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

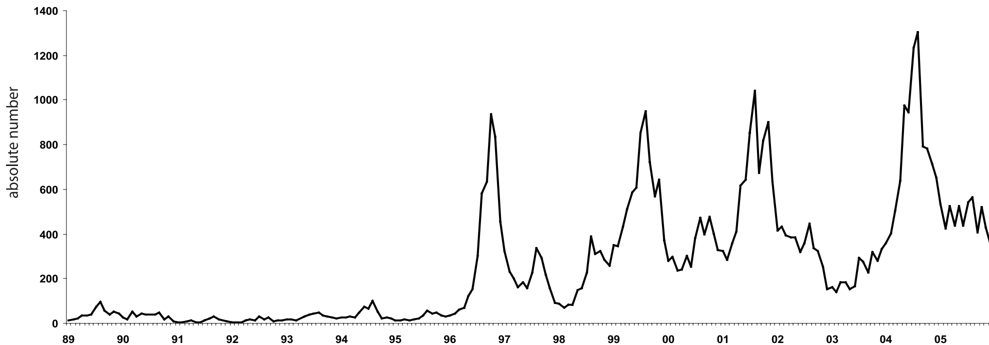

Fig.1 Reported pertussis per month in the Netherlands for the period 1989-2005. Notifications are corrected for the first day of illness (Source: National Institute of Public Health and the Environment).

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] *B. pertussis*	Gene expression[a] *B. bronchiseptica*	Protein expression[a] *B. pertussis*	Protein expression[a] *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˙ 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).

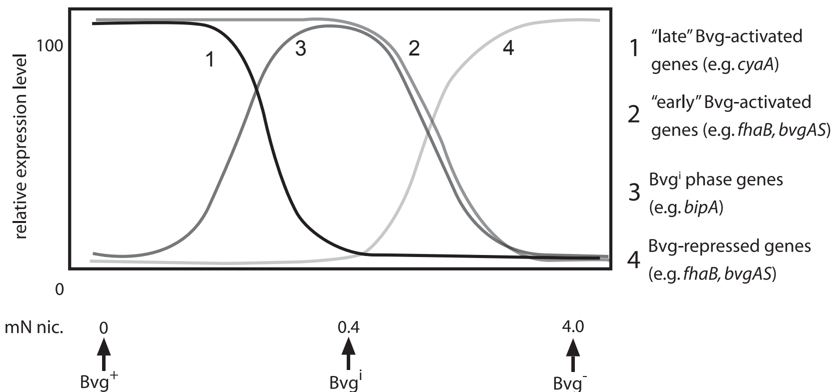

Fig. 2 Expression curves for the four classes of genes regulated by BvgAS. Genes expressed maximally in the Bvg^+ phase (such as *cyaA*) are referred to as "late" Bvg-activated genes and are represented by the black curve (curve 1). Genes that are expressed maximally under both Bvg^+ and Bvg^i phase 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.

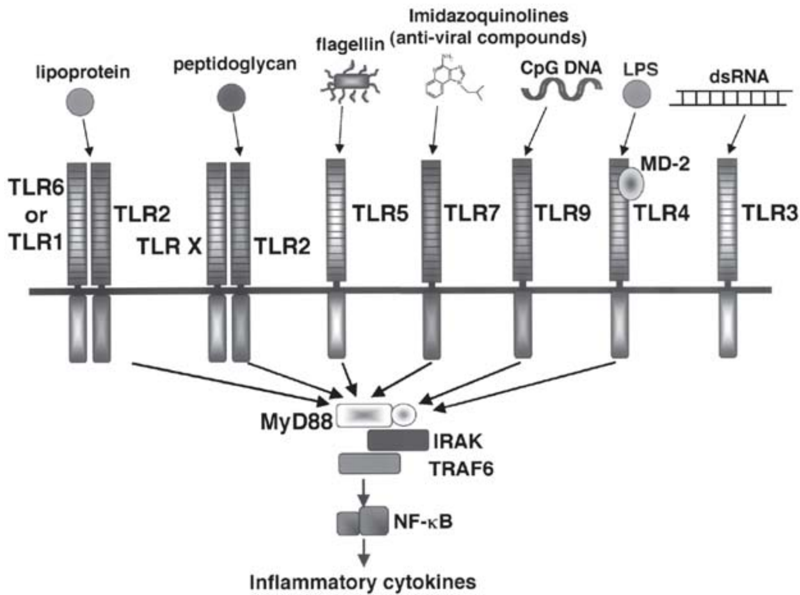

Fig. 3 Summary of ligands recognised by TLR family. The responses to all ligands, except for dsRNA, are dependent on MyD88, as assayed by inflammatory cytokine production. MyD88 does not appear to be involved in dsRNA-dependent TLR3 signalling. X, unknown. Adapted from Akira *et al.*, 2003.

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

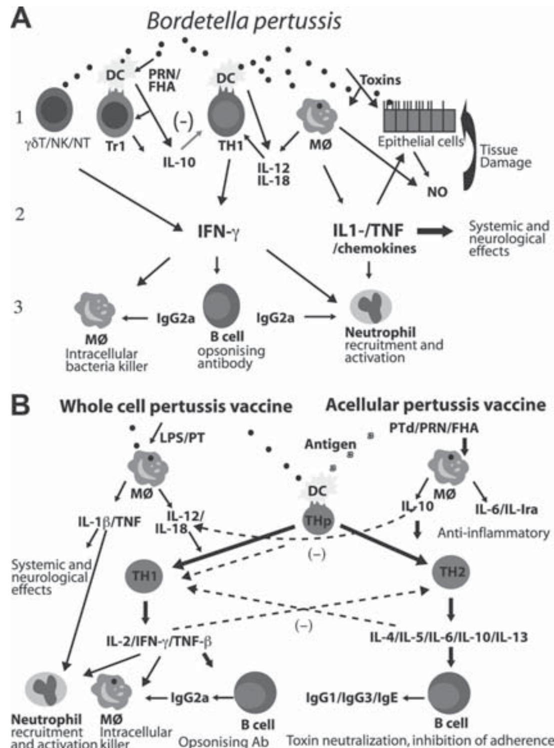

Fig. 4 (A) Immunity to *B. pertussis* in a naïve host: evidence from the murine respiratory challenge model. (1) Recognition of bacterial components by cells of the innate and acquired immune system, (2) Production of soluble mediators and (3) recruitment and activation of effector cells and molecules. 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), $\gamma\delta$ 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,

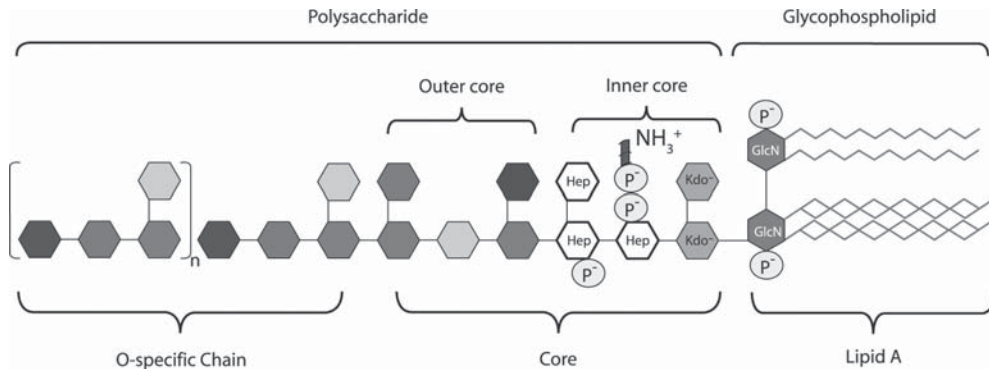

Fig. 5 Schematic representation of the chemical structure of enterobacterial LPS. Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; P, phosphate; EtN, ethanolamine; zigzag lines, fatty acids. Adapted from Caroff *et al.*, 2002.

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).

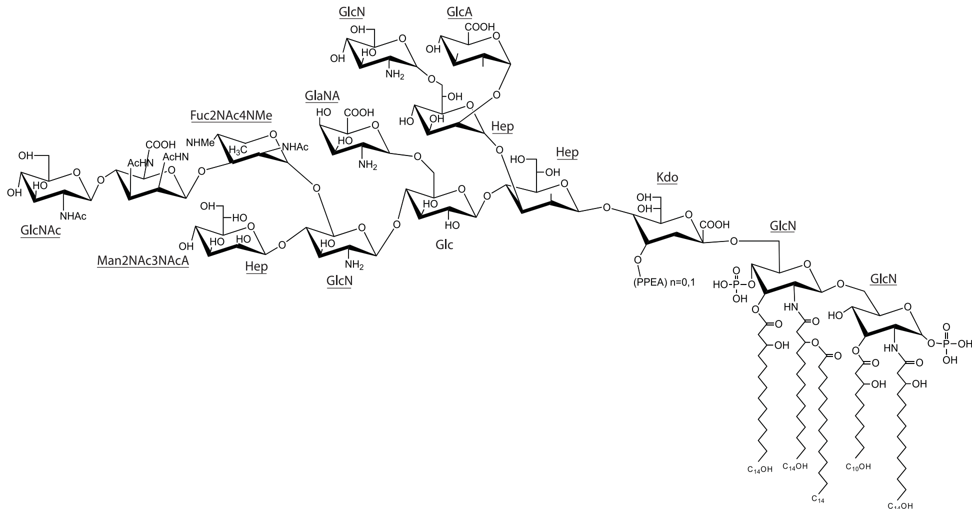

Fig. 6 Structure of the LPS of *B. pertussis* 1414. Abbreviations used: glucose (Glc), L-glycero-D-manno-heptose (Hep), 2-keto-3-deoxyoctulosonic acid (Kdo), glucosamine (GlcN), glucuronic acid (GlcA), *N*-acetyl glucosamine (GlcNAc), 2-acetamido-4-*N*-methyl-2,4-dideoxy-fucose (Fuc2NAc4NMe), 2,3-diacetamido-2,3-dideoxy-mannuronic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

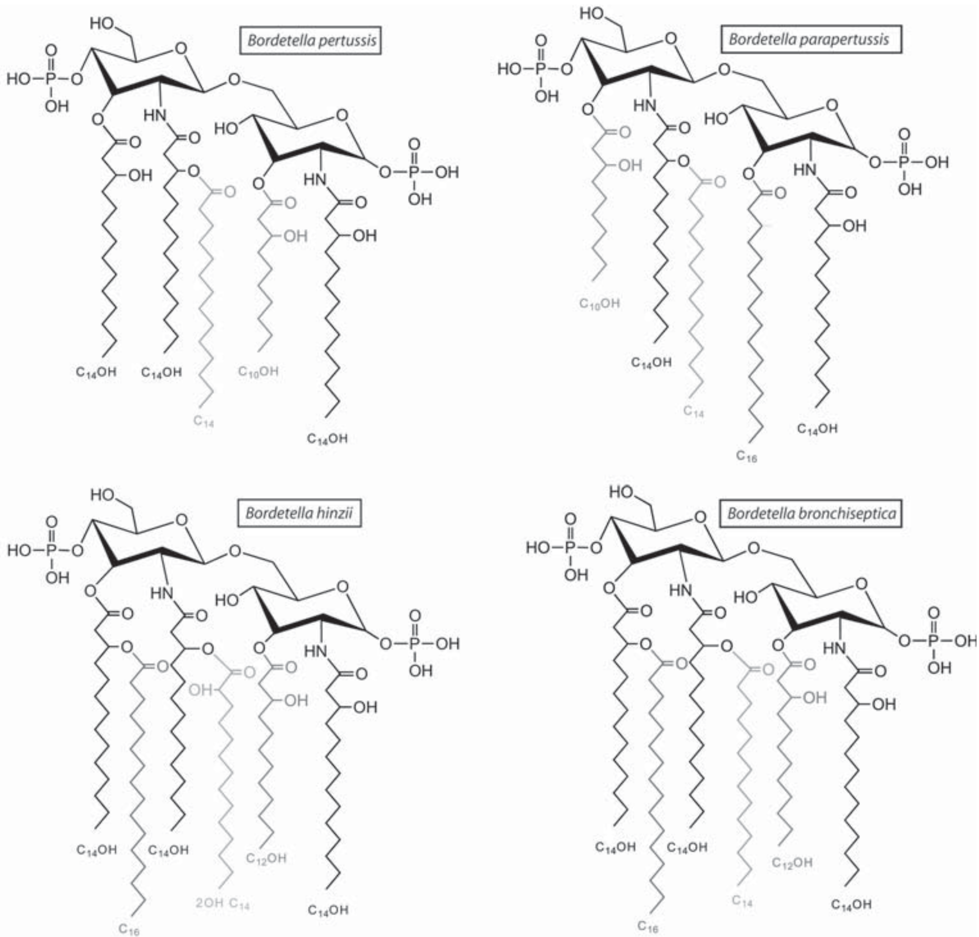

Fig. 7 Examples of lipid A structures from different *Bordetella* strains. Each colour indicates a specific fatty acid. Adapted from Caroff *et al.*, 2002.

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

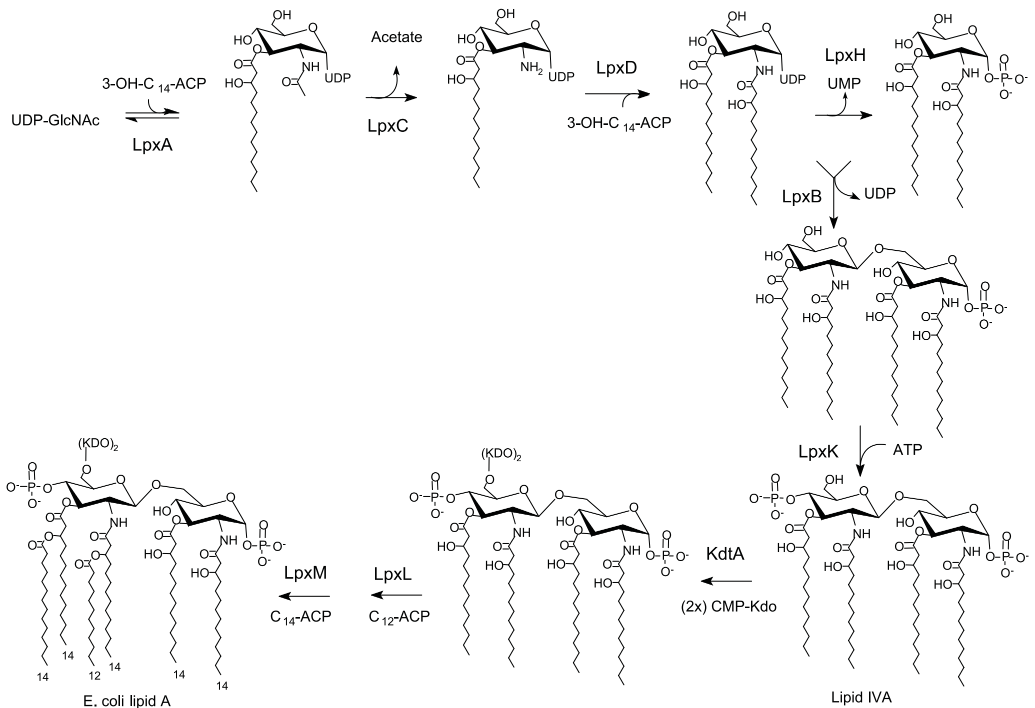

Fig. 8 Lipid A biosynthesis route of *E. coli*.

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

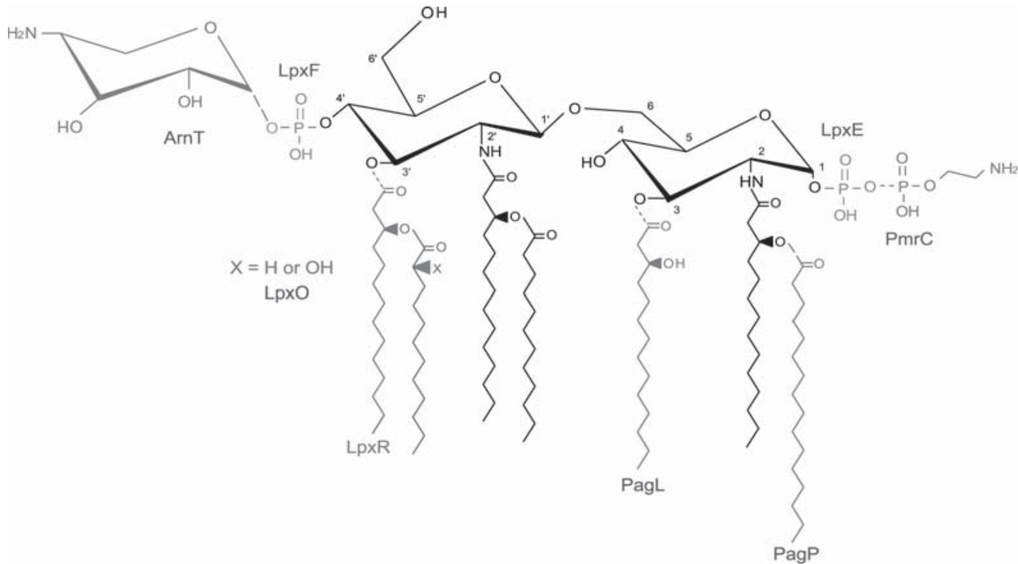

Fig. 9 Regulated modifications of lipid A. LpxE and LpxF function as 1- and 4'-phosphatases, respectively. Substitution of the phosphate moieties with L-Ara4N or pEtN is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary 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).

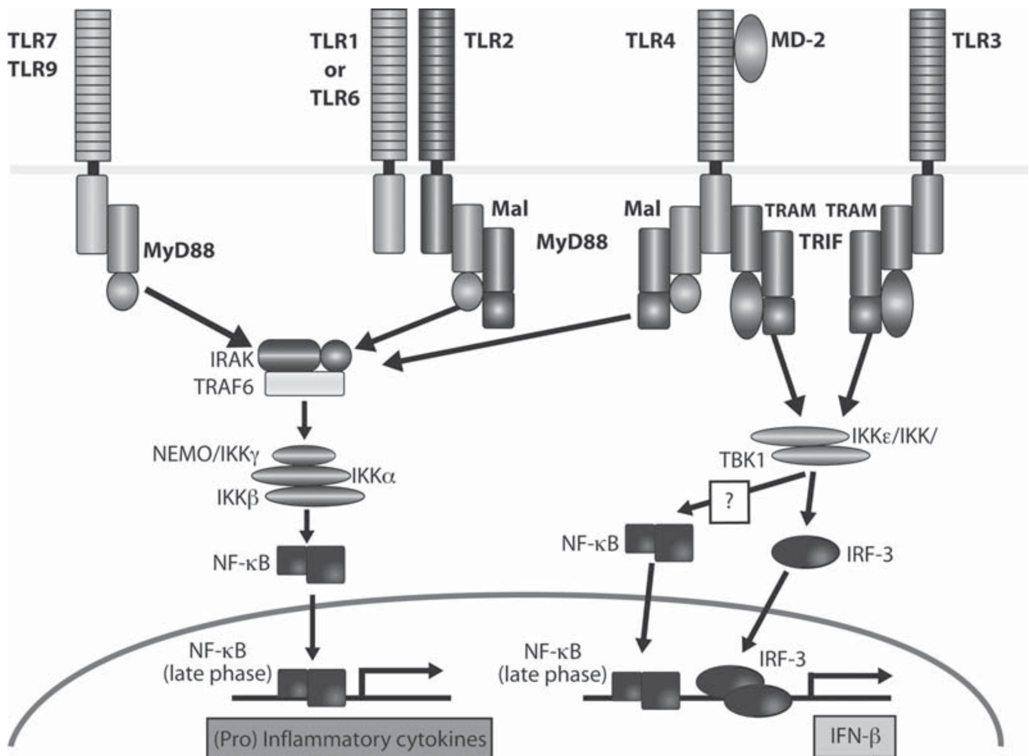

Fig. 10 TIR domain-containing adaptors and TLR signalling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via the majority of TLRs. Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. 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°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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