Chapter 4

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

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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).

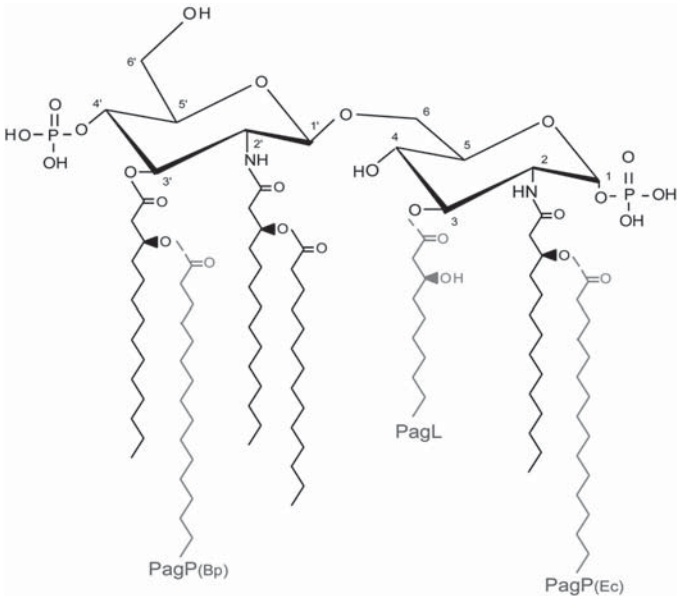

Fig. 1. *B. pertussis* lipid A architecture and modification by PagP and PagL. *B. pertussis* lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2' fatty acyl chain is 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

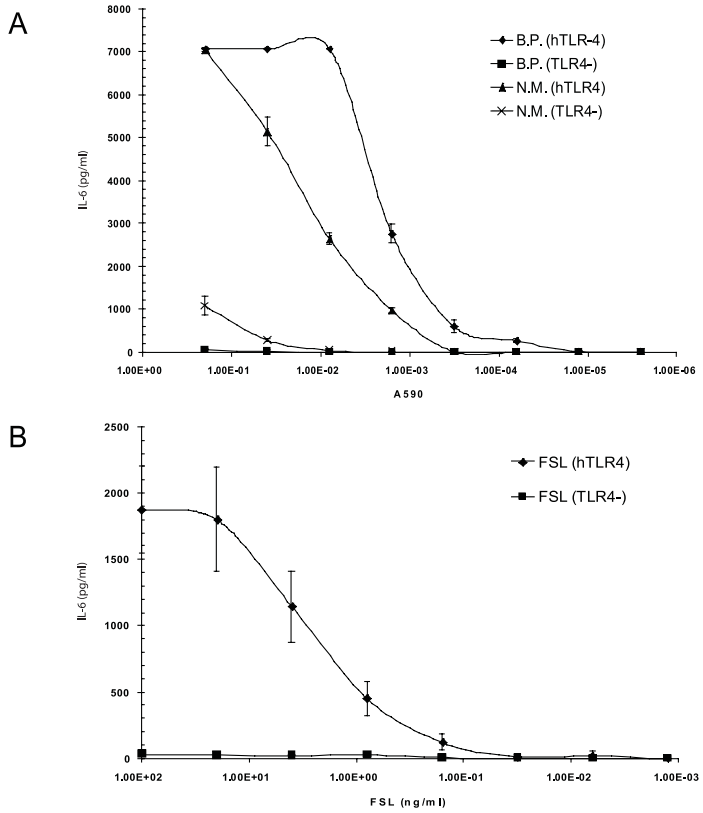

Fig. 2. Contribution of LPS to the endotoxic activity of whole *B. pertussis* cells. Stimulation of IL-6 production by (A) wild-type, whole *B. pertussis* cells (B.P.) or LPS-deficient *N. meningitidis* cells (N.M.) or by (B) the TLR2/6 ligand FSL was tested with the TLR4-deficient mouse macrophage cell 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).

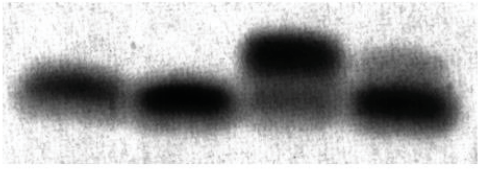

Fig. 3. Analysis by Tricine-SDS-PAGE of *in vivo* LPS modification. LPS was isolated from wild-type (WT) and PagP- or PagL-expressing *B. pertussis* strain Tohama and analysed by Tricine-SDS-PAGE.

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

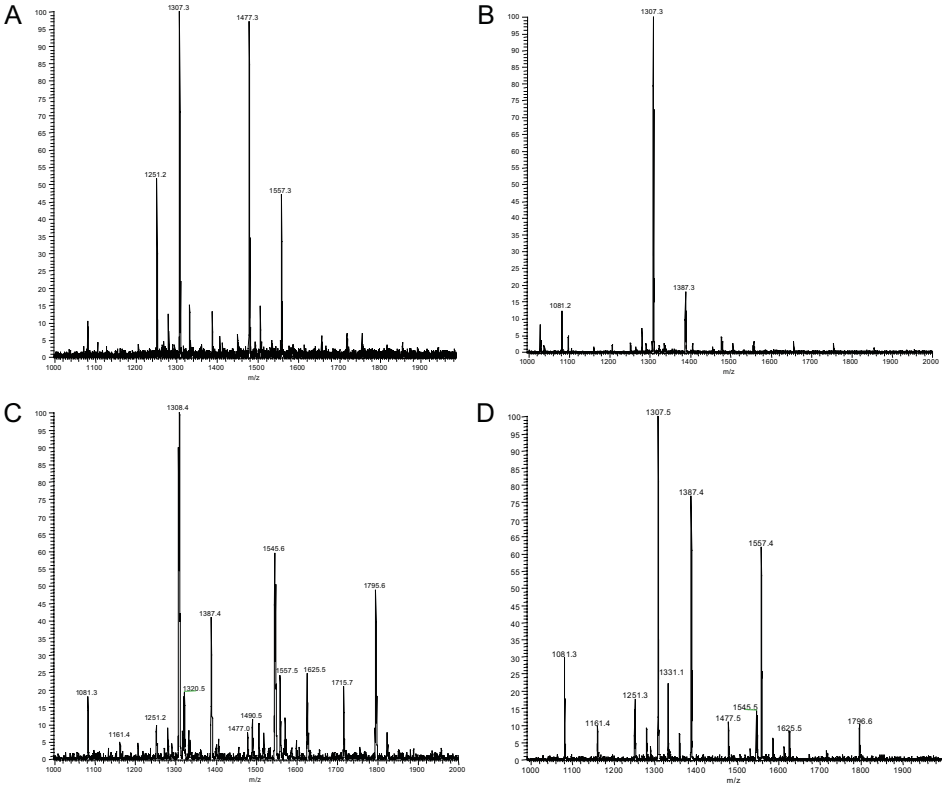

Fig. 4. Structural analysis by ESI-MS of purified *B. pertussis* LPS. Lipid A species from wild-type *B. pertussis* strain Tohama (A) and its derivatives expressing $PagL_{(Bb)}$ (B), $PagP_{(Ec)}$ (C), and $PagP_{(Bp)}$ (D) were analyzed by ESI-MS. Major peaks at *m/z* 1557, 1477, 1387, 1307, 1251, 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).

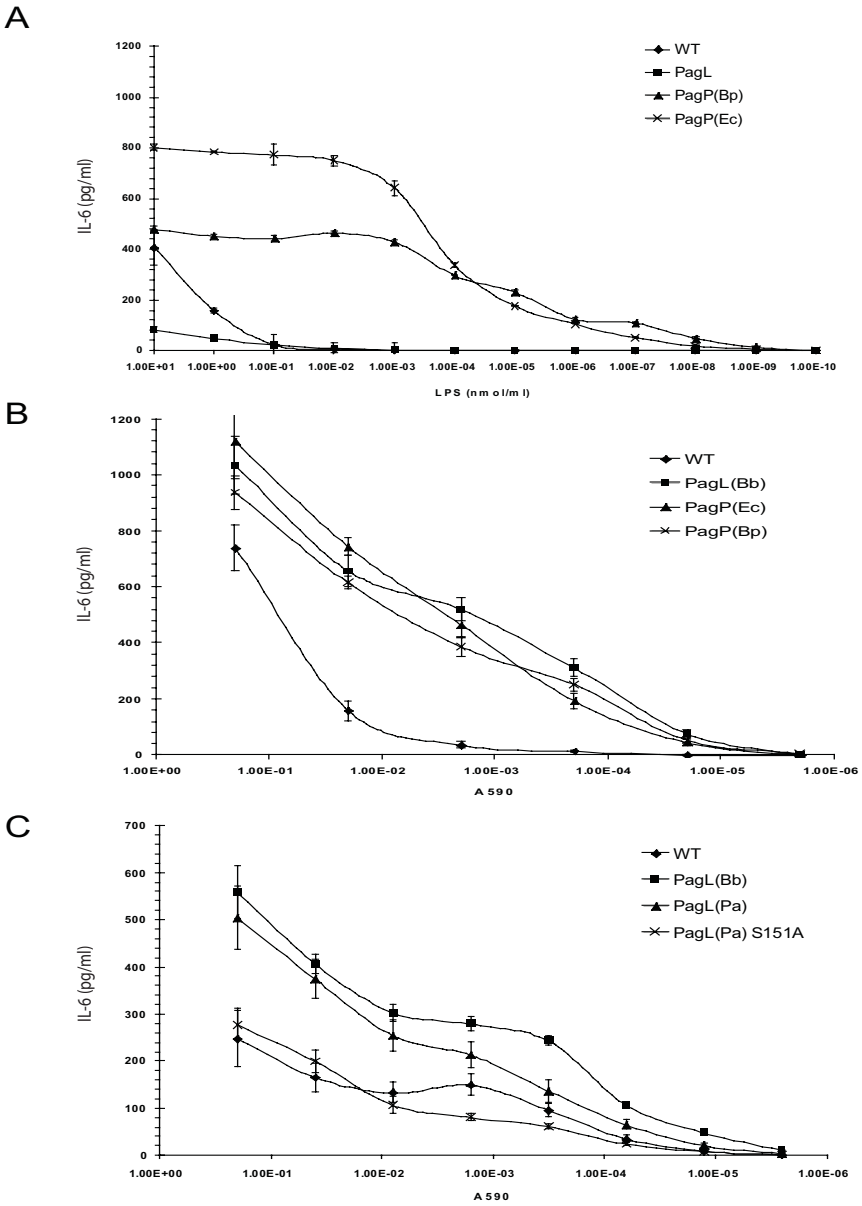

Fig. 5. IL-6 induction by purified *B. pertussis* LPS and whole cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of purified wild-type (WT) or PagP- or PagL-modified LPS from *B. pertussis* strain Tohama, (B) a whole-cell 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.

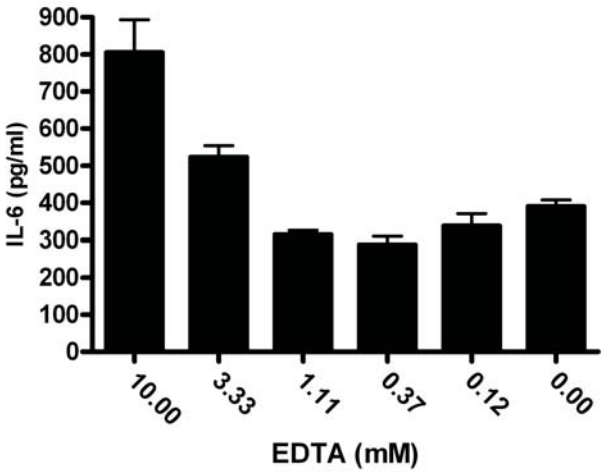

Fig. 6. Stimulation of IL-6 production by a *B. pertussis* whole-cell suspension in the presence of EDTA. The production of IL-6 by the human macrophage cell line MM6 was stimulated with a whole-cell suspension in the presence of various amounts of EDTA. The IL-6 concentration in the culture supernatant 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

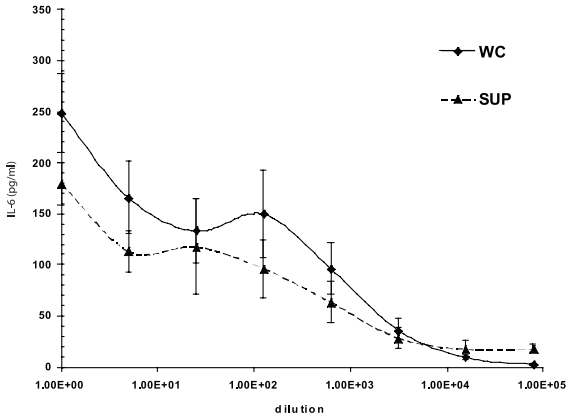

Fig. 7. Released LPS determines endotoxicity of *B. pertussis* whole-cell suspensions. Production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type *B. pertussis* (WC) or with the cell-free supernatant of such a suspension (SUP). 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.

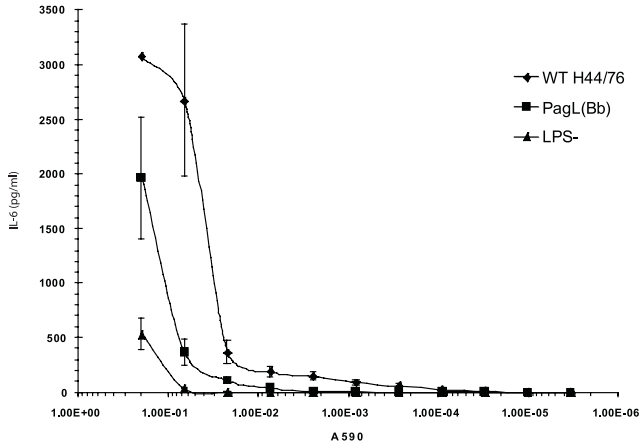

Fig. 8. PagL modification reduces endotoxic activity of *N. meningitidis* cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type *N. meningitidis* cells (WT H44/76), *N. meningitidis* cells expressing $PagL_{(Bb)}$ (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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References

Akerley, B. J., Monack, M. D., Falkow, S., and Miller, J. F. (1992) The *bvgAS* locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. *J. Bacteriol.* **174:** 980-990.

Allen, A., and Maskell, D. (1996) The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. *Mol. Microbiol.* **19:** 37-52.

Amador, C., Chiner, E., Calpe, J. L., Ortiz de la Table, V., Martinez, C., and Pasquau, F. (1991) Pneumonia due to *Bordetella bronchiseptica* in a patient with AIDS. *Rev. Infect. Dis.* **13:** 771-772.

Amano, K., Fukushi, K., and Watanabe, M. (1990) Biochemical and immunological comparison of lipopolysaccharides from *Bordetella* species. *J. Gen. Microbiol.* **136:** 481-487.

Ausiello, C. M., Urbani, F., la Sala, A., Lande, R., and Cassone, A. (1997) Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. *Infect. Immun.* **65:** 2168-2174.

Banus, S. A., Vandebriel, R. J., de Ruiter, H., Dormans, J. A. M. A., Nagelkerke, N. J., Mooi, F. R., Hoebee, B., van Kranen, H. J., and Kimman, T. G. (2006). Host genetics of *Bordetella pertussis* infection in mice: significance of Toll-like receptor 4 in genetic susceptibility and pathobiology. *Infect. Immun.* **74:** 2596-2605.

Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. H. (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. *EMBO J.* **19:** 5071-5080.

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.

Boyd, A. P., Ross, P. J., Conroy, H., Mahon, N., Lavelle, E. C., and Mills, K. H. G. (2005) *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. *J. immunol.* **175:** 730-738.

Caroff, M., Deprun, C., Richards, J. C., and Karibian, D. (1994) Structural characterization of the lipid A of *Bordetella pertussis* 1414 endotoxin. *J. Bacteriol.* **176:** 5156-5159.

Caroff, M., Karibian, D., Cavaillon, J-M., and Haeffner-Cavaillon, N. (2002) Structural and functional analyses of bacterial lipopolysaccharides. *Microbes. Infect.* **4:** 915-926.

Chaby, R., and Caroff, M. (1988) Lipopolysaccharide of *Bordetella pertussis* endotoxin. p. 247-271. In A. C. Wardlaw, and R. Parton (eds). Pathogenesis and immunity in pertussis. John Wiley, New York.

Cotter, P. A., and Jones, A. M. (2003) Phosphorelay control of virulence gene expression in *Bordetella*. *Trends Microbiol.* **11:** 367-373.

Cotter, P. A., and Miller, J. F. (1997) A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol. Microbiol.* **24:** 671-685.

Fürste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48:** 119-131.

Gangloff, S. C., Hijiya, N., Haziot, A., and Goyert, S. M. (1999) Lipopolysaccharide structure influences the macrophage response via CD14-independent and CD14-dependent pathways. *Clin. Infect. Dis.* **28:** 491-496.

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.

Goodnow, R. A. (1980) Biology of *Bordetella bronchiseptica***.** *Microbiol. Rev.* **44:** 722-738.

Gueirard, P., Weber, C., Le Coustumier, A., and Guiso, N. (1995) Human *Bordetella bronchiseptica* infection related to contact with infected animals: persistence of bacteria in host. *J. Clin. Microbiol.* **33:** 2002-2006.

Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95:** 189-198.

Haeffner-Cavaillon, N., Carroff, M., and Cavaillon, J-M. (1989) Interleukin-1 induction by lipopolysaccharides: structural requirements of the 3-deoxy-D-manno-2-octulosonic acid (KDO). *Mol. Immunol.* **26:** 485-494.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557-580.

Homma, J. Y., Matsuura, M., Kanegasaki, S., Kawakubo, Y., Kojima, Y., Shibukawa, N., Kumazawa, Y., Yamamoto, A., Tanamoto, K., and Yasuda, T. (1985) Structural requirements of lipid A responsible for the functions: a study with chemically synthesized lipid A and its analogues. *J. Biochem.* **98:** 395-406.

Janusch, H., Brecker, L., Lindner, B., Alexander, C., Gronow, S., Heine, H., Ulmer, A. J., Rietschel, E. T., and Zähringer, U. (2002) Structural and biological characterization of highly purified hepta-acyl lipid A present in the lipopolysaccharide of the *Salmonella enterica* sv. Minnesota Re deep rough mutant strain R595. *J. Endotoxin Res.* **8:** 343-356.

Jia, W., Zoeiby, A. E., Petruzziello, T. N., Jayabalasingham, B., Seyedirashti, S., and Bishop, R. E. (2004) Lipid trafficking controls endotoxin acylation in outer membranes of *Escherichia coli*. *J. Biol. Chem.* **279:** 44966-44975.

Karbarz, M. J., Kalb, S. R., Cotter, R. J., and Raetz, C. R. H. (2003) Expression cloning and biochemical characterization of a *Rhizobium leguminosarum* lipid A 1-phosphatase. *J. Biol. Chem.* **278:** 39269-39279.

Kawasaki, K., Ernst, R. K., and Miller, S. I. (2004) 3-*O*-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of *Salmonella typhimurium*, modulates signaling through Toll-like receptor 4. *J. Biol. Chem.* **279:** 20044-20048.

Kawasaki, K., Ernst, R. K., and Miller, S. I. (2005) Inhibition of *Salmonella enterica* serovar Typhimurium lipopolysaccharide deacylation by aminoarabinose membrane modification. *J. Bacteriol.* **187:** 2448-2457.

Leive, L. (1965) Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochem. Biophys. Res. Commun.* **21:** 290–296.

Lesse, A. J., Campagnari, A. A., Bittner, W. E., and Apicella, M. A. (1990) Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Immunol. Methods* **126:** 109-117.

Loppnow, H., Brade, H., Durrbaum, I., Dinarello, C. A., Kusumoto, S., Rietschel, E. T., and Flad, H. D. (1989) IL-1 induction-capacity of defined lipopolysaccharide partial structures. *J. Immunol.* **142:** 3229-3238.

Mann, P. B., Elder, K. D., Kennett, M. J., and Harvill, E. T. (2004). Toll-like receptor 4-dependent early elicited tumor necrosis factor alpha expression is critical for innate host defense against *Bordetella bronchiseptica*. *Infect. Immun.* **72:** 6650-6658.

Mann, P. B., Kennett, M. J., and Harvill, E. T. (2004) Toll-like receptor 4 is critical to innate host defense in a murine model of bordetellosis. *J. Infect. Dis.* **189:** 833-836.

Mattoo, S., and Cherry, J. D. (2005) Molecular pathogenesis, Epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev.* **18:** 326-382.

Miller, J. F., Johnson, S. A., Black, W. J., Beattie, D. T., Mekalanos, J. J., and Falkow, S. (1992) Constitutive sensory transduction mutations in *Bordetella pertussis bvgS* gene. *J. Bacteriol.* **174:** 970-979.

Mirlashari, M. R., and Lyberg, T. (2003) Expression and involvement of Toll-like receptors (TLR)2, TLR4, and CD14 in monocyte TNF-α production induced by lipopolysaccharides from *Neisseria meningitidis*. *Med. Sci. Monit.* **9:** BR316-324.

Munford, R. S., Hall, C. L., Lipton, J. M., and Dietschy, J. M. (1982) Biological activity, lipoprotein-binding behavior, and *in vivo* disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. *J. Clin. Invest.* **70:** 877-888.

Okusawa, T., Fujita, M., Nakamura, J., Into, T., Yasuda, M., Yoshimura, A., Hara, Y., Hasebe, A., Golenbock, D. T., Morita, M., Kuroki, Y., Ogawa, T., and Shibata, K. (2004) Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. *Infect. Immun.* **72:** 1657-1665.

Pålsson-Mcdermott, E. M., and O'Neill, L. A. J. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor 4. *Immunology* **113:** 153-162.

Peppler, M. S. (1984) Two physically and serologically distinct lipopolysaccharide profiles in strains of *Bordetella pertussis* and their phenotype variants. *Infect. Immun.* **43:** 224-232.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000) Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. *Proc. Natl. Acad. Sci. U S A* **97:** 2163-2167.

Post, D., Zhang, D., Eastvold, J., Teghanemt, A., Gibson, B. W., and Weiss, J.P. (2005) Biochemical and functional characterization of membrane blebs purified from *Neisseria meningitidis* sergroup B. *J. Biol. Chem.* **280:** 38383-38394.

Preston, A., Maxim, E., Toland, E., Pishko, E. A., Harvill, E. T., Caroff, M., and Maskell, D. J. (2003) *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. *Mol. Microbiol.* **48:** 725-736.

Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71:** 635-700.

Reina, J., Bassa, A., Llompart, I., Borrell, N., Gomez, J., and Serra, A. (1991) Pneumonia caused by *Bordetella bronchiseptica* in a patient with a thoracic trauma. *Infection* **19:** 46-48.

Reynolds, C. M., Ribeiro, A. A., McGrath, S. C., Cotter, R. J., Raetz, C. R. H., and Trent, M. S. (2006) An outer membrane enzyme encoded by *Salmonella typhimurium lpxR* that removes the 3'-acyloxyacyl moiety of lipid A. *J. Biol. Chem.* **281:** 21974-21987

Ross, P. J., Lavelle, E. C., Mills, K. H. G., and Boyd, A. P. (2004) Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infect. Immun.* **72:**1568-1579.

Rutten, L., Geurtsen, J., Lambert, W., Smolenaers, J. J. M., Bonvin, A., 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.

Ryan, M., Gothefors, L., Storsaeter, J., and Mills, K. H. G. (1997) *Bordetella pertussis*-specific Th1/Th2 cells generated following respiratory infection or immunization with an acellular vaccine: comparison of the T cell cytokine profiles in infants and mice. *Dev. Biol. Stand.* **89:** 297-305.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmitt, H. J. (1996) Acellular pertussis vaccines: the final countdown. *Eur. J. Pediatr.* **155:** 74-76.

Smith, A. M., Guzmán, C. A., and Walker, M. J. (2001) The virulence factors of *Bordetella pertussis*: a matter of control. *FEMS Microbiol. Rev.* **25:** 309-333.

Steeghs, L., den Hartog, R., de Boer, A., Zomer, B., Roholl, P., and van der Ley, P. (1998) Meningitis bacterium is viable without endotoxin. *Nature* **392:** 449-450.

Steeghs, L., Berns, M., ten Hove, J., de Jong, A., Roholl, P., van Alphen, L., Tommassen, J., and van der Ley, P. (2002) Expression of foreign LpxA acyltransferases in *Neisseria meningitidis* results in modified lipid A with reduced toxicity and retained adjuvant activity. *Cell. Microbiol.* **4:** 599-611.

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.

Thalen, M., van den lJssel, J., Jiskoot, W., Zomer, B., Roholl, P., de Gooijer, C., Beuvery, C., and Trampen, J. (1999) Rational medium design for *Bordetella pertussis*: basic metabolism. *J. Biotechnol.* **75:** 147-159.

Tommassen, J., van Tol, H., and Lugtenberg, B. (1983) The ultimate localization of an outer membrane protein of *Escherichia coli* K-12 is not determined by the signal sequence. *EMBO J.* **2:** 1275-1279.

Trent, M. S., Pabich, W., Raetz, C. R. H., and Miller, S. I. (2001) A PhoP/PhoQ-induced lipase (PagL) that catalyzes 3-*O*-deacylation of lipid A precursors in membranes of *Salmonella typhimurium*. *J. Biol. Chem.* **276:** 9083-9092.

Trent, M. S. (2004) Biosynthesis, transport, and modification of lipid A. *Biochem. Cell Biol.* **82:** 71-86.

Tsai, C. M., and Frasch, C. E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119:** 115-119.

Watanabe, M., Takimoto, H., Kumazawa, Y., and Amano, K. (1990) Biological properties of lipopolysaccharides from *Bordetella* species. *J. Gen. Microbiol.* **136:** 489-493.

Welch, D. F. (1991) Applications of cellular fatty acid analysis. *Clin. Microbiol. Rev.* **4:** 422-438.

Westphal, O., and Jann, J. K. (1965) Bacterial lipopolysaccharides, extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.* **5:** 83-91.

Wilm, M., and Mann, M. (1996) Analytical properties of the nanoelectrospray ion source. *Anal. Chem.* **68:** 1-8.

Zarrouk, H., Karibian, D., Bodie, S., Perry, M. B., Richards, J. C., and Caroff, M. (1997) Structural characterization of the lipids A of three *Bordetella bronchiseptica* strains: variability of fatty acid substitution. *J. Bacteriol.* **179:** 3756-3760.

Ziegler-Heitbrock, H. W., Thiel, E., Futterer, A., Herzog, V., Wirtz, A., and Riethmuller, G. (1998) Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer* **41:** 456-461.