag) or 3 mg/ml aluminum hydroxide (Serva), 40 μg/ml MPL (Sigma-Aldrich), or 40 μg/ml *N. meningitidis* LpxL2 LPS (van der Ley *et al.*, 2001) was added as an adjuvant.

Immunisation and intranasal challenge

All animal experiments were performed at the Netherlands Vaccine Institute in accordance with the Dutch national guidelines for animal experimentation. Groups of 4- to 8-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml vaccine (prepared as described above), or with 0.5 ml PBS/$AlPO_4$, PBS/$Al(OH)_3$, PBS/MPL, or PBS alone as controls, at days 0 and 14. At day 28, the mice were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *B. pertussis* strain B213 in 40 μl medium as described (Willems *et al.*, 1998).

Autopsy and collection of bronchoalveolar lavage fluid cells

Mice were sacrificed at day 3, 5, or 7 after infection. Animals were anesthetised with ketamine, rompun, and atropine, and blood was collected from the orbital plexus. Perfusion was performed with 2 ml of PBS supplemented with 3.5% heat-inactivated Fetal Calf Serum (FCS; PAA, Linz, Austria) in the right heart ventricle. The lungs and spleen were excised, and used either to obtain bronchial LN, splenocytes, lung lobes for CFU determination, lung lobes for histological examinations, or bronchoalveolar lavage fluid (BALF) cells. BALF cells were obtained by placing a cannula intratracheally and fixing it using a suture. The lungs were placed in a 50-ml tube and one ml PBS was brought into the lungs and sucked up. This was repeated twice. BALF cells were pelleted by centrifugation, resuspended in PBS, counted using a Coulter Counter Z2 (Beckman Coulter B.V.), and cytospin preparations were made using a cytospin centrifuge (Shandon) and cells were visually differentiated after staining according to May-Grunwald and Giemsa.

CFU determination

Left lung lobes were collected in 1 ml of Verwey medium (Tritium) and homogenised using a tissue homogeniser (Pro-200, ProScientific, Monroe, CT, USA) at maximum speed for 10 s. The homogenates were diluted 10- and 100-fold for the

immunised mice and 1000-fold for the control mice, and 100-μl aliquots of the dilutions were plated on BG plates supplemented with 30 μg/ml streptomycin and incubated at 35°C for 5 days. Results are reported as log protection (LOG_{Prot}) values, which allows for comparison of vaccine efficacies. The LOG_{Prot} was calculated using the following equation: LOG_{Prot} = 10log(mean CFU of PBS/$AlPO_4$-treated mice) - 10log(CFU of each individual immunised mouse).

Histological examination

Left lung lobes were fixed intratracheally with 4% formalin for 24 h. After overnight dehydration, they were embedded in paraffin. Five-μm sections were cut and stained with haematoxylin/eosin. Histological lesions were semi-quantitatively scored as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. This score incorporates the frequency as well as the severity of the lesions.

Pertussis Toxin-specific IgG

Total serum IgG antibody titers against PT were determined in an enzyme-linked immunosorbent assay (ELISA) as described (de Melker *et al.*, 2000). In short, flat-bottom 96-well microtiter plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated overnight at room temperature with 10 μg/ml PT (RIVM) in PBS. Antibody titers were measured for individual sera using horseradish peroxidase (HRP)-labelled goat anti-mouse secondary antibodies (SouthernBiotech). A four-parameter curve fit was made for optical density values of 3-fold serial dilutions, and the antibody titers were calculated in reciprocal dilutions that gave 50% of the maximum absorbance.

Total serum IgE

Blood was allowed to clot at 4°C overnight and centrifuged for 2 min at 16,100 x *g*. Total serum IgE was measured as previously described (Brewer *et al.*, 1996). Briefly, 96-well plates (Nunc-Immuno Plate) were coated with 2 μg/ml anti-mouse IgE (mAb R35-72; Pharmingen) in coating buffer (0.04 M carbonate buffer, pH 9.6). After overnight incubation at 4°C, the plates were washed (3 times with 0.05% Tween-20 in PBS), incubated in blocking buffer (10% FCS in PBS) for 1 h at 37°C, and washed. Normal mouse serum (Dako) was used as a standard. Standard and serial dilutions of sera were added. The plates were incubated for 2 h at 37°C and washed. Biotinylated anti-mouse IgE (0.1 μg/ml in blocking buffer; monoclonal antibody 23G3; Southern Biotechnology Associates) was added and incubated for 1 h at 37°C. The plates were washed and incubated for 20 min at room temperature with streptavidine-HRP (200 times diluted; R&D). To detect HRP, the

plates were washed and incubated for 3 min at room temperature in 10% sodium acetate, 1.66% tetramethylbenzidine, and 0.02% hydrogen peroxidase. Stop solution (R&D) was added and the plates were read at 450 nm.

Cell culture

The culture medium used was RPMI-1640 (Gibco) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Cell suspensions were made by pressing the LN or spleens through a cell strainer (Falcon). Cells were counted using a Coulter Counter (Beckman Coulter). LN cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A (MP Biomedicals) in flat-bottom 12-well culture plates (Costar) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Spleen cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A or *B. pertussis* ($1.0x10^5$ heat-inactivated bacteria per well) in 96-well tissue culture plates (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Bacteria were heat-inactivated at 56°C during 30 min.

Cytokine determination

Interleukin-6 (IL-6) concentrations in the sera were quantified with an ELISA against mouse IL-6 according to the manufacturer's instructions (eBioscience). To determine cytokine concentrations in the supernatant of lung homogenates, an 11-plex panel containing beads for mouse IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-□, TNFα, and GM-CSF (Bio-Rad) was used. 96-wells filter bottom plates (Bio-Rad) were pre-wet with 100 μl of Bio-Plex assay buffer (Bio-Rad). After each step, buffer was removed by vacuum. Beads were diluted in assay buffer, and 50 μl of the solution was added per well. The plates were washed twice with 100 μl of Bio-Plex wash buffer (Bio-Rad). The cytokine standards were diluted in Verwey medium to a range of 32,000 to 0.18 pg/ml. Of standards and samples, 50 μl/well were added. The plates were incubated for 30 min; each incubation step consisted of vortexing the plates at 1100 rpm for 30 s, followed by incubation with shaking at 300 rpm. The plates were washed 3 times with 100 μl of wash buffer. Detection antibody was diluted in detection antibody diluent (Bio-Rad), and 25 μl/well was added. The plates were incubated for 30 min, and washed 3 times with 100 μl of assay buffer. Streptavidin-phycoerythrin was diluted in assay buffer, and 50 μl/well was added. The plates were incubated for 10 min and washed 3 times with 100 μl of wash buffer. The beads were resuspended in 125 μl of assay buffer and analysed on a Bio-Plex (Bio-Rad). The results shown were obtained at low photo multiplier tube settings. All steps were performed at room temperature.

Statistics

One-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test was performed (GraphPad). Alternatively, ANOVA followed by the T-Test was used (GraphPad). Histological data were analysed using the non-parametric Wilcoxon test (SPSS).

Results

Protection against *B. pertussis* challenge after immunisation with acellular pertussis vaccines

To evaluate the protection offered by the DTaP vaccines, groups of 9 BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a HD of the vaccines and subsequently challenged with *B. pertussis* strain B213. At day 5 after challenge, lung CFU numbers were determined. All three vaccines conferred significant protection against colonisation by the bacteria. When mutually compared, immunisation with the MPL-adjuvated vaccine (LOG_{Prot}~4.89 ±SD 1.28) and the LpxL2-adjuvated vaccine (LOG_{Prot}~4.78 ±SD 1.36) provided a significantly better protection, with *p* values of 0.014 and 0.028, respectively, than did immunisation with the $AlPO_4$-adjuvated vaccine (LOG_{Prot}~3.5 ±SD 0.63), indicating that the supplementation with LPS analogs improved vaccine efficacy.

PT-specific antibody responses

As PT-specific IgG antibody titers have been shown to correlate with protection (Cherry *et al.*, 1998), anti-PT IgG levels at days 3 and 7 after challenge were determined in groups immunised with 1/5 HD of the $Al(OH)_3$-adjuvated vaccine or the MPL-adjuvated vaccine using PT-specific ELISAs. High PT-specific serum IgG titers were detected in both groups (Fig. 1A). However, the titers were significantly higher in the mice immunised with the MPL-adjuvated vaccine both at day 3 (5.4-fold) and at day 7 (2.6-fold).

Total serum IgE levels

Traditionally, an increased serum IgE level forms one of the hallmarks of type-I hypersensitivity. Total serum IgE antibody titers were determined at day 3 after challenge in the groups immunised with 1/5 HD of the $Al(OH)_3$- or the MPL-adjuvated vaccine, or with the adjuvants alone as controls. As compared to aluminum or MPL alone, DTaP vaccination elicited high serum IgE titers (Fig. 1B). However, when mutually compared, no significant differences between the two DTaP-vaccinated groups were found.

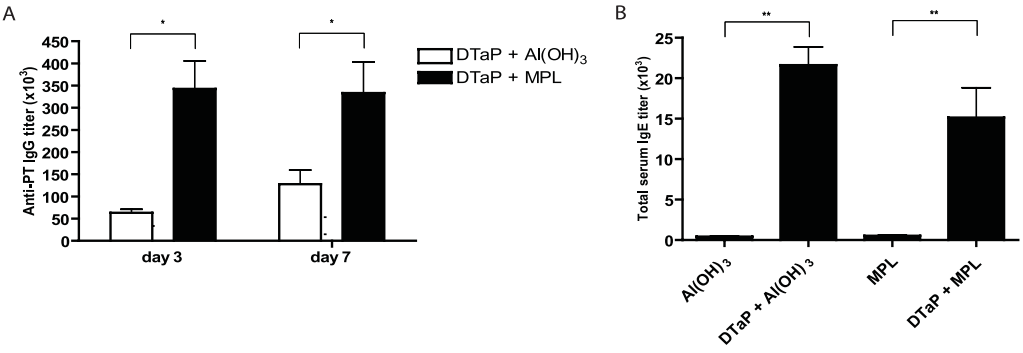

Fig. 1. Antibody responses. Anti-PT IgG (A) and total serum IgE (B) titers were measured in mice immunised with the $Al(OH)_3$- or MPL-adjuvated DTaP vaccines, or with $Al(OH)_3$ or MPL alone as controls using ELISA. Results are expressed as mean antibody titers (± SEM) from six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Evaluation of histological changes

A second parameter for type-I hypersensitivity is lung eosinophilia. Hence, lung eosinophilia was quantified by histological examination of the left lung lobes at day 3 after challenge in the groups immunised with 1/5 HD of the $Al(OH)_3$- or the MPL-adjuvated vaccine, or with the adjuvants alone as controls. The lungs of the mice immunised with the MPL-adjuvated vaccine showed significantly reduced eosinophilia, as compared to all other groups (Fig. 2). Further histological examination showed that immunisation with the $Al(OH)_3$- ($p=0.009$) or the MPL-adjuvated ($p=0.016$) vaccine induced significant perivasculitis. Immunisation with both DTaP vaccines also resulted in minor increases in peribronchiolitis, hypertrophy of the bronchiolar mucus cells, and alveolitis (data not shown).

Bronchoalveolar lavage fluid cells

To determine whether the various adjuvants affected cell-type distribution in BALF samples, BALF cells collected from mice immunised with 1/10 HD were gathered and visually differentiated at day 5 after challenge. The percentages and numbers of macrophages, neutrophils, and lymphocytes were similar in all groups, and also the total number of BALF cells was not differentially affected (data not shown). However, the group immunised with the $AlPO_4$-adjuvated vaccine showed a significantly higher eosinophil fraction than the groups immunised with the MPL-adjuvated vaccine, the LpxL2-adjuvated vaccine, or PBS alone (Fig. 3), again indicating that the vaccines adjuvated with the LPS analogs evoked lower type-I hypersensitivity than did the aluminum-adjuvated vaccine.

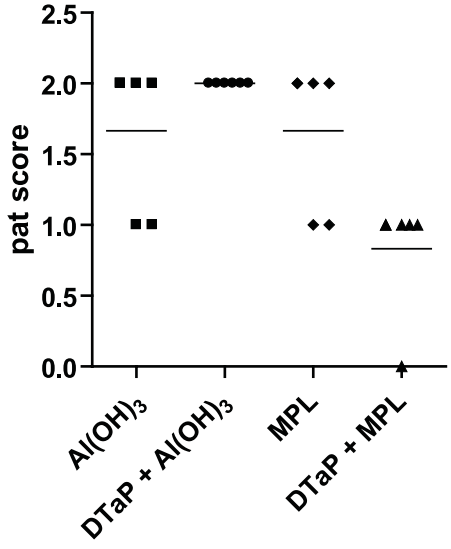

Fig. 2. Lung eosinophilia. Histological lesions in lungs from mice immunised with 1/5 HD of the $Al(OH)_3$- or MPL-adjuvated DTaP vaccine, or with adjuvants alone as controls were semi-quantitatively scored (pat-score) as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. Each symbol represents an individual mouse; horizontal lines represent the group average.

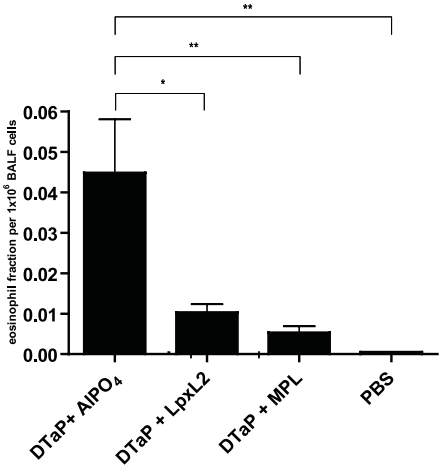

Fig. 3. BALF eosinophil numbers. Mice were immunised with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. At day 5 after challenge, the eosinophil fraction in the lungs was determined. Results are expressed as the mean fraction of eosinophils per $1x10^6$ BALF cells (± SEM). The data represent the averages of six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Cytokine production by splenocytes and bronchial LN cells

To evaluate whether the vaccines elicited a Th1- or Th2-type of response, splenocytes and bronchial LN cells from the groups immunised with 1/10 HD of the DTaP vaccines or with PBS as a control were isolated at day 5 after challenge and stimulated with heat-inactivated *B. pertussis* cells and Concanavalin A, respectively, after which their cytokine secretion profiles were analysed. Mutual vaccine comparison did not reveal major differences for most of the cytokines analysed, i.e., IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-13, TNFα, IFN-γ, and GM-CSF. Yet, bronchial LN cells from the group immunised with the $AlPO_4$-adjuvated vaccine secreted higher amounts of IL-4 and IL-5 (both indicative of a Th2-type response) than did the bronchial LN cells from the groups immunised with the MPL- or LpxL2-adjuvated vaccines, or with PBS alone (Fig. 4A). For IL-4, these differences were not significant, but for IL-5 they were. Also the splenocytes from the group immunised with the $AlPO_4$-adjuvated vaccine secreted higher amounts of both IL-4 and IL-5 than did the splenocytes from mice of any of the other groups (Fig. 4B). However, most of the differences were not significant (Fig. 4B).

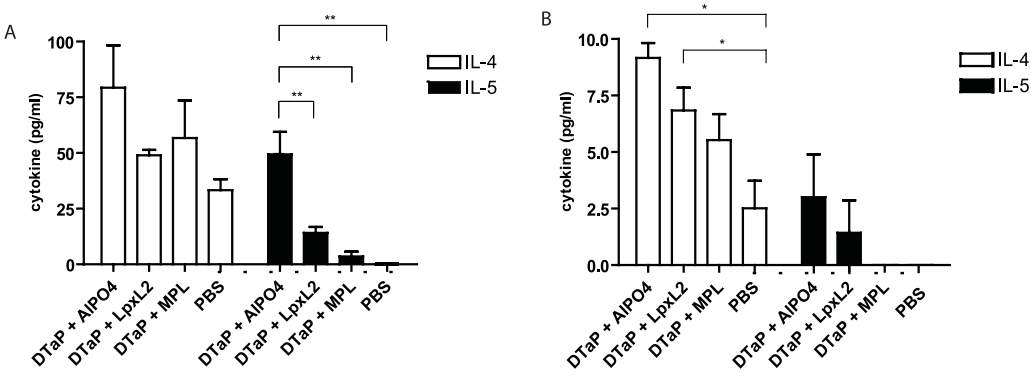

Fig. 4 Cytokine expression profiles. Mice were immunised with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. Bronchial LN cells (A) and splenocytes (B) were isolated at day 5 after challenge and stimulated with concanavalin A and heat-inactivated *B. pertussis*, respectively. IL-4 and IL-5 concentrations in the cell-culture supernatants were measured. Results are expressed as mean cytokine concentrations (± SEM) from six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Serum pro-inflammatory cytokines

To address the *in vivo* reactogenicity of the DTaP vaccines, we analysed the concentration of the pro-inflammatory cytokine IL-6 in serum samples taken 4 h after the primary or booster immunisation (Fig. 5). As controls, serum IL-6 levels were also

measured in mice immunised with a wP vaccine or with PBS alone. Consistent with the relatively high reactogenicity of wP vaccines, immunisation with the wP vaccine elicited significantly higher serum IL-6 levels than did immunisation with the DTaP vaccines or with PBS alone. When compared to the PBS control group, primary immunisation with the $AlPO_4$-adjuvated DTaP vaccine evoked similarly low serum IL-6 levels. Yet, when this vaccine was used for booster immunisation, significantly higher IL-6 levels were found. As compared with the $AlPO_4$-adjuvated vaccine, the MPL- and LpxL2 LPS-adjuvated vaccines displayed higher reactogenicity at both the primary and the booster vaccination, as indicated by the significantly higher serum IL-6 levels in these groups (Fig. 5). Nonetheless, these IL-6 levels were clearly lower than those measured in the group immunised with the wP vaccine.

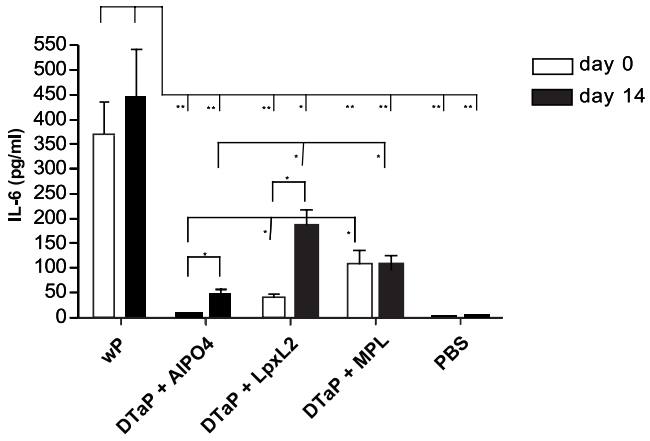

Fig. 5 Serum pro-inflammatory cytokines. Mice were immunised (at day 0 and day 14) with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. Serum IL-6 concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from seven mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Discussion

This study was aimed at comparing the efficacy, immunogenicity, and reactogenicity of aluminum-, MPL-, or LpxL2 LPS-adjuvated DTaP vaccines. We showed that all three formulations tested conferred significant protection against a challenge with *B. pertussis*. Importantly, we found that the DTaP vaccines supplemented with the LPS-analogs provided a significantly better protection than did the vaccine adjuvated with aluminum. For LpxL2 LPS, this result was unexpected since it was previously shown that it has only poor immune-stimulating activity (van der Ley *et al.*, 2001). This discrepancy may be explained by the notion that van der Ley *et al.* (2001) measured adjuvant activity by analysing serum bactericidal antibody titers, whereas in our study, the exhibited adjuvant activity was based upon vaccine efficacy.

Cellular and humoral immunity have complementary roles in protective mechanisms against *B. pertussis* (Mills, 2001). To gain more insight into the consequences of the use of different adjuvants on immune effector mechanisms, we first analysed the PT-specific serum IgG levels. The results revealed that immunisation with the MPL-supplemented vaccine elicited significantly higher anti-PT antibody titers than did immunisation with the aluminum-adjuvated vaccine. This observation suggests that an increased vaccine efficacy may correlate with elevated anti-PT antibody titers. Consistently, it has been reported that PT forms an important protective antigen in both humans and mice (reviewed in Mattoo and Cherry, 2005; Mills, 2001).

It has been shown previously that especially aP vaccines induce or augment parameters of type-I hypersensitivity, such as increased total serum IgE levels, increased lung eosinophilia and BALF eosinophil numbers, and increased Th2 cytokine production by the bronchial LN cells (Vandebriel *et al.*, 2007). In the present study we showed that, as compared to vaccination with the aluminum-adjuvated vaccine, immunisation with the MPL- or LpxL2 LPS-adjuvated vaccine reduced lung eosinophilia (only determined for the MPL-adjuvated vaccine), BALF eosinophil numbers, and Th2 cytokine production by both splenocytes and bronchial LN cells. However, the total serum IgE levels were not influenced by the type of adjuvants used. Of note, total serum IgE levels differ from the other type-I hypersensitivity parameters, since it is systemic and not influenced by *B. pertussis* infection (Vandebriel *et al.*, 2007). Together our results demonstrate that the addition of LPS analogs, instead of aluminum, to the DTaP vaccine re-directed the immune response towards a more Th1-type response and generally reduced type-I hypersensitivity, except for the serum IgE levels.

Analysis of serum samples taken 4 h after primary and booster immunisation revealed that MPL or LpxL2 LPS in the vaccine significantly increased the levels of the pro-inflammatory cytokine IL-6. This result shows that these adjuvants increased the reactogenicity of the vaccine. However, as compared to the wP vaccine, the exhibited reactogenicity was still low. Unexpectedly, we found that the IL-6 levels, evoked by the aluminum-adjuvated vaccine, were significantly higher after booster immunisation than after primary immunisation. Since the vaccine composition was the same, this observation suggests that booster immunisation, also in the absence of strong immune-stimulatory molecules, such as LPS, may elicit a stronger pro-inflammatory cytokine response than primary immunisation. Similar results were observed after immunisation with the LpxL2-adjuvated vaccine, but not with the MPL-adjuvated vaccine.

The results presented here show that the use of MPL or LpxL2 LPS rather than aluminum as an adjuvant in DTaP vaccines reduces vaccine-induced type-

I hypersensitivity and re-directs the evoked immune response towards a Th1-type response. Furthermore, these adjuvants significantly increased vaccine efficacy. It is tempting to speculate that this increased efficacy is due to an improved balance between the cellular (Th1) and humoral (Th2) response. In any case, these results indicate that the use of LPS analogs as adjuvants may be a useful strategy to improve aP vaccines. Since MPL is registered for clinical use as an adjuvant already, clinical testing of combinations of MPL and current aP vaccines should be feasible within a reasonable time frame. Obviously, the data presented here are not only important in the field of pertussis vaccinology, but may also offer opportunities for the development of vaccines against other microorganisms.

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Chapter 8

Identification and functional characterisation of *Bordetella pertussis lpxL* homologues

Jeroen Geurtsen, Eline Angevaare, Marlieke Janssen, Hendrik-Jan Hamstra, Jan ten Hove, Alex de Haan, Betsy Kuipers, Jan Tommassen, and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial outer membrane and is a potent stimulator of the host innate immune response. The biosynthesis of the lipid A moiety of LPS is a complex process, in which multiple gene products are involved. Two late lipid A acyl transferases, LpxL and LpxM, were first identified in *Escherichia coli* and shown to be responsible for the addition of secondary acyl chains to the 2' and 3' positions of lipid A, respectively. Here, we describe the identification of two *lpxL* homologues in the genome of *Bordetella pertussis*. We show that one of them, LpxL2, is responsible for the addition of the secondary myristate group that is normally present at the 2' position of *B. pertussis* lipid A, whereas the other one, LpxL1, mediates the addition of a previously unrecognised secondary 2-hydroxy laurate. In addition, we show that an *lpxL1*-deficient mutant of *B. pertussis* displays alterations in outer membrane integrity and haemolytic activity, as well as a defect in the infection of human macrophages.

Introduction

Pertussis or whooping cough is a severe acute respiratory illness that is characterised by paroxysmal coughing and a distinctive “whooping” sound when air is subsequently inhaled. The disease is highly contagious and most severe in neonates and children younger than one year. Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*. While the genus *Bordetella* currently encompasses nine species, apart from *B. pertussis* only three other members, i.e., *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella holmesii*, have been associated with respiratory infections in humans and other mammals (Mattoo and Cherry, 2005).

The Gram-negative bacterial cell envelope is composed of two membranes, the inner and the outer membrane, which are separated by the periplasm. The inner membrane is a symmetrical bilayer composed of phospholipids, whereas the outer membrane is asymmetric and consists of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. LPS, which is also known as endotoxin, consists of three distinct structural domains: lipid A, the core, and the O-antigen (Raetz and Whitfield, 2002). The first domain, lipid A, functions as a hydrophobic membrane anchor and forms the bioactive component of the molecule (Takada and Kotani, 1989). The structure of lipid A is conserved among different bacterial groups, indicating its importance for the correct functioning of the outer membrane. Generally, lipid A consists of a β-1,6-linked D-glucosamine (GlcN) disaccharide carrying ester- and amide-linked 3-hydroxyl fatty acids at the C-2, C-3, C-2’, and C-3’ positions, and phosphate groups at positions C-1 and C-4’. The endotoxic activity of LPS is based on the recognition of lipid A by the TLR4/MD-2 complex of the host, which leads to the activation of NF-κB and, consequently, to an increased production and secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α (Pålsson-McDermott and O’Neill, 2004).

Current knowledge about lipid A biosynthesis mainly comes from studies in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), where the biosynthetic pathway has been completely elucidated. It consists of nine enzymes that work in a successive order. In the first step, an acyl chain is transferred from the *R*-3-hydroxytetradecanoic acid (3OH C14)-acyl carrier protein to the GlcN 3 position of UDP-N-acetyl glucosamine (GlcNAc) by the acyltransferase LpxA (Crowell *et al.*, 1986; Coleman and Raetz, 1988). Then, the acylated UDP-GlcNAc is de-acetylated by the LpxC enzyme (Young *et al.*, 1995), after which LpxD adds a 3-hydroxyl acyl chain at this position (Kelly *et al.*, 1993), resulting in a UDP-2,3-diacylGlcN molecule. Next, UMP is removed from a proportion of the UDP-2,3-diacylGlcN pool by LpxH (Babinski *et al.*, 2002), before a tetra-acylated GlcN disaccharide is formed by LpxB (Crowell *et al.*,

1986). After 4'-phosphorylation by LpxK, creating a molecule known as lipid IV_A (Garrett *et al.*, 1997), two 2-keto-3-deoxyoctulosonic acid (Kdo) residues are added by KdtA (Clementz and Raetz, 1991), and finally the secondary acyl chains are added by the late acyltransferases LpxL and LpxM (Clementz *et al.*, 1996; Clementz *et al.*, 1997).

The late acyltransferase LpxL of *E. coli* was found to be responsible for the addition of a secondary laurate moiety to the 2' position of lipid A (Karow *et al.*, 1991; Clementz *et al.*, 1996). LpxL homologues have been identified in several other Gram-negative bacteria, including *Haemophilus influenza* (Lee *et al.*, 1995), *Neisseria meningitidis* (van der Ley *et al.*, 2001), *S.* Typhimurium (Sunshine *et al.*, 1997), and *Yersinia pestis* (Rebeil *et al.*, 2006). The second late acyltransferase, LpxM, is closely related to LpxL and was initially described as a multicopy suppressor of an *lpxL* mutation (Karow and Georgopoulos, 1992). In *E.* coli, LpxM is responsible for the addition of a secondary myristate (C14) chain at the 3' position of lipid A (Clementz *et al.*, 1997). Bacteria with mutations in LpxL and LpxM harbour underacylated LPS species, which display a reduced biological activity (Low *et al.*, 1998; van der Ley *et al.*, 2001; Cognet *et al.*, 2003).

The structure of *B. pertussis* lipid A (Fig. 1) resembles that of *E. coli*. It typically consists of a GlcN disaccharide substituted with 3OH C14 residues at positions 2, 2', and 3' via ester or amide linkage and with an *R*-3-hydroxydecanoic acid (3OH C10) residue at the 3 position via ester linkage. A secondary C14 replaces the hydroxyl group of 3OH C14 at the 2' position (Fig. 1) (Caroff *et al.*, 1994). Limited information on the genetics of *Bordetella* lipid A biosynthesis is currently available and detailed analyses have only been performed for the acyl transferase LpxA and the Kdo transferase KdtA (Sweet *et al.*, 2002; Isobe *et al.*, 1999).

The goal of the present study was to identify the gene encoding the enzyme responsible for the attachment of the secondary acyl chain to *B. pertussis* LPS with the eventual goal to inactivate this gene and create a less reactogenic vaccine strain. We identified a locus of two *lpxL* homologues in the genome of *B. pertussis*, which raised the question which of these genes is responsible for the attachment of the secondary acyl chain and what the function of the other LpxL homologue might be. The study resulted in the identification of new LPS forms in *B. pertussis*, required for invasion of or survival within macrophages.

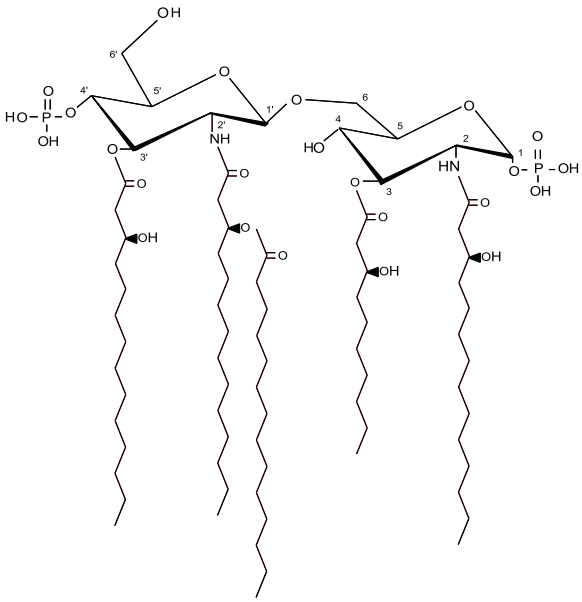

Fig. 1. *B. pertussis* lipid A architecture. *B. pertussis* lipid A consists of a bisphosphorylated GlcN disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2' fatty-acyl chain is esterified with myristate (Caroff *et al.*, 1994).

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used are described in Table 1. Typically, the *E. coli* strains were grown at 37°C in a modified Luria-Bertani broth, designated LB (Tommassen *et al.*, 1983), supplemented with 0.2% glucose or at either 30°C or 42°C in a synthetic minimal medium (SV) (Winkler and de Haan, 1948) supplemented with 0.5% glucose, while shaking at 200 rpm. When appropriate, the media were supplemented with 100 μg/ml ampicillin, 10 μg/ml tetracycline, 10 μg/ml gentamicin, 50 μg/ml nalidixic acid, or 300 μg/ml streptomycin, for plasmid maintenance or strain selection. *B. pertussis* was grown at 35°C on Bordet-Gengou (BG) agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading). To induce the overexpression of the *lpxL1* and *lpxL2* genes from plasmids in *B. pertussis*, the bacteria were grown in synthetic THIJS medium (Thalen *et al.*, 1999) supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 35°C while shaking at 175 rpm.

TABLE 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
B. pertussis		
B213	A streptomycin resistant derivative of *B. pertussis* strain Tohama	Kasuga *et al.*, 1953
B213 Δ*lpxL1*	*lpxL1* mutant of B213 strain, Str^R, Gm^R	This study
E. coli		
TOP10F'	*F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	*F- Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	Hanahan, 1983
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir* R6K Kan^R	N.V.I.[a]
W3110	Wild-type strain, F^-, λ^-	N.V.I.[a]
MLK53	*htrB1*::Tn10, Tet^R derivative of W3110	Karow and Georgopoulos, 1992
Plasmids		
pCRII-TOPO	*E. coli* cloning vector, Amp^R Kan^R	Invitrogen
pET-11a	*E. coli* high-copy expression vector, Amp^R, T7 promotor	Novagen
pMMB67EH	Broad-host-range expression vector, Amp^R	Fürste *et al.*, 1986
pKAS32	Allelic exchange suicide vector, Amp^R	Skorupski and Taylor, 1996
pBSL141	*E. coli* vector harbouring gentamicin-resistance cassette, Amp^R Gm^R	Alexeyev *et al.*, 1995
pLpxL1	pET-11a derivative harbouring *B. pertussis lpxL1*	This study
pLpxL2	pET-11a derivative harbouring *B. pertussis lpxL2*	This study
pMMB67EH-LpxL1	pMMB67EH derivative harbouring *B. pertussis lpxL1*	This study
pMMB67EH-LpxL2	pMMB67EH derivative harbouring *B. pertussis lpxL2*	This study
pCRII-LpxL1$_{up}$	pCRII derivative harbouring *lpxL1*-upstream sequence	This study
pCRII-LpxL1$_{down}$	pCRII derivative harbouring *lpxL1*-downstream sequence	This study
pKAS32-LpxL1$_{KO}$	pKAS32 derivative harbouring *lpxL1* knock out construct, Amp^R, Gm^R	This study

[a] Netherlands Vaccine Institute, Bilthoven, The Netherlands

Recombinant DNA techniques

All plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega Wizard®*Plus* SV Minipreps system. Calf-intestine alkaline phosphatase and restriction endonucleases were used according to the instructions of the manufacturer (Fermentas). DNA fragments were isolated from agarose gels using the Qiagen quick gel extraction kit. Ligations were performed using the rapid DNA ligation kit (Roche).

The *lpxL1* and *lpxL2* genes from *B. pertussis* strain B213 were obtained by PCR. The chromosomal template DNA was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x*g*, after which the supernatant was used as template DNA. The sequences of the forward primers, which contained an NdeI site (underlined), including an ATG start codon, were 5'-AACATATGCTCGTCACCCTGTTA-3' (*lpxL1*) and 5'-AACATATGAGCCAATTCAAGA-3' (*lpxL2*). The sequences of the reverse primers, which contained a BamHI site (underlined) and included a stop codon, were 5'-AAGGATCCTCATCGTTCGGGTTCCTG-3' (*lpxL1*)

and 5'-AAGGATCCTCAGTACAGCTTGGGCTT-3' (*lpxL2*). The PCRs were performed under the following condition: 50 μl total reaction volume, 25 pmol of each primer, 0.2 mM dNTPs, 3 μl of template DNA solution, 1.5% dimethylsulfoxide, 1.75 units Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche). The temperature program was as follows: 95°C for 3 min, a cycle of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C, repeated 30 times, followed by 10 min at 72°C and subsequent cooling to 4°C. The PCR products were purified from agarose gel and subsequently cloned into pCRII-TOPO. Plasmid DNA from a correct clone was digested with NdeI and BamHI, and the LpxL-encoding fragments were ligated into NdeI- and BamHI–digested pET-11a. The ligation mixture was used to transform *E. coli* DH5α using the $CaCl_2$ method (Sambrook *et al.*, 1989). Plasmids containing the correct inserts were designated pLpxL1 and pLpxL2. The nucleotide sequences of the cloned genes were confirmed by sequencing in both directions.

To allow for expression in *B. pertussis*, the *lpxL1* and *lpxL2* genes were subcloned into the broad host-range, low copy-number vector pMMB67EH. To this end, pLpxL1 and pLpxL2 were digested with XbaI and HindIII, and the relevant fragments were ligated into XbaI- and HindIII-digested pMMB67EH. The ligation mixture was used to transform *E. coli* DH5α. Plasmids with the correct inserts were designated pMMB67EH-LpxL1 and pMMB67EH-LpxL2 (Table 1). The pMMB67EH-based plasmids were used to transform *E. coli* SM10(λpir), which allowed for subsequent transfer of the plasmids to *B. pertussis* by conjugation. Furthermore, the plasmids were used to transform *E. coli* strains W3110 and MLK53 (Table 1) for complementation experiments.

To construct a *B. pertussis lpxL1* mutant strain, we amplified part of the DNA upstream of *lpxL1* from *B. pertussis* strain B213 by using primers 5'-AAATTCGCTCTGGCGCTGCAC-3' and 5'-AATCAGCACGCGTCTGACCGATGCGAA TGAAAGGGCGG-3', containing a MluI site (underlined). Additionally, a DNA fragment downstream of *lpxL1* was obtained by PCR with primers 5'-AAGTCAGACGCGTGCTGAGACAGCGCGCGGCAGGAACC-3', containing a MluI site (underlined), and 5'-AATCCACGTGATAGCGCCCGGT-3'. Both PCR products were cloned into pCRII-TOPO, resulting in plasmids pCRII-LpxL1$_{up}$ and pCRII-LpxL1$_{down}$, respectively. A MluI–XbaI fragment of pCRII-LpxL1$_{down}$ was ligated into MluI–XbaI-restricted pCRII-LpxL1$_{up}$. The resulting plasmid was cut with MluI to allow for insertion of the gentamicin-resistance cassette from plasmid pBSL141 obtained by MluI digestion. Finally, an XbaI-SacI fragment of the construct obtained was ligated into the XbaI-SacI-restricted suicide plasmid pKAS32. The final construct, designated pKAS32-LpxL1$_{KO}$, contained the gentamicin-resistance cassette in the reverse orientation relative to the transcription direction of the *lpxL1* gene and was used to construct a *B. pertussis lpxL1*

mutant by allelic exchange. Transformants were screened by PCR using various primer sets.

Isolation and analysis of LPS

LPS was isolated using the hot phenol/water extraction method (Westphal and Jann, 1965) with slight modifications (Geurtsen *et al.*, 2006). The fatty acid composition was analysed using a 6890 Agilent gas chromatograph (Welch, 1991). The lipid A moiety of LPS was isolated as described (Geurtsen *et al.*, 2006) and used for structural analysis by nanoelectrospray tandem MS on a Finnigan LCQ in the negative ion mode (Wilm and Mann, 1996).

Endotoxic activity assays

The human macrophage cell line MM6 (Ziegler-Heitbrock *et al.*, 1998) was stimulated with serial dilutions of whole bacterial cell suspensions or purified LPS as described (Geurtsen *et al.*, 2006). The bacterial cell suspensions were prepared by collecting the cells from cultures by centrifugation, after which they were resuspended in PBS at an A_{590} of 1.0, heat-inactivated for 10 min in the presence of 8 mM formaldehyde, and stored at 4°C. Following stimulation, IL-6 concentrations in the culture supernatants were quantified with an Enzyme-linked Immunosorbent Assay (ELISA) against human IL-6 according to the manufacturer's instructions (PeliKine Compact™).

Determination of antibiotic sensitivity

B. pertussis strains were grown for 2 days on blood agar plates. Ten colonies of each strain were resuspended in 600 μl THIJS medium, after which 150 μl of the suspension was spread on fresh blood agar plates. Filter paper Sensi-discs™ (BD Biosciences) containing kanamycin (30 μg), ampicillin (10 μg), erythromycin (15 μg), tetracycline (10 μg), rifampicin (5 μg), or chloramphenicol (30 μg), were placed on the agar surface. After 4 days of incubation at 35°C, the growth inhibition halo was measured.

Infection of human macrophages

For infection of human macrophages, bacteria were grown for 16 h on fresh BG blood agar plates, after which they were washed once with PBS and resuspended in 1 ml of IMDM medium (Gibco BRL). Bacteria were added to $5x10^5$ MM6 cells, which were maintained in 0.25 ml of pre-warmed IMDM medium in 24-well tissue culture plates, at a multiplicity of infection of 10 (final volume = 500 μl). After 2 h of incubation (5%

CO_2, 37°C), 100 µg/ml of colistin sulphate (end concentration) was added to the wells, after which the plates were incubated further for 2 h at 37°C. Then, the MM6 cells were collected by centrifugation and washed once with IMDM medium, after which they were lysed (1 min, 22°C) in 0.15 ml PBS containing 0.1% Triton X-100. The lysed cells were plated onto BG blood agar plates, and the number of viable intracellular bacteria was estimated by colony forming unit (CFU) counting after 72 h of growth. Infection experiments were repeated three times. The colistin sensitivity of the wild-type and mutant strain was determined by growing them in the presence of various concentrations of colistin and, after diluting the suspensions and plating them on BG blood agar plates, counting the amount of CFU.

Results

Identification of late lipid A acyltransferase homologues in *B. pertussis*

The 306- and 323-amino acid residue sequences of the *E. coli* K-12 LpxL and LpxM proteins with GenBank Accession Numbers NP_415572 and NP_416369, respectively, were used to identify putative *lpxL* and *lpxM* homologues in the complete *B. pertussis* genome sequence present in the NCBI database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). BLAST search (Altschul *et al.*, 1990) revealed the presence of two homologues of *lpxL* and *lpxM*, i.e., BP3072 and BP3073 with GenBank Accession Numbers NP_881643 and NP_881644, respectively. BP3072 and BP3073 show a sequence identity of 21% and 29% to *E. coli* LpxM, respectively, of 23% and 31% to *E. coli* LpxL, respectively, and of 25% to each other. Since both proteins show a higher sequence identity to *E. coli* LpxL than to *E. coli* LpxM, BP3072 and BP3073 were designated *lpxL1* and *lpxL2*, respectively. The open reading frames (ORFs) are adjacent to one another, with the stop codon of *lpxL1* overlapping the start codon of *lpxL2*, and, therefore, seem to form an operon. Upstream, in the reverse orientation, and downstream of the operon, genes are located putatively encoding a homologue of the S-adenosylmethionine synthetase MetK and of the diaminopimelate epimerase DapF, respectively. Further BLAST analysis revealed the presence of *lpxL1* and *lpxL2* homologues in *B. parapertussis*, i.e., BPP0191 and BPP0190, with GenBank Accession Numbers NP_882552 and NP_882551, respectively, and in *B. bronchiseptica*, i.e., BB0194 and BB0193, with GenBank Accession Numbers NP_886744 and NP_886743, respectively. The mutual sequence identity between the *Bordetella* proteins is 97% for the LpxL1 proteins and 98% for the LpxL2 proteins. Furthermore, the genetic organisation of the *lpxL1*/*lpxL2* operon is conserved among the *Bordetella* strains.

Cloning of *lpxL* genes and complementation of the *E. coli lpxL* mutant phenotype

E. coli lpxL mutants show a growth defect on nutrient broth above 32°C (Karow *et al.*, 1991). To test whether the identified *B. pertussis lpxL* homologues can complement this phenotype, we cloned the *lpxL1* and *lpxL2* genes into the broad host-range low copy-number vector pMMB67EH under the control of the *tac* promoter and used the resulting plasmids to transform *E. coli lpxL* mutant strain MLK53 (Karow and Georgopoulos, 1992). As controls, both MLK53 and the parental *E. coli* strain W3110 were transformed with vector pMMB67EH. The strains were first grown to early log phase in minimal medium at 30°C, after which the bacteria were transferred to LB and further incubated at 42°C. As shown in Fig. 2, the growth defect of the *E. coli lpxL* mutant was complemented by the plasmid harbouring *lpxL2*. The plasmid encoding LpxL1 did not complement the phenotype and its presence, as compared to the empty vector control, seemed to hamper growth even further (Fig. 2).

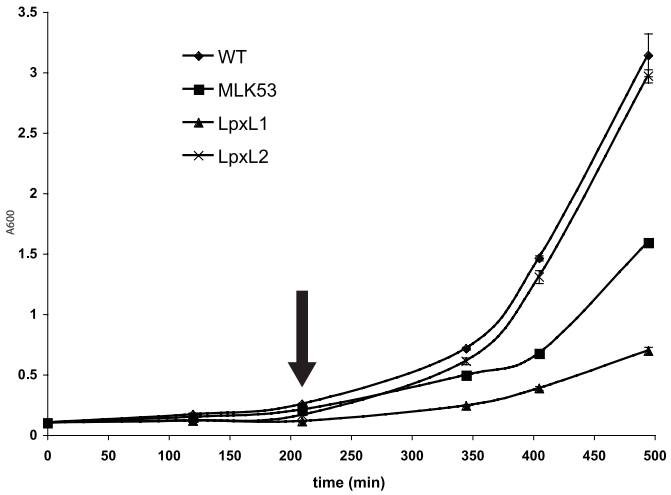

Fig. 2. Complementation of the temperature-sensitive growth phenotype of *E. coli lpxL* mutant MLK53. Bacteria were grown to early log phase in minimal medium at 30°C. After 3½ h (arrow), the bacteria were transferred to LB and further incubated at 42°C. WT, wild-type *E. coli* strain W3110 harbouring the empty pMMB67EH vector; MLK53, *E. coli lpxL* mutant strain harbouring the empty pMMB67EH vector; LpxL1, *E. coli* MLK53 expressing *B. pertussis lpxL1*; LpxL2, *E. coli* MLK53 expressing *B. pertussis lpxL2*. Absorbance was measured at 600 nm.

Overexpression of LpxL1 and LpxL2 in *B. pertussis*

The effect on LPS composition of *lpxL1* and *lpxL2* overexpression was studied in *B. pertussis* strain B213 after introduction of the pMMB67EH-derived plasmids. No

obvious effect of *lpxL1* and *lpxL2* overexpression was observed upon Tricine-SDS-PAGE analysis of isolated LPS (data not shown). To evaluate possible alterations in LPS composition in more detail, the lipid A moieties of the strains were analysed by ESI-MS in the negative-ion mode (Fig. 3). This analysis revealed the presence of four major lipid A species in wild-type LPS (Fig. 3A). The peak at *m/z* 1557 represents the characteristic penta-acylated bis-phosphate species that is typically found in *B. pertussis* (Caroff et al., 1994), whereas the peak at *m/z* 1477 corresponds to a penta-acylated mono-phosphate species. The two remaining peaks at *m/z* 1307 and 1251 represent deacylated lipid A species of the molecular ion at *m/z* 1477 that miss the primary 3OH C10 residue at the 3 position and a primary 3OH C14 residue, probably at the 3' position (Geurtsen *et al.*, 2006), respectively. Besides these four major lipid A species, several minor species were detected. The peaks at *m/z* 1331 and 1387 correspond to the *bis*-phosphorylated forms of the molecular ions at *m/z* 1251 and 1307, respectively, whereas the peak at *m/z* 1081 corresponds to a mono-phosphate form missing both the 3OH C10 and a 3OH C14 residue. Additional minor species included molecular ions at *m/z* 1279, 1450, and 1505. Mass calculations indicate that these species most likely correspond to the molecular ions at *m/z* 1081, 1251, and 1307 respectively, substituted with an additional hydroxyl C12 chain (*m/z* 198).

Upon overexpression of LpxL1 and LpxL2 (Fig. 3B and C, respectively), no major changes in the spectrum of lipid A species were detected. The only new lipid A species detected was a peak at *m/z* 1675, which appeared after overexpression of LpxL1 (Fig. 3B). The mass of this peak corresponds to the molecular ion at *m/z* 1477, however, with an additional hydroxyl C12 chain present. Interestingly, in the *lpxL1*-overexpressing strain, the relative abundance of lipid A species containing an hydroxyl C12 chain, i.e., the molecular ions at *m/z* 1279, 1450, 1505, and 1675, increased from ~9% in wild-type LPS to ~19% in the LPS from the strain overexpressing LpxL1 (Table 2). This result indicates that upon overexpression of LpxL1, the fraction of hydroxyl C12-containing lipid A species increased. No obvious differences were observed upon *lpxL2* overexpression.

To determine the nature of hydroxyl C12 chain identified above, the fatty acid content of the isolated LPS was analysed by GC/MS. The results revealed that LPS from the *lpxL1*-overexpressing strain contained an increased amount of 2-hydroxyl C12 (2OH C12), but not 3OH C12 (Fig. 4), implying that the hydroxyl C12 chain identified above harbours its hydroxyl group at the 2 position. The presence of minor amounts of 3OH C12 in wild-type *B. pertussis* LPS can probably be explained by the relaxed acyl chain specificity of the *B. pertussis* LpxA enzyme (Sweet *et al.*, 2002).

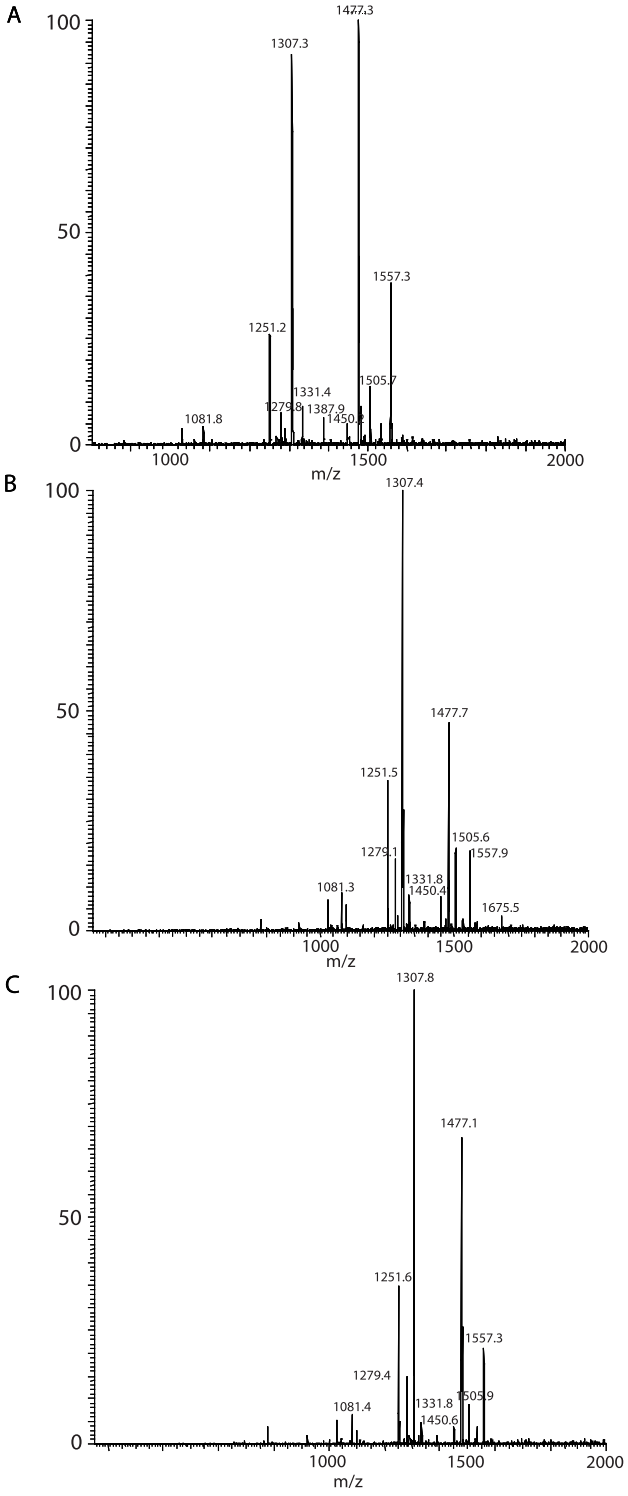

Fig. 3. Structural analysis by ESI-MS of purified *B. pertussis* LPS. Lipid A species from wild-type *B. pertussis* strain B213 (A) and its derivatives overexpressing *lpxL1* (B) or *lpxL2* (C) were analysed by ESI-MS. Major peaks at *m/z* 1557, 1477, 1387, 1307, 1251, and 1081 were interpreted as the characteristic penta-acylated *bis*-phosphate species that is typically found in *B. pertussis*, the corresponding penta-acylated *mono*-phosphate species, the deacylated lipid A species of the molecular ion at *m/z* 1557 missing the primary 3OH C10 residue at the 3 position, the deacylated lipid A species of the molecular ion at *m/z* 1477 missing the primary 3OH C10 residue at the 3 position, the deacylated lipid A species of the molecular ion at *m/z* 1477 missing a primary 3OH C14 residue, and the deacylated lipid A species of the molecular ion at *m/z* 1477, missing both the primary 3OH C10 residue at the 3 position and a primary 3OH C14 residue, respectively. The peaks at *m/z* 1279, 1450, 1505, and 1675 correspond to the molecular ions present at *m/z* 1081, 1251, 1307, and 1477, but contain an extra 2-hydroxyl C12 group.

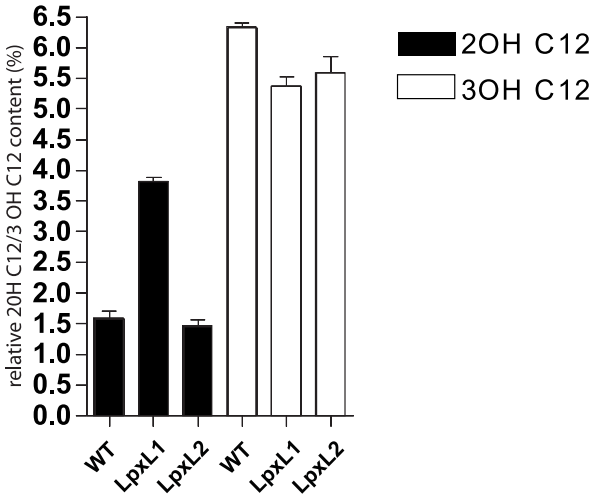

Fig. 4. GC/MS analysis of LPS from wild-type, *lpxL1*-, and *lpxL2*-overexpressing *B. pertussis* strains. GC/MS analysis of LPS purified from wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 overexpressing *lpxL1* (LpxL1), and *B. pertussis* strain B213 overexpressing *lpxL2* (LpxL2). Indicated are the normalised 2OH C12 and 3OH C12 contents with the 3OH C14 content set at 100.

TABLE 2

Relative abundance of lipid A molecular ions as determined by ESI-MS

	1081 -C14-3OH -C10-3OH -PO4	1251 -C14-3OH -PO4	1279 -C14-3OH -C10-3OH -PO4 +C12-2OH	1307 -C10-3OH -PO4	1331 -C14-3OH	1387 -C10-3OH	1450 -C14-3OH -PO4 +C12-2OH	1477 -PO4	1505 -C10-3OH -PO4 +C12-2OH	1557	1675 -PO4 +C12-2OH	% +C12-2OH
Wild-type	1.5	8.5	2.6	30.3	3.1	2.1	1.8	32.8	4.6	12.6	0.0	9.0
LpxL1	3.4	13.0	6.7	37.2	3.4	0.0	3.2	17.9	7.1	6.7	1.5	18.5
LpxL2	2.6	13.2	5.8	37.7	1.9	0.0	1.5	25.6	3.4	8.3	0.0	10.7

Endotoxic activity of LPS and whole bacterial cells

To assess the effect of *lpxL* overexpression on the endotoxic activity of LPS, the potency of the purified LPS to stimulate the production of IL-6 by the human macrophage cell line MM6 was tested. As compared to wild-type LPS, the purified LPS from the strain overexpressing *lpxL1* had a strongly increased potency to stimulate the macrophages, as can be expected from its increased amount of hexa-acyl lipid A (Fig. 5A). Similarly,

whole-cell suspensions of *B. pertussis* cells overexpressing *lpxL1* showed, as compared to *B. pertussis* cells containing the empty vector, an increased potency to stimulate the macrophages (Fig. 5B). Interestingly, such an increase, although not as robust, was also observed for the cells overexpressing *lpxL2* (Fig. 5B). Since purified LPS of the latter strain did not show an increased stimulation potency (Fig. 5A), its increased biological activity of the whole cell preparation is currently not understood.

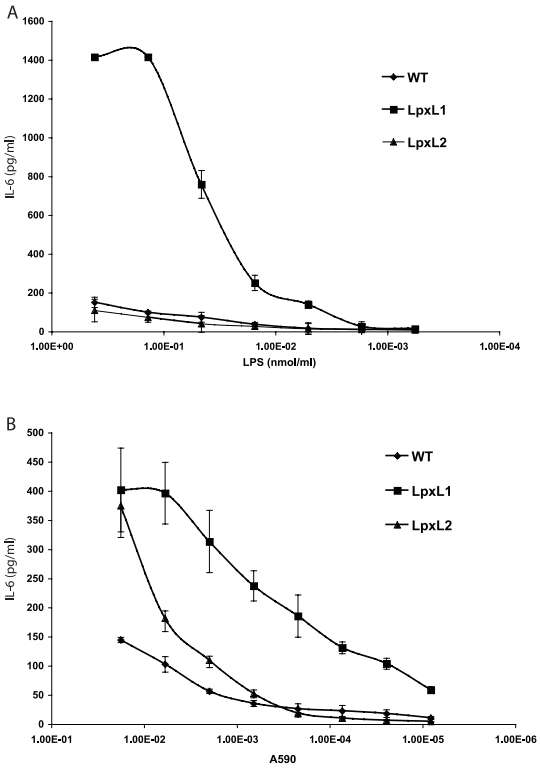

Fig. 5. IL-6 induction by purified *B. pertussis* LPS and whole cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of LPS purified from a wild-type (WT), a *lpxL1*-, or a *lpxL2*-overexpressing *B. pertussis* strain, or (B) a whole-cell suspension of *B. pertussis* cells overexpressing *lpxL1* or *lpxL2*, or containing the empty vector pMMB67EH (WT). IL-6 concentration in the culture supernatant was quantified in an ELISA against human IL-6. The data represent the averages of three individual experiments.

Construction and phenotype of a *B. pertussis lpxL1* mutant strain

One of our initial aims was to create *B. pertussis* mutant strains in which *lpxL1*, *lpxL2*, or the complete *lpxL1*/*lpxL2* operon, are deleted. To achieve this goal, we made several plasmid constructs that were suitable for replacing the target sequence on the

B. pertussis chromosome with an antibiotic-resistance cassette via allelic exchange. Following this strategy, we easily obtained clones in which the *lpxL1* gene was replaced by a gentamicin-resistance marker. However, this was not the case for *lpxL2* or the *lpxL1*/*lpxL2* operon. We have tried various strategies, using different constructs, different markers, and different selection temperatures, but up till now, we have not been able to isolate a viable *lpxL2* or *lpxL1/lpxL2 B. pertussis* mutant.

To characterise the *lpxL1* mutant obtained, we first studied its growth characteristics. At both 35°C and at 28°C, the growth of the mutant strain in synthetic THIJS medium was comparable to that of the wild-type strain (data not shown). Additionally, Western blots showed that the expression of the virulence factors pertactin, fimbriae, pertussis toxin, and filamentous haemagglutinin was unaffected in the mutant strain (data not shown). We also tested whether the mutant strain would have a decreased capability of inducing the production of pro-inflammatory cytokines, since overexpression of *lpxL1* led to increased stimulatory activity (Fig. 5B). Curiously, as shown in Fig. 6, also the mutant cells showed in increased capacity of inducing IL-6 production by the macrophages, due to an unknown pleiotropic effect.

Normally, *B. pertussis* exhibits haemolytic activity in its virulent Bvg^+ phase, as is indicated by the formation of haloes around the colonies on blood agar plates. The mutant strain exhibited a reduced capacity to form such haloes. Haloes were still formed, but they were smaller, took longer to arise, and were less intense (data not shown). In contrast, the *lpxL1*-overexpressing strain formed larger and more intense haemolytic haloes than the wild-type strain. These observations suggest that the presence of a 2OH C12 moiety in *B. pertussis* lipid A is somehow linked to haemolytic activity of the bacteria.

To determine whether the *lpxL1* mutation affected the integrity of the outer membrane, susceptibility to various antibiotics was measured. As shown in Fig. 7, the mutant strain was as susceptible to most antibiotics as was the wild-type strain. However, the diameter of the growth inhibition zone around filter paper discs containing rifampicin was twice as large as that in case of the wild-type strain.

Infection of human macrophages by the *B. pertussis lpxL1* mutant strain

B. pertussis is capable of invading and surviving within various eukaryotic cell types, including human macrophages (Friedman *et al.*, 1992). In a previous study, it was shown that an *lpxM* mutant of *Neisseria gonorrhoeae* was impaired in its survival inside urethral epithelial cells (Post *et al.*, 2002). To test whether the *B. pertussis lpxL1* mutant is affected in its ability to infect human macrophages, we determined the number of

bacteria that could be recovered from the intracellular compartment after 2 h of infection. However, we first tested whether the wild-type and mutant strain exhibited differences in their ability to survive within medium or for their susceptibility to colistin, but this was not the case (data not shown). As compared to the wild-type strain (2381 ±92 CFU/well), the number of bacteria recovered for the *lpxL1* mutant strain was about more than 15-fold lower (138 ±62 CFU/well). Thus, apparently, the activity of LpxL1 is indeed required for the efficient infection of human macrophages by *B. pertussis*.

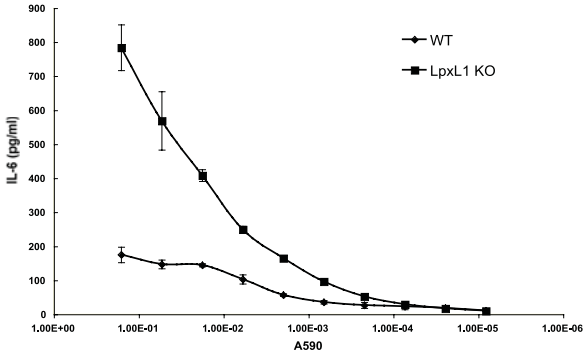

Fig. 6. IL-6 induction by whole cells of wild-type *B. pertussis* and the *lpxL1* mutant of *B. pertussis*. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type or *lpxL1*-mutant *B. pertussis* cells. The IL-6 concentration in the culture supernatant was quantified in an ELISA against human IL-6. The data represent the averages of three individual experiments.

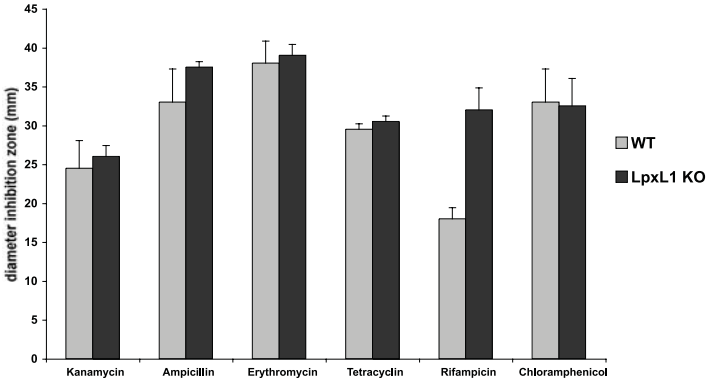

Fig. 7. Antibiotic susceptibility of wild-type *B. pertussis* and the *lpxL1* mutant of *B. pertussis*. Shown is the diameter in millimetres of the inhibition zone around filter paper discs containing each one of six different antibiotics. The data shown represents the averages of three individual experiments.

Discussion

B. pertussis lipid A has been reported to consist of penta-acylated lipid A species containing four primary hydroxylated acyl chains and one secondary acyl chain, i.e., a C14 acyl chain at the 2' position (Caroff *et al.*, 1994). The goal of this study was to identify and characterise the enzyme responsible for the addition of the secondary C14 acyl chain. BLAST searches using the *E. coli* late acyl transferases LpxL and LpxM as leads identified two homologues in the *B. pertussis* chromosome. This finding raised the questions which of the two *lpxL* homologues in *B. pertussis* mediates the addition of the secondary C14 chain to the LPS and what the function of the other *lpxL* homologue might be. We found that LpxL2, but not LpxL1, was capable of fully restoring the temperature-sensitive phenotype of an *E. coli lpxL* mutant, indicating that the function of *B. pertussis* LpxL2 resembles that of *E. coli* LpxL, and, thus, that LpxL2 mediates the addition of the secondary C14 acyl chain to the 2' position of *B. pertussis* lipid A. This conclusion was further supported by mass spectrometry and endotoxic activity data, which showed that *lpxL2* overexpression in *B. pertussis* did not lead to changes in the composition or toxicity of the LPS. Since already 100% of the *B. pertussis* lipid A species contain a secondary C14 acyl chain at the 2' position, overexpression of the responsible enzyme was not expected to affect LPS structure or toxicity. The observation that LpxL1 was unable to complement the *E. coli lpxL* mutant phenotype was a first indication that LpxL1 has a different function. We showed that *lpxL1* overexpression in *B. pertussis* leads to the accumulation of a novel, previously undetected lipid A species in *B. pertussis* containing an extra secondary 2OH C12 chain. Thus, either LpxL1 functions as a 2-hydroxyl lauryl transferase, which directly attaches a 2OH C12 moiety to lipid A, or, alternatively, as a regular lauryl transferase, which adds a C12 acyl chain that is later on hydroxylated by a another enzyme, for instance LpxO, for which a homologue has been found in the *B. pertussis* genome (Gibbons *et al.*, 2000). Also the exact position at which LpxL1 attaches an acyl chain remains to be determined. We tried to resolve this by using MS/MS analysis, but the spectra obtained were inconclusive (data not shown). However, it can be concluded that LpxL1 does not add a secondary acyl chain at the lipid A 3 position, since lipid A species without primary 3OH C10 acyl chain at this position, but with a 2OH C12 moiety (Fig 3B, peak at *m/z* 1505) could be detected. We detected also a lipid A species that lacks one of the primary 3OH C14 chains, and, previously, we demonstrated that this chain is probably missing at the 3' position (Geurtsen *et al.*, 2006). Thus, the observation that lipid A species containing an extra 2OH-C12, but missing a 3OH C14 could be detected (Fig 3B, peaks at *m/z* 1279 and 1449) suggests that the substituted 2OH C12 is present at the C-2 position. Another question is why

lpxL1 expression in *E. coli* MLK53 did not restore the mutant phenotype, since it is known that overexpression of a lipid A-acyl transferase with a different specificity than LpxL, such as LpxM, can also restore the mutant phenotype (Karow and Georgopoulos, 1992). Possible answers to this question are that *lpxL1* expression in *E. coli* was not high enough to support complementation, that LpxL1 only functions after a secondary acyl chain has been attached to the 2' position, which is not the case in the *E. coli lpxL* mutant LPS, or that the acyl chain donor was not sufficiently present. The latter possibility can probably only be true when LpxL1 directly transfers a 2OH C12 chain.

In the wild-type *B. pertussis* strain, the majority of lipid A was mono secondary acylated and only a small subset of the lipid A species (<10%) harboured an extra secondary 2OH C12 group. Thus, under the growth conditions applied, LpxL1 activity is low, whereas LpxL2 is highly active. Since *lpxL1* and *lpxL2* almost certainly constitute an operon, an important question is how the bacterium is capable of controlling LpxL1 activity. One possibility is that the LPS is normally only a poor substrate for LpxL1 and is therefore modified with low efficiency. Alternatively, *lpxL1* and *lpxL2* expression is differentially controlled at the level of translation initiation. Analysis of the nucleotide sequence upstream of the ATG start codons of *lpxL1* and *lpxL2* supports the latter hypothesis. The *lpxL2* start codon is preceded by a sequence -$_{+13}$AGGAAC$_{+8}$- that resembles the consensus Shine-Dalgarno (SD) sequence -AGGAGG-. However, upstream of *lpxL1*, such an SD sequence could not be found. Also analysis of the -18 to +18 nucleotides around the ATG start codon applying the algorithm described by Kolaskar and Reddy (1985), revealed that the *lpxL1* ATG start codon is inadequate. Thus, the relatively low abundance of lipid A species carrying an 2OH C12 may be explained by a poor translation initiation of *lpxL1* mRNA.

One of our initial goals was to obtain *B. pertussis* mutants in which the *lpxL* homologues, either separately, or as the complete operon, were knocked out. Since mutations in late acyl transferases have often been shown to reduce the toxicity of the LPS, we reasoned that *B. pertussis lpxL* mutant strains may form a good basis for the development of less reactogenic whole-cell pertussis vaccines. The only viable mutant obtained was an *lpxL1* knock out. Since we were not able to isolate *lpxL2* or *lpxL1*/*lpxL2* mutants, *lpxL2* appears essential for *B. pertussis* viability.

The *lpxL1* mutant strain obtained exhibited a clearly reduced haemolytic activity, while a *B. pertussis* strain that overexpressed *lpxL1* displayed the opposite phenotype and exhibited an increased haemolytic activity. The haemolytic activity of *B. pertussis* is caused by the activity of the secreted adenylate cyclase (CyaA), a member of the RTX (repeat in toxin) family of bacterial pore-forming toxins (Ladant and Ullmann, 1999).

Although CyaA, whose expression is regulated by the *Bordetella* Bvg two-component system, is efficiently secreted via a type 1 secretion mechanism, a large proportion of the proteins secreted stays attached to the bacterial cell surface (Hewlett *el al.*, 1976). For a long time, it was though that this membrane-bound CyaA is the active form, but, recently, it was shown that only the freshly secreted, newly synthesised toxin molecules, are able to penetrate target cells and elicit effector functions (Gray *et al*., 2004). Although the exact reason for the reduced haemolytic activity of the mutant strain remains to be elucidated, it is tempting to speculate that the capacity of CyaA to remain attached to the surface is inversely correlated to the amount of hexa-acylated LPS.

Increased susceptibility to rifampicin, a hydrophobic antibiotic, has been associated with decreased outer membrane integrity (Abadi *et al*., 1996). Hence, the observed increase in rifampicin susceptibility of the *lpxL1* mutant strain is probably indicative for decreased outer membrane integrity. Another striking observation was that *lpxL1* mutant cells displayed an increased capacity to induce cytokine release by macrophages. This observation was unexpected since LPSs from late acyl transferase mutants generally show a reduced reactogenicity (Low *et al.*, 1998; van der Ley *et al.*, 2001; Cognet *et al.*, 2003). However, as we demonstrated in a previous study (Geurtsen *et al.*, 2006), a decrease in the integrity of the *B. pertussis* outer membrane can potentially lead to an increased LPS release, which can counter balance the effect of a reduced LPS toxicity. A second possibility, which is consistent with the observation that the *lpxL1* mutant strain displayed a decreased haemolytic activity, would be that CyaA is more easily released from wild-type cells than from the mutant cells. Since CyaA has been shown to have immune suppressive activity (Mattoo and Cherry, 2005; Mills, 2001), a reduced CyaA release can be expected to result in an increased toxicity of the *B. pertussis* cells. A third possibility is that the *lpxL1* mutant strain displays unknown pleiotropic effects.

Whereas LpxL1 activity is apparently required for maintaining the integrity of the outer membrane, a low activity is apparently already sufficient for this purpose. This situation is probably beneficial for the bacterium, since the presence of large quantities of hexa-acylated LPS species would rapidly activate the host innate immune system and thereby evoke the clearance of the bacterium. However, it is known that *B. pertussis* also has a partial intracellular life cycle (Cheers and Gray, 1969). Inside a cell, a bacterium encounters a completely different environment implicating that the requirements to survive may also be different. It was previously shown that a late acyl transferase mutant of *N. gonorrhoeae* displayed a decreased survival inside urethral epithelial cells (Post *et al.*, 2002). Therefore, we hypothesised that the presence of an

extra secondary acyl chain may be important for an efficient infection of eukaryotic cells by *B. pertussis*. Indeed, we showed that the recovery rate of the *lpxL1* mutant strain was much lower than that of the wild-type strain after infection of human macrophages. This result suggests that the activity of LpxL1 is indeed required for an efficient infection of and/or survival within human macrophages and may therefore form an important factor for successful host infection by *B. pertussis*. Interestingly, Gibbons *et al.* (2000) proposed a model in which lipid A-2-hydroxylation functions in the suppression of host cell signalling, permitting a more prolonged survival of bacteria in the host cell. Furthermore, it will be interesting to determine whether *lpxL1* expression is upregulated within the macrophages.

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Chapter 9

Gene cluster involved in lipopolysaccharide-core biosynthesis and identification of a novel lipid A modification in *Bordetella pertussis*

Jeroen Geurtsen, Monika Dzieciatkowska, Liana Steeghs, Annemarie Boleij, Kelly Broen, Hendrik-Jan Hamstra, Jianjun Li, Jim Richards, Jan Tommassen, and Peter van der Ley

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Abstract

Lipopolysaccharide (LPS), also known as endotoxin, is one of the main constituents of the Gram-negative bacterial outer membrane. Whereas its lipid A part is generally seen as the main determinant for endotoxic activity, the oligosaccharide moiety plays an important role in the interaction with professional antigen-presenting cells, such as dendritic cells. Here, we describe a novel four-gene cluster involved in the biosynthesis of the *Bordetella pertussis* core oligosaccharide. By insertionally inactivating the genes and studying the resulting LPS structures, we show that at least two of the genes encode active glycosyltransferases. In addition, we demonstrate that mutations in the operon differentially affect dendritic cell maturation and macrophage activation. Interestingly, we also found a previously unknown modification of lipid A with a hexosamine.

Introduction

LPS is an amphiphilic molecule located in the outer leaflet of the outer membrane of Gram-negative bacteria. LPS possesses both endotoxic activity and adjuvant activity. Both properties are based upon its recognition by the host TLR4/MD-2 receptor complex (reviewed in Pålsson-McDermott and O'Neill, 2004; O'Neill, 2006). LPS consists of three distinct structural domains: lipid A, the core, and the O-antigen. Lipid A functions as a hydrophobic membrane anchor and forms the bioactive component of the molecule (Takada and Kotani, 1989). The core region consists of a complex oligosaccharide, which, as compared to the O-antigen, shows only limited structural variability. In some bacteria, e.g., *Enterobacteriaceae*, the core oligosaccharide (core OS) can be divided into an inner core and an outer core. The outer core primarily consists of pyranosidic hexoses, e.g., D-glucose, D-galactose, and D-glucosamine, whereas the inner core primarily consists of octulosonic acids and heptopyranoses. In the vast majority of Gram-negative bacteria, the core domain is connected to the lipid A domain by a specific carbohydrate, 2-keto-3-deoxyoctulosonic acid (Kdo) (Raetz and Whitfield, 2002). The O-antigen comprises the most variable part of the LPS and confers bacteria serotype specificity. It is composed of repeating sugar subunits of one to eight sugars. Each O-chain can contain up to 50 of these subunits. The O-antigen has been implicated in bacterial immune escape, especially the escape from serum complement-mediated lysis (Raetz and Whitfield, 2002).

In contrast to the LPS of *Bordetella bronchiseptica* and *Bordetella parapertussis*, the LPS of *Bordetella pertussis* never contains an O-antigen domain (Peppler, 1984; Di Fabio *et al.*, 1992). Therefore, *B. pertussis* LPS is often referred to as lipooligosaccharide. *B. pertussis* produces two dominant LPS forms, band A and band B LPS (Peppler, 1984). Band B LPS is composed of lipid A and a core oligosaccharide consisting of 9 carbohydrates (Caroff *et al.*, 2000). Addition of a terminal trisaccharide, consisting of *N*-acetyl glucosamine, 2,3-diacetamido-2,3-dideoxy-mannuronic acid, and 2-acetamido-4-*N*-methyl-2,4-dideoxy-fucose, to band B LPS forms the LPS referred to as band A.

In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, the core OS biosynthesis gene cluster consists of three operons, designated the *gmhD*, *waaQ*, and *WaaA* operons. The *gmhD* operon consists of four genes, *gmhD* and *waaFCL*, which are involved in the synthesis of the inner core (Schnaitman and Klena, 1993). The *gmhD*, *waaF*, and *waaC* genes encode proteins involved in the biosynthesis and transfer of Heptoses I and II to Kdo_2-lipid A (Schnaitman and Klena, 1993), whereas the *waaL* gene product is a ligase that is involved in the attachment of the O-antigen (MacLachlan *et al.*, 1991). The *waaQ* operon is the largest of the three operons and encodes proteins

that are involved in the biosynthesis of the outer core and in modification/decoration of the core OS. The number and types of genes present within in the *waaQ* operon differs per strain, which explains the strain-specific differences in core composition (Heinrichs *et al.*, 1998). The *waaA* operon often encodes only one protein, KdtA. Only in *E. coli* K-12, an additional non LPS-related open reading frame (ORF) is present (Raetz and Whitfield, 2002). The *kdtA* gene of *Enterobacteriaceae* encodes the bifunctional Kdo transferase that adds the two Kdo residues in the Kdo_2-lipid A biosynthesis (Clementz and Raetz, 1991).

Although the *Bordetella* and *E. coli* core OS show some resemblance, the exact composition and configuration of residues display marked differences. For example, the *Bordetella* core OS contains only one Kdo residue, instead of the two or three residues that are found in most other Gram-negative bacteria, including *E. coli*. Recently, this was shown to be due to the functioning of *Bordetella* KdtA as a monofunctional, rather than as a bifunctional Kdo transferase (Isobe *et al.*, 1999). Like in *E. coli*, the *Bordetella* core OS starts with two heptose residues attached to Kdo. The responsible glycosyltransferases were identified and shown, as expected, to be homologues of the WaaC and WaaF enzymes, respectively (Allen *et al.*, 1998a; Sisti *et al.*, 2002). Additionally, the *wlb* locus encompassing the genes responsible for the addition of the terminal trisaccharide in band A LPS has been identified (Allen and Maskell, 1996; Allen *et al.*, 1998b). The enzymes responsible for the synthesis of the remaining portion of the *Bordetella* core OS are currently unknown and await further identification.

Although its lipid A part is generally seen as the main determinant for the biological activity of LPS through the activation of the TLR4/MD-2 receptor complex, the oligosaccharide region can also play an important role in its interaction with antigen-presenting cells (APCs). Receptors implicated in this type of LPS recognition include the complement receptor CR3 and the scavenger receptor SR-A (van Amersfoort *et al.*, 2003; Plüddemann *et al.*, 2006). In the case of *Neisseria meningitidis*, the LPS oligosaccharide region has been shown to be a critical determinant for the bacterial interaction with dendritic cells (DCs) (Uronen-Hansson *et al.*, 2004; Kurzai *et al.*, 2005; Steeghs *et al.*, 2006). Interestingly, among a panel of mutants with a truncated LPS oligosaccharide chain, the *lgtB* mutant lacking only the terminal galactose residue of the lacto-*N*-neotetraose unit showed a strongly increased association with DCs, also resulting in higher uptake of the bacteria (Steeghs *et al.*, 2006). This interaction was shown to be entirely mediated by the C-type lectin DC-SIGN (Steeghs *et al.*, 2006). By analogy, *B. pertussis* mutants with an altered oligosaccharide chain might also be affected in their interaction with DCs. Specific targeting to APCs, such as DCs, could conceivably affect the outcome of the immune response against a whole-cell pertussis

vaccine. As a first step towards improvement of whole-cell vaccines by this route, we have now identified a gene cluster involved in LPS oligosaccharide biosynthesis in *B. pertussis*, identified the LPS alterations in the knockout mutants, and studied the effects of the mutations on interaction with DCs and endotoxic activity. Interestingly, during our analysis, we also found a previously unknown modification of lipid A.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used are described in Table 1. Typically, the *E. coli* strains were grown at 37°C in Luria-Bertani broth while shaking at 200 rpm. When appropriate, bacteria were grown in the presence of 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 10 μg/ml gentamicin, for plasmid maintenance or strain selection. *B. pertussis* was grown in synthetic THIJS medium (Thalen *et al.*, 1999) or on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood (Tritium) at 35°C.

TABLE 1
Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
B. pertussis		
B213	Streptomycin resistant derivative of *B. pertussis* strain Tohama	Kasuga et al. (1953)
B213 ΔBP2328	BP2328 mutant of strain B213, Str^R, Km^R	This study
B213 ΔBP2329	BP2329 mutant of strain B213, Str^R, Km^R	This study
B213 ΔBP2331	BP2331 mutant of strain B213, Str^R, Km^R	This study
E. coli		
TOP10F'	*F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	*F$^-$ Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	Hanahan (1983)
SM10(λpir)	*thi thr leu fhyA lacY supE recA*::RP4-2-Tc::Mu λ *pir* R6K Km^R	N.V.I.[a]
Plasmids		
pGEM-T Easy	*E. coli* cloning vector Amp^R	Promega
pUC4K	*E. coli* vector harbouring kanamycin-resistance cassette, Amp^R Km^R	Viera and Messing, 1982
pSS1129	Allelic exchange vector, *bla gen rpsL oriVColE1 oriT λ cos*	Stibitz, 1994
pGEM-BP2328$_{up}$	pGEM-T Easy derivative harbouring BP2328 upstream sequence	This study
pGEM-BP2328$_{down}$	pGEM-T Easy derivative harbouring BP2328 downstream sequence	This study
pGEM-BP2329$_{up}$	pGEM-T Easy derivative harbouring BP2329 upstream sequence	This study
pGEM-BP2329$_{down}$	pGEM-T Easy derivative harbouring BP2329 downstream sequence	This study
pGEM-BP2331	pGEM-T Easy derivative harbouring BP2331 sequence	This study
pSS1129-BP2328$_{KO}$	pSS1129 derivative harbouring BP2328 knock out construct, Km^R	This study
pSS1129-BP2329$_{KO}$	pSS1129 derivative harbouring BP2329 knock out construct, Km^R	This study
pSS1129-BP2331$_{KO}$	pSS1129 derivative harbouring BP2331 knock out construct, Km^R	This study

[a] Netherlands Vaccine Institute, Bilthoven, The Netherlands

Recombinant DNA techniques

All plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega Wizard®*Plus* SV Minipreps system. Restriction endonucleases were used according to the instructions of the manufacturer (Roche). DNA fragments were isolated from agarose gels using the Promega Wizard® SV Gel and PCR Clean-Up system. Ligations were performed using the rapid DNA ligation kit (Roche).

All primers used are described in Table 2. Chromosomal template DNA for PCR reactions was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x *g*, after which the supernatant was used as template DNA. To construct *B. pertussis* mutant strains B213ΔBP2328 and ΔBP2329, we amplified DNA segments encompassing the 5' region and upstream sequences of the corresponding ORFs by using primers BP2328_FW_{up}, BP2329_FW_{up}, and primers BP2328_REV_{up} and BP2329_REV_{up}, which both contained a BamHI site. Additionally, DNA fragments containing the 3' regions and downstream sequences of the ORFs were obtained by PCR with primers BP2328_FW_{down}, BP2329_FW_{down}, both containing a BamHI site, and primers BP2328_REV_{down} and BP2329_REV_{down}. To construct a *B. pertussis* BP2331 mutant strain, the corresponding ORF was amplified by using primers BP2331_FW and BP2331_REV. The PCRs were performed using pure Taq Ready-to-go PCR beads (Amersham Biosciences) in a 25-μl total reaction volume with 5 pmol of each primer. The temperature program was as follows: 95°C for 3 min, 30 cycles of 15 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by 7 min at 72°C and subsequent cooling to 4°C. The PCR products were purified from agarose gel and subsequently cloned into pGEM-T Easy resulting in plasmids pGEM-BP2328$_{up}$, pGEM-BP2328$_{down}$, pGEM-BP2329$_{up}$, pGEM-BP2329$_{down}$, and pGEM-BP2331, respectively. The BamHI–SpeI

TABLE 2

Primers

Name	Sequence (5'-3')[a]
BP2328_FW_{up}	TTCCGCACTTACTGGCTGAG
BP2328_FW_{down}	GGATCCTCGCGGTACGACAGCACAT
BP2328_REV_{up}	GGATCCTGTTGCGCGAGATGCTGGAG
BP2328_REV_{down}	CCTCATCGCCAAGGTCAATC
BP2329_FW_{up}	TCACCTTCGACGACGGATAC
BP2329_FW_{down}	GGATCCGTGCGCATCTACCTGATCC
BP2329_REV_{up}	GGATCCGAATCGACCACGATGAAC
BP2329_REV_{down}	GATCCAGCTTGGCCTGGTTG
BP2331_FW	GTGACGTGGTGGTACATCAG
BP2331_REV	TGGTCTACCGCAGGAACAAT

[a] BamHI restriction sites are underlined

fragments of pGEM-BP2328$_{down}$ and pGEM-BP2329$_{down}$ were ligated into *Bam*HI–*Spe*I-restricted pGEM-BP2328$_{up}$ and pGEM-BP2329$_{up}$, respectively. The resulting plasmids and plasmid pGEM-BP2331 were cut with BamHI and EcoRV, respectively, to allow for insertion of the kanamycin-resistance cassette from plasmid pUC4K obtained by BamHI and HinDII digestion, respectively. Finally, EcoRI fragments of the constructs obtained were ligated into the EcoRI-restricted suicide plasmid pSS1129. The final constructs, designated pSS1129-BP2328$_{KO}$, pSS1129-BP2329$_{KO}$, and pSS1129-BP2331$_{KO}$, respectively, contained the kanamycin-resistance cassette in the same orientation as the transcription direction of the operon. The pSS1129-based plasmids were used to transform *E. coli* SM10(λpir), which allowed for subsequent transfer of the plasmids to *B. pertussis* and construction of *B. pertussis* BP2328, BP2329, and BP2331 mutants by allelic exchange. Transformants were screened by PCR using various primer sets.

LPS isolation and preparation of de-*O*-acylated LPS

LPS was isolated using the hot phenol/water extraction method (Westphal and Jann, 1965) with slight modifications (Geurtsen *et al.*, 2006). De-*O*-acylation of LPS was achieved by mild hydrazinolysis (Holst, 2000). Briefly, LPS was dissolved in anhydrous hydrazine (200 μl), and incubated at 37°C for 50 min with constant stirring to release the *O*-linked fatty acyl chains. The mixture was cooled and 600 μl of cold acetone were added in small portions to convert hydrazine to acetone hydrazone. The precipitate of the de-*O*-acylated LPS was collected by centrifugation (4000 x *g*, at 7°C for 30 min). The pellet was washed twice with 600 μl of cold acetone, centrifuged and dissolved in water before lyophilisation.

Capillary electrophoresis-electrospray mass spectrometry

A Prince CE system (Prince Technologies) was coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1.0 μl/min. Separations were obtained on a ~90-cm length bare fused-silica capillary using 15 mM ammonium acetate in deionised water, pH 9.0. The 5 kV and –5 kV of electrospray ionisation voltage were used for positive and negative ion mode detections, respectively. For all the mass spectrometric experiments, nitrogen was used as curtain and collision gas. In the MS^2 (enhanced product ion scan or EPI) and MS^3 experiments, the scan speed was set to 4000 Da/s with Q_0 trapping, the trap fill time was set as “dynamic” and the resolution of Q1 was set as “unit”. For MS^3 experiments, the excitation coefficient was set at a value to excite only the first isotope for a single charged precursor with excitation time set at 100 ms.

LPS analysis by Tricine-SDS-PAGE

Approximately 10^9 bacteria were suspended in 50 μl of sample buffer (Laemmli, 1970), and 0.5 mg/ml proteinase K (end concentration) was added. The samples were incubated for 60 min at 55°C, followed by 10 min at 95°C to inactivate proteinase K. The samples were then diluted 10 fold by adding sample buffer, after which 2 μl of each sample were applied to a Tricine-SDS-PAGE gel (Lesse *et al.*, 1990). The bromophenol blue was allowed to run into the separating gel at 35 V, after which the voltage was increased to 105 V. After the front reached the bottom of the gel, electrophoresis was continued for another 45 min. The gels were fixed overnight in water/ethanol/acetic acid 11:8:1 (v/v/v) and subsequently stained with silver as described (Tsai and Frasch, 1982).

Preparation of bacterial cell suspensions

Bacteria were inactivated in 0.5% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min and washed thoroughly in RPMI 1640 medium without phenol red (Gibco). Bacterial suspensions with an optical density at 600 nm (A600) of 1, corresponding to ~10^9 bacteria/ml, were prepared in RPMI 1640 medium without phenol red.

Human DC generation and culture

Immature human DC were generated from human peripheral blood mononuclear cells (PBMCs) as described previously with minor modifications (Sallusto and Lanzavecchia, 1994). Briefly, PBMCs were isolated from heparinised blood from healthy volunteers using density-gradient centrifugation over a Ficoll gradient (Amersham Biosciences). Recovered PBMC fractions were washed three times in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS) (Bodinco BV). Next, monocytes were prepared from PBMCs by centrifugation over a three-layer Percoll gradient (GE Healthcare Bio-Sciences AB) (60%, 47.5%, and 34% Percoll in RPMI 1640, 10% FCS). Monocytes were harvested from the upper interface and washed three times with RPMI 1640, 10% FCS medium and incubated in a six-well plate (4 ml per well, 0.5x10^6 cells/ml) in RPMI 1640, 10% FCS, supplemented with 2.4 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Gibco), 100 ng/ml of human recombinant GM-CSF (Peprotech), and 50 ng/ml of human recombinant IL-4 (Strathmann-Biotec AG). After six days of culture, immature DC (imDC) were harvested, which were negative for CD14 and CD83, expressed low levels of CD86 and HLA-DR, and expressed high levels of CD40 and CD11c as assessed by flow cytometry.

DC stimulation

ImDC were washed and resuspended at a concentration of $5x10^5$ cells/ml in RPMI 1640 10% FCS, and co-incubated with either PFA-fixed *B. pertussis* cells at a multiplicity of infection (MOI) of 10 or 100, or purified LPS at a concentration of 10 or 1000 ng/ml. Unstimulated imDC served as control in all experiments. DC were harvested after 24 h and directly stained for expression of cell surface markers; the supernatants were stored at -80°C before cytokine measurements.

Flow cytometric analysis of cell surface markers

Surface expression of DC maturation markers and co-stimulatory molecules was assessed by flow cytometry. Immature or stimulated DC were harvested, washed in RPMI 1640, 10% FCS and resuspended in filter-sterilised PBS containing 0.1% bovine serum albumin (FACS buffer). Next, cells were incubated for 30 min at 4°C with either one of the following antibodies: FITC-conjugated anti-human CD11c (mIgG1) and CD83 (mIgG1), phycoerythrin-conjugated anti-human CD86 (mIgG1) and CD40 (mIgG1), allophycocyanin-conjugated anti-human CD14 (mIgG1) and HLA-DR (mIgG2b) and appropriate fluorochrome-labelled isotype controls (CD11c, CD40 and CD14 from eBioscience; CD83, CD86 and HLA-DR from BD Pharmingen). Cells were washed twice with FACS buffer and analysed using flow cytometry (FACScan, Becton Dickinson).

Cytokine measurements

Human IL-10 and IL-12p70 concentrations in the supernatants of stimulated DCs were determined using an Enzyme-linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (BD Biosciences Pharmingen).

Endotoxic activity assays

The human macrophage cell line MM6 (Ziegler-Heitbrock *et al.*, 1988) was stimulated with serial dilutions of whole bacterial cell suspensions or purified LPS as described (Geurtsen *et al.*, 2006). The bacterial cell suspensions were prepared by collecting the cells from cultures by centrifugation, after which they were resuspended in PBS at an OD_{590} of 1.0, heat-inactivated for 10 min in the presence of 8 mM formaldehyde, and stored at 4°C. Following stimulation, IL-6 concentrations in the culture supernatants were quantified with an ELISA against human IL-6 according to the manufacturer's instructions (PeliKine Compact™).

Results

Identification of a novel LPS-biosynthesis operon in *B. pertussis*

As a glucose (β1-4) heptose linkage is a common feature of the LPS inner core in many bacteria including *B. pertussis*, we used genes encoding glycosyltransferases with this specificity from *N. meningitidis* (*lgtF/icsB*), among others, to identify homologous sequences in the *B. pertussis* Tohama genome sequence. In this way we found a cluster of four genes (BP2328 to BP2331, GenBank Accession Numbers NP_880966 to NP_880969), three of which showed high sequence similarity to LPS glycosyltransferases from various bacteria, i.e., BP2328, BP2329 and BP2331. BP2330 shows the highest similarity to a polysaccharide deacetylase from *Xylella fastidiosa*. The four ORFs are close to each other and in some cases even overlap, suggesting that they constitute an operon (Fig. 1A). The genes upstream and, in the reverse orientation, downstream of the operon, putatively encode homologues of the DNA polymerase III subunit alpha DnaE and of the putative sulfatase YhbX of *E. coli*, respectively. In order to study the role of the putative LPS glycosyltransferases, we made constructs in suicide plasmid pSS1129 carrying the individual BP2328, BP2329, and BP2331 genes interrupted by a kanamycin-resistance cassette for insertional inactivation by allelic exchange. Using

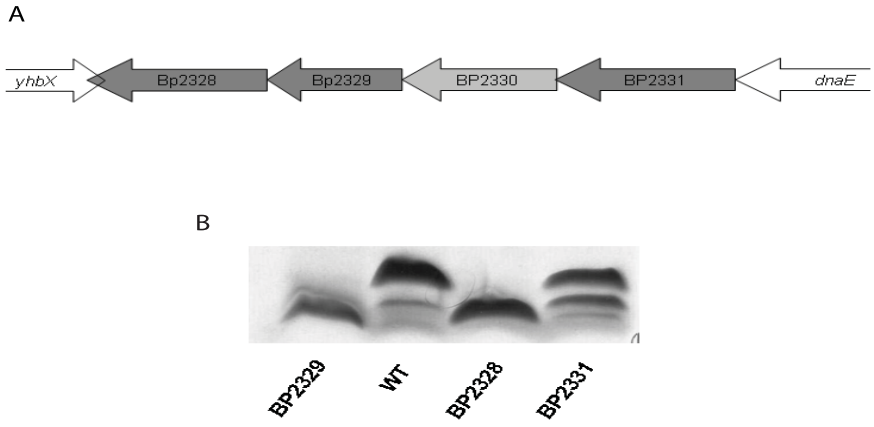

Fig. 1. (A) Schematic representation of the identified glycosyltransferase operon. Dark gray arrows indicate the genes that encode putative glycosyltransferases, whereas the light grey and white arrows indicate the gene encoding a putative monosaccharide deacetylase and the flanking ORFs, respectively. (B) Analysis of LPS profiles from the wild-type *B. pertussis* strain (WT), and the BP2329-, BP2328-, and BP2331-mutant strains by Tricine-SDS-PAGE.

this approach, knockout mutants for all three genes could be readily obtained in *B. pertussis* strain B213. Analysis of their LPS by Tricine-SDS-PAGE of whole-cell lysates showed clearly truncated LPS for the BP2328 and BP2329 mutants (Fig. 1B). In contrast, the LPS of the BP2331 mutant strain was more heterogenic and consisted of multiple bands, including the wild-type length.

LPS structural analysis

To determine their structure, LPS from the wild-type and BP2328-, BP2329-, and BP2331-mutant strains was isolated, *O*-deacylated, and analysed by ESI-MS in the negative-ion mode (Fig. 2). The proposed LPS compositions are summarised in Table 3. The spectrum of wild-type LPS (Fig. 2A) revealed a major triply-charged ion at *m/z* 1108.5 corresponding to full-length *B. pertussis* LPS with the composition GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH. Additional ions were present at *m/z* 770.1 ($[M-3H]^{3-}$), 811.1 ($[M-4H]^{4-}$), 831.4 ($[M-4H]^{4-}$), 888.3 ($[M-3H]^{3-}$), 951.8 ($[M-H]^{-}$), 987.1 ($[M-2H]^{2-}$), 1081.7 ($[M-3H]^{3-}$), 1121.1 ($[M-3H+K]^{3-}$), 1155.0 ($[M-2H]^{2-}$), and 1162.1 ($[M-3H]^{3-}$). Most of these ions corresponded to dephosphorylated or truncated glycoforms; however, the triply-charged ion at *m/z* 1162.1 corresponded to full-length *B. pertussis* LPS substituted with an additional hexosamine moiety (Table 3).

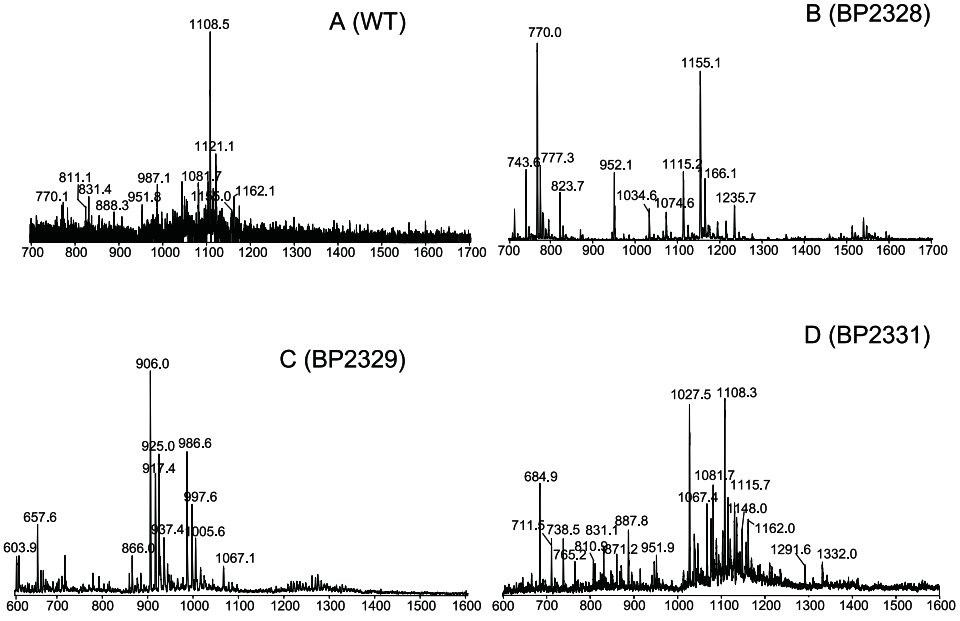

Fig. 2. Negative ion ESI-MS of *O*-deacylated LPS of wild-type *B. pertussis* (A) and *B. pertussis* mutant strains BP2328 (B), BP2329 (C) and BP2331 (D).

TABLE 3

Negative ion ESI-MS data and proposed compositions for *O*-deacylated LPS of wild-type *B. pertussis* and *B. pertussis* mutant strains BP2331, BP2328, and BP2329. Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: glucose (Glc), 162.14; heptose (Hep), 192.17; 2-keto-3-deoxyoctulosonic acid (Kdo), 220.18; phosphate (*P*), 79.98; glucosamine (GlcN),161.17; hexosamine (HexN), 161.17; glucuronic acid (GlcA), 176.13; *N*-acetyl-glucosamine (GlcNAc), 203.19; 2-acetamido-4-*N*-methyl-2,4-dideoxy-fucose (Fuc2NAc4NMe), 200.12; 2,3-acetamido-2,3-dideoxy-mannuronic acid (Man2NAc3NAcA), 258.09; galactosaminuronic acid (GalNA),175.13 and lipid A-OH, 953.02. Table does not include sodium and potassium adducts and singly-charged lipid A-OH ions (*m/z* 952 ($[M-H]^-$)).

Sample	*Observed ions [m/z]*			*Molecular mass [Da]*		*Relative amount*	*Proposed composition*
	$[M-4H]^{4-}$	$[M-3H]^{3-}$	$[M-2H]^{2-}$	Observed	Calculated		
WT			987.1	1976.2	1975.8	0.30	Glc•GlcA•Hep_2•*P*•Kdo•lipid A-OH
		770.1	1155.0	2312.7	2312.1	0.20	GalNA•Glc•GlcN•GlcA•Hep_2•*P*•Kdo•lipid A-OH
		888.3		2667.9	2665.4	0.18	GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH
	811.1	1081.7		3248.3	3246.9	0.42	GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•Kdo•lipid A-OH
	831.4	1108.5		3329.0	3326.8	1.0	GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH
		1162.1		3489.3	3488.0	0.32	HexN•GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH
BP2328		743.6	1115.2	2233.1	2232.1	0.29	GalNA•Glc•GlcN•GlcA•Hep_2•Kdo•lipid A-OH
		770.0	1155.1	2312.6	2312.1	1.0	GalNA•Glc•GlcN•GlcA•Hep_2•*P*•Kdo•lipid A-OH
		823.7	1235.7	2473.8	2473.3	0.24	GalNA•Glc•$GlcN_2$•GlcA•Hep_2•*P*•Kdo•lipid A-OH
			1034.6	2071.2	2070.8	0.06	GalNA•Glc•GlcA•Hep_2•Kdo•lipid A-OH
			1074.6	2151.2	2150.8	0.05	GalNA•Glc•GlcA•Hep_2•*P*•Kdo•lipid A-OH
BP2329			866.0	1734.0	1733.7	0.3	GlcA•Hep_2•Kdo•lipid A-OH
		603.9	906.0	1814.4	1813.6	1.0	GlcA•Hep_2•*P*•Kdo•lipid A-OH
			937.4	1876.8	1876.8	0.35	GlcN•GlcA•Hep_2•*P*•Kdo•lipid A-OH –H_2O
		657.6	986.6	1975.5	1974.8	0.82	GlcN•GlcA•Hep_2•*P*•Kdo•lipid A-OH
			1067.1	2136.2	2136.0	0.24	$GlcN_2$•GlcA•Hep_2•*P*•Kdo•lipid A-OH
BP2331		684.9	1027.5	2057.4	2057.0	0.82	Glc•GlcN•GlcA•Hep_2•Kdo•lipid A-OH
		711.5	1067.4	2137.2	2137.0	0.36	Glc•GlcN•GlcA•Hep_2•*P*•Kdo•lipid A-OH
		738.5		2218.5	2218.2	0.11	Glc•$GlcN_2$•GlcA•Hep_2•Kdo•lipid A-OH
		765.2	1148.0	2298.3	2298.1	0.25	Glc•$GlcN_2$•GlcA•Hep_2•*P*•Kdo• lipid A-OH
			1115.7	2233.4	2232.1	0.45	GalNA•Glc•GlcN•GlcA•Hep_2•Kdo•lipid A-OH
			1291.6	2585.2	2585.5	0.21	GalNA•Glc•$GlcN_2$•GlcA•Hep_3•Kdo•lipid A-OH
		887.8	1332.0	2666.2	2665.4	0.34	GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH
	810.9	1081.7		3247.9	3246.9	0.58	GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•Kdo•lipid A-OH
	831.1	1108.3		3328.2	3326.8	1.0	GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH
	871.2	1162.0		3488.9	3488.0	0.44	HexN•GlcNAc•Man2NAc3NAcA•Fuc2NAc4NMe•GalNA•Glc•$GlcN_2$•GlcA•Hep_3•*P*•Kdo•lipid A-OH

The ESI-MS spectrum of the BP2328-mutant LPS (Fig. 2B) showed triply-charged ions at *m/z* 743.6, 770.0, and 823.7, together with their corresponding doubly-charged ions at *m/z* 1115.2, 1155.1, and 1235.7. Additional peaks were present at *m/z* 777.3 ([M-3H+Na]$^{3-}$), 952.1 ([M-H]$^{-}$), 1034.6 ([M-2H]$^{2-}$), 1074.6 ([M-2H]$^{2-}$), and 1166.1 ([M-2H+Na]$^{2-}$). Assignment of the peaks revealed that the most complete core OS structure was represented by the ions at *m/z* 823.7 and 1235.7 corresponding to the composition GalNA•Glc•$GlcN_2$•GlcA•Hep_2•*P*•Kdo•lipid A-OH. BP2329 mutant LPS (Fig. 2C) showed triply charged ions at *m/z* 603.9 and 657.6, together with their corresponding doubly-charged ions at *m/z* 906.0 and 986.6. In addition, sodium and potassium adducts of these ions were present at *m/z* 917.4 and 997.6, and *m/z* 925.0 and 1005.6, respectively. Additional peaks were present at *m/z* 866.0 ([M-2H]$^{2-}$), 937.4 ([M-2H-H_2O]$^{2-}$), and 1067.1 ([M-2H]$^{2-}$). In this case, the most complete core structure was represented by the doubly-charged ion at *m/z* 1067.1 corresponding to the composition $GlcN_2$•Glc A•Hep_2•*P*•Kdo•lipid A-OH. BP2331 mutant LPS (Fig. 2D) showed a large number of peaks, including triply-charged ions at *m/z* 1108.3 and 1162.0 corresponding to full-length *B. pertussis* LPS and full-length *B. pertussis* LPS substituted with an additional hexosamine, respectively.

To resolve the location of the additional hexosamine moiety, which was observed in both wild-type and BP2331-mutant LPS, ESI-MS^2 studies were performed in negative-ion mode (Fig. 3). MS/MS spectra of the ions at *m/z* 1108.3 (Fig. 3A) and 1162.0 (Fig. 3B) both showed a singly charged fragment ion at *m/z* 951.5, which revealed that lipid A-OH, resulting from the cleavage between the Kdo-lipid A bond under collision-induced dissociation, consisted of a β-(1→6)-linked disaccharide of *N*-acylated (3OH C14) glucosamine residues, each residue being substituted with a phosphate group. The spectrum of ion at *m/z* 1162.0 also showed an additional ion at *m/z* 1112.6, which indicates that the extra hexosamine residue was directly attached to lipid A. MS^3 on *m/z* 1112.6 further supported this conclusion (Fig. 3C).

Dendritic cell activation by *B. pertussis* LPS mutants

To determine the influence of the LPS mutations on DC activation, immature DCs were co-cultured with PFA-fixed *B. pertussis* wild-type and mutant bacteria at an MOI of 10 and 100. DC activation was monitored by analysis of maturation marker (CD83 and HLA-DR) and co-stimulatory molecule (CD86 and CD40) expression by flow cytometry (Fig. 4A) and IL-10 and IL12p70 induction by ELISA (Fig. 4B). Wild-type and all mutant bacteria induced CD83, HLA-DR, CD86, and CD40 expression, demonstrating that all strains were capable of activating DCs. However, the BP2329- and BP2331-mutant bacteria were clearly less and more stimulatory, respectively, than

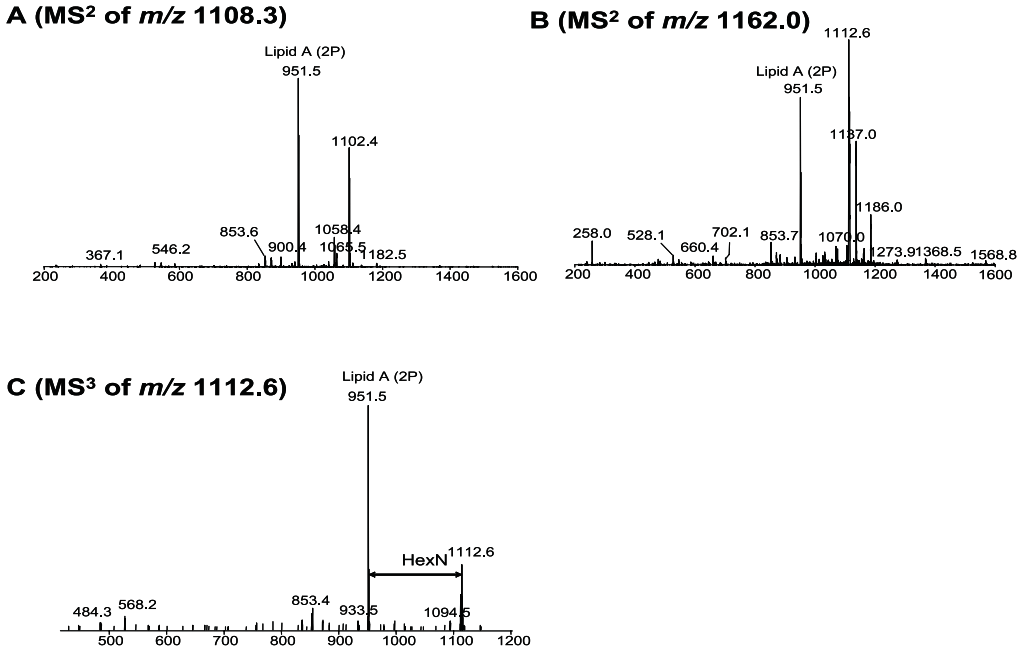

Fig. 3. Negative mode tandem mass spectrometric analysis of *O*-deacylated LPS from the BP2331-mutant strain. (A) extracted MS/MS spectrum of the ion at *m/z* 1108.3, (B) extracted MS/MS spectrum of the ion at *m/z* 1162.0, (C) extracted MS^3 spectrum of the ion at *m/z* 1112.6 from the ion at *m/z* 1162.0.

the wild-type bacteria, whereas the BP2328-mutant strain was as efficient as the wild type. The lower DC maturation observed in the case of the BP2329-mutant strain was accompanied by lower induction of IL-10 and IL-12p70 (Fig. 4B). Similarly, the BP2331 mutant, which displayed an enhanced DC-maturation capacity, induced higher amounts of IL-10 and IL-12p70. The wild-type strain and the BP2328-mutant strain induced comparable levels of IL-10, which is in agreement with the equal expression of co-stimulatory molecules and maturation markers on the DCs in response to these strains. However, whereas the wild-type strain clearly induced IL-12p70 production, this was hardly the case for the BP2328-mutant strain (Fig. 4B), suggesting that IL-10 and IL-12p70 expression can be differentially regulated.

To assess whether the observed differences in DC activation capacity between the wild-type and mutant strains are directly related to the differences in the LPS composition, DC activation studies were performed with 10 and 1000 ng/ml of purified LPS. In contrast to the high increase in expression of maturation markers and co-stimulatory molecules on DCs in response to wild-type, BP2328-, and BP2331-mutant bacteria, only minor increases in CD83, CD86, and CD40 expression (Fig. 5A) and no

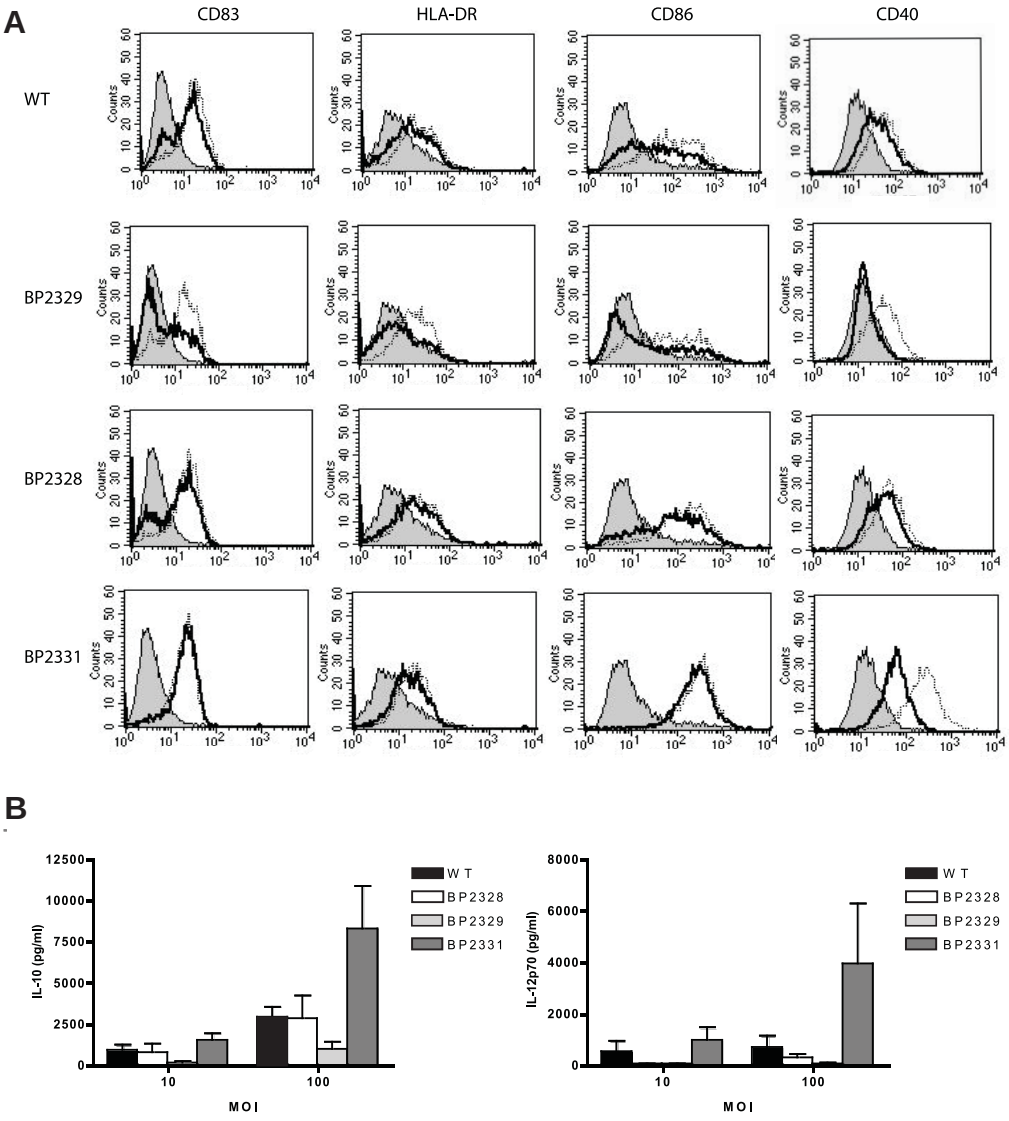

Fig. 4. DC activation after stimulation with the wild-type and mutant *B. pertussis* cells. (A) Analysis of CD83, HLA-DR, CD86, and CD40 cell-surface expression in human DCs after 24 h stimulation with PFA-fixed wild-type and mutant *B. pertussis* cells at MOI 10 (black line) or 100 (dashed line). Unstimulated DCs served as control (grey-filled histogram). Shown are FACS histograms for the indicated *B. pertussis* strains from 5,000 events counted. The vertical axis represents the cell number, while the horizontal axis represents the intensity of staining. (B) IL-10 and IL-12p70 production by cultured human DCs after stimulation with PFA-fixed wild-type and mutant *B. pertussis* cells at MOI 10 or 100. Results are expressed as mean cytokine concentrations (± SD).

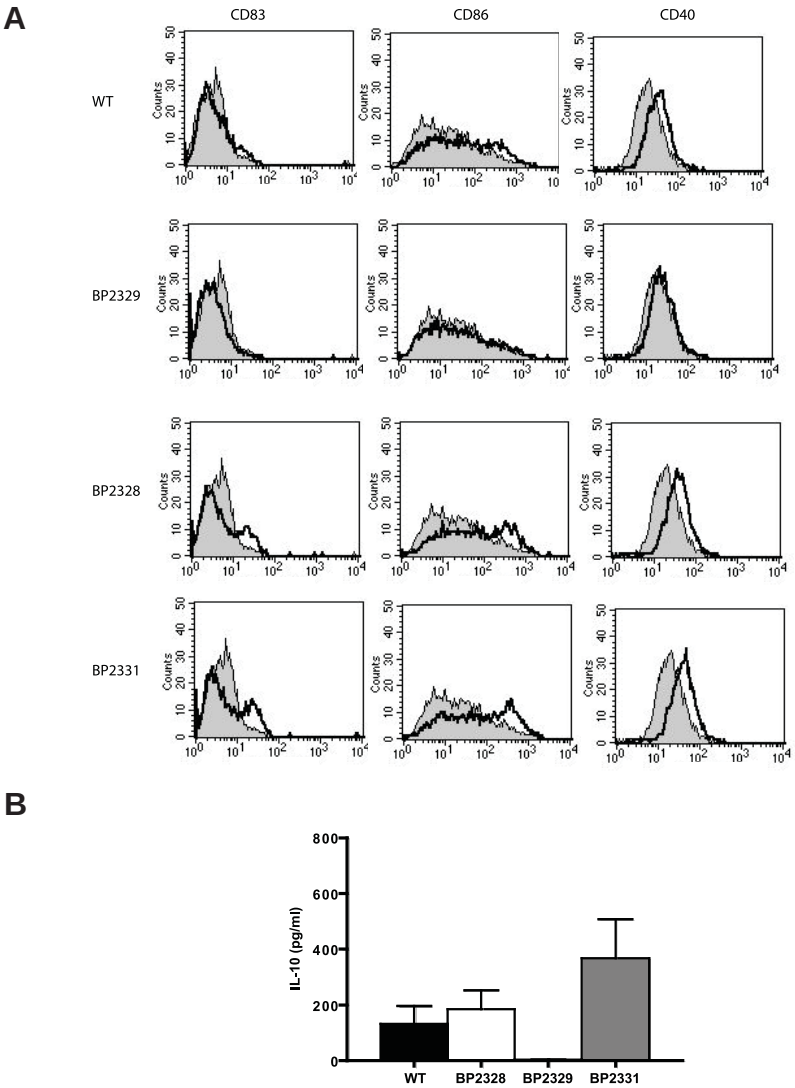

Fig. 5. DC activation after stimulation with purified wild-type and mutant *B. pertussis* LPS. (A) Analysis of CD83, CD86, and CD40 cell-surface expression in human DCs after 24 h stimulation with 1 μg/ml purified LPS. Unstimulated DCs served as control (grey-filled histogram). Shown are FACS histograms for the LPS of the indicated *B. pertussis* strains from 5,000 events counted. The vertical axis represents the cell number, while the horizontal axis represents the intensity of staining. (B) IL-10 production by cultured human DCs after stimulation with 1 μg/ml purified LPS. Results are expressed as mean cytokine concentrations (± SD).

increase in HLA-DR expression (data not shown) was found even with 1000 ng/ml LPS of these strains. Similarly, IL-10 induction was low (Fig. 5B) and IL-12p70 could not be detected in supernatants of DCs stimulated with LPS (data not shown). Nevertheless, mutual comparison (Figs. 5A and 5B) demonstrated that, in accordance with the results obtained with intact bacteria, the highest DC activation capacity was found for the LPS isolated from the BP2331-mutant strain, followed by those of the BP2328-mutant strain and the wild-type strain, whereas that of the BP2329-mutant strain was incapable of maturing DCs. Thus, the alterations in the LPS structure of the mutants differentially affect DC activation capacity.

Endotoxic activity of LPS and whole bacterial cells

To assess the consequences of the LPS mutations on the endotoxic activity of LPS, the potency of the purified LPS to stimulate the human macrophage cell line MM6 for IL-6 production was tested. As compared with wild-type LPS, purified LPS from the BP2331-mutant strain had a strongly increased potency to stimulate the macrophages (Fig. 6A). In contrast, LPS from the BP2329-mutant strain had a reduced potency to stimulate IL-6 production, whereas LPS from the BP2328 mutant was similarly active as wild-type LPS (Fig. 6A). Only at the two highest LPS concentrations tested, the latter LPS was more active than wild-type LPS was. Consistent with the data obtained with purified LPS, whole-cell suspensions of the BP2331 mutant showed, as compared to wild-type cells, a clearly increased potency to stimulate the macrophages (Fig. 6B). However, also the BP2328-mutant cells showed this effect (Fig. 6B), while BP2329-mutant cells had similar activity as the wild-type cells in spite of their less active purified LPS (Fig. 6A).

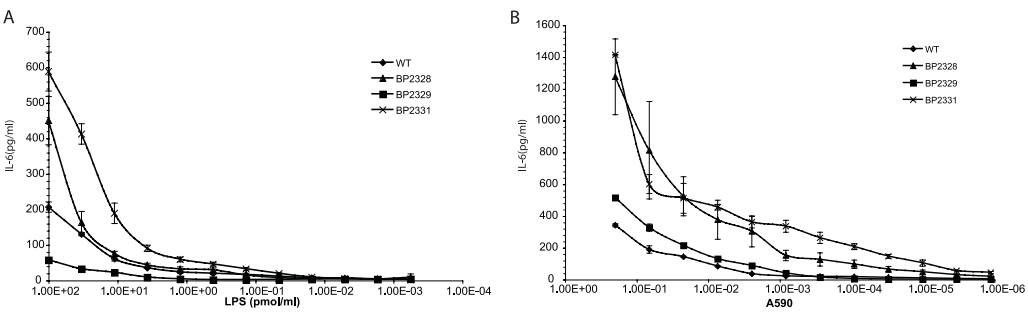

Fig. 6. IL-6 induction by purified *B. pertussis* LPS and whole bacterial cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of stock solutions of purified LPS (A) or whole bacterial cells (B) from the wild-type *B. pertussis* strain (WT), or the BP2328-, BP2329-, and BP2331-mutant strains. IL-6 concentrations in the culture supernatants were quantified in an ELISA against human IL-6. The data represent the averages of three individual experiments.

Discussion

The goal of the present study was to identify new LPS glycosyltransferases in the *B. pertussis* genome. By using sequences of known LPS glycosyltransferases as leads, we were able to identify a four-gene operon. In a previous study, in which the genome sequence of the poultry pathogen *Bordetella avium* was compared to the genome sequences of other *Bordetellae*, an gene cluster homologous to the one here identified was described as being involved in LPS biosynthesis (Sebaihia *et al.*, 2006). However, no functional studies were reported which could confirm this assignment.

To study the role of this operon in *B. pertussis* LPS biosynthesis, we inactivated the putative glycosyltransferase genes by allelic exchange and compared the LPS profiles of the wild-type and mutant strains using Tricine-SDS-PAGE and ESI-MS. Unexpectedly, we found that the wild-type strain not only contained full-length *B. pertussis* LPS, but also harboured a full-length species substituted with an extra hexosamine moiety, which, as we showed, was directly attached to lipid A. Substitution of *B. pertussis* lipid A with hexosamine has previously not been observed and therefore represents a novel modification of *B. pertussis* lipid A.

The proposed truncated oligosaccharide structures for the BP2328- and BP2329- mutant strains are summarised in Fig. 7. The most complete core OS structure in the BP2328 mutant strain consisted of GalNA•Glc•$GlcN_2$•GlcA•Hep_2•*P*•Kdo attached to lipid A-OH, suggesting that the BP2328 mutant strain lacks the terminal trisaccharide and heptose residues. Since it has been demonstrated that addition of the trisaccharide is determined by the *wlb* locus (Allen and Maskell, 1996; Allen *et al.*, 1998b), the BP2328-encoded protein could function as a heptosyltransferase responsible for the attachment of the terminal heptose (Fig. 7. option 1). If this assumption is correct, it would implicate that the trisaccharide can only be added to the 6 position of the GlcN after the heptose has been added to the 4 position by the BP2328-encoded enzyme. Alternatively, because we identified here a novel modification of *B. pertussis* lipid A with hexosamine, it is also possible that one of the GlcN residues in the structure mentioned above is actually the novel hexosamine attached to lipid A. If this assumption is correct, it would implicate that the BP2328-mutant strain misses, besides the terminal trisaccharide and heptose, also a GlcN residue from the core OS and, thus, that the BP2328-encoded protein functions as a GlcN(1-4) to Glc transferase (Fig. 7, option 2). From the results obtained, it is impossible to discriminate between these two alternatives and further MS analysis will be required to determine the precise location of the HexN residue in question. Analysis of the BP2329-mutant LPS showed that this LPS was further truncated and that its most complete structure consisted of $GlcN_2$•GlcA•Hep_2•*P*•Kdo•lipid A-OH. Since this

structure misses the Glc to which the second GlcN of the core OS should be connected, one of the two GlcN residues indicated in the structure mentioned must represent the novel HexN residue attached to lipid A. Therefore, this composition suggests that the BP2329-encoded protein functions as a glucosyltransferase that attaches Glc to the first heptose subunit (Fig. 7). This would agree with the high homology of this gene product with glucose (β1-4) heptose transferases, such as *rfaK* and *lgtF/icsB*, which were used to identify the gene in the first place. The most complicated phenotype was observed in the case of the BP2331 mutant. Although the protein shows high sequence similarity to various LPS glycosyltransferases, full-length *B. pertussis* LPS was still present in the mutant strain. This observation suggests either that the BP2331 gene does not encode an active LPS glycosyltransferase or that the encoded enzyme shows redundancy. Consistent with this last option, we have identified a gene, i.e., BP3671 with GenBank Accession Number CAE43928, in the genome of *B. pertussis* which encodes for a protein that shows 69% identity to the BP2331-encoded protein. Albeit the LPS profiles of the wild-type and BP2331-mutant strain were more or less comparable, one striking observation was that the mutant LPS was more heterogenic. Although the exact reason for this phenomenon remains to be elucidated, one possible explanation could be that the BP2331 mutant somehow displays an increased non-stoichiometrical substitution of its LPS, possibly with hexosamine. Of note, besides the three glycosyltransferase homologs described above, the here identified gene cluster contains a fourth gene, i.e., BP2330, which encodes for a deacetylase. Modification of lipid A with amino sugars has

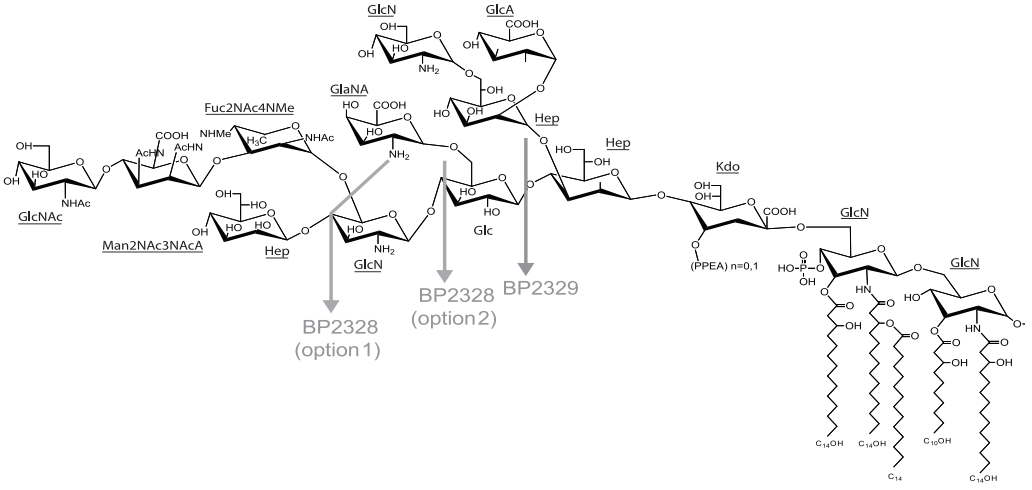

Fig. 7. Structure of *B. pertussis* LPS. Proposed truncated core OS structures of the BP2328- and BP2329-mutant strains are indicated by red arrows. Adapted from Caroff *et al*. (2000).

been described in various bacteria, e.g., substitution with 4-aminoarabinose in *E. coli* and *Salmonella* (Trent *et al.*, 2001b), and with galactosamine in *Francisella tularensis* (Phillips *et al.*, 2004). The aminoarabinose pathway has been studied in detail in *E. coli* and has been shown to involve the assembly of the sugar moiety on a separate undecaprenyl phosphate carrier prior to its transfer to lipid A (Trent *et al.*, 2001a). This pathway includes the ArnD deformylase required for freeing the amino group. Since it is conceivable that insertion of the kanamycin-resistance cassette in BP2331 has increased the expression of the downstream BP2330 gene, and one could speculate that the BP2330-encoded protein functions, by analogy, as the deacetylase responsible for releasing acetate from the amino group of hexosamine before it is attached to lipid A, it is tempting to speculate that an increased BP2330 expression may have led to an increased hexosamine modification of lipid A, and, consequently, an increased LPS heterogeneity in the BP2331-mutant cells. Further quantitative analysis of the presence of this modification, as well as the construction of mutants altered in the dedicated biosynthetic pathway, will be required to test this hypothesis.

After having addressed the structure of the LPS, purified LPS and whole bacterial cells were tested for their ability to induce maturation of DCs and to stimulate the production of pro-inflammatory cytokines by human macrophages. The results showed that, as compared to the wild-type strain, the BP2331-mutant strain displayed an increased capacity to induce DC maturation and pro-inflammatory cytokine production. Similar outcomes were obtained with purified LPS. In contrast, whole bacterial cells and purified LPS from the BP2328- and BP2329-mutant strains displayed a similar and decreased capacity to maturate DCs and stimulate macrophages, respectively. These results show that alterations in LPS core OS-composition differentially affect the biological properties of *B. pertussis* LPS. From the perspective of vaccine development, this is an interesting finding, since this may allow for the development of strains that more efficiently prime immune responses. Furthermore, mutants that display an increased LPS heterogeneity, such as the BP2331-mutant strain, may elicit a larger variety of anti-LPS antibodies, which, on itself, may positively influence vaccine efficacy.

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Chapter 10

General and Summarising Discussion

Pertussis or whooping cough is a highly contagious respiratory tract disease that is caused by the Gram-negative bacterium *Bordetella pertussis*. Since the introduction of pertussis mass-vaccination in the mid 1900s, pertussis incidence, morbidity, and mortality have been reduced dramatically in most Western nations and to a lesser extent in many developing countries. However, the success of pertussis vaccination is not as complete as that of other vaccines and pertussis is frequently the least well-controlled disease in childhood vaccination programs. Furthermore, as of the 1980s, pertussis incidence is rising again in many countries and most importantly, whereas pertussis first was a typical disease of young children, it is nowadays predominantly found among adolescents, adults, and, of most concern, small infants. The exact reasons for this re-emergence and altered age distribution are currently not well understood and remain under debate.

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial outer membrane and is also known as endotoxin due to its ability to induce endotoxic shock in higher organisms (Raetz and Whitfield, 2002). It is one of the major causes of the relatively high reactogenicity of whole-cell pertussis (wP) vaccines. At present, the majority of developing countries use wP vaccines because they are cheap, effective, and easy to produce, whereas most industrialised countries have switched to less reactogenic, but more expensive acellular pertussis (aP) vaccines. These latter vaccines do not contain LPS and were shown to be comparably effective. Yet, aP vaccines have, as compared to wP vaccines, some important drawbacks. An example of this is the increased cost/benefit ratio. Since the production costs of aP vaccines are high, administration in the developing world forms an important “economical” problem. In addition, the currently used aP vaccines are based upon a small number of purified antigens, which might potentially result in a rapid selection of escape mutants. Another important issue is that aP vaccines evoke an immunologically different response (Th2-type response) than do wP vaccines and natural infection, which elicit Th1-type immune responses (reviewed in Mills, 2001). Th2-type immune responses have been linked to type-1 hypersensitivity diseases, such as asthma, atopic dermatitis, and anaphylactic shock (Wills-Karp, 1999; Foster *et al.*, 2002). Especially the consequences of aP vaccination for young, immunologically immature infants are currently not well understood. These notions, together with the problems described above with respect to pertussis re-emergence and alterations in age distribution, emphasise the idea that the development of improved pertussis vaccines remains important. In this thesis, we explored the possibilities of improving pertussis vaccines by altering their LPS composition, either with the help of LPS-modifying enzymes (**chapters 2** to **5**), by adding non-toxic LPS

derivatives (**chapters 6** and **7**), or by changing LPS biosynthesis (**chapters 8** and **9**). The work was mainly focused on the development of a less-reactogenic wP vaccine; however, as demonstrated in **chapter 7**, LPS (derivatives) may also be useful for the improvement of aP vaccines.

LPS-modifying enzymes

Gram-negative bacteria are able to modify the structure of lipid A. These modifications can promote resistance to host cationic antimicrobial peptides and alter recognition by TLR4/MD-2 (Ernst *et al.*, 2001). Regulated lipid A modifications, and the enzymes responsible, were first characterised in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Later on, they were also identified in other Gram-negative bacteria. It is hypothesised that selective pressure on both pathogen and host resulted in marked variations in lipid A structures and the host responses through TLR4, and this diversity is likely to have played a role in the evolution of infectious diseases. Modification of lipid A can be induced under various conditions, such as growth in the presence of antimicrobial peptides or by growing under cation depletion (Bader *et al.*, 2005). Other environmental conditions, such as temperature and anaerobiosis, promote modification of lipid A in *Yersinia pestis* and *Pseudomonas aeruginosa*, respectively (Miller *et al.*, 2005). In *S.* Typhimurium, lipid A modifications are regulated by the environmental sensor-kinase transcriptional regulatory system PhoP/PhoQ (Ernst *et al.*, 2001). It has been shown that modification of LPS often reduces its endotoxic activity (Guo *et al.*, 1997; Kawasaki *et al.*, 2005). Hence, we hypothesised that LPS-modifying enzymes may be useful tools for reducing the reactogenicity of *B. pertussis* LPS and, thereby, for the development of safer wP vaccines. One of the primary candidates for this application was an enzyme known as PagL.

PagL is a PhoP/PhoQ-regulated lipid A 3-*O*-deacylase that was initially identified in *S.* Typhimurium (Trent *et al.*, 2001). At that moment, no obvious *pagL* homologs could be found in the nonredundant or unfinished microbial databases, except in the closely related strains *S. enterica* serovars Typhi and Paratyphi (Trent *et al.*, 2001). Therefore, PagL was postulated to be a *Salmonella*-specific virulence factor (Trent *et al.*, 2001). However, it was then already known that also other Gram-negative bacteria contain 3-*O*-deacylated lipid A species. We started this project by searching the genomes of Gram-negative bacteria for homologs of *S.* Typhimurium *pagL*. As shown in **chapters 2** and **3**, we found that *pagL* homologs were much more widely disseminated among Gram-negative bacteria than initially described. Interestingly, we also identified *pagL*

homologs in several non-pathogenic (soil) bacteria, excluding its role as a dedicated virulence factor. Of note, this of course does not eliminate the option that PagL contributes to the virulence of some species. Nevertheless, there are still Gram-negative bacteria which are known to contain 3-*O*-deacylated lipid A species, but that do not seem to harbour a *pagL* homolog, e.g., *Rhizobium leguminosarum* and *Rhizobium etli*. In a previous study, it was shown that 3-*O*-deacylase activity in these latter species, unlike PagL (**chapter 3**), is dependent on divalent cations (Basu *et al.*, 1999). Possibly, the sequence homology of the *Rhizobium* enzymes to the genuine PagL enzymes is too low to be detected. Another possibility is that in *Rhizobium* species, but maybe also in other Gram-negative bacteria, structurally distinct lipid A 3-*O*-deacylases exist. From this last perspective, it would be tempting to speculate that in some bacteria, lipid A deacylation may be mediated by a homolog of the outer membrane phospholipase OMPLA. As we showed in **chapter 3**, the active-site architecture of PagL very much resembles that of OMPLA, suggesting that OMPLA, in theory, may be able to function as a lipid A deacylase. Hence, if OMPLA by spontaneous mutations has gained affinity for LPS in particular bacteria, such as *R. leguminosarum* and *R. etli*, this may explain the presence of 3-*O*-deacylated lipid A species in the absence of PagL. Consistently, OMPLA has been shown to be calcium-dependent (Ubarretxena-Belandia *et al.*, 1998), which could explain the cation-dependency of lipid A deacylation. Interestingly, we also found a bacterium that contains multiple *pagL* genes, i.e., *Pseudomonas putida*. Apart from the homolog encoded by locus PP_0737 identified in **chapter 2**, also locus PP_3154 on the chromosome of *P. putida* KT2440 encodes a PagL homolog (J.G., unpublished observation). The two *P. putida* homologs show a mutual sequence identity (based on the amino acid sequence) of 58%, which makes it unlikely that the two copies are the result of a recent gene duplication (J.G., unpublished observation). When tested in an *in vitro* activity assay similar to the one used for *P. aeruginosa* PagL (**chapter 3**), both *P. putida* homologs showed 3-*O*-deacylase activity (J.G., unpublished result), suggesting that the enzymes show redundancy. The reason why *P. putida* has two *pagL* homologs remains to be elucidated and may, for instance, be related to differential gene regulation or differences in affinity for particular subsets of LPS species.

When we screened the *Bordetella* genomes, we found that the *B. pertussis* genome also contains a *pagL* homolog; however, the open reading frame was disrupted by a frame-shift mutation. In contrast, in the closely related species *Bordetella bronchiseptica* and *Bordetella parapertussis*, the ORFs were found to be intact (**chapter 2**). The loss of *pagL* expression in *B. pertussis* may thus be an example of host adaptation. To gain more insight into the dissemination of this frame-shift mutation

among *B. pertussis* isolates, we screened twenty *B. pertussis* strains that were isolated in the period 1949-2000. All tested strains contained the frame-shift mutation (J.G., unpublished result), suggesting that the loss of *pagL* expression is a common feature among *B. pertussis* strains. We have recently developed a *B. pertussis* strain in which the chromosomal *pagL* ORF has been restored. Preliminary results show that this *B. pertussis* strain expresses *pagL* and contains fully deacylated LPS (J.G., unpublished result). It will be interesting to investigate how this strain behaves in a murine infection model. Furthermore, identification of the *Bordetella pagL* homologs enabled us to use *Bordetella* PagL, in stead of PagL from more distantly related species, for expression in *B. pertussis*, thereby greatly reducing the risk of problems concerning for instance awkward codon-usage and protein instability.

During our BLAST searches, we observed that the mutual sequence identity between the newly identified PagL homologs was very low. This made us wonder about what enzymatic mechanism underlies PagL activity. Hence, we decided, before continuing with the experiments in *B. pertussis*, to try to find out how the enzyme actually works. Elucidation of the PagL crystal structure revealed that monomeric PagL has all features needed for activity. As discussed in **chapter 3**, this raised the interesting question how PagL activity can be regulated. In a recent paper, it was shown that PagL activity was increased in environmental isolates of *P. aeruginosa* by growth in medium limited for magnesium and decreased by growth at low temperature in laboratory-adapted strains of *P. aeruginosa* (Ernst *et al.*, 2006). Although these results demonstrate that *pagL* expression can be regulated, they do not provide a possible mechanism for silencing of PagL, once produced. Therefore, this question remains to be elucidated and further experiments, which, for instance, address the relevance of dimerisation for PagL inactivation or the possible role of other LPS modifications, should be conducted. Another interesting observation was that after expression of PagL in *E. coli* BL21 Star, the LPS underwent PagP-mediated re-modification with palmitate (**chapter 2**). PagP is an outer-membrane localised lipid A palmitoyl transferase (Bishop *et al.*, 2000). It is known that PagP becomes activated upon disturbance of the outer membrane, for instance upon addition of EDTA (Jia *et al.*, 2004). Thus, this observation suggests that deacylation of LPS reduces the integrity of the outer membrane. This conclusion is consistent with the observation that deacylation increased LPS release from *B. pertussis* membranes (**chapter 4**). However, when *pagL* was heterologously expressed in *E. coli* XL1-Blue or *S.* Typhimurium, such re-modification with palmitate was not observed (Ernst *et al.*, 2006). Possibly, different strain backgrounds or expression levels used are responsible for the different results. An interesting hypothesis is that in *S.* Typhimurium and *E. coli*

XL1-Blue, which is an *E. coli* K-12 derivative, the LPS core domain compensates for the PagL-mediated decrease in outer membrane integrity, whereas this is not the case in *E. coli* BL21 Star, an *E. coli* B derivative, which has a substantially smaller LPS core domain. It has previously been shown that mutations in the LPS outer-core region can influence outer membrane integrity (Skurnik *et al.*, 1999). Interestingly, when we expressed enzymatically inactive PagL in *E. coli* BL21 Star and analysed the LPS profiles, bands with lower electrophoretic mobility than that of wild-type LPS were observed (**chapter 2**, Fig. 9B). It is tempting to speculate that these bands correspond to PagP-modified LPS. If this is indeed the case, also inactive PagL can apparently activate PagP and thus the presence of the PagL β-barrel alone would already be enough to disturb the outer membrane. Possibly, the tilted membrane-orientation of PagL (**chapter 3**) creates membrane disturbances by which phospholipids can flip to the outer leaflet of the outer membrane and become a substrate for PagP. Of note, besides PagL, PagP is the only known outer-membrane protein with a tilted orientation in the membrane (Ahn *et al.*, 2004). It would be very interesting to test whether PagL can induce phospholipid flip-flop across a lipid bilayer. Another question that needs to be answered is how the specificity of PagL for the lipid A 3 position is mediated. Since amino acid conservation is very low, it is unlikely that a dedicated LPS-binding motif is involved. However, it is conceivable that for instance positively charged residues in the extracellular loops, which can interact with the negatively charged lipid A backbone, play a role or that the barrel displays some sort of steric specificity.

The biological function of PagL remains unknown, given that *S.* Typhimurium and *P. aeruginosa pagL* mutants do not display obvious phenotypes (Trent *et al.*, 2001; **chapter 2**). Nevertheless, recent studies have provided some interesting insights that may help to resolve this question. It has, for instance, been shown that deacylation of LPS reduces its endotoxic activity, which may prevent recognition by the host innate immune system (Kawasaki *et al.*, 2005; **chapter 4**). In addition, it has been shown that *pagL* expression is increased in *P. aeruginosa* isolated from cystic fibrosis (CF) infants as compared to environmental isolates, whereas deacylated LPS species were not observed in some clinical *P. aeruginosa* isolates from patients with severe pulmonary disease, suggesting that loss of PagL function can occur during long-term adaptation to the CF airway (Ernst *et al*, 2006). These observations suggest that PagL may function in modulating the host immune response. However, the observation that *pagL* is also found in non-pathogenic (soil) bacteria suggests that besides a role in pathogenesis, PagL also must have a broader function. Since *pagL* expression influences the integrity of the outer membrane (**chapters 2** and **4**), it could well be that PagL has a function in

controlling, for instance, outer membrane fluidity and thereby adaptation to environmental conditions.

Role of LPS composition in the endotoxic activity of *B. pertussis* LPS and whole bacterial cells

One of the main targets of this thesis was to lower the reactogenicity of *B. pertussis* LPS and, thereby, of the bacterium itself. Less-reactogenic *B. pertussis* strains could then function as the basis for the development of safer wP vaccines. The endotoxic activity of LPS is based upon its recognition by the host innate immune system, activation of NF-κB, and subsequent up regulation of pro-inflammatory cytokines. During the late 1980s, experiments with LPS and LPS derivatives revealed that lipid A forms the bioactive component of LPS, responsible for the majority of IL-1 induction and immuno-regulation in human mononuclear cells (Loppnow *et al.*, 1989; Loppnow *et al.*, 1990). To date, a wide range of synthetic LPS structures has been tested for their biological activity and much is known regarding the contribution of specific elements to the overall toxicity of the molecule. It has become clear that the number, length, and positioning of the acyl chains and the phosphorylation status of the disaccharide backbone are important. For instance, dephosphorylated LPS shows a ~1000-fold lower endotoxic activity than wild-type *E. coli* lipid A (Rietschel *et al.*, 1987). However, not the phosphate groups themselves, but rather the negative charge they represent seems to be the determining factor, since lipid A substituted with a phosphono-oxyethyl group has an activity comparable to phosphate-substituted lipid A (Ulmer *et al.*, 1992). Furthermore, glucosamine monosaccharide preparations carrying phosphates and acyl chains at various positions in general lack biological activity (Aschauer *et al.*, 1990), suggesting that the disaccharide backbone is also important.

Like other amphiphatic molecules, LPS and lipid A form large supra-molecular aggregates in aqueous environments above the critical micellar concentration, which depends, amongst others, on the hydrophobicity (Brandenburg and Wiese, 2004). The structure of these aggregates is dependent on the chemical composition and influenced by pH, temperature, water content, and the presence of cations. LPS/lipid A aggregates can adopt two main phases, the gel phase and the liquid-crystalline phase. At a characteristic temperature, a reversible transition between these two phases takes place. Given the complex structure of LPS/lipid A (aggregates), the consequences of alterations in chemical composition on transition temperature are currently not fully understood.

Some striking correlations between the phase-transition temperature and

structure of the LPS/lipid A aggregates and exhibited endotoxic activity have been described. However, it is hard to distinguish whether the observed effects are due to alterations in the fluidity or structure of the aggregates, or a combination of both. For instance, it has been shown that deep rough LPS in the presence of Zn^{2+} has an enhanced capability to induce pro-inflammatory cytokine secretion at 37°C (Wellinghausen *et al.*, 1996). The authors found that an increased acyl chain order facilitated stronger interactions between LPS and LPS-binding protein and thereby enhanced the transport of LPS to the host TLR4/MD-2 complex. Thus, the phase transition temperature determines, for an important part, what kind of supramolecular structure LPS/lipid A aggregates adopt and, thereby, their endotoxic activity (Schromm *et al.*, 2000; Seydel *et al.*, 2000). So-called cylindrical-shaped lipid A species, such as those from *Rhodobacter capsulatus* and *Chromobacterium violaceum*, but also tetra- and penta-acyl species from *Enterobacteriacae*, adopt a lamellar structure and are poorly active. These lipid A species were found to work as efficient LPS antagonists if a sufficiently high negative backbone charge was present (Schromm *et al.*, 1998). In contrast, partially conical-shaped lipid A species that adopt a mixed lamellar/cubic structure, such as MPL and lipid A from *Campylobacter jejuni*, had an intermediate activity, whereas conical-shaped lipid A species, such as enterobacterial hexa-acyl lipid A, were highly active (Brandenburg and Wiese, 2004). Thus, in conclusion, the effect of one particular structural alteration on the toxicity of one particular LPS/lipid A species is relatively easy to predict. However, LPS preparations are often heterogeneous, meaning that the exhibited biological activity is the sum of all separate species including agonists, antagonists, and inactive forms. It becomes even more complicated when one realises that the interest is often in the toxicity of complex biological samples, such as wP vaccines, which contain besides LPS also additional (immune-modulating) components.

A clear example that changes in LPS composition can sometimes lead to unexpected results was provided in **chapter 4**. In that chapter, we explored the consequences of PagL and PagP expression on the *in vitro* toxicity of *B. pertussis* LPS and whole bacterial cells. Previously, it was shown that wild-type *B. pertussis* LPS already is a poor cytokine inducer in human monocytes, as can be expected from its cylindrical-shaped structure (Haeffner-Cavaillon *et al.*, 1989). We showed that deacylation by PagL further reduces the endotoxic activity of the LPS. This suggests that the number of acyl chains influences the endotoxic activity of *B. pertussis* LPS. Consistently, the PagP-mediated addition of an extra acyl chain led to an increased endotoxic activity of the LPS. Although modification of hexa-acylated *Salmonella* LPS by PagP was earlier reported to result in LPS with a reduced endotoxic activity (Janusch

et al., 2002; Kawasaki *et al.*, 2005), the increase in endotoxic activity of *B. pertussis* LPS we observed is not surprising, since *B. pertussis* LPS is a mixture of tetra- and penta-acylated forms, which, after modification by PagP, will be converted into more active penta- and hexa-acylated forms.

After having addressed the toxicity of purified *B. pertussis* LPS and its PagP- and PagL-modified derivatives, our next goal was to determine how these modifications influenced the toxicity of whole bacterial cells. To our surprise, we found that cells expressing either *B. bronchiseptica* or *P. aeruginosa* PagL, in contrast to the purified LPS preparations, had an increased potency to induce the production of IL-6 by human macrophages. It is well established that soluble LPS aggregates, on a molar basis, are biologically more active than LPS present in membrane structures such as in bacterial cells, outer membrane vesicles or liposomes (Post *et al.*, 2005). As deacylation of LPS may weaken LPS-LPS interactions, we hypothesised that the increased endotoxic activity of the PagL-expressing cells could potentially be due to an increased LPS release and subsequent formation of soluble aggregates. Such a mechanism could then counter-balance the effect of the reduced endotoxic activity of purified deacylated LPS. Consistent with this hypothesis, we observed that PagL expression increased the release of LPS from the bacterial cells into the medium. Furthermore, as anticipated, a treatment known to result in LPS release, i.e., incubation in the presence of EDTA, increased the IL-6 inducing capacity of a *B. pertussis* cell suspension. Therefore, increased release of deacylated LPS could eventually lead to a net increase in the endotoxic activity of PagL-expressing cells. However, it cannot be excluded that also other factors contribute to the observed increase in endotoxic activity, since it is conceivable that LPS deacylation also facilitates the release of other (immune-modulating) compounds. Remarkably, a similar discrepancy between the effects of an LPS modification on purified LPS and on whole bacterial cells was observed in **chapter 9**, in which we identified a new LPS glycosyltransferase operon in the *B. pertussis* genome. By insertionally inactivating the genes and studying the effects on LPS structure, we demonstrated that at least two of the genes in this operon encode active LPS glycosyltransferases. When we tested the purified wild-type and mutant LPSs and whole cells for their endotoxic activity, we found that the purified LPS from the ΔBP2329 mutant strain exhibited a reduced biological activity, whereas the mutant cells instead showed a slightly increased endotoxic activity. Thus, changes in LPS composition influence the endotoxic activity at different levels, i.e., not only directly through altering the LPS per se, but also indirectly through the effect on LPS accessibility. In the case of PagL and the ΔBP2329 mutant strain, these effects worked in opposite directions. Altogether, these results suggest that *B. pertussis*

has a relatively fragile outer membrane and that alterations in LPS composition can easily disturb its integrity, thereby promoting the release of LPS. This conclusion is consistent with the finding that also an *lpxL1*-deficient mutant of *B. pertussis* displays decreased outer membrane integrity. As demonstrated in **chapter 8**, LpxL1 mediates the addition of a previously unrecognised secondary 2-OH C12 moiety to *B. pertussis* lipid A. We showed that this moiety is present in wild-type *B. pertussis* LPS, yet, only at a low frequency (<10% of the total LPS species). Nevertheless, when we knocked out the *lpxL1* gene, we found that the mutant strain exhibited an increased sensitivity towards rifampicin, which is indicative for decreased outer membrane integrity. This result supports the conclusion that relatively small changes in LPS composition can have dramatic effects on the stability of the *B. pertussis* outer membrane and that this phenomenon can diminish the effect of reducing LPS toxicity. Perhaps, this may also be one of the reasons why *B. pertussis* is unable to survive outside the human host. Possible solutions to the problem are the addition of membrane-stabilising agents or the removal of soluble LPS aggregates. It would for instance be interesting to determine whether replacing aluminum phosphate by aluminum hydroxide in wP vaccines would beneficially influence vaccine reactogenicity since the binding affinity of soluble LPS aggregates for aluminum hydroxide is much higher than for aluminum phosphate (Shi *et al.*, 2001).

Role of LPS in pertussis vaccination

The role of LPS in *Bordetella* pathogenesis and immunity is unclear. It has been shown that *Bordetella* LPS is highly immunogenic, acts as an immunological adjuvant, and exhibits the properties that are expected of an endotoxin (Amano *et al.*, 1990; Chaby and Caroff, 1988; Watanabe *et al.*, 1990). Furthermore, *Bordetella* LPS and its recognition by the TLR4/MD-2 complex have been shown to be important determinants in the course of infection in mice (Mann *et al.*, 2004a; Mann *et al.*, 2004b). In addition, antibodies against *B. pertussis* LPS have been shown to play a role in the protection against *B. pertussis* infection (Granstrom and Granstrom, 1993; Shahin *et al.*, 1994), and they have also been shown to bind to *B. parapertussis* and inhibit their adherence to respiratory tract epithelial cells, suggesting cross-protection between the two *Bordetella* species (van den Berg *et al.*, 1999). Thus, apparently, *B. pertussis* LPS plays a dual role; on the one hand as an immune-stimulating compound and on the other hand as a protective antigen. In **chapter 4**, we showed that expression of the LPS-modifying enzymes PagP and PagL modulates the *in vitro* toxicity of *B. pertussis* LPS and whole bacterial cells. The next goal was to determine how these alterations in

LPS composition affect the efficacy and reactogenicity of wP vaccines (**chapter 5**). Mice were immunised with different wP vaccines and then challenged with *B. pertussis*. We found that expression of PagL, but not of PagP, significantly increased vaccine efficacy without altering vaccine reactogenicity. The latter conclusion deviates from that of the *in vitro* experiments which indicated that PagL-expressing *B. pertussis* cells exhibited an increased toxicity (**chapter 4**). Thus, apparently, *in vitro* toxicity data cannot predict the outcomes of *in vivo* experiments, emphasising the importance of both *in vitro* and *in vivo* analyses. Possible explanations for this discrepancy are differences between human (**chapter 4**) and murine (**chapter 5**) cells or the use of only a single cell type in the *in vitro* experiments, i.e., macrophages, whereas *in vivo* experiments comprise many different cell types simultaneously. Also, the availability of LBP and CD14 probably differs in the two settings. Since an increased vaccine potency probably allows for a reduction of the vaccine dosage, these data imply that PagL-expressing *B. pertussis* strains may form a good basis for the development of less reactogenic wP vaccines, as lower vaccine dosages will probably also reduce vaccine side-effects. Thus, in conclusion, we provided proof of principle that LPS-modifying enzymes may indeed form promising tools for the development of safer wP vaccines. Obviously, this observation is not only significant in the field of pertussis vaccinology, but may also be applicable for the development of vaccines against other Gram-negative bacteria. Furthermore, these data showed, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

In **chapter 6**, we took a somewhat different approach and explored the possibilities of improving wP efficacy and reducing reactogenicity by supplementing the vaccine with LPS analogs that are capable of modulating immune responses. We showed that addition of monophosphoryl lipid A (MPL) increased vaccine efficacy without changing the reactogenicity. Furthermore, we demonstrated that *Neisseria meningitidis* LpxL2 LPS functions as an LPS antagonist and that supplementation of the wP vaccine with this LPS not only decreased the reactogenicity, but also increased the efficacy of the vaccine. Thus, apparently, the LPS derivatives can influence both the efficacy and reactogenicity of the wP vaccines and their application may therefore be a useful strategy for vaccine improvement. However, more detailed studies using different dosages and different experimental backgrounds should be conducted before definite conclusions concerning the application of such an approach can be drawn. Furthermore, it may be worthwhile to test a broader range of LPS derivatives.

Interestingly, as shown in **chapter 7**, LPS analogs may not only be beneficial for improving wP vaccines, but also for improving aP vaccines. One of the main problems

concerning aP vaccination is that it drives the immune response towards a Th2-type response (Mills, 2001). Th2-type immune responses are correlated with type-1 hypersensitivity and probably provide only suboptimal protection, since it is known that in both mice and man cellular (Th1) and humoral (Th2) immunity have complementary roles in protection against *B. pertussis* infection (Mills, 2001). Since LPS has strong Th1-skewing properties, we hypothesised that supplementation of a DTaP vaccine with LPS analogs would probably alter the Th1/Th2 balance by tipping it towards more a Th1-type response, thereby decreasing type-1 hypersensitivity and simultaneously increasing vaccine efficacy. Indeed, we found in a mouse model that addition of LPS derivatives to the DTaP vaccine re-directed the immune response towards a Th1-type response. Furthermore, it decreased parameters, such as lung eosinophilia, indicative for type-1 hypersensitivity and, most importantly, it improved vaccine efficacy. These results support the hypothesis that LPS derivatives can modulate aP-induced immune responses. In addition, we analysed the effect of LPS supplementation on vaccine reactogenicity and found that MPL and LpxL2 LPS significantly increased the post-immunisation serum IL-6 levels as compared to an aluminum-adjuvated vaccine. However, as compared to a wP vaccine, the serum IL-6 levels evoked were still low. Thus, the application of LPS derivatives may form a useful strategy for improving aP vaccines, since they re-directed the immune response towards to a more Th1-type response, decreased type-1 hypersensitivity, and improved vaccine efficacy. Of course, such a strategy will not provide a solution for the problems concerning aP production-costs and escape-mutant selection.

The results described in this thesis demonstrate that efficacy and reactogenicity of both wP and aP vaccines can be changed in the right direction by modulating the LPS content. We have shown that alterations in LPS composition, i.e., in case of the PagL/expressing strain (**chapter 5**), or addition of LPS derivatives (**chapters 6** and **7**) can increase vaccine efficacy. Further analysis revealed that the increased vaccine efficacy observed did not correlate with higher anti-*B. pertussis* total IgG, IgG1, or IgG2a antibody titers (**chapters 5** and **6**). However, the increased efficacy of the DTaP vaccines supplemented with MPL or LpxL2 LPS did coincide with higher anti-pertussis toxin antibody titers, suggesting that these antibodies may be important for aP-mediated protection (**chapter 7**). Nevertheless, alterations in LPS composition can modulate antibody responses, as was revealed, for example, by the decreased IgG1/IgG2a ratio in the mice immunised with the PagP- and PagL-expressing strains, as compared to the mice immunised with the wild-type strain (**chapter 5**). Analysis of the cytokine concentrations in the lung homogenates revealed no major differences between

the immunised groups and the control groups, except for gamma-interferon (IFN-γ) (**chapters 5** and **6**). The importance of IFN-γ for protection against *Bordetella* infection has been shown repeatedly (Mills *et al.*, 1998; Mahon *et al.*, 1997; Barbic *et al.*, 1997; Pilione and Harvill, 2006). IFN-γ induces the transcription of several activation markers, various cell surface receptors, chemokine receptors, and co-stimulatory receptors involved in antigen presentation (Dalton *et al.*, 1993; Ellis *et al.*, 2004; Janeway, 2001). Furthermore, IFN-γ activates and recruits neutrophils (Ellis *et al.*, 2004; Burch *et al.*, 2006). Consistently, we observed that the wP-immunised mice exhibited an increased lung neutrophil-fraction. Neutrophils have been shown to play an important role in *Bordetella* clearance (Kirimanjeswara *et al.*, 2003; Kirimanjeswara *et al.*, 2005; Pishko *et al.*, 2004) Overall, our data suggest a central role for both IFN-γ and neutrophils in the clearance of *B. pertussis* from the lungs. Thus, analysis of these parameters may allow the reliable prediction of vaccine efficacy in mice. Whether this is also true for humans remains to be determined. Interestingly, in a recent study, it was shown that besides IFN-γ-producing cells, also IL-17-producing T cells are important for protective cellular immunity against *B. pertussis* (Higgins *et al.*, 2006). It would therefore be interesting to also study IL-17 responses in future experiments.

Prospects for pertussis vaccination

Pertussis causes nearly 300,000 deaths in children every year. Current pertussis vaccines have proved to be effective and relatively safe in large-scale clinical trials. At present, most developing countries use wP vaccines because they are cheap, effective, and easy to produce, whereas most industrialised countries have switched to less reactogenic, but more expensive aP vaccines. In general, pertussis vaccination comprises a three-dose immunisation series starting at 6 weeks to 3 months after birth and is usually completed by the age of 6 months. In addition, many schedules include a toddler booster, and a fourth or fifth dose at 4–6 years of age. Pertussis vaccination provides protection against death and admission to hospital, although cases of pertussis disease in recently vaccinated people are sporadically observed.

Despite high vaccination coverage, pertussis is still prevalent and increasing and is frequently the least well-controlled disease in childhood vaccination programs, possibly as a result of waning immunity. Addition of new antigens, such as adenylate cyclase, to aP vaccines might improve some aspects of the immune response to vaccination, but is unlikely to significantly increase the duration of protection. Potentially more interesting would be supplementation of aP vaccines with LPS analogs, which, as we showed, increases vaccine efficacy and drives the immune response towards a more protective

Th1-type response without substantially increasing vaccine reactogenicity (**chapter 7**). Since MPL has already been registrated for use in a human vaccine (hepatitis B), MPL-supplemented aP vaccines might be the least complicated to introduce from a regulatory perspective. However, this approach, of course, does not provide a solution for the problems concerning the high production costs of aP vaccines and their risk to select for escape mutants more rapidly. Today, many scientists from around the world are putting tremendous effort in trying to improve pertussis vaccines. These efforts include the development of live-attenuated vaccines (Mielcarek *et al.*, 2006), DNA vaccines (Kamachi *et al.*, 2003), and safer wP vaccines (**this thesis**). Here, we provided proof of principle that LPS engineering can be a useful strategy for improving pertussis vaccines. However, at this moment, it is too early to tell which approach will be most promising and should thus be pursued. Currently, I would say that the most promising candidate for an improved pertussis vaccine would be a wP vaccine supplemented with non-toxic LPS antagonists, such as LpxL2 LPS. We have shown that this approach led to a less-reactogenic vaccine with an increased efficacy. However, it is very well imaginable that such an approach will stumble on regulatory difficulties since it requires the addition of extra LPS to wP vaccines. Therefore, the use of the PagL-expressing *B. pertussis* strains may also be a good option. In addition, it would be worthwhile to proceed with testing other vaccine strains, such as the core OS mutants (**chapter 9**) or the *lpxL1*-deficient mutant (**chapter 8**), additional LPS-modifying enzymes, such as the lipid A 3'-*O*-deacylase LpxR and the lipid A 1- and 4'-phosphatases LpxE and LpxF, additional LPS mutations, and adjuvants, e.g., aluminum phosphate vs. aluminum hydroxide. In any case, introducing new pertussis vaccines will take a substantial amount of time, and thus, in the meanwhile, the potential of currently available pertussis vaccines should be maximised. As pertussis has persisted in a huge adult and adolescent reservoir during the past three decades (Mattoo and Cherry, 2005), rationalisation of pertussis boosters in childhood and introduction of widespread repeat booster vaccination in adolescents and adults can be expected to lessen disease prevalence and morbidity among susceptible infants. Complementary to this, it might be possible to protect the youngest infants, for instance by immunising pregnant mothers or by vaccinating directly after birth.

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Appendices

Nederlandse Samenvatting

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Nederlandse Samenvatting

Kinkhoest is een ernstige infectieziekte van de bovenste luchtwegen veroorzaakt door de Gram-negatieve bacterie *Bordetella pertussis*. Aanvankelijk is een kinkhoestbesmetting niet te onderscheiden van een verkoudheid, maar na enige tijd ontstaan de voor kinkhoest typische, zeer langdurige en hevige hoestbuien, waarbij de patiënt tot stikkens toe hoest en daarna met gierende ademhaling weer lucht schept: de zogenaamde “whoop” uit de Engelse benaming “whooping cough”. De kinkhoestbacterie verspreidt zich door middel van aerosolen, die tijdens het hoesten vrij komen. Kinkhoest heeft een incubatieperiode van 7 tot 10 dagen. Tijdens deze periode koloniseert de bacterie eerst de keelholte en luchtpijp, waarna de bacterie zich snel vermeerdert en verder verspreidt. De productie van toxines veroorzaakt beschadiging van de slijmvliezen, waardoor uiteindelijk de voor kinkhoest zo karakteristieke symptomen ontstaan.

De introductie van kinkhoestvaccins in de jaren 40 en 50 van de vorige eeuw heeft ervoor gezorgd dat de kinkhoestincidentie, almede de morbiditeit en mortaliteit, enorm verlaagd zijn in nagenoeg alle westerse landen en vele ontwikkelingslanden. De eerste kinkhoestvaccins waren zogeheten cellulaire vaccins en bestonden uit hele, afgedode bacteriën. Na het van start gaan van de vaccinatiecampagnes werd echter snel duidelijk dat deze vaccins relatief veel bijwerkingen geven. Dit wordt veroorzaakt door de aanwezigheid van sterk immuun-stimulerende componenten, waaronder het lipopolysaccharide (LPS) molecuul. Dit molecuul is een belangrijke component van Gram-negatieve bacteriën en staat erom bekend sepsis (endotoxische shock) te kunnen veroorzaken. Om deze reden staat LPS ook wel bekend als endotoxine. Daarnaast heeft LPS nog een andere eigenschap: het functioneert als een immunologisch adjuvant. Dit houdt in dat de door vaccinatie opgewekte immuunrespons krachtiger zal zijn in aanwezigheid van LPS. De problemen betreffende de veiligheid van de cellulaire vaccins hebben geleid tot de ontwikkeling van minder toxische kinkhoestvaccins, de zogeheten a-cellulaire kinkhoestvaccins, die in het begin van de jaren tachtig van de vorige eeuw werd geïntroduceerd en sinds 2005 ook in het Nederlandse vaccinatieprogramma worden toegepast. Deze vaccins bevatten geen LPS en bestaan uit een combinatie van gezuiverde kinkhoestantigenen. A-cellulaire kinkhoestvaccins geven duidelijk minder bijwerkingen en lijken over het algemeen even goed te beschermen als de cellulaire vaccins. Een probleem van deze vaccins is echter dat de productiekosten relatief hoog zijn wat de toepasbaarheid in ontwikkelingslanden bemoeilijkt. Verder is het aannemelijk dat het gebruik van a-cellulaire vaccins zal leiden tot een versnelde selectie van mutanten, die niet meer goed herkend worden door het immuunsysteem; door het schaarse aantal antigenen waar deze vaccins op gebaseerd zijn bestaat er een relatief grote kans op het ontstaan van mutaties die de herkenning van de bacterie door het

immuunsysteem doen verminderen. Een ander belangrijk nadeel van deze vaccins is dat ze een ander soort immuniteit opwekken dan de cellulaire kinkhoestvaccins of een natuurlijke kinkhoestbesmetting. Terwijl de cellulaire vaccins en natuurlijke besmetting een zogenoemde Th1 respons opwekken, wekken a-cellulaire kinkhoestvaccins een zogenaamde Th2 respons op. Uit onderzoek is gebleken dat een te eenzijdige Th2 response kan leiden tot een verhoogd risico op allergische aandoeningen. Zeker over de lange termijn effecten van het vaccineren van immunologisch immature individuen (lees: zuigelingen) met a-cellulaire kinkhoestvaccins is op dit moment onvoldoende bekend.

Vandaag de dag maken de meeste ontwikkelingslanden gebruik van cellulaire kinkhoestvaccins, omdat deze vaccins niet alleen relatief goed werken, maar ook eenvoudig en goedkoop te produceren zijn. Daarentegen maken de meeste westerse landen op dit moment gebruik van de veiliger-geachte, maar duurdere a-cellulaire kinkhoestvaccins. Ondanks dat kinkhoestvaccinatie over het algemeen als een succes wordt gezien en de incidentie relatief laag is, is kinkhoest altijd in de samenleving aanwezig gebleven. Zeker in vergelijking met andere vaccineerbare ziektes is kinkhoest, als ziekte, het minst onder controle. Hier komt bij dat vanaf de jaren 80 van de vorige eeuw de kinkhoestincidentie in vele landen, inclusief Nederland, weer aan het stijgen is. Misschien nog zorgwekkender is dat, waar kinkhoest vroeger voornamelijk voorkwam bij kleuters, de ziekte tegenwoordig steeds vaker gevonden wordt bij pubers, volwassenen en belangrijker, bij pasgeborenen, die nog geen goed afweersysteem hebben. De bovenstaande redenen laten zien dat de ontwikkeling van verbeterde kinkhoestvaccins erg belangrijk is. Een van de belangrijkste obstakels hierbij, in ieder geval vanuit het perspectief van een verbeterd cellulair kinkhoestvaccin, is dat deze vaccins dus relatief veel bijwerkingen veroorzaken. Een van de voornaamste oorzaken hiervan is, zoals eerder gezegd, de aanwezigheid van LPS in het vaccin . Zoals de naam “lipopolysaccharide” al zegt, bestaat LPS uit een lipide gedeelte (het zogenaamde lipid A) en een polysaccharide (suiker) gedeelte. Lipid A is op zichzelf weer opgebouwd uit twee suikermoleculen (glucosamines), die ieder nog een fosfaatgroep hebben. Verder zijn aan deze twee glucosamines nog een aantal vetzuurstaarten gekoppeld (zie Fig. 6 van de General Introduction). Het is bekend dat de toxiciteit van het LPS voor het overgrote deel afhankelijk is van de samenstelling van het lipid A gedeelte en dan vooral van de hoeveelheid en de lengte van de vetzuurstaarten en de aanwezigheid van de fosfaatgroepen. Tevens is bekend dat het mogelijk is kleine veranderingen in het LPS te maken en dat deze veranderingen de toxiciteit van het LPS kunnen beïnvloeden. Zo is bijvoorbeeld aangetoond dat het LPS van de hersenvliesontstekingsbacterie

Neisseria meningitidis beduidend minder toxisch wordt als er vetzuurstaarten vanaf worden gehaald. Daar komt bij dat de adjuvant activiteit (de goede eigenschap van LPS) van deze veranderde LPS moleculen soms grotendeels behouden bleek te zijn. Dit minder toxische LPS met een behouden adjuvant activiteit is uiteraard uitermate geschikt voor gebruik in humane vaccins, omdat je dan wel de goede maar niet de slechte eigenschappen van het LPS benut.

Het voornaamste doel van het hier beschreven onderzoek was om veranderingen aan te brengen in het LPS van *B. pertussis* en zo te komen tot een minder toxische kinkhoeststam, die dan gebruikt zou kunnen worden als basis voor een verbeterd cellulair vaccin. Voor het aanbrengen van de veranderingen in het LPS maakten we enerzijds gebruik van enzymen die het LPS op specifieke plekken kunnen veranderen (**hoofdstukken 2 t/m 5**) en anderzijds grepen we in in de biosynthese route van het LPS (**hoofdstukken 8 & 9**). Verder hebben we onderzocht of het toevoegen van speciale LPS moleculen (deze zijn in staat de toxiciteit van andere LPS moleculen beïnvloeden) aan een cellulair kinkhoestvaccin een goede strategie is om de vaccins te verbeteren (**hoofdstuk 6**). In het verlengde hiervan hebben we ook bekeken of de toevoeging van deze speciale LPS moleculen de werking en effectiviteit van a-cellulaire kinkhoestvaccins beïnvloedt, onder andere omdat het bekend is dat LPS een Th2 immuunrespons om kan buigen naar een Th1 immuunrespons. Dit is interessant aangezien een van de belangrijkste nadelen van a-cellulaire kinkhoestvaccins was, dat ze een Th2 in plaats van een Th1 immuunrespons opwekken (**hoofdstuk 7**). Aangezien aangetoond is dat het reduceren van het aantal vetzuurstaarten aan LPS in het algemeen leidt tot verminderde toxiciteit was een enzym, PagL, dat vetzuurstaarten van LPS af kan knippen een van de primaire kandidaten voor toepassing in onze strategie. We zijn daarom eerst begonnen met het bestuderen van dit enzym (**hoofdstukken 2 & 3**).

Hoofdstuk 2 beschrijft de identificatie van PagL homologen in een groot aantal Gram-negatieve bacteriën. Het PagL enzym werd als eerste gevonden in de bacteriesoort *Salmonella enterica*. Toentertijd dacht men dat PagL uniek was voor deze ene soort. Toen wij echter gingen zoeken in een database waarin alle bekende genoomsequenties van bacteriesoorten opgenomen zijn, bleek al snel dat aan PagL gelijkende eiwitten ook in veel andere bacteriesoorten te vinden zijn. Nieuwe homologen werden onder andere geïdentificeerd in de genomen van verschillende *Bordetella* en *Pseudomonas* soorten, inclusief de kinkhoestbacterie *B. pertussis* en de opportunistische humane pathogeen *Pseudomonas aeruginosa* (*P. aeruginosa* is vooral bekend om zijn rol bij taaislijmziekte, waarbij de bacterie vaak infecties veroorzaakt). Interessant genoeg vonden we dat de *pagL* sequentie in het genoom van *B. pertussis*, in tegenstelling tot de andere *Bordetella*

soorten, verstoord is door een frame-shift mutatie, waardoor deze bacterie het PagL enzym niet meer kan maken. Wellicht is het muteren van *pagL* door *B. pertussis* dus wel een manier geweest om zich aan te passen aan de menselijke gastheer. Verder konden we op basis van sequentievergelijkingen een voorspelling doen welke aminozuren belangrijk zouden kunnen zijn voor de activiteit van het PagL enzym. We kwamen hierbij uit op twee aminozuurresiduen: één serine en één histidine. Door te laten zien dat het vervangen van deze aminozuren leidt tot een niet actief enzym, konden we aantonen dat ze inderdaad belangrijk zijn voor de activiteit van PagL.

In **hoofdstuk 3** gingen we nog een stap verder en werden nog een aantal nieuwe PagL homologen geïdentificeerd. Daarnaast beschrijft dit hoofdstuk de kristalstructuur van het PagL enzym van *P. aeruginosa*. Deze structuur liet zien dat het enzym een β-barrel is opgebouwd uit acht β-strands. Verder vonden we dat het enzym waarschijnlijk scheef in de membraan zit en konden we, nu we met veel meer detail konden kijken, een betere voorspelling doen over welke aminozuren belangrijk zijn voor de enzym activiteit. Naast de al in **hoofdstuk 2** geïdentificeerde serine en histidine residuen konden we nu ook een derde aminozuur, een glutamaat, aanwijzen als onderdeel van het enzymatisch centrum van het eiwit. Daarnaast werden nog drie andere belangrijke residuen gevonden: een aspartaat (voor substraat herkenning), een fenylalanine (voor substraat positionering) en een asparagine (betrokken bij de formatie van het oxyanion hole).

Na deze uitgebreide analyse van het PagL enzym stapten we in **hoofdstuk 4** over op de mogelijke toepassing van dit enzym voor de ontwikkeling van minder toxische kinkhoeststammen. Daartoe brachten we het *pagL* gen van *Bordetella bronchiseptica* in *B. pertussis* tot expressie. We kozen voor PagL van *B. bronchiseptica*, omdat we in **hoofdstuk 2** hadden laten zien dat dit enzym inderdaad actief is, en omdat we, gezien de nauwe verwantschap tussen *B. pertussis* en *B, bronchiseptica*, hierbij de minste problemen verwachtten met betrekking tot de expressie en biogenese van het eiwit en de herkenning van het LPS substraat. Behalve de PagL-producerende stam werden ook nog twee PagP-producerende *B. pertussis* stammen geconstrueerd. PagP is ook een LPS-modificerend enzym, maar het plakt een extra vetzuurstaart aan lipid A, in plaats van er een af te halen zoals PagL dat doet. Eerst lieten we zien dat het LPS dat deze drie *B. pertussis* stammen maken een veranderde toxiciteit heeft. Het LPS van de PagL-producerende stam was minder toxisch, terwijl het LPS van de PagP-producerende stammen juist toxischer was. Dit klopte met onze verwachtingen, aangezien de algemene regel luidde: hoe minder vetzuurstaarten aanwezig, hoe minder toxisch het LPS. Vervolgens bestudeerden we de toxiciteit van hele bacteriecellen. Zoals verwacht

waren de PagP-producerende cellen, net als het LPS van deze bacteriën, toxischer geworden, maar verrassenderwijs was dit ook het geval voor de PagL-producerende bacteriën. Dus, hoewel het LPS minder toxisch geworden was, vonden we dat de hele bacteriecellen juist toxischer geworden waren. In het vervolg van het hoofdstuk lieten we zien dat dit resultaat wellicht deels verklaard kan worden, doordat gedeacyleerd LPS makkelijker loslaat uit de bacteriemembraan. Het is bekend dat vrij LPS toxischer is dan eenzelfde hoeveelheid membraan-gebonden LPS. Het zou dus kunnen dat het makkelijker vrijkomen van het LPS uit de PagL-producerende bacteriën de verlaagde toxiciteit van het LPS te niet doet.

In **hoofdstuk 5** maakten we de overstap naar een diermodel. Tot dan toe hadden we toxiciteit alleen getest *in vitro* en nu wilden we graag de effecten van de LPS modificaties testen in levende dieren. Hiervoor werden de in **hoofdstuk 4** geconstrueerde *B. pertussis* stammen en een controle (wildtype) stam verwerkt tot cellulaire kinkhoestvaccins en gebruikt voor vaccinatie van muizen. Na twee vaccinaties werden de muizen geïnfecteerd met wildtype *B. pertussis*, waarna geanalyseerd werd hoe goed de muizen in staat waren om de infectie te weerstaan. Hieruit kwam naar voren dat zowel het vaccin gebaseerd op de PagP-producerende stam als het vaccin gebaseerd op de PagL-producerende stam een betere bescherming boden dan het controle vaccin. Naast deze effectiviteitsanalyse werd ook gekeken naar de hoeveelheid *B. pertussis*-specifieke antilichamen in het bloed en naar een specifieke klasse van eiwitten, genaamd cytokines, die een belangrijke rol spelen binnen het immuunsysteem. Doel was te bepalen wat voor een type immuunrespons de vaccins hadden opgewekt en hoe toxisch de vaccins waren. Voor dit laatste werd onder andere de interleukine-6 (IL-6) concentratie in het bloed bepaald. IL-6 is een ontstekingsopwekkend cytokine, dat wordt geproduceerd zodra een persoon geïnfecteerd wordt met bacteriën. Normaal gesproken is dit een goede zaak, aangezien een ontstekingsreactie nodig is om een bacterie-infectie op te ruimen, maar in het geval van bijvoorbeeld het cellulaire kinkhoestvaccin, wanneer zeer lokaal een grote dosis bacteriën wordt ingespoten, kan de reactie hierop wel eens te heftig zijn, wat dan resulteert in ongewilde bijwerkingen. Uit de analyse van de IL-6 concentraties in het bloed bleek dat de vaccins gebaseerd op de PagP- en PagL-producerende stammen qua toxiciteit gelijk waren aan het controle vaccin. Dit laatste was onverwacht aangezien we in **hoofdstuk 4** gezien hadden dat de productie van PagP en PagL de bacteriecellen toxischer maakte. Blijkbaar zijn resultaten die verkregen worden *in vitro* dus niet automatisch te vertalen naar uitkomsten van dierexperimenten. Deze conclusie onderstreept het belang van het uitvoeren van beide soorten experimenten. De vaccintoxiciteit werd ook nog op andere manieren bepaald.

Dit gebeurde in een zogeheten muistoxiciteitstest, waarbij na vaccinatie het gewicht van de muizen en de hoeveelheid witte bloedcellen in het bloed bepaald werden. Daarbij geldt dat hoe meer gewicht de muizen verliezen en hoe meer witte bloedcellen aanwezig, hoe toxischer de vaccins. Uit deze analyse bleek dat alle geteste vaccins leidden tot evenveel gewichtsverlies, maar dat in de muizen gevaccineerd met de PagP-producerende stam duidelijk grotere hoeveelheden witte bloedcellen aanwezig waren dan in de muizen gevaccineerd met de PagL-producerende stam of de controle stam. Deze resultaten laten zien dat de PagL-producerende stam, en in mindere mate misschien ook de PagP-producerende stam, veelbelovende kandidaten zijn voor de ontwikkeling van een verbeterd cellulair kinkhoest vaccin. Aangezien vaccinatie met de PagL-producerende stam een betere bescherming opleverde dan vaccinatie met de controle stam kan voor eenzelfde vaccineffectiviteit waarschijnlijk een lagere vaccin dosis gebruikt worden, wat zal resulteren in een reductie van bijwerkingen.

In **hoofdstuk 6** zijn we overgegaan op een andere aanpak. In dit hoofdstuk hebben we bestudeerd of een tweetal specifieke LPS analogen de effectiviteit en toxiciteit van cellulaire kinkhoestvaccins gunstig kunnen beïnvloeden. Hiervoor werden monophosphoryl lipid A (MPL) en *N. meningitidis* LpxL2 LPS gebruikt. MPL heeft een lage toxiciteit en wordt gebruikt als immunologisch adjuvant. LpxL2 heeft ook een lage toxiciteit, maar bezit daarnaast, zoals beschreven in dit hoofdstuk, ook de capaciteit om de toxiciteit van andere LPS vormen te verlagen (het werkt als een zogeheten LPS antagonist). Het idee was dat toevoeging van MPL waarschijnlijk zou leiden tot een verhoogde vaccineffectiviteit (MPL werkt immers als een adjuvant) zonder de toxiciteit van het vaccin te verhogen. De resultaten lieten zien dat dit inderdaad het geval was en dat toevoeging van MPL aan cellulaire kinkhoestvaccins, net als het gebruik van de PagL-producerende stam, waarschijnlijk een verlaging van de vaccindosis mogelijk maakt en dus het aantal en de ernst van de bijwerkingen zal verlagen. De resultaten met het LpxL2 gesupplementeerde vaccin waren nog meer veelbelovend. LpxL2 LPS werkt als een LPS antagonist wat inhoudt dat toevoeging aan een cellulair vaccin de vaccintoxiciteit zal verlagen. En inderdaad, na vaccinatie met het LpxL2 gesupplementeerde vaccin waren de IL-6 concentraties in het bloed significant lager dan na vaccinatie met het controle vaccin. Hier komt bij dat de effectiviteitsanalyse liet zien dat het LpxL2 gesupplementeerde vaccin ook nog eens een betere bescherming gaf. Het mes snijdt hier dus aan twee kanten; de toxiciteit wordt verlaagd en de effectiviteit gaat omhoog. Deze resultaten laten zien dat toevoeging van LpxL2 LPS (of LPS vormen met vergelijkbare eigenschappen) aan cellulaire kinkhoestvaccins een uitermate veelbelovende strategie is om uiteindelijk te komen tot een effectiever en

minder toxisch cellulair kinkhoestvaccin.

In **hoofdstuk 7** hebben we net als in **hoofdstuk 6** gebruikt gemaakt van de LPS analogen MPL en LpxL2 LPS. Dit keer werden de analogen echter toegevoegd aan a-cellulaire kinkhoest vaccins. De achterliggende gedachte was dat toevoeging van LPS, waarvan het bekend is dat het de immuunrespons richting Th1 duwt, de Th2 respons, die normaal door a-cellulaire kinkhoestvaccins wordt opgewekt, om zou kunnen buigen naar een Th1 respons. Tevens hebben MPL en LpxL2 LPS een adjuverende werking, wat betekent dat zij de vaccineffectiviteit waarschijnlijk zullen verhogen. De resultaten lieten zien dat toevoeging zowel van MPL als van LpxL2 LPS inderdaad leidde tot een sterkere Th1 respons en een verhoogde vaccineffectiviteit. Deze bevindingen ondersteunen de conclusie dat het gebruik van LPS analogen potentieel een goede strategie is om in ieder geval sommige van de problemen met a-cellulaire kinkhoestvaccins op te kunnen lossen.

In de laatste twee hoofdstukken zijn we weer terug gegaan naar het aanbrengen van veranderingen in het LPS. Hiervoor maakten we deze keer geen gebruik van LPS-modificerende enzymen maar grepen we, door het aanbrengen van mutaties, direct in in de LPS biosynthese route. **Hoofdstuk 8** beschrijft de ontdekking van twee *B. pertussis* genen, *lpxL1* en *lpxL2*, die coderen voor eiwitten die lijken op enzymen die in andere bacteriën verantwoordelijk zijn voor het aanplakken van secundaire vetzuurstaarten aan LPS. Aangezien *B. pertussis* LPS normaal gesproken maar 1 secundaire vetzuurstaart heeft (een C14 vetzuurstaart; zie Fig. 6 van de General Introduction) was de vraag welke van de twee gecodeerde enzymen verantwoordelijk is voor het aanzetten van deze vetzuurstaart en wat de functie van het andere enzym is. Uit onze analyses bleek dat de secundaire C14 vetzuurstaart wordt aangezet door het LpxL2 enzym, terwijl het andere enzym, LpxL1, verantwoordelijk is voor het aanzetten van een tot nu toe voor *B. pertussis* onbekende secundaire hydroxy-C12 vetzuurstaart. Verder hebben we laten zien dat een *B. pertussis lpxL1* mutant onder andere een veranderde membraan integriteit heeft en minder goed in staat is macrofagen te infecteren. Verder onderzoek zal moeten uitwijzen wat het effect van de *lpxL1* mutatie op het vaccinpotentieel van *B. pertussis* is. Tot slot hebben we in **hoofdstuk 9** een viertal nieuwe *B. pertussis* genen geïdentificeerd die mogelijk coderen voor eiwitten betrokken bij de biosynthese van het suikergedeelte van het LPS. Door deze genen één voor één uit te schakelen konden we laten zien dat in ieder geval twee van de vier genen hier inderdaad bij betrokken zijn. De functie van de andere twee genen is nog onduidelijk. Het suikergedeelte van het LPS is onder andere belangrijk voor de binding van het LPS (en dus van de hele bacterie) aan immuuncellen. Deze binding is belangrijk in twee opzichten. Enerzijds zorgt het

ervoor dat de bacteriën efficiënt kunnen worden opgenomen, wat belangrijk is voor het opbouwen van een goede immuunrespons. Anderzijds zorgt het er ook voor dat de immuuncellen zich verder ontwikkelen (matureren). Om deze reden hebben we de maturatie van dendritische cellen (een speciale klasse van immuuncellen) onderzocht na incubatie met onze suikermutanten. Hierbij kwam naar voren dat veranderingen in het suikergedeelte van het LPS inderdaad invloed hebben op de maturatie van de dendritische cellen. Mutaties kunnen zowel leiden tot een slechtere maturatie (ΔBP2329 mutant) als tot een betere maturatie (ΔBP2331 mutant). Deze resultaten laten zien dat niet alleen veranderingen in het lipid A gedeelte van het LPS bruikbaar kunnen zijn voor de ontwikkeling van een verbeterd kinkhoestvaccin, maar dat ook zeker aanpassingen in het suikergedeelte tot de opties behoren. Om hier meer duidelijkheid over te krijgen zullen deze mutanten getest moeten worden in een muizen beschermingsmodel.

Op dit moment eist kinkhoest wereldwijd jaarlijks ongeveer 300.000 kinderlevens waarvan het overgrote deel onder de niet gevaccineerde populatie. Kinkhoestvaccins hebben bewezen betrekkelijk veilig en effectief te zijn. In de meeste landen bestaat kinkhoestvaccinatie uit een primaire serie van drie immunisaties, startend tussen de 6 weken en 3 maanden na geboorte, gevolgd door een vierde immunisatie wanneer de kinderen ongeveer 1 jaar oud zijn. In sommige landen wordt ook op latere leeftijd (tussen de 4 en 6 jaar) nog een vijfde immunisatie gegeven. Ondanks een hoge vaccinatiegraad komt kinkhoest nog steeds relatief vaak voor. Een van de mogelijke oorzaken hiervan is dat kinkhoestvaccinatie maar voor een beperkte tijd bescherming biedt en dat op op latere leeftijd dus niet meer voldoende immuniteit aanwezig is om infectie te voorkomen. Deze redenering is consistent met de observatie dat kinkhoest tegenwoordig steeds vaker gevonden wordt bij oudere kinderen en volwassenen. Aanpassingen van huidige a-cellulaire kinkhoestvaccins, bijvoorbeeld door het toevoegen van extra antigenen, zou tot verbetering van de effectiviteit kunnen leiden, maar het is twijfelachtig of deze aanpassingen ook zullen leiden tot een langere beschermingsduur. Het is wellicht interessanter om te kijken naar de mogelijkheid van het toevoegen van LPS analogen. Zoals we in **hoofdstuk 7** hebben laten zien leidt deze strategie tot het opwekken van een ander soort immuniteit: Th1 immuniteit in plaats van Th2 immuniteit. In het achterhoofd houdende dat een natuurlijke kinkhoestinfectie wel voor langere tijd bescherming biedt en dat natuurlijke infectie ook Th1 immuniteit opwekt, zou dit kunnen betekenen dat het toevoegen van LPS analogen aan a-cellulaire kinkhoestvaccins niet alleen, zoals we hier hebben laten zien, de effectiviteit verhoogd, maar wellicht ook langer bescherming biedt. Dergelijke aanpassingen zullen echter niet alle problemen van de a-cellulaire kinkhoestvaccins, bijvoorbeeld de hoge productiekosten, oplossen. Daarom zullen ook

andere mogelijkheden onderzocht moeten worden. Een flink aantal wetenschappers is op dit moment bezig met het ontwikkelen van verbeterde kinkhoestvaccins. Hierbij wordt onder andere gewerkt aan geattenueerde pertussis vaccins, welke bestaan uit verzwakte, maar levende kinkhoestbacteriën, en vaccins gebaseerd op DNA. In dit proefschrift hebben we laten zien dat het aanpassen van de LPS samenstelling ook een goede strategie kan zijn om cellulaire kinkhoestvaccins te verbeteren. Het is op dit moment echter te vroeg om te zeggen op welke manier dit dan het beste kan. Het meest veelbelovend lijkt op dit moment de optie van het toevoegen van LPS antagonisten, zoals bijvoorbeeld LpxL2 LPS. We hebben laten zien dat deze strategie leidt tot een effectiever vaccin dat minder toxisch is. Het is echter wel denkbaar dat het toevoegen van extra LPS aan kinkhoestvaccins zal stuiten op bezwaren met betrekking tot de registreerbaarheid van een dergelijk vaccin, aangezien LPS wordt gezien als een toxisch bestanddeel en het dus raar is om iets wat in principe toxisch is toe te voegen aan een vaccin dat je minder toxisch wilt maken. Het is daarom wellicht interessanter het gebruik van bijvoorbeeld de PagL-producerende stam te overwegen. Daarnaast is het belangrijk door te gaan met het testen van andere *B. pertussis* stammen, bijvoorbeeld de suikermutanten en de *lpxL1* mutant, andere LPS-modificerende enzymen, andere LPS mutaties en andere soorten adjuvantia. Niettemin zal het introduceren van nieuwe kinkhoestvaccins nog een flinke tijd in beslag nemen, onder andere omdat het registreren van nieuwe vaccins erg tijdrovend is. Het is daarom van belang het gebruik van de huidige kinkhoestvaccins verder te optimaliseren. Hierbij zou onder andere het invoeren van (vrijwillige) kinkhoestvaccinatie voor pubers en volwassenen of voor zwangere vrouwen (factoren die bescherming bieden tegen kinkhoest kunnen van moeder op kind worden overgedragen via de placenta) overwogen kunnen worden. Verder zou het wellicht mogelijk zijn om zéér kort na geboorte al een eerst kinkhoestvaccinatie te geven.

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Ria T. (nog bedankt voor de leuke baby spulletjes), Ria K., Robin, Ronald, Stefanie, Virginie en Wieke (die kip in mosterd-dragon saus eet ik af en toe nog steeds). Verder zijn er natuurlijk ook nog een aantal collega's die inmiddels vertrokken zijn: de pokeraars Boris en Peter B. (die 5 euro biljetjes hadden toch ook wel wat), Bouke, Basssie (was gezellig toen in Groningen), Chechu, Cécile-Marie, Jeroentje (wanneer gaan we nou vissen?), Romé (thanx for the supervision on the Omp85 project) en Viviane. Allen bedankt! Naast de collegae van West-4 zijn er binnen het Kruyt nog een aantal andere mensen die ik graag wil bedanken. Ten eerste Lucy. Ik vond het erg fijn om met je samen te werken. Ik denk dat hoofdstuk 3 van mijn proefschrift een mooi voorbeeld is hoe een goede samenwerking tot een uitstekend resultaat kan leiden. Ik wens je alle succes toe in je verdere carrière en ik hoop je zeker nog eens tegen te komen. Hiernaast gaat mijn dank ook uit naar Adri Thomas (we hebben elkaar -gelukkig maar- niet al te vaak gezien, maar de keren dat ik je gesproken heb bevielen de mentorgesprekken prima), Suat (ondanks dat de NMR experimenten uiteindelijk niet het gewenste resultaat opgeleverd hebben vond ik onze discussies altijd erg boeiend), Mohamed, Kees Kruithof en Birgit Wieczorek (nog bedankt voor de zoektocht naar *in vitro* PagL substraten, al hebben we deze nooit gevonden), Maarten Egmond en Piet Gros (bedankt voor de discussies), de dames van het secretariaat, Ton en Cor, de afdeling beeldverwerking en vormgeving en natuurlijk de mensen van de bacteriologische keuken. Zoals ik zei ben ik werkzaam geweest op meerdere labs. Na 2,5 jaar universiteit heb ik mijn spullen gepakt en ben ik verhuisd naar de afdeling VO van het Nederlands Vaccin Instituut. Voor deze tijd was ik hier ook al sporadisch aanwezig, maar mijn bezoekjes waren over het algemeen van korte duur waardoor sommige mensen zich wellicht afvroegen wie die jongen nou eigenlijk was en wat die nou toch steeds kwam doen? Nadat ik definitief in Bilthoven gevestigd was leerden we elkaar pas wat beter kennen en ontdekte ik dat het NVI een zeer fijne werkomgeving biedt, ondanks dat het af en toe toch wel heel anders was dan wat ik op de universiteit gewend was. Uiteraard zijn er hier ook een hoop mensen die ik persoonlijk wil bedanken. Ten eerste Hendrik-Jan en Betsy. Dankzij jullie grote labervaring zijn jullie van enorme waarde geweest bij het uitvoeren van mijn soms toch iets wat uitgebreide experimenten. Jullie zijn allebei toppertjes! Daarnaast wil ik ook Jan en Alex bedanken voor het uitvoeren van de MS analyses. Zoals jullie zien staan jullie namen boven een hoop hoofdstukken wat jullie inbreng nogmaals onderstreept. Grote dank gaat ook uit naar Wilma. Dankzij haar tips en vooral ook grote inspanning ziet dit boekje er weer helemaal gelikt uit. Ook de rest van de VO mensen wil ik bedanken en ook hier doe ik het weer op alfabet: Cécile (bedankt voor het beantwoorden van al mijn immunologische vragen), Claire, Elvira, Ernst, Floris (onze eigen assistent-

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Bilthoven, 15 februari 2007

Curriculum Vitae

Jeroen Geurtsen was born on June 23rd 1980 in Utrecht, the Netherlands. After finishing high school (VWO) at the Oosterlicht College in Nieuwegein in 1998, he studied Biology at Utrecht University. During this study, he completed two internships. The first one in the Molecular Microbiology group of prof. dr. Jan Tommassen, where he studied the function of an evolutionary conserved outer-membrane protein of *Neisseria meningitidis*, i.e., Omp85 (12 months), and the other one in the Medical Microbiology group of prof. dr. Ben Berkhout in the Amsterdam Medical Centre (AMC), University of Amsterdam, where he investigated the role of RNA interactions in the translation initiation of the human immuno-deficiency virus GAG polypeptide (6 months). After receiving the Master of Science degree in September 2002, he started as a PhD student on a collaborative project between the Department of Molecular Microbiology of the faculty of Biology of Utrecht University, the Netherlands under the supervision of prof. dr. Jan Tommassen and the Unit Research and Development of the Netherlands Vaccine Institute in Bilthoven, the Netherlands under the supervision of dr. Peter van der Ley. As of January 1st 2007, he is working as a post-doc in the same groups for a short period and as of early spring 2007, he will start working as a post-doc at the Free University of Amsterdam in the Medical Microbiology group of dr. Ben Appelmelk on an EU-granted project, which is aimed at developing an improved vaccine against *Mycobacterium tuberculosis*.

List of Publications and Patents

Publications

1. **Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M., and Tommassen, J.** (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299:** 262-265.

2. **Bos, M. P., Tefsen, B., Geurtsen, J., and Tommassen, J.** (2004) Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. *Proc. Natl. Acad. Sci. U S A* **101:** 9417-9422.

3. **Tefsen, B., Geurtsen, J., Beckers, F., Tommassen, J., and de Cock, H.** (2005) Lipopolysaccharide transport to the bacterial outer membrane in spheroplasts. *J. Biol. Chem.* **280:** 4504-4509.

4. **Geurtsen, J., Steeghs, L., ten Hove, J., van der Ley, P., and Tommassen, J.** (2005) Dissemination of lipid A deacylases (pagL) among gram-negative bacteria: identification of active-site histidine and serine residues. *J. Biol. Chem.* **280:** 8248-8259.

5. **Rutten, L.*, Geurtsen, J.*, Lambert, W., Smolenaers, J. J., Bonvin, A. M., de Haan, A., van der Ley, P., Egmond, M. R., Gros, P., and Tommassen, J.** (2006) Crystal structure and catalytic mechanism of the LPS 3-*O*-deacylase PagL from *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U S A* **103:** 7071-7076.
   *L.R. and J.G. equally centributed to this work

6. **Geurtsen, J., Steeghs, L., Hamstra, H-J., ten Hove, J., de Haan, A., Kuipers, B., Tommassen, J., and van der Ley, P.** (2006) Expression of LPS-modifying enzymes PagP and PagL modulates the endotoxic activity of *Bordetella pertussis*. *Infect. Immun.* **74:** 5574-5585.

7. **Geurtsen, J., Vandebriel, R. J., Gremmer, E. R., Kuipers, B., Tommassen, J., and van der Ley, P.** (2007) Consequences of the expression of lipopolysaccharide-modifying enzymes for the efficacy and reactogenicity of whole-cell pertussis vaccines. *Submitted for publication*.

8. **Geurtsen, J., Dzieciatkowska, M., Steeghs, L., Boleij, A., Broen, K., Hamstra, H-J., Li, J., Richards, J., Tommassen, J., and van der Ley, P.** (2007) Gene cluster involved in lipopolysaccharide-core biosynthesis and identification of a novel lipid A modification in *Bordetella pertussis*. *Submitted for publication.*

9. **Geurtsen, J., Banus, S. A., Gremmer, E. R., Ferguson, H., Vermeulen, J. P., Dormans, J. A. M. A., Tommassen, J., van der Ley, P., Mooi, F. R., and Vandebriel, R. J.** (2007) Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccines and reduce type-I hypersensitivity. *Submitted for publication.*

10. **Geurtsen, J., Fransen, F., Vandebriel, R. J., Gremmer, E. R., de la Fonteyne-Blankestijn, L. J. J., Kuipers, B., Tommassen, J., and van der Ley, P.** (2007) Supplementation of whole-cell pertussis vaccines with lipopolysaccharide analogs: a novel strategy for modulating vaccine efficacy and reactogenicity. *Submitted for publication.*

11. **Geurtsen, J., Angevaare, E., Janssen, M., Hamstra, H-J., ten Hove, J., de Haan, A., Kuipers, B., Tommassen, J., and van der Ley, P.** (2007) Identification and functional characterization of *Bordetella pertussis lpxL* homologues. *Submitted for publication*.

Patents

1. **Geurtsen, J., van der Ley, P., and Tommassen, J.** Deacylation of lipopolysaccharide in Gram-negative bacteria. Patent no.: *P218195EP*.

2. **Geurtsen, J., van der Ley, P., and Tommassen, J.** Improved vaccines against *Bordetella pertussis* based on lipopolysaccharide-glycosyltransferase mutants. Patent no.: *P6014031EP.*

Colour figures

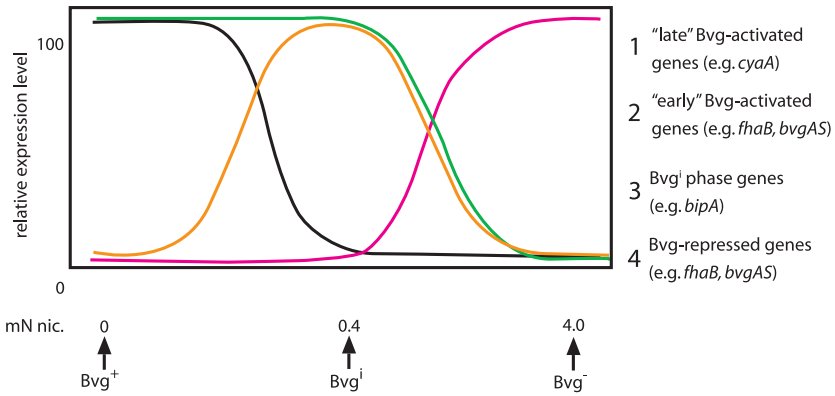

Fig. 2 Expression curves for the four classes of genes regulated by BvgAS. Genes expressed maximally in the Bvg$^+$ phase (such as *cyaA*) are referred to as "late" Bvg-activated genes and are represented by the black curve (curve 1). Genes that are expressed maximally under both Bvg$^+$ and Bvg^i phase conditions (such as *fhaB*) are referred to as "early" Bvg-activated genes and are represented by the green curve (curve 2). Genes expressed maximally only under Bvg^i phase conditions (such as *bipA*) are represented by the gold curve (curve 3). Finally, genes that are repressed by BvgAS and expressed maximally only under Bvg$^-$ phase conditions are represented by the red curve (curve 4). Abbreviation: nic, nicotinic acid. Adapted from Mattoo and Cherry, 2005.

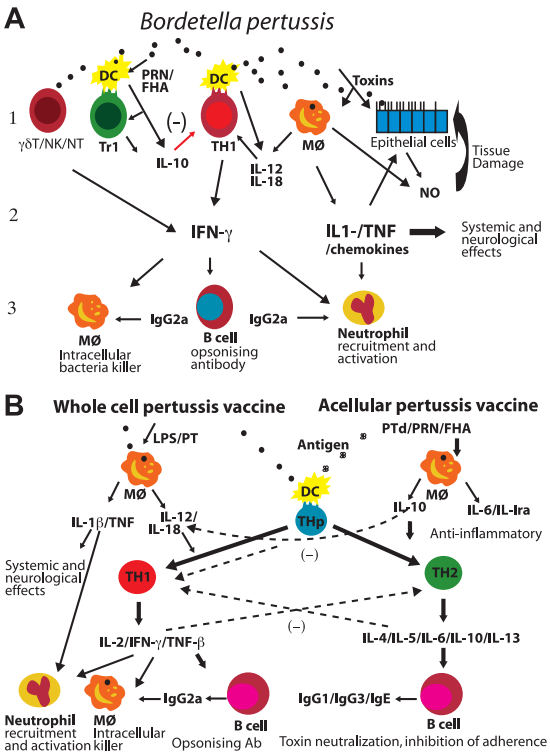

Fig. 4 (A) Immunity to *B. pertussis* in a naïve host: evidence from the murine respiratory challenge model. (1) Recognition of bacterial components by cells of the innate and acquired immune system, (2) Production of soluble mediators and (3) recruitment and activation of effector cells and molecules. When *B. pertussis* enters the respiratory tract, the bacteria bind to ciliated epithelial cells, but are also recognised and taken up by cells of the innate immune system, such as macrophages (Mϕ), dendritic cells (DCs), γδ T cells, natural killer (NK) or natural T cells (NT). DCs process and present bacterial antigens to T cells. The production of IL-12 and IL-18 by innate cells results in polarisation of the T-cell response to the Th1 subtype. However, early in infection the local Th1 response is suppressed due to the effects of IL-10 secreted by antigen-stimulated Tr1 cells or by FHA-stimulated macrophages and DCs. Nitric oxide (NO) and the pro-inflammatory cytokines, IL-1β and TNF-α induced by bacterial toxins, especially LPS, tracheal colonisation factor (TCT) and pertussis toxin (PT), as well as contributing to bacterial elimination, also mediate local lung pathology and are responsible for many of the systemic and neurological consequences of the infection. IFN-γ secreted early in infection by cells of the innate immune system, and later in infection by Th1 cells, stimulates recruitment and activation of macrophages and neutrophils and provides help for B cells to secrete opsonising and complement-fixing antibody (IgG2a in the mouse). Opsonised or non-opsonised bacteria are taken up by neutrophils and macrophages, which are killed by NO or reactive oxygen intermediates. (B) Distinct mechanisms of immunity induced with whole cell and acellular vaccines: a central role of Th1 and Th2 cells in regulation of the acquired immune response. wP include residual bacterial toxins, such as LPS, which activates IL-1β, TNF-α, IL-12 and IL-18 production by macrophages and other cells of the innate immune system. These pro-inflammatory cytokines regulate the selective induction of Th1 cells from the precursor T cell (Thp), but also contribute to the side effects of immunisation with wP. Cytokines secreted by Th1 cells, especially IFN-γ, provide help for opsonising antibody production and activate macrophages and neutrophils to take up and kill intracellular bacteria. In contrast, aP are devoid of bacterial toxins that stimulate IL-12 and IL-18, but include components such as FHA, which stimulate IL-10 production, and consequently have anti-inflammatory activity and preferentially induce Th2 cells. Th2 cells provide help for B cells to secrete IgE and murine IgG1, IgG3 antibodies, which neutralise toxins and prevent adherence of bacteria in the respiratory tract. Adapted from Mills *et al.*, 2001.

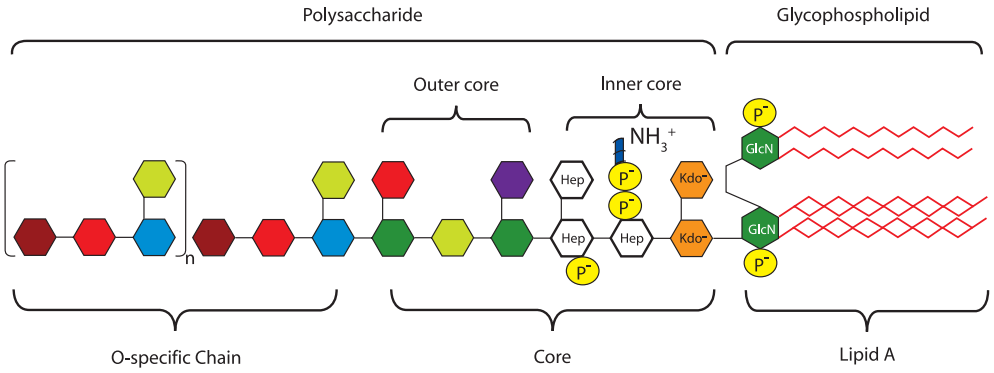

Fig. 5 Schematic representation of the chemical structure of enterobacterial LPS. Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; P, phosphate; EtN, ethanolamine; zigzag lines, fatty acids. Adapted from Caroff *et al.*, 2002.

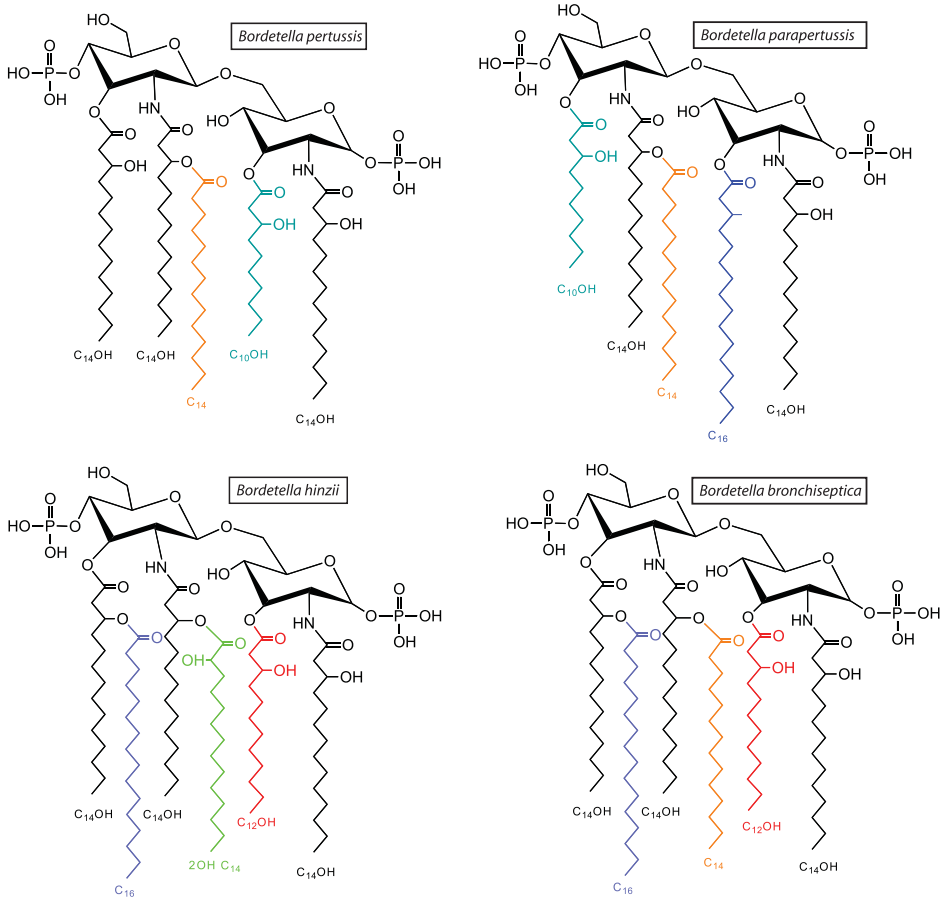

Fig. 7 Examples of lipid A structures from different *Bordetella* strains. Each colour indicates a specific fatty acid. Adapted from Caroff *et al.*, 2002.

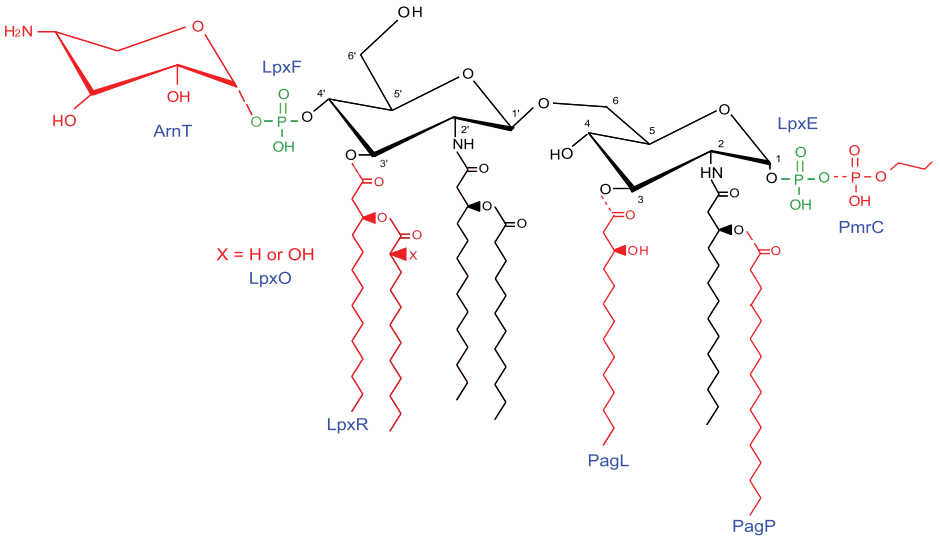

Fig. 9 Regulated modifications of lipid A. LpxE and LpxF function as 1- and 4'-phosphatases, respectively. Substitution of the phosphate moieties with L-Ara4N or pEtN is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary palmitoyl chain at the 2-position by PagP, and the removal of the acyl chain moieties at the 3- and 3' position by PagL and LpxR, respectively.

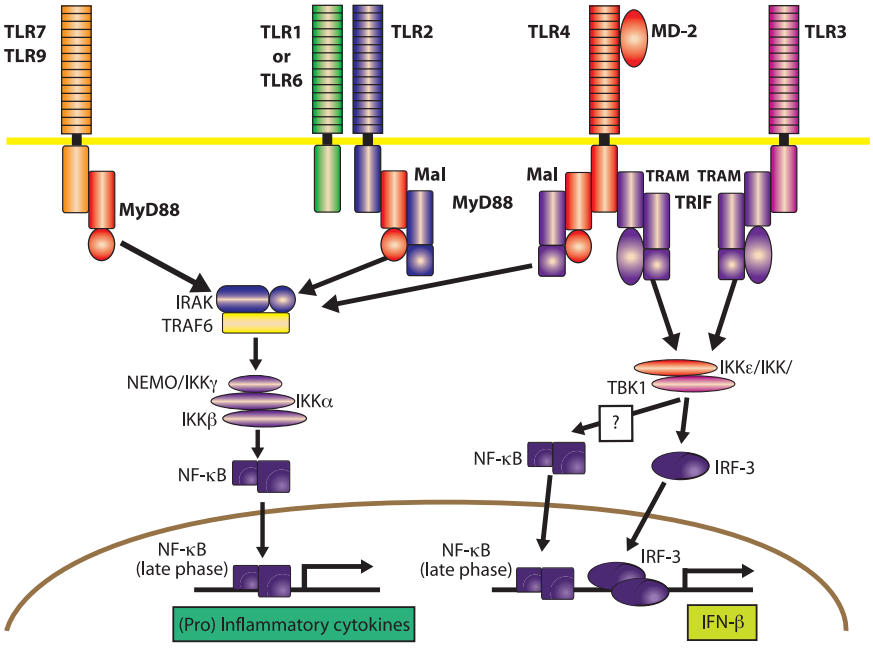

Fig. 10 TIR domain-containing adaptors and TLR signalling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via the majority of TLRs. Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In the TLR4- and TLR3-mediated signalling pathways, a MyD88-independent pathway exists that leads to activation of IRF-3 via TBK1 and IKKε/IKK*i*. The TIR domain-containing adaptor TRIF and the TRIF-related adapter molecule TRAM mediate this MyD88-independent pathway. Adapted from Takeda an Akira, 2004.

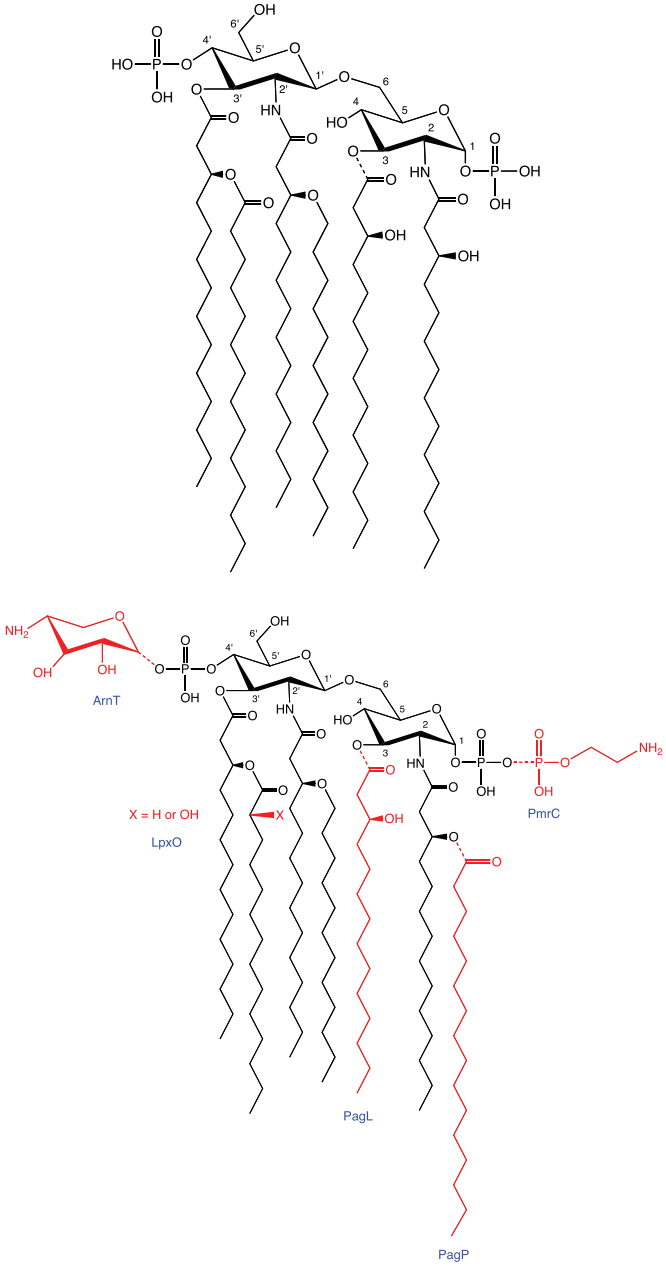

Fig. 1 Lipid A architecture. (A) *E. coli* lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with four *R*-3-hydroxymyristoyl moieties, of which the 2'- and 3'-fatty-acyl chains are esterified with laurate and myristate, respectively. (B) regulated modifications of *Salmonella* lipid A. Substitution of the phosphate moieties with L-Ara4N or phosphoethanolamine is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary palmitoyl chain at the 2 position by PagP, and the removal of the 3-hydroxymyristoyl moiety at the 3 position by PagL are shown.

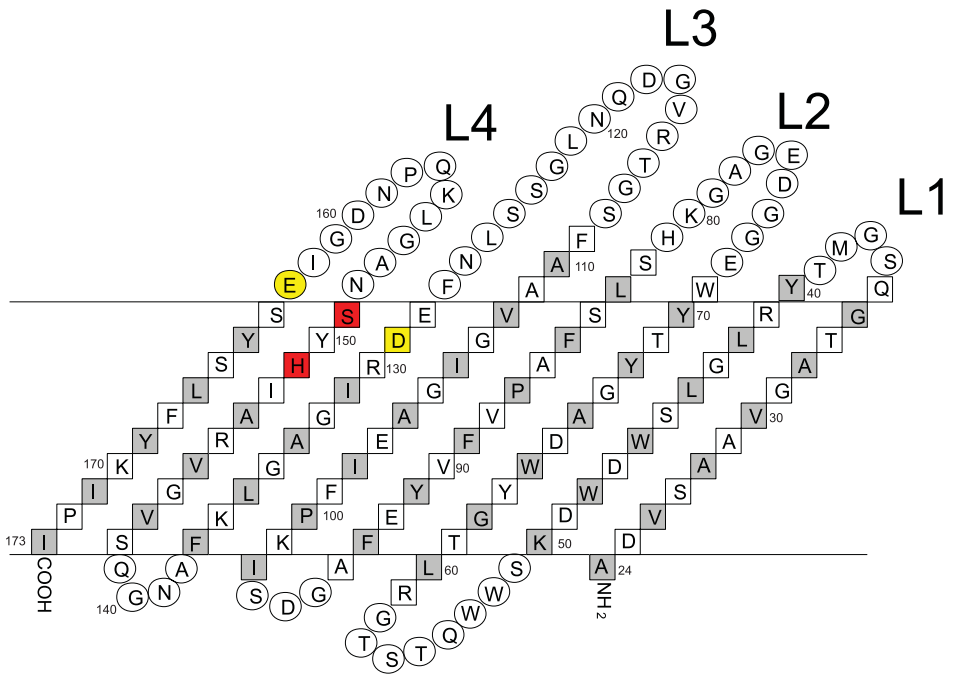

Fig. 8 Topology model for PagL from *P. aeruginosa*. A model for the topology of $PagL_{(Pa)}$ was constructed using the general rules of outer membrane protein architecture as described by Jacobs *et al*. (2003). The proposed model consists of an eight-stranded β-barrel with four loops (L1–4) extending into the external environment. Residues in the postulated β-strands are shown in diamonds, which are shaded for residues that are exposed to the lipid bilayers. His-149 and Ser-151 (marked in red; position in the $PagL_{(Pa)}$ precursor) are absolutely conserved (Fig. 2) and are suggested to be part of a classical catalytic triad of a serine hydrolase. Potential candidates for the acidic residue of the catalytic triad are indicated in yellow. Numbers refer to the positions of the residues in the precursor sequence.

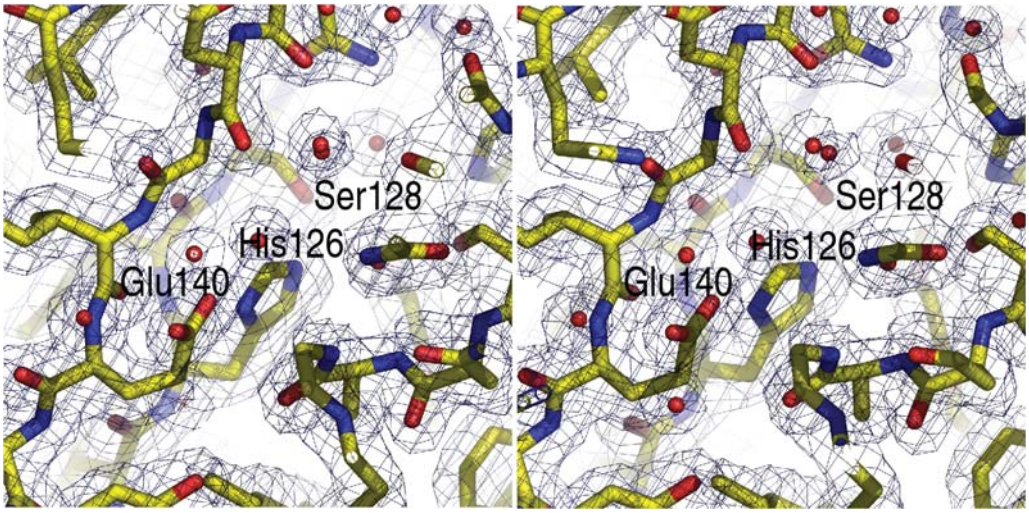

Fig. 1. Stereoview of the catalytic site with the final $2F_o$-F_c electron density map, at 2.0 Å resolution and contoured at 1σ, shown as chicken wire. Protein is shown as sticks. Carbon atoms are shown in yellow, oxygen in red and nitrogen in blue. Catalytic triad residues are labelled.

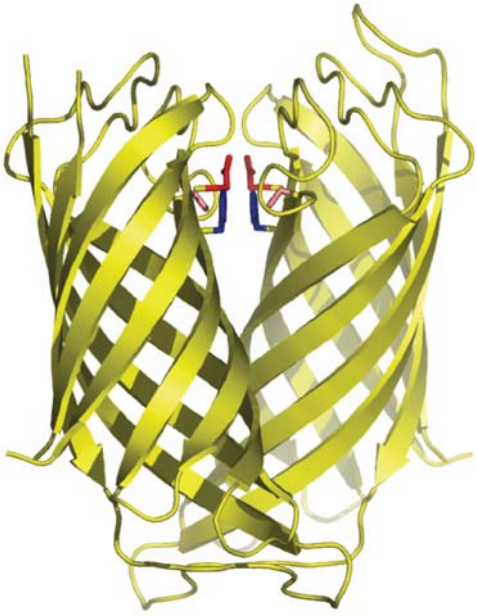

Fig. 4. PagL dimer in the crystal structure. The active-site residues Ser-128, His-126, and Glu-140 are coloured pink, blue, and red, respectively.

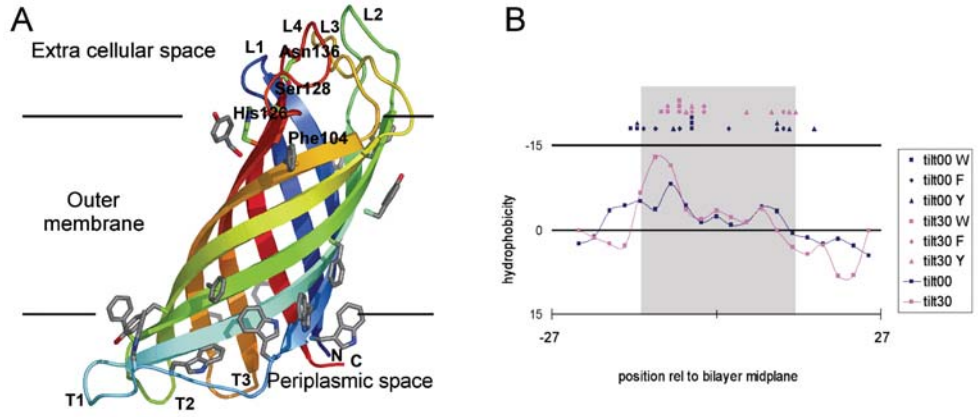

Fig. 5. PagL structure and membrane orientation. (A) Ribbon representation of PagL. The N and C termini are labelled and coloured blue and red, respectively, with gradient colours in between. The four extracellular loops are labelled L1–L4, and the three periplasmic turns are labelled T1–T3. Aromatic residues located at the presumed membrane boundaries are shown in gray, with nitrogen and oxygen atoms shown in blue and red, respectively. The only four completely conserved residues among PagL homologs are labelled. (B) Hydrophobicity profiles for the outward-facing PagL residues as a function of membrane position (periplasmic side left, extracellular side right) are shown as solid lines. Negative $\Sigma(\Delta G)$ values indicate regions that are more hydrophobic. The blue line and symbols present results for the positions with the β-barrel axis aligned along the membrane normal, whereas the magenta line and symbols are for the protein tilted by 30°. The symbols represent the Cγ positions of Trp (squares), Tyr (circles), and Phe (triangles) residues that form the inner and outer aromatic girdles. The image shown in (A) was prepared with PYMOL (www.pymol.org).

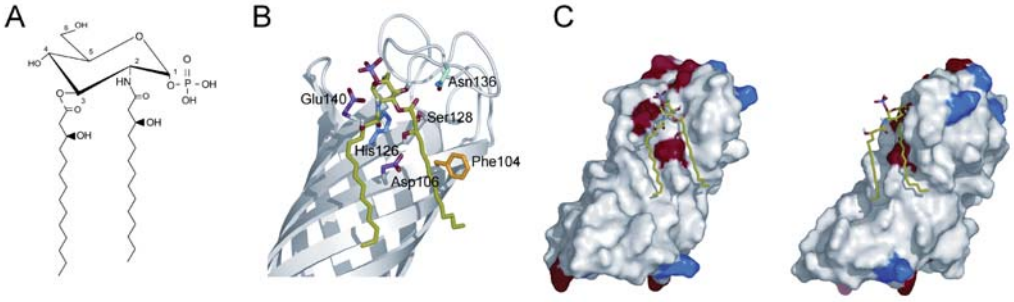

Fig. 8. Modelling of lipid X onto the active site of PagL. (A) Schematic representation of lipid X. (B) Lipid X modelled onto the active site of PagL. PagL is represented as a gray ribbon diagram. Lipid X is shown as green sticks with oxygen atoms in red and a phosphate atom in magenta. The hydrogen atoms from hydroxyl groups are shown in gray. Some amino acid residues important for PagL activity are shown as sticks and are labelled. (C) Two views (~90° rotated) of the electrostatic surface potential of PagL with lipid X. Positively and negatively charged residues are coloured blue and red, respectively. Lipid X is shown as green sticks. The images in *B* and *C* were prepared with PYMOL (www.pymol.org).

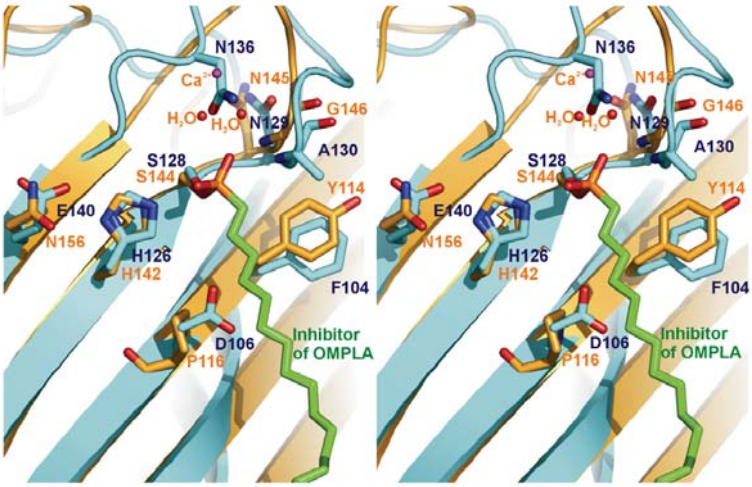

Fig. 9. Stereo diagram of the active site Ser-128 and His-126 of PagL superposed on the active site Ser-144 and His-142 of OMPLA. PagL is shown in cyan, whereas OMPLA is represented in orange. Residues and atoms that may have an important role for activity are shown as sticks and are labelled with cyan and orange text for PagL and OMPLA, respectively. The hexadecanesulfonyl moiety of an OMPLA inhibitor is covalently attached to Ser-144 of OMPLA and coloured green. The image was prepared by using PYMOL (www.pymol.org).

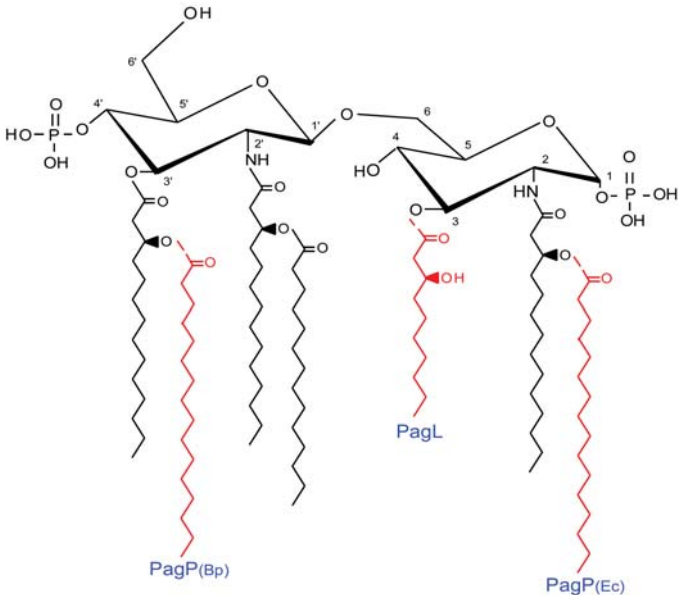

Fig. 1. *B. pertussis* lipid A architecture and modification by PagP and PagL. *B. pertussis* lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2' fatty acyl chain is esterified with myristate. *B. pertussis* PagP (PagP$_{(Bp)}$) and *E. coli* PagP (PagP$_{(Ec)}$) add a secondary palmitoyl chain at the 3' and 2 positions, respectively. Removal of the 3-hydroxydecanoic acid moiety at the 3 position is mediated by PagL.

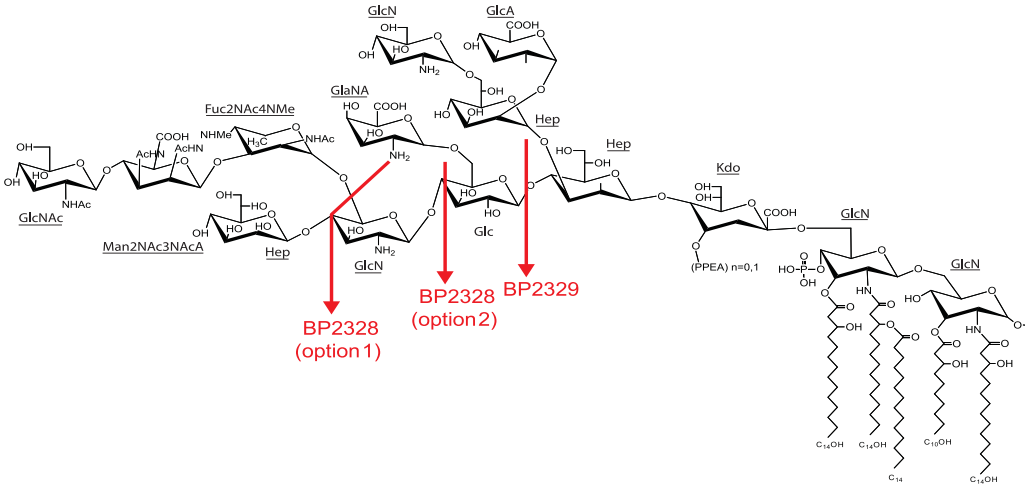

Fig. 7. Structure of *B. pertussis* LPS. Proposed truncated core OS structures of the BP2328- and BP2329-mutant strains are indicated by red arrows. Adapted from Caroff *et al*. (2000).