Expression of *Legionella pneumophila* paralogous lipid A biosynthesis genes under different growth conditions

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*Legionella pneumophila* is an opportunistic pathogen that in the environment colonizes biofilms and replicates within amoebae. The bacteria employ the intracellular multiplication/defective organelle trafficking (Icm/Dot) type IV secretion system to grow intracellularly in a specific vacuole. Using *Acanthamoeba castellanii* as a host cell, we have previously identified *lcsC* (*Legionella cytotoxic suppressor*), a paralogue of the lipid A disaccharide synthase *lpxB*, as a cytotoxic factor of *L. pneumophila*. A bioinformatic analysis of the genome revealed that *L. pneumophila* is unique in harbouring two paralogues of *lpxB* and two and three paralogues of the lipid A biosynthesis acyltransferases *lpxA* and *lpxD*, respectively. *LcsC* (*lpxB1*) forms a transcriptional unit with *glnA*, encoding a putative UDP-GlcNAc oxidase in the biosynthetic pathway leading to 3-aminoglucomosine analogues of lipid A. *LpxB2* clusters with *lpxD2*, *lpxA2* and *lpxL* paralogues, encoding secondary acyltransferases. *LcsC/lpxB1* and *lpxB2* were found to partially complement the growth defect of an *Escherichia coli* *lpxB* conditional mutant strain, indicating that both corresponding enzymes possess lipid A disaccharide synthase activity. The two *L. pneumophila* *lpxB* paralogues are not functionally equivalent, since expression of *lcsC/lpxB1* but not *lpxB2* in an *L. pneumophila icmG* mutant is cytotoxic for *A. castellanii*, and LPS purified from the two strains triggers CD14-dependent tumour necrosis factor (TNF)α production by macrophages with a different potency. The *lpxB* and *lpxA* paralogues are expressed under various growth conditions, including broth, biofilms and in *A. castellanii*. While the flagellar gene flaA is mainly expressed in late stationary phase, the *lpxB* and *lpxA* paralogues are preferentially expressed in the exponential and early stationary phases. Upon exposure to hypotonic stress and nutrient deprivation, *lpxA1, and to a lesser extent lcsC/lpxB1*, is upregulated. The differential regulation of *lpxB* or *lpxA* paralogues in response to changing environmental conditions might allow *L. pneumophila* to adapt its lipid A structure.

**Abbreviations:** ACES, *N*-((2-acetamido)-2-aminoethanesulfonic acid; ACP, acyl carrier protein; Icm/Dot, intracellular multiplication/defective organelle trafficking; GlcN, 2-amino-2,3-dideoxy-3-deoxy-o-glucopyranose (glucosamine); GlcN3N, 2,3-diamino-2,3-dideoxy-2-deoxy-o-glucopyranose; GlcNAc, 2-acetamido-2,3-dideoxy-2-deoxy-a-d-glucopyranose (N-acetylgalactosamine); GlcNAc3N, 2-acetamido-3-amino-2,3-dideoxy-2-deoxy-a-d-glucopyranose; Kdo, 3-deoxy-d-manno-oct-2-ulosonic acid; lcs, *Legionella* cytotoxic suppressor; PI, propidium iodide; TNF, tumour necrosis factor.

Supplementary tables listing bacterial strains and plasmids, oligonucleotides used in this study, paralogues of LpxA–C in different bacteria and genomic arrangements of selected lipid A biosynthesis genes in three *L. pneumophila* strains, and a supplementary figure showing cotranscription of *lcsC/lpxB1* and *glnA*, are available with the online version of this paper.

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INTRODUCTION

Legionella pneumophila is a Gram-negative bacterium that colonizes different niches in the environment. The bacteria not only survive and replicate in numerous amoebae and ciliates (Fields, 1996; Hilbi et al., 2007; Steinert et al., 2002), but also form and colonize biofilms (Mampel et al., 2006; Murga et al., 2001). Furthermore, L. pneumophila is an opportunistic human pathogen that upon inhalation of contaminated aerosols may cause the life-threatening pneumonia Legionnaires’ disease. The bacteria grow within macrophages from different sources, including human alveolar macrophages (Nash et al., 1984), primary macrophages from A/J mice (Spörri et al., 2006; Yamamoto et al., 1988) and several macrophage-like cell lines (Fields, 1996).

Phagocytosis and intracellular replication of L. pneumophila is promoted by the intracellular multiplication/defective organelle trafficking (Icm/Dot) type IV secretion system (T4SS) (Hilbi et al., 2001; Segal et al., 1998; Vogel et al., 1998), a conjugation apparatus which translocates more than 40 putative ‘effector’ proteins into host cells (Brüggemann et al., 2006; Hilbi, 2006; Nagai & Roy, 2003). The Icm/Dot T4SS is also required for L. pneumophila to survive and grow on agar plates impregnated with Acanthamoeba castellanii (Albers et al., 2005). This ‘amoebae plate test’ was recently used to screen an L. pneumophila chromosomal library for multicopy suppressors of the partial growth defect of an L. pneumophila icmG mutant strain. In this screen, a paralogue of the lipid A disaccharide synthase lpxB was identified and termed lcsC (Legionella cytotoxic suppressor). Expression of lcsC in the icmG mutant strain rendered L. pneumophila more cytotoxic against A. castellanii, but did not enhance intracellular replication of the bacteria (Albers et al., 2005).

Lipid A (endotoxin) is the hydrophobic membrane anchor of bacterial LPS, and consists of a non-repeating ‘core’ oligosaccharide and a distal polysaccharide, the ‘O antigen’ (Raetz & Whitfield, 2002). Lipid A biosynthesis is probably similar in L. pneumophila and Escherichia coli, since the genomes of the L. pneumophila strains Philadelphia (Chien et al., 2004), Paris and Lens (Cazalet et al., 2004) encode orthologues of E. coli lipid A biosynthetic genes. However, several features distinguish L. pneumophila lipid A from the corresponding enterobacterial compounds (Fig. 1). The backbone of L. pneumophila lipid A contains 2,3-diamino-2,3-dideoxyglucose (GlcN3N) and lacks glucosamine (GlcN) (Zähringer et al., 1995). The initial step in the biosynthesis of this lipid A analogue is the conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) to its 3-amino derivative UDP-GlcNAc3N, which in Acidithiobacillus ferrooxidans is catalysed by the NAD+ -dependent oxidase GnnA and the L-glutamate-dependent aminotransferase GnnB (Sweet et al., 2004). Moreover, lipid A of L. pneumophila is substituted with unusual long-chain

Fig. 1. Putative pathway of lipid A biosynthesis in L. pneumophila. The putative pathway is based on homologous enzymes found in A. ferrooxidans (GnnA, -B) and E. coli (LpxA, -B, -C, -D, -H, -K, -L, WaaA). Two paralogues of LpxA, LpxB or GnnB, and three paralogues of LpxD or LpxL, but no homologue of LpxM, are present in L. pneumophila. Primary and secondary acyl chains are indicated as identified for L. pneumophila. The reaction catalysed by a specific enzyme is highlighted in red. 2-KG, 2-kgutoglutarate.
[28:0(27-oxo) and 27:0-dioic], branched and dihydroxylated fatty acids (Moll et al., 1992; Zähringer et al., 1995). These fatty acids render lipid A from L. pneumophila very hydrophobic, and possibly account for its low endotoxic activity due to the failure to interact with the soluble LPS receptor CD14 (Neuhammer et al., 1998).

The core oligosaccharide of L. pneumophila LPS has hydrophobic properties due to N- and O-acetyl moieties, and the inner core contains 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) but lacks heptose and phosphate groups (Zähringer et al., 1995). Finally, the O antigen of L. pneumophila serogroup 1 LPS is a homopolymer of a unique monosaccharide unit, termed legiosaminic acid. The hydroxyl and amino groups of legiosaminic acid are substituted by acetyl and acetimidoyl groups, respectively, rendering the O chain highly hydrophobic also (Knirel et al., 1994; Zähringer et al., 1995).

Lipid A modifications alter the physical properties of the outer membrane and profoundly affect various interactions between pathogenic bacteria and their environment, including resistance against antibiotics and cationic peptides, growth within host cells and endotoxic activity (Miller et al., 2005; Raetz & Whitfield, 2002). A prominent example is Salmonella enterica serovar Typhimurium, which, dependent on PhoP–PhoQ and other two-component systems, regulates a number of lipid A-modifying enzymes. The acyl transferase PagP, for example, is expressed under the low magnesium concentrations encountered inside a phagosome and catalyses the addition of palmitate (C16) to the primary acyl chain at position 2 of lipid A (Bishop et al., 2000; Guo et al., 1998). Thus, a hepta-acylated LPS species is formed, and S. enterica serovar Typhimurium exhibits increased resistance to cationic antimicrobial peptides (CAMPs). L. pneumophila harbours the pagP-like gene rcp (resistance to cationic antimicrobial peptides), conferring resistance to CAMPs in low-magnesium medium (Röbye et al., 2001). Similar to a pagP mutant, an rcp mutant also showed decreased resistance to CAMPs. Moreover, the rcp allele was defective for growth in Hartmannella vermiformis amoebae and U937 macrophages, as well as for lung colonization of A/J mice.

Reversible LPS phase variation is a frequently adopted strategy of pathogenic bacteria to alter their surface carbohydrate pattern and thus adapt to changes in the environment (immune evasion, colonization of new niches). Phase-variable expression of L. pneumophila serogroup 1 LPS coincides with decreased virulence in a guinea pig infection model, reduced intracellular replication within macrophage-like HL60 cells or A. castellanii, and impaired serum resistance (Lüneberg et al., 1998). While these features are apparently disadvantageous to infection of a mammalian host, colonies of the phase-variant strain appear sticky and slimy, and thus LPS phase variation might facilitate adherence to surfaces and colonization of biofilms (Lüneberg et al., 2001). Phase variation in L. pneumophila has been found to be due to the reversible chromosomal excision of a 30 kb genetic element (Lüneberg et al., 2001). While the virulent wild-type strain RC1, the 30 kb element is integrated into a specific site in the chromosome, in the phase-variant strain 811, the element is excised and replicates as a high-copy-number plasmid. The 30 kb element does not harbour genes related to LPS biosynthesis, yet in the phase-variant strain, N-linked methylation of legiosaminic acid in the O-chain polysaccharide is absent (Kooistra et al., 2002b), and the profile of fatty acids attached to lipid A is shifted towards shorter-chain moieties (3-hydroxylated saturated C16–18 fatty acids instead of 3-hydroxylated saturated C20–22 fatty acids; Kooistra et al., 2002a).

Here we report that L. pneumophila is unique among a number of bacteria in harbouring paralogues of multiple lipid A biosynthesis genes. We provide evidence that the previously identified cytotoxic L. pneumophila lipid A disaccharide synthase lcsC (lpxB1), as well as its paralogue lpxB2, is involved in lipid A biosynthesis, but only lcsC/lpxB1 is cytotoxic for amoebae. Moreover, LPS isolated from L. pneumophila overexpressing either lpxB1 or lpxB2 triggers tumour necrosis factor (TNF)α production by macrophages to a different extent. Finally, expression analysis of the lpxA and lpxB paralogues indicates that the genes are expressed under different environmental conditions.

**METHODS**

**Bacteria, amoebae and reagents.** The bacterial strains used in this study are listed in Supplementary Table S1. L. pneumophila was grown on charcoal–yeast extract (CYE) agar plates (Feeley et al., 1979) or in ACES-buffered yeast extract (AYE) broth, supplemented with chloramphenicol (Cm; 5 μg ml⁻¹) or kanamycin (Km; 50 μg ml⁻¹), if necessary. E. coli strains were cultured in LB medium, supplemented with Cm (30 μg ml⁻¹), ampicillin (Ap; 100 μg ml⁻¹) or 0.2% L-(−)-arabinose, if required. A. castellanii (ATCC 30234) was grown in proteose yeast extract glucose (PYG) medium at 30 °C (Albers et al., 2005; Moffat & Tompkins, 1992; Segal & Shuman, 1999) and split once or twice a week. High-gel-strength agar was from Serva, protease peptone from Becton Dickinson Biosciences and Bacto yeast extract from Difco. All other reagents were from Sigma–Aldrich.

**Construction of plasmids.** DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits from Qiagen or Macherey–Nagel. The plasmids used and constructed are listed in Supplementary Table S1. To construct plasmid pBCR-lcsC (pMMB207C-RBS-lcsC), the lcsC/lpxB1 gene (lpc1371) including the RBS was released from plasmid pUA26 (pMMB207-RBS-lcsC) by digestion with EcoRI and BamHI, filling in with Klenow polymerase and religation with T4 DNA polymerase. To construct plasmid pBCR-lpxB (pMMB207C-RBS-lpxB), the corresponding ORF (lpc2945) was amplified by PCR using chromosomal DNA as template and the oligonucleotides lpxB2lo and lpxB2re (see Supplementary Table S2), respectively. The PCR fragment was cut with Ndel and Psfl and inserted into pMMB207C-RBS-lcsC cut with the same enzymes, thus replacing lcsC/lpxB1 with lpxB2. All constructs containing PCR fragments were sequenced.
Chromosomal deletion of lcsC/lpxB. We attempted to generate a chromosomal insertion deletion of lcsC/lpxB in L. pneumophila strain JR32 by allelic exchange using the suicide vector pLAW344 (Wia
ter et al., 1994). To construct the suicide vector, plasmid pUA9 containing an in-frame partial deletion in lcsC/lpxB (Albers et al., 2005) was cut with BbsI (a unique site 23 bp downstream of the site joining the 5’ and 3’ remnants of lcsC/lpxB) and blunted, and the Km-resistance cassette from pUC4K was released with BanHI, blunted and inserted to yield pUA44. The insert with the Km cassette in lcsC/lpxB was released by EcoRI, blunted and inserted in pLAW344 digested with EcoRV to yield the allelic-exchange vector pUA46. This construct consists of a 318 bp region homologous to 5’ and upstream sequences of lcsC, followed by a 23 bp 3’ region of lcsC, the Km-resistance cassette, and a 597 bp region homologous to 3’ and downstream sequences of lcsC. The allelic exchange was performed as described previously (Wia
ter et al., 1994; Tiaden et al., 2007). Briefly, pUA46 was electroporated into strain JR32 and grown for 6 days on CYE/Km plates. Single colonies were then cultured overnight in AYE/BSA/Km medium, counter-selected on CYE/Km/2 % sucrose plates and streaked on CYE/Km and CYE/Cm plates to control for the desired resistance pattern. Colonies (Km^6, Suc^5, Cm^5) were further analysed by PCR using the primers oUA7, oUA29, oKmfo and oKmre in different combinations.

Identification of homologues of L. pneumophila lipid A biosynthesis genes. To identify enzymes involved in L. pneumophila lipid A biosynthesis, a BLASTP search (Altschul et al., 1997) was performed in the genomes of L. pneumophila strains Philadelphia-1 (Chien et al., 2004), Lens and Paris (Cazalet et al., 2004) and other bacterial genomes (see Supplementary Table S3). The following proteins were used as the query: E. coli K12 (LpxA, accession number P0A722; LpxB, P10441; LpxC, P0A725; LpxD, P21645; LpxH, P43341; LpxK, AAC74001; LpxL, AAC74138; LpxM, AAC74925; WaaA, AAC76657) and A. ferrooxidans (GnnA, AA548421; GnnB, AA548422).

Complementation of a conditional E. coli lpxB mutant strain with L. pneumophila lpxB paralogues. The ability of the L. pneumophila lpxB paralogues to complement an E. coli lpxB mutant was examined using the temperature-sensitive lpxB mutant MN7 (Nishijima et al., 1981). The mutant was transformed by electro
toration with empty vectors (pUC18, pMMB207C-RBS), with plasmid pSR8 expressing E. coli lpxB (Crowell et al., 1986), or with plasmids expressing the L. pneumophila lpxB paralogues (pMMB207C-RBS-lcsC, pMMB207C-RBS-lpxB). Cultures grown overnight were transferred to an OD_{600} of 1.0, and appropriate dilutions were plated on LB agar containing 80 µg Ap ml^{-1} (pUC18, pSR8) or 20 µg Cm ml^{-1} (pMMB207C and derivatives). On the plates, expression of E. coli lpxB or L. pneumophila lpxB paralogues was induced with 0.2 % arabinose or 10 µM IPTG, respectively. Higher concentrations of IPTG tended to inhibit growth of the bacteria, suggesting that the L. pneumophila lpxB paralogues were toxic for E. coli. The plates were incubated at 30 or 42 °C, and c.f.u. were determined after 24 and 48 h.

Cytotoxicity and intracellular multiplication of L. pneumophila overexpressing lpxB paralogues. Cytotoxicity and intracellular replication of the L. pneumophila icmG mutant strain MW635 expressing lpxB paralogues were assayed as described previously (Albers et al., 2005). Briefly, 4 × 10^8 A. castellanii per well (24-well plates) or 2.5 × 10^8 A. castellanii per well (96-well plates) in PYG were seeded and grown overnight. Before infection, the PYG medium was replaced with Ac buffer (Moffat & Tompkins, 1992), which for growth experiments contained Cm (5 µg ml^{-1}) and IPTG (0.2 mM). L. pneumophila icmG mutants harbouring the lpxB paralogues were grown on CYE/Cm agar plates for 3 or 4 days in the presence of 0.2 or 0.05 mM IPTG to induce expression of lcsC/lpxB1 or lpxB2, respectively. The bacteria were resuspended and diluted in sterile water to infect the amoebae at an m.o.i. of 50–100. The infection was synchronized by centrifugation (880 g, 5 min), and the plates were incubated at 30 °C. To determine cytotoxicity, 2 days post-infection propidium iodide (PI) solution at a final concentration of 1 µg ml^{-1} was added to the wells. After several minutes’ incubation, the amoebae were viewed in bright-field or epifluorescence microscopy with an inverse microscope (Zeiss Axiovert 200M, × 20 objective), and cytotoxicity was quantified by determining the percentage of PI-positive (dead) amoebae per field of view (500–1000 amoebae). Intracellular growth of L. pneumophila was determined by plating appropriately diluted supernatant of infected amoebae on CYE agar plates at the time points indicated.

LPS preparation. L. pneumophila LPS was purified as described elsewhere (Jürgens & Fehrenbach, 1997). Briefly, bacteria grown on CYE agar plates were resuspended in water and adjusted to the same OD_{500} = 40–50. The bacteria were lysed by boiling (30 min) and sonication (1 min, maximum power). After addition of DNase (15 min, 37 °C), the sample was split into portions of 200 µl diluted 1:2 with SDS loading buffer (2 % SDS, 10 %, v/v, glycerol, 100 mM Tris, 0.002 % Bromphenol Blue, 1 % β-mercaptoethanol), boiled (10 min) and digested with 20 µl proteinase K (Roche) for 1 h at 60 °C. Finally, the sample was precipitated in 2 vols 0.375 M MgCl2 in 95 % ethanol/5 % water (overnight, −20 °C), centrifuged (30 min, 4 °C, 1500 g) and resuspended in 200 µl water. LPS from E. coli O111:B4 (Sigma–Aldrich) was repurified by phenol: water extraction (Manthey et al., 1994). Each LPS preparation was quantified by a chromogenic Limulus amoebocyte lysate assay kit (HyCult Biotechnology, Tebu-Bio). The samples were diluted and analysed as instructed by the manufacturer, using a Multiscan Ascent ELISA reader (Thermo Fisher Scientific).

Isolation of murine peritoneal macrophages and determination of TNFα. Wild-type BALB/c and BALB/c CD14−/− mice (Gangloff et al., 2005; Haziot et al., 1996) were 5–7 weeks old, grown without pain and distress, and euthanized by CO2 inhalation, following protocols approved by the institutional ethical committee for animal experiments (Université de Reims Champagne Ardenne). The mice were injected with 3 ml 3 % (w/v) Brewer’s thioglycollate broth (bioMérieux). Four days later, cells were harvested by peritoneal lavage with 10 ml RPMI 1640 containing 2 mM L-glutamine, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) (all reagents from Invitrogen). The cells were washed twice in RPMI 1640, resuspended in 2 ml medium supplemented with 1 % autologous serum, added to the wells of a 24-well cell-culture plate at a density of 0.5 × 10^6 cells per 500 µl and incubated for 3 h at 37 °C in a humidified 5 % CO2 incubator to allow the macrophages to adhere. After washing the cells twice with 2 ml RPMI 1640, the macrophages were stimulated for 4 h at 37 °C in a humidified 5 % CO2 incubator with 10^{-10} ng ml^{-1} of the different LPS preparations. The cell supernatants were harvested 4 h after treatment to analyse TNFα production by ELISA according to the manufacturer’s instructions (R&D Systems).

RNA extraction from L. pneumophila grown under different conditions. To quantify the expression of lipid A biosynthesis genes, RNA was extracted with the RNeasy Midi Prep kit (Qiagen) according to the manufacturer’s instructions and quantified by measuring the OD_{260}. Contaminating genomic DNA was digested with DNase RQ1 (Promega), which was removed with the RNeasy Mini kit (Qiagen) using the clean-up protocol. L. pneumophila was grown for 3–4 days on CYE agar plates followed by growth in AYE medium and the following additional protocols:

(i) Gene expression of L. pneumophila grown in AYE broth was quantified in 3 ml cultures, which were inoculated at an OD_{600} of 0.1.
At the time points indicated (exponential, early or late stationary phase), the bacteria were harvested, resuspended in RNAProtect reagent (Qiagen) and processed for RNA isolation. Co-transcription of the gnnA and lscCl/lpxB1 genes was analysed in exponential-phase cultures (OD600 0.8). Gene expression under hypotonic stress and nutrient deprivation was determined in stationary-phase cultures (24 h, OD600 2.8–3.3), which were washed once with distilled water and resuspended in distilled water or, as a control, in spent AYE medium and incubated for another 4-5 h at room temperature.

(ii) To quantify RNA from bacteria growing intracellularly in amoebae, 2 x 10^8 A. castellani were seeded in a 10 cm dish the day before the experiment, infected with L. pneumophila in early stationary phase at a m.o.i. of 40 and incubated at 30 °C. At 1.5 h post-infection, gentamicin (50 μg ml⁻¹) was added for 1.5 h to kill extracellular bacteria. The antibiotic was removed by medium exchange, and immediately, or after an additional 20 h, the supernatant was aspirated, the plates were chilled on ice, and the amoebae were scraped into 1.75 ml RNAprotect reagent. To disrupt the host cells, the suspension was passed four times through a ball homogenizer (Isobiotec) with a clearance of 6 μm. Host-cell debris was removed by centrifugation (2 min, 250 g). To pellet bacteria, the supernatant was spun again (10 min, 5200 g), and the RNA was extracted.

(iii) Expression of lipid A biosynthesis genes in sessile L. pneumophila was determined by growing biofilms in 96-well plates in a batch system for 5 days, as described previously (Mampel et al., 2006). Planktonic bacteria were carefully aspirated, pooled, harvested and resuspended in RNAProtect reagent. Sessile bacteria were washed once with distilled water and resuspended in RNAProtect.

**Expression analysis of lipid A biosynthesis genes by RT-PCR.**

RNA isolated under the different growth conditions described above was reverse-transcribed using the 3’ primers listed in Supplementary Table S2. The primers were annealed to the RNA for 10 min at 70 °C. The reaction mixture contained 200 ng RNA (400 ng RNA from bacteria infecting A. castellani), 20 pmol 3’ primer, 0.5 mM dNTP and 80 U M-MLV reverse transcriptase in 25 μl 1 x reaction buffer (Promega), and was incubated for 60 min at 42 °C. PCR was performed with 2.5 μl reverse transcription mixture as a template, 50 pmol each of the 3’ and 5’ primers (Supplementary Table S2), 0.2 mM each of dNTP and 1 μl Taq polymerase. Cotranscription of gnnA and lscCl/lpxB1 was analysed using primer oUA50 to synthesize cDNA and primers oUA50 and oUA55 for PCR. The primers were designed to have a melting temperature of 58–60 °C and to yield a 480–520 bp product with 40–60 mol% G+C content. The PCR conditions were: 95 °C (30 s), 55 °C (30 s), 72 °C (40 s, or 45 s after the first 10 cycles). Samples were taken after the number of cycles indicated, and the amounts of the PCR products were determined on agarose gels.

**RESULTS**

L. pneumophila harbours multiple paralogues of lipid A biosynthesis genes

The L. pneumophila lscCl/lpxB1 gene has recently been identified as a gene that upon overexpression is cytotoxic for A. castellani (Albers et al., 2005). LscC is 34 % identical to the E. coli lipid A disaccharide synthase LpxB, which catalyses the glycosyl transferase reaction between UDP-2,3-diacylglycosamine and 2,3-diacylglycosamine-1-phosphate to form the tetra-acylated lipid IVₐ in the course of lipid A biosynthesis (Fig. 1). The L. pneumophila genome harbours another lpxB gene (lpxB2), which is 43 % identical to E. coli lpxB and 31 % identical to L. pneumophila lscCl/lpxB1, respectively, at the amino acid level.

In order to gain more insight into lipid A biosynthesis in L. pneumophila, we examined the genomes of L. pneumophila Philadelphia-1, Paris and Lens for the presence of lipid A biosynthesis genes. Interestingly, the L. pneumophila strains were found to harbour not only single orthologues of the E. coli genes required for the constitutive, GlcNAc-based lipid A biosynthesis pathway [lpxA, -B, -C, -D, -H, -K, -L and WaaA (KdA)], but also multiple copies of some of the genes (Fig. 1, Table 1). Moreover, in agreement with the earlier finding that L. pneumophila synthesizes a GlcN3N-derivative of lipid A (Zähringer et al., 1995), orthologues of the Acidithiobacillus ferooxidans gnnA and gnnB genes are also present. These genes encode an NAD⁺-dependent oxidase and a pyridoxal phosphate-dependent transaminase, respectively, which convert UDP-GlcNAc into its 3-amino derivative UDP-GlcNAc3N (Sweet et al., 2004).

L. pneumophila is unique among a number of bacteria in containing multiple paralogues of the disaccharide synthase LpxB, and also of the primary acyl transferases LpxA (Acyl-ACP: UDP-GlcNAc O-acyltransferase) and LpxD [Acyl-ACP: UDP-3-O-(3-hydroxyacyl)-GlcN N-acyltransferase] (ACP, acyl carrier protein) (Supplementary Table S3). The bacteria examined include Coxiella burnetii, the closest relative of L. pneumophila, and other pathogenic or symbiotic species from different taxonomic groups. Among the bacteria analysed, only the intracellular

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*The proteins harbour of L. pneumophila strain Philadelphia-1 are indicated. The corresponding genes are also organized in similar clusters in L. pneumophila strains Paris and Lens (see Supplementary Table S4).

†Gene clusters are labelled with different symbols (●, ▲, ■). Percentage identity to the orthologous enzymes from E. coli or A. acidiferrooxidans (GnnA, -B) is shown in parentheses.

Table 1. Paralogues of lipid A biosynthesis proteins in L. pneumophila
pathogenic bacterium *Francisella tularensis* also harbours two paralogues of *lpxD*. All three *L. pneumophila* strains contain two paralogues of *lpxA* and *lpxB*, and three *lpxD* paralogues, respectively. The genes are clustered similarly in the three *L. pneumophila* strains, yet their order varies to some extent (see Supplementary Table S4, available with the online version of this paper).

Kdo₂-lipid IVₐ is converted into lipid A in *E. coli* by the addition of lauroyl and myristoyl residues to the primary acyl chains. These reactions are catalyzed by the ACP-dependent acyl transferases LpxL and LpxM, respectively (Fig. 1). While the *L. pneumophila* genome contains three *lpxL* homologues (Table 1), no gene with significant homology to LpxM was identified.

### L. pneumophila lcsC/lpxB1 forms an operon with gnnA and complements a conditional *E. coli* lpxB mutant strain

Both *L. pneumophila* lpxB paralogues are located adjacent to other lipid A biosynthesis genes in the genome. *LcsC/lpxB1* (*lpg1371*) is located immediately downstream of *gnnA* (*lpq1372*), which encodes the putative first enzyme of the lipid A biosynthesis pathway in *L. pneumophila*. To examine whether the two genes are cotranscribed, RNA was isolated, and RT-PCR was performed using primers hybridizing to *gnnA* and *lcsC/lpxB1*, respectively. The specific amplification of a 926 bp PCR product demonstrates that *gnnA* and *lcsC/lpxB1* are cotranscribed (see Supplementary Fig. S1, available with the online version of this paper), supporting the idea that both enzymes participate in the same biosynthetic pathway. *LpxB2* (*lpq2945*) lies next to *lpxD2* (*lpq2944*) and *lpxA2* (*lpq2943*) (Table 1), but is encoded on the opposite DNA strand, and therefore does not form an operon with these genes.

The genomic organization of the *L. pneumophila* lpxB genes suggests that both paralogues are involved in lipid A biosynthesis. To test whether the *lpxB* paralogues function as lipid A disaccharide synthases, a complementation assay was performed. Deletion of *lpxB* is lethal for *E. coli*; however, a temperature-sensitive mutant strain (MN7) is available (Nishijima et al., 1981). The strain was transformed with *L. pneumophila lcsC/lpxB1* or *lpxB2* expression vectors and grown on plates at 30 °C (permissive temperature) or 42 °C (non-permissive temperature) for 24 or 48 h. Under these conditions, expression of *E. coli lpxB* on plasmid pSR8 restored growth at 42 °C to a level similar to that at 30 °C. Expression of *L. pneumophila lcsC/lpxB1*, or to a lesser extent *lpxB2*, complemented growth at the non-permissive temperature of 42 °C (Fig. 2), indicating that both *L. pneumophila* lpxB paralogues show lipid A disaccharide synthase activity. Expression of *L. pneumo-

### Cytotoxicity of *L. pneumophila* lpxB paralogues towards *A. castellanii*

Since both *L. pneumophila* lpxB paralogues show lipid A disaccharide synthase activity, the genes might substitute for each other, contrary to the situation in *E. coli*, where the single *lpxB* gene is essential (Raetz & Whitfield, 2002). Therefore, we attempted to chromosomally delete the *lcsC/lpxB1* gene by allelic exchange. However, in several attempts, we did not obtain a strain lacking *lcsC/lpxB1*, suggesting that the gene might be essential for *L. pneumophila*.

Since *lcsC/lpxB1* was identified as a result of its ability to increase the cytotoxicity of an *L. pneumophila icmG* mutant strain towards *A. castellanii*, we tested whether the expression of *lpxB2* is also toxic for amoebae. *A. castellanii* was infected with an *icmG* mutant strain expressing either *lcsC/lpxB1* or *lpxB2*, and cytotoxicity was determined by PI
uptake 2 days post-infection (Albers et al., 2005). Under these conditions, overexpression of lcsC/lpxB1, but not lpxB2, was cytotoxic for A. castellanii (Fig. 3a). While the expression of lcsC/lpxB1 in an L. pneumophila icmG mutant background killed approximately 27% of the amoebae within 2 days, expression of lpxB2 did not increase cytotoxicity above background level (Fig. 3b). This finding suggests that the two L. pneumophila lpxB paralogues are not functionally equivalent. Upon induction of the lpxB paralogues in L. pneumophila grown in broth or on plates, growth of bacteria overexpressing lcsC/lpxB1 or lpxB2 was impaired, suggesting that the expression of the lpxB paralogues is toxic to some extent for the bacteria (data not shown). However, the colony-forming efficiency of L. pneumophila strains overexpressing lcsC/lpxB1 or lpxB2 was not lower than that of a control strain harbouring the empty plasmid, and within A. castellanii these strains survived and grew similarly to the control strain (Fig. 3c). These results indicate that expression of lcsC/lpxB1 but not lpxB2 kills the amoebae by a mechanism not related to intracellular replication. Furthermore, under the conditions used, the extra- and intracellular viability of L. pneumophila was not affected by overexpression of either lpxB parologue.

Fig. 3. Cytotoxicity of L. pneumophila lpxB paralogues to A. castellanii. Amoebae were infected at an m.o.i. of 50 with L. pneumophila icmG mutant strains harbouring either the control plasmid pMMB207C-RBS (pBCR, □), the complementing plasmid pGS-Lc-63-14 (plcmG, ■), or plasmids pBCR-lcsC (pLcsC, ●) or pBCR-lpxB (pLpxB, ○), which express lcsC/lpxB1 or lpxB2, respectively. (a) Cytotoxicity was assayed 2 days post-infection (30 °C) by uptake of the fluorescent dye PI. Bright-field (upper panel) and fluorescence micrographs (lower panel) are shown. Bar, 20 μm. (b) Quantification of cytotoxicity data, representing means and SDs of triplicate fields of view (500–1000 amoebae). A two-tailed Student’s t test was used to determine statistical significance (P<0.05). Similar results were obtained in three independent experiments. (c) Intracellular growth of L. pneumophila within A. castellanii was determined by counting c.f.u. in supernatants of infected amoebae.
CD14-dependent and -independent induction of TNFα by purified LPS from L. pneumophila expressing lpxB paralogues

TNFα production by macrophages exposed to LPS is known to be very sensitive to the structure and concentration of the LPS (Gangloff et al., 1999, 2005). Confirming previous observations, we found that wild-type mouse peritoneal macrophages were 4–5 orders of magnitude less sensitive to purified LPS from L. pneumophila JR32 (serogroup 1) or an isogenic icmG mutant strain, compared to purified smooth LPS from E. coli (serogroup O111: B4), and this effect was dependent on the presence of the LPS receptor CD14 (Fig. 4; Neumeister et al., 1998). To test the effects of lcsC/lpxB1 and lpxB2 on TNFα production, we purified LPS from an L. pneumophila icmG mutant strain expressing one of the two lpxB paralogues, which show a different degree of cytotoxicity towards amoebae (Fig. 3). Stimulation of CD14-expressing macrophages with purified LPS from the LPS receptor CD14-dependent and -independent induction of TNFα (1.5 μg ml⁻¹) (Fig. 4a). In contrast, compared to smooth E. coli LPS, similar amounts of purified LPS from L. pneumophila JR32 or the icmG mutant strain were required to trigger TNFα release from CD14-negative macrophages, and less than 10-fold more purified LPS_Lc LPS icmG produced similar amounts of TNFα compared to E. coli LPS (Fig. 4b). Interestingly, at the same concentration, LPS_Lc was more potent than LPS_Lb in triggering TNFα production by CD14-positive macrophages, and to a lesser extent, also by CD14-negative macrophages, suggesting that the LPS structures produced upon overexpression of lcsC/lpxB1 or lpxB2 are different.

Expression of L. pneumophila lpx genes during growth in a complex medium

Kdo-lipid A is essential for most Gram-negative bacteria, implying that single copies of the corresponding biosynthetic genes are essential and constitutively expressed. In contrast, in response to changing environmental conditions, the expression of a number of acyl transferases and other lipid A-modifying genes is regulated, leading to structurally different lipid A molecules (Miller et al., 2005; Raetz & Whitfield, 2002). L. pneumophila harbours multiple paralogues of lipid A biosynthesis genes, which are predicted to modulate the acylation state of lipid A and might be regulated in response to environmental conditions. To determine the conditions under which L. pneumophila lipid A biosynthesis genes are transcribed, we examined by RT-PCR the gene-expression levels in response to growth rate and hypotonic stress, and in biofilms or amoebae.

The RT-PCR method was validated using L. pneumophila genes with known expression profiles during different growth phases in liquid medium: 16S rRNA was used as a control for RNA present at constitutively high levels, rpoA encodes the α subunit of RNA polymerase, which is maximally expressed during the exponential phase, and flaA encodes the major flagellum component (flagellin) that is strongly upregulated during the stationary phase, which accords with the observation that L. pneumophila becomes motile upon entry into stationary phase (Byrne & Swanson, 1998; Heuner et al., 1999). As expected, 16S rRNA was constitutively expressed at high levels, rpoA was expressed in all growth phases, but moderately downregulated in late stationary phase, and the expression of flaA was strongly upregulated in late stationary phase.
(Fig. 5a). In the absence of reverse transcriptase, only faint bands from the target genes were visible after 32 PCR cycles, indicating that the amount of contaminating DNA in the samples was low.

The expression levels of the lpxB and lpxA paralogues were examined in the exponential, early stationary and late stationary phases. Under the conditions used, the amount of lcsC/lpxB1 and lpxB2 RNA was rather low in all growth phases, and expression of lcsC/lpxB1 was further reduced in late stationary phase. The two lpxA paralogues were expressed at comparable levels, preferentially in the exponential and early stationary phases. Since the lpxB and lpxA paralogues are synchronously expressed during growth of L. pneumophila in a rich medium, the genes seem to be used in parallel under these conditions (Fig. 5a).

Expression of L. pneumophila lpx genes under hypotonic stress and nutrient deprivation

To analyse the expression of L. pneumophila lipid A biosynthesis genes during osmotic stress or nutrient deprivation, a liquid culture in stationary phase (OD 600
3.3) was split and suspended either in distilled water or in spent AYE medium. L. pneumophila tolerates exposure to distilled water, and gene expression was examined 5 h after incubation at room temperature. While expression of flaA was not affected upon transition of L. pneumophila from rich medium to distilled water, the expression of lpxA1 and, to a lesser extent, lcsC/lpxB1 was upregulated, while the expression of lpxB2 was slightly down-regulated under these conditions (Fig. 5b). The expression of lpxA2 in late stationary phase was very low and not induced upon incubation of L. pneumophila in distilled water. The inverse regulation of lcsC/lpxB1 and lpxB2 and the upregulation of lpxA1 suggest that under osmotic stress or nutrient deprivation, L. pneumophila preferentially employs lcsC/lpxB1 and lpxA1 to modulate its lipid A structure.

Expression of L. pneumophila lpx genes during intracellular growth in A. castellanii and in biofilms

Pathogenic bacteria respond to the challenges of the intracellular environment of phagocytes by modulating their lipid A (Miller et al., 2005). To examine lpx gene expression during residence in a phagocyte, A. castellanii amoebae were infected with L. pneumophila, and bacterial gene expression was analysed at different time points post-infection. Due to intracellular bacterial replication, the proportion of bacterial versus amoebic RNA is higher in the 24 h sample than in the 4 h sample. Therefore, the method used here allows comparison of different genes within the same RNA preparation (at a given time point), but does not allow direct comparison of expression levels at different time points.

At 24 h post-infection, the lpxA and lpxB paralogues, as well as lpxC, were abundantly expressed at comparable levels by L. pneumophila residing within A. castellanii, while at 4 h post-infection, lpxA1 was slightly more strongly expressed than lpxA2 (Fig. 6a). This finding suggests that lpxA1 is required earlier than lpxA2 during the transition of L. pneumophila from the transmissive (post-exponential) phase to the replicative phase in A. castellanii. Notably, lpxA1 displayed a similar pattern of expression upon incubation of the bacteria in distilled water (Fig. 5b), and thus the gene appears to be similarly regulated under several environmental conditions.

Finally, to determine whether the substratum plays a role for the expression of L. pneumophila lipid A biosynthesis genes, we assayed the transcription of lpxA and lpxB in sessile and planktonic L. pneumophila grown to stationary phase in a batch biofilm system run with AYE medium (Mampel et al., 2006). Five days after inoculation, the lpxA and lpxB paralogues were expressed to a similar extent in sessile L. pneumophila adhering to the polystyrene surface of 96-well plates (Fig. 6b). Similarly, the lpxA and lpxB paralogues were expressed in the planktonic bacteria residing in the supernatant of the biofilm. Thus, under the conditions used, the lpxA and lpxB paralogues do not seem to be differentially regulated in biofilms formed by L. pneumophila.

DISCUSSION

A bioinformatic search for genes with significant homology to E. coli and A. ferrooxidans lipid A biosynthesis genes revealed that L. pneumophila harbours genes required for the conversion of GlcN3Ac to GlcN3Ac3N (gnaA and -B), several paralogues of acyl transferases (lpxA, lpxD and lpxL), as well as two paralogues of the lipid A disaccharide synthase lpxB (Fig. 1, Table 1). The first gene of the L. pneumophila GlcN3Ac3N-dependent lipid A biosynthesis pathway, gnaA, and lcsC/lpxB1 were found to be co-transcribed (Supplementary Fig. S1). This gene organization is analogous to that of E. coli and many other bacteria employing a GlcN3Ac-dependent lipid A biosynthesis pathway, where the first gene in the pathway, lpxA, and lpxB are co-transcribed (Crowell et al., 1986, 1987; Raetz & Whitfield, 2002).

Both L. pneumophila lpxB paralogues partially complemented an E. coli conditional lpxB mutant, indicating that the L. pneumophila lpxB genes indeed function as lipid A disaccharide synthases (Fig. 2). The observed low efficiency of lpxB complementation is likely to be due to the fact that the L. pneumophila and E. coli substrates of LpxB are structurally different. While E. coli LpxB uses GlcN-based substrates, L. pneumophila LpxB is predicted to condense GlcN3N-based lipid A precursors, which are absent in E. coli (Sweet et al., 2004). Moreover, the primary fatty acid chains of E. coli and L. pneumophila lipid A precursors differ considerably in length (C14 and C20, respectively). These structural differences in the LpxB substrates likely result in a reduced efficiency of lipid A synthesis upon
heterologous complementation of the conditional E. coli lpxB mutant.

The multiple paralogues of the putative acyltransferases LpxA, LpxD and LpxL may have different substrate specificities and might preferentially use fatty acids with distinct chain lengths, branches or other modifications, which have been identified in L. pneumophila lipid A (Moll et al., 1992; Zähringer et al., 1995). Moreover, the two LpxB lipid A disaccharide synthases might condense differently acylated substrates generated by the acyltransferase paralogues, resulting in lipid IVα moieties that vary in primary fatty acid composition. Alternatively, the LpxB paralogues might use either GlcN3N- or GlcN-based precursors as a substrate to form mixed lipid A species containing both GlcN3N and GlcN, as described for A. ferrooxidans and other bacteria (Sweet et al., 2004). In agreement with the notion that L. pneumophila produces lipid A moieties with different structures, the lpxB paralogues were found to be functionally distinct, as overexpression of lcsC/lpxB1, but not lpxB2, was cytotoxic for amoebae (Fig. 3), and LPS preparations of lcsC/lpxB1 or lpxB2 expressed by an L. pneumophila icmG mutant induced TNFα in peritoneal macrophages with different potencies (Fig. 4).

The LPS pattern of L. pneumophila grown intracellularly in Acanthamoeba polyphaga has been found to differ from the LPS pattern of bacteria grown in broth (Barker et al., 1993), and recently, LPS-containing vesicles released from the outer membrane of growing L. pneumophila have been shown to inhibit phagosome–lysosome fusion in infected macrophages independently of the Icm/Dot secretion system (Fernandez-Moreira et al., 2006). Interestingly, only vesicles derived from the transmissive/infective phase, and not the replicative phase, inhibited phagosome–lysosome fusion, and the structure of LPS was developmentally regulated, indicating that LPS modifications might be involved in altering host-cell vesicle trafficking. Thus, L. pneumophila produces distinct lipid A structures in response to different environmental conditions.

To analyse whether differences in the LPS structure correlate with a differential regulation of lpx genes, we analysed the transcription of these genes under various conditions. During growth in nutrient broth, there were no large differences between the expression of lpxA1 and lpxA2 or between the expression of lcsC/lpxB1 and lpxB2 (Fig. 5a). However, upon transfer of L. pneumophila from nutrient broth into distilled water, expression of lpxA1 and, to a lesser extent, lcsC/lpxB1 was upregulated, while expression...
of lpxB2 was slightly down-regulated (Fig. 5b). Furthermore, shortly after infection of A. castellanii by L. pneumophila, lpxA1 was somewhat more strongly expressed than its parologue lpxA2 (Fig. 6a). This regulation pattern suggests a role for LcsC/LpxB1 and LpxA1 in L. pneumophila exposed to salt- and nutrient-free medium, possibly mimicking a nutritionally poor natural environment, and lpxA1 might represent a lipid A biosynthesis gene preferentially required during the transmissive/infective phase of L. pneumophila (Molofsky & Swanson, 2004). At 24 h post-infection, lpxA1 and lpxA2, as well as lcsC/lpxB1 and lpxB2, were expressed at a similar level, indicating a role in lipid A biosynthesis for all lpxA and lpxB paralogues at later stages of the infection, during which L. pneumophila is in the replicative phase. Accordingly, the lpxA and lpxB paralogues were also expressed at similar levels during exponential replication in broth and in early, but not in late stationary phase (Fig. 5a).

Structural modifications of lipid A may also significantly affect the course of infection in multicellular host organisms. Lipid A (and especially the secondary acyl chains) is the active part of endotoxin, which is recognized by the innate immune system by binding to the CD14 receptor and toll-like receptor (TLR)4 (Miller et al., 2005; Raetz & Whitfield, 2002). L. pneumophila LPS fails to bind CD14 (Neumeister et al., 1998) and TLR4 (Girard et al., 2003), probably due to the unusually long and branched fatty acid residues of its lipid A. The specific composition of L. pneumophila lipid A fatty acids, possibly attached by the different lpx paralogues, might contribute to avoidance of the recognition of the bacteria by the innate immune system. Thus, due to the involvement of L. pneumophila lipid A in stress protection, immune recognition and intracellular life, modification of its structure contributes to the adaptation of L. pneumophila to its diverse niches.

Fig. 6. Expression of L. pneumophila lpx genes during intracellular growth within A. castellanii or in biofilms. (a) RNA from uninfected A. castellanii amoebae (0 h) or from amoebae infected for 4 or 24 h was extracted, and expression of lipid A biosynthesis genes was assayed by RT-PCR. The number of PCR cycles is indicated on the right. G, genomic DNA as PCR template; −, without reverse transcriptase (RT); +, with reverse transcriptase. (b) To quantify lipid A biosynthesis gene expression in biofilms, RNA from sessile or planktonic L. pneumophila was extracted after 5 days’ growth in a 96-well plate in AYE medium. Similar results were obtained in three (a) or two (b) experiments.
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