Chapter 8

Identification and functional characterisation of *Bordetella pertussis lpxL* homologues

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Abstract

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

Introduction

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

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

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

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

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

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

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

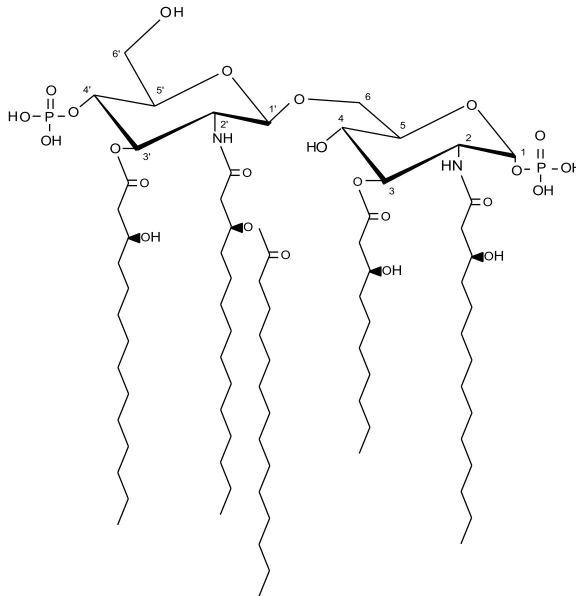

Fig. 1. *B. pertussis* lipid A architecture. *B. pertussis* lipid A consists of a bisphosphorylated GlcN disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2' fatty-acyl chain is esterified with myristate (Caroff *et al.*,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

Materials and Methods

Bacterial strains and growth conditions

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

TABLE 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
B. pertussis		
B213	A streptomycin resistant derivative of *B. pertussis* strain Tohama	Kasuga *et al.*, 1953
B213 Δ*lpxL1*	*lpxL1* mutant of B213 strain, Str^R, Gm^R	This study
E. coli		
TOP10F'	*F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	*F⁻ Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	Hanahan, 1983
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir* R6K Kan^R	N.V.I.[a]
W3110	Wild-type strain, F^-, $λ^-$	N.V.I.[a]
MLK53	*htrB1*::Tn10, Tet^R derivative of W3110	Karow and Georgopoulos, 1992
Plasmids		
pCRII-TOPO	*E. coli* cloning vector, Amp^R Kan^R	Invitrogen
pET-11a	*E. coli* high-copy expression vector, Amp^R, T7 promotor	Novagen
pMMB67EH	Broad-host-range expression vector, Amp^R	Fürste *et al.*, 1986
pKAS32	Allelic exchange suicide vector, Amp^R	Skorupski and Taylor, 1996
pBSL141	*E. coli* vector harbouring gentamicin-resistance cassette, Amp^R Gm^R	Alexeyev *et al.*, 1995
pLpxL1	pET-11a derivative harbouring *B. pertussis lpxL1*	This study
pLpxL2	pET-11a derivative harbouring *B. pertussis lpxL2*	This study
pMMB67EH-LpxL1	pMMB67EH derivative harbouring *B. pertussis lpxL1*	This study
pMMB67EH-LpxL2	pMMB67EH derivative harbouring *B. pertussis lpxL2*	This study
pCRII-$LpxL1_{up}$	pCRII derivative harbouring *lpxL1*-upstream sequence	This study
pCRII-$LpxL1_{down}$	pCRII derivative harbouring *lpxL1*-downstream sequence	This study
pKAS32-$LpxL1_{KO}$	pKAS32 derivative harbouring *lpxL1* knock out construct, Amp^R, Gm^R	This study

[a] Netherlands Vaccine Institute, Bilthoven, The Netherlands

Recombinant DNA techniques

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

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

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

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

To construct a *B. pertussis lpxL1* mutant strain, we amplified part of the DNA upstream of *lpxL1* from *B. pertussis* strain B213 by using primers 5'-AAATTCGCTCTGGCGCTGCAC-3' and 5'-AATCAGCACGCGTCTGACCGATGCGAATGAAAGGGCGG-3', containing a MluI site (underlined). Additionally, a DNA fragment downstream of *lpxL1* was obtained by PCR with primers 5'-AAGTCAGACGCGTGCTGAGACAGCGCGGCAGGAACC-3', containing a MluI site (underlined), and 5'-AATCCACGTGATAGCGCCCGGT-3'. Both PCR products were cloned into pCRII-TOPO, resulting in plasmids pCRII-$LpxL1_{up}$ and pCRII-$LpxL1_{down}$, respectively. A MluI–XbaI fragment of pCRII-$LpxL1_{down}$ was ligated into MluI–XbaI-restricted pCRII-$LpxL1_{up}$. The resulting plasmid was cut with MluI to allow for insertion of the gentamicin-resistance cassette from plasmid pBSL141 obtained by MluI digestion. Finally, an XbaI-SacI fragment of the construct obtained was ligated into the XbaI-SacI-restricted suicide plasmid pKAS32. The final construct, designated pKAS32-$LpxL1_{KO}$, contained the gentamicin-resistance cassette in the reverse orientation relative to the transcription direction of the *lpxL1* gene and was used to construct a *B. pertussis lpxL1*

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

Isolation and analysis of LPS

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

Endotoxic activity assays

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

Determination of antibiotic sensitivity

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

Infection of human macrophages

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

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

Results

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

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

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

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

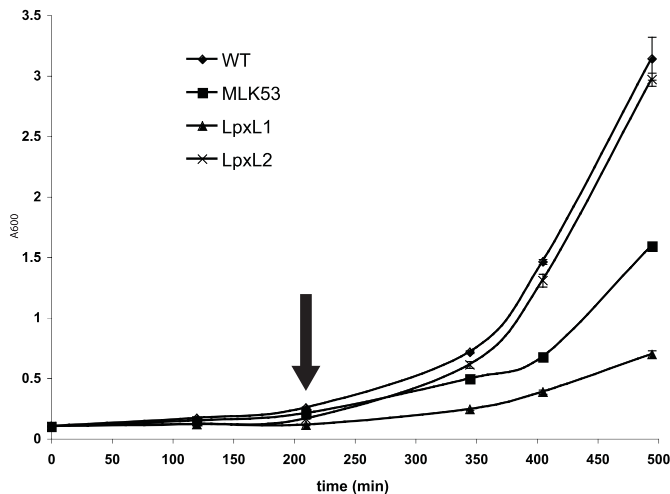

Fig. 2. Complementation of the temperature-sensitive growth phenotype of *E. coli lpxL* mutant MLK53. Bacteria were grown to early log phase in minimal medium at 30°C. After 3½ h (arrow), the bacteria were transferred to LB and further incubated at 42°C. WT, wild-type *E. coli* strain W3110 harbouring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

Overexpression of LpxL1 and LpxL2 in *B. pertussis*

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

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

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

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

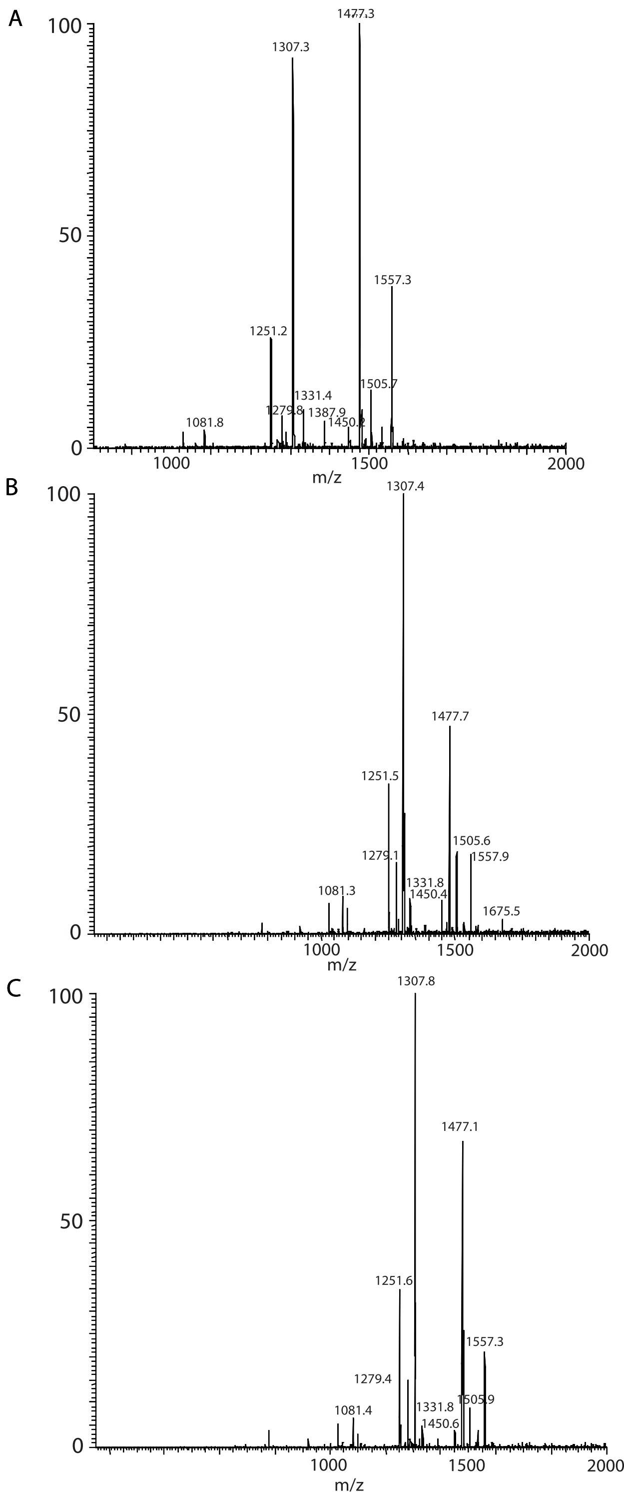

Fig. 3. Structural analysis by ESI-MS of purified *B. pertussis* LPS. Lipid A species from wild-type *B. pertussis* strain B213 (A) and its derivatives overexpressing *lpxL1* (B) or *lpxL2* (C) were analysed by ESI-MS. Major peaks at *m/z* 1557, 1477, 1387, 1307, 1251, and 1081 were interpreted as the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

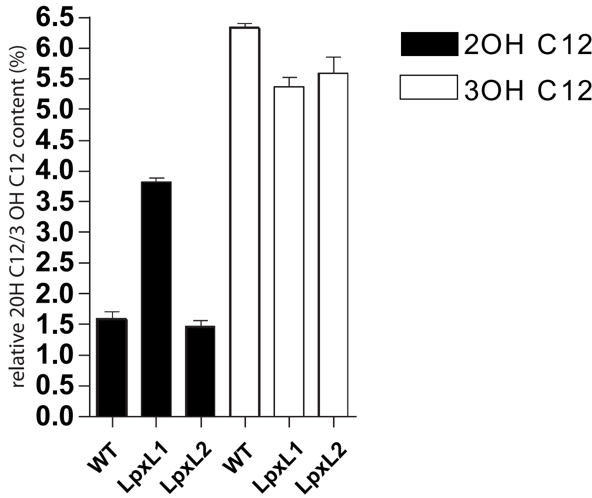

Fig. 4. GC/MS analysis of LPS from wild-type, *lpxL1*-, and *lpxL2*-overexpressing *B. pertussis* strains. GC/MS analysis of LPS purified from wild-type *B. pertussis* strain B213 (WT), *B. pertussis* strain B213 overexpressing *lpxL1* (LpxL1), and *B. pertussis* strain B213 overexpressing *lpxL2* (LpxL2).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

TABLE 2

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

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

Endotoxic activity of LPS and whole bacterial cells

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

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

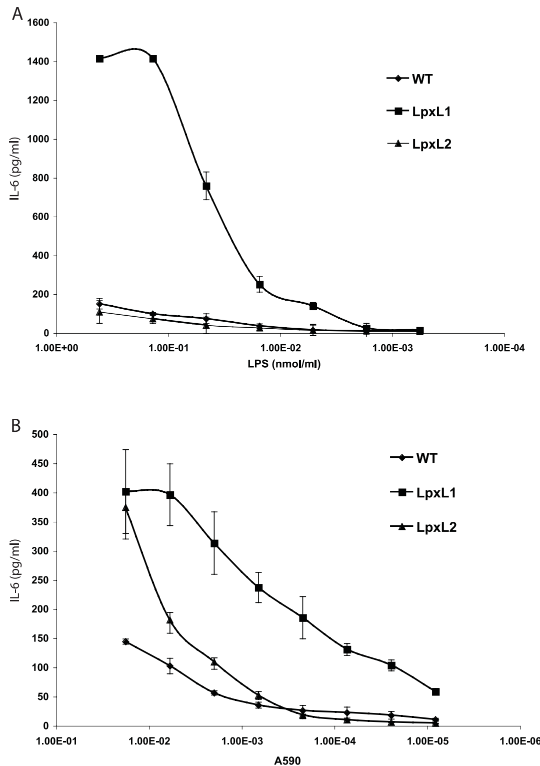

Fig. 5. IL-6 induction by purified *B. pertussis* LPS and whole cells. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of LPS purified from a wild-type (WT), a *lpxL1*-, or a *lpxL2*-overexpressing *B. pertussis* strain, or (B)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

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

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

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

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

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

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

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

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

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

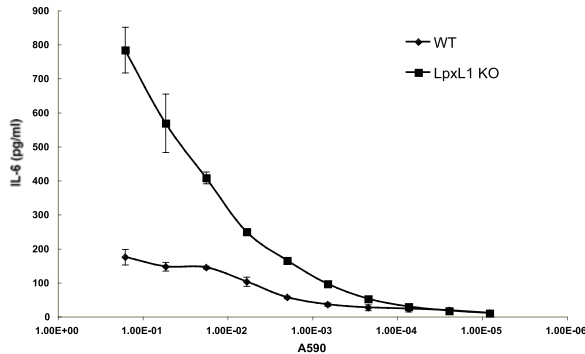

Fig. 6. IL-6 induction by whole cells of wild-type *B. pertussis* and the *lpxL1* mutant of *B. pertussis*. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of a whole-cell suspension of wild-type or *lpxL1*-mutant *B. pertussis* cells. The IL-6 concentration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

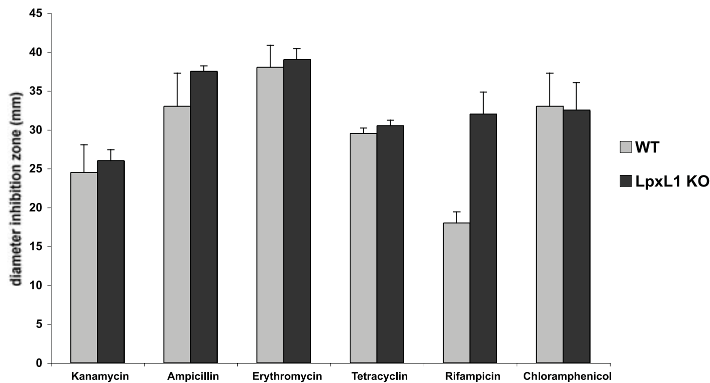

Fig. 7. Antibiotic susceptibility of wild-type *B. pertussis* and the *lpxL1* mutant of *B. pertussis*. Shown is the diameter in millimetres of the inhibition zone around filter paper discs containing each one of six different antibiotics. The data shown represents the averages of three individual experiments.

Discussion

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

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

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

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

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

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

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

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

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

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