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 (lcm/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 *gnaA*, encoding a putative UDP-GlcNAc oxidase in the biosynthetic pathway leading to 3-aminoglucosamine 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* *lcmG* 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.

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Abbreviations: ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; ACP, acyl carrier protein; lcm/Dot, intracellular multiplication/defective organelle trafficking; GlcN, 2-amino-2,3-dideoxy- α -D-glucopyranose (glucosamine); GlcN3N, 2,3-diamino-2,3-dideoxy- α -D-glucopyranose; GlcNAc, 2-acetamido-2,3-dideoxy- α -D-glucopyranose (*N*-acetylglucosamine); GlcNAc3N, 2-acetamido-3-amino-2,3-dideoxy- α -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 *gnaA*, are available with the online version of this paper.

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. α -KG, α -ketoglutarate.

[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 a failure to interact with the soluble LPS receptor CD14 (Neumeister *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-ulonic 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 legionaminic acid. The hydroxyl and amino groups of legionaminic 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 lipid A 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 (Robey *et al.*, 2001). Similar to a *pagP* mutant, an *rcp* mutant also showed decreased resistance to CAMPs. Moreover, the *rcp* mutant 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 in 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 legionaminic 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 C_{16–18} fatty acids instead of 3-hydroxylated saturated C_{20–22} 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 $\mu\text{g ml}^{-1}$) or kanamycin (Km; 50 $\mu\text{g ml}^{-1}$), if necessary. *E. coli* strains were cultured in LB medium, supplemented with Cm (30 $\mu\text{g ml}^{-1}$), ampicillin (Ap; 100 $\mu\text{g ml}^{-1}$) 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, proteose 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 (*lpg1371*) including the RBS was released from plasmid pUA26 (pMMB207-RBS-*lcsC*) by digestion with *EcoRI* and *BamHI* and inserted into the same restriction sites of pMMB207C. Plasmid pTS-10 (pMMB207C-RBS) was constructed by cutting pMMB207C-RBS-*lcsC* with *NdeI* and *BamHI*, filling in with Klenow polymerase and religation with T4 DNA polymerase. To construct plasmid pBCR-*lpxB* (pMMB207C-RBS-*lpxB*), the corresponding ORF (*lpg2945*) was amplified by PCR using chromosomal DNA as template and the oligonucleotides oLpxB2fo and oLpxB2re (see Supplementary Table S2), respectively. The PCR fragment was cut with *NdeI* and *PstI* 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/lpxB1*. We attempted to generate a chromosomal insertion deletion of *lcsC/lpxB1* in *L. pneumophila* strain JR32 by allelic exchange using the suicide vector pLAW344 (Wiater *et al.*, 1994). To construct the suicide vector, plasmid pUA9 containing an in-frame partial deletion in *lcsC/lpxB1* (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/lpxB1*) and blunted, and the Km-resistance cassette from pUC4K was released with *Bam*HI, blunted and inserted to yield pUA44. The insert with the Km cassette in *lcsC/lpxB1* was released by *Eco*RI, blunted and inserted in pLAW344 digested with *Eco*RV 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 (Wiater *et al.*, 1994; Tieden *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^R, Suc^R, Cm^S) were further analysed by PCR using the primers oUA7, oUA29, oKmfo and oKmr 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, AAS48421; GnnB, AAS48422).

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 electroporation 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 adjusted to an OD₆₀₀ 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 × 10⁴ *A. castellanii* per well (24-well plates) or 2.5 × 10⁴ *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 by 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 OD₆₀₀, ~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 × 10⁶ 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 10⁻¹–10⁴ 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 OD₂₆₀. 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 OD₆₀₀ 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 *gmnA* and *lcsC/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, 2×10^6 *A. castellanii* were seeded in a 10 cm dish the day before the experiment, infected with *L. pneumophila* in early stationary phase at an 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 bacteria 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 and cooled on ice before the other components were added. The final reaction mixture contained 200 ng RNA (400 ng RNA from bacteria infecting *A. castellanii*), 20 pmol 3' primer, 0.5 mM of each dNTP and 80 U M-MLV reverse transcriptase in 25 µl 1 × 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 of each dNTP and 1 µl Taq polymerase. Cotranscription of *gmnA* and *lcsC/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* *lcsC/lpxB1* gene has recently been identified as a gene that upon overexpression is cytotoxic for *A. castellanii* (Albers *et al.*, 2005). *LcsC* is 34 % identical to the *E. coli* lipid A disaccharide synthase *LpxB*, which catalyses the glycosyl transferase reaction between UDP-2,3-diacylglucosamine and 2,3-diacylglucosamine-1-phosphate to form the tetra-acylated lipid IV_A 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* *lcsC/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* (*KdtA*)], 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 ferrooxidans* *gmnA* and *gmnB* 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

Table 1. Paralogues of lipid A biosynthesis proteins in *L. pneumophila*

Protein	Paralogue*		
	1	2	3
GnnA	Lpg1372 (39 %) ●†		
GnnB	Lpg1424 (39 %)	Lpg0755 (34 %)	
LpxA	Lpg0511 (47 %) ▲	Lpg2943 (45 %) ■	
LpxB	Lpg1371 (34 %) ●	Lpg2945 (43 %) ■	
LpxC	Lpg2608 (58 %)		
LpxD	Lpg0508 (36 %) ▲	Lpg2944 (33 %) ■	Lpg0100 (30 %)
LpxH	Lpg1552 (38 %)		
LpxK	Lpg1818 (42 %)		
LpxL	Lpg2940 (24 %) ■	Lpg2941 (18 %) ■	Lpg0363 (24 %)
LpxM	Not present		
WaaA	Lpg2340 (43 %)		

*The proteins harboured by *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.

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 IV_A is converted into lipid A in *E. coli* by the addition of lauroyl and myristoyl residues to the primary acyl chains. These reactions are catalysed 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 *gmnA* 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 *gmnA* (*lpg1372*), 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 *gmnA* and *lcsC/lpxB1*, respectively. The specific amplification of a 926 bp PCR product demonstrates that *gmnA* 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* (*lpg2945*) lies next to *lpxD2* (*lpg2944*) and *lpxA2* (*lpg2943*) (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. pneumophila lcsC/lpxB1* or *lpxB2* complemented growth at the non-permissive temperature of 42 °C fully or partially, respectively, as compared to the corresponding empty plasmid pBCR (pMMB207C-RBS) (Fig. 2). These results

Fig. 2. The *L. pneumophila lpxB* paralogues participate in lipid A biosynthesis. To assay the function of *L. pneumophila lpxB* paralogues, the conditional *E. coli lpxB* mutant strain MN7 was transformed with plasmid pUC18 (vector control), pSR8 (*E. coli lpxB*), pBCR (pMMB207C-RBS, vector control), pLcsC (pBCR-*lcsC*, *L. pneumophila lcsC/lpxB1*) or pLpxB (pBCR-*lpxB*, *L. pneumophila lpxB2*), and the strains were grown on agar plates at the permissive (30 °C, white bars) or non-permissive (42 °C, black bars) temperature. At the non-permissive temperature, the plating efficiency of strains containing derivatives of plasmid pBCR was three orders of magnitude lower than that of strains harbouring derivatives of pUC18. The data represent means and SDs of triplicates. A two-tailed Student's *t* test was used to determine statistical significance ($P < 0.01$). Similar results were obtained in three independent experiments.

indicate that both *L. pneumophila lpxB* paralogues show lipid A disaccharide synthase activity.

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*.

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

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* paralogue.

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 μ m. (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 TNF α by purified LPS from *L. pneumophila* expressing *lpxB* paralogues

TNF α 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 TNF α 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 *icmG* mutant strain expressing *lcsC/lpxB1* (LPS_{LcsC}) or *lpxB2* (LPS_{LpxB2}) required approximately 7000 and 60 000 times more LPS, respectively, than purified smooth *E. coli* LPS to produce an equivalent concentration of TNF α (1.5 $\mu\text{g ml}^{-1}$) (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 TNF α release from CD14-negative macrophages, and less than 10-fold more purified LPS_{LcsC} or LPS_{LpxB2} produced similar amounts of TNF α compared to *E. coli* LPS (Fig. 4b). Interestingly, at the same concentration, LPS_{LcsC} was more potent than LPS_{LpxB2} in triggering TNF α 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

Fig. 4. CD14-dependent and -independent induction of TNF α by purified LPS from *L. pneumophila* expressing *lpxB* paralogues. Murine peritoneal macrophages from (a) wild-type (CD14-expressing) BALB/c mice, or from (b) CD14-deficient BALB/c mice were stimulated with purified, smooth LPS from *E. coli* O111:B4 ($\circ$), purified LPS from *L. pneumophila* JR32 wild-type or *icmG* mutant strains harbouring the empty plasmid pMMB207C (JR32/pBC, $\triangle$; *icmG*/pBC, $\square$), or plasmids expressing *lcsC/lpxB1* (*icmG*/pLcsC, $\blacktriangle$) or *lpxB2* (*icmG*/pLpxB2, $\blacksquare$), and the production of TNF α was measured. The results are representative of four independent experiments.

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 down-regulated 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 (OD₆₀₀ 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 GlcNAc to GlcNAc3N (*gmnA* 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* GlcNAc3N-dependent lipid A biosynthesis pathway, *gmnA*, 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 GlcNAc-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

Fig. 5. Expression of *L. pneumophila* *lpx* genes in liquid cultures. (a) The expression of genes with known expression profiles and *lpxB* or *lpxA* paralogues was assayed using RT-PCR in *L. pneumophila* grown in AYE broth. The number of PCR cycles is indicated on the right. EX, exponential phase (OD₆₀₀ 0.8, 6 h incubation); ES, early stationary phase (OD₆₀₀ 2.4, 16 h); LS, late stationary phase (OD₆₀₀ 3.4, 26 h); G, genomic DNA as PCR template; –, without reverse transcriptase (RT); +, with reverse transcriptase. (b) To analyse expression of *L. pneumophila* *lpx* genes under osmotic stress and nutrient deprivation, bacteria from stationary-phase liquid cultures were split and incubated either in spent AYE medium or in distilled water for 5 h at room temperature. RNA was extracted and expression levels were assayed by RT-PCR. Similar results were obtained in three experiments.

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 IV_A 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 TNF α 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

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.

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 paralogue *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.

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