Identification, characterization and differential expression of a cytotoxic *Legionella pneumophila* lipid A disaccharide synthase paralogue

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Abstract

*Legionella pneumophila* replicates intracellularly in a specific vacuole in phagocytic cells. The major virulence determinant is the Icm/Dot type IV secretion system which is required for intracellular growth. An *icmG* mutant strain shows a partial growth defect in presence of the amoeba *Acanthamoeba castellanii* on agar plates and was used to screen a genomic *L. pneumophila* library to identify proteins involved in virulence. Thus, suppressors of the growth defect of the *icmG* mutant strain were selected. The genes responsible for the suppression were identified on the library plasmid by deletion analysis. Increased survival in presence of amoebae was found not to be caused by more efficient intracellular growth but by an enhanced cytotoxicity for amoebae. Therefore, the genes were termed *Legionella* cytotoxic suppressors (*lcs*). Cytotoxic genes identified showed similarities to hydrolases (*lcsA*), NlpD-related metalloproteases (*lcsB*), lipid A disaccharide synthases (*lcsC*), and ABC transporters (*lcsD*). Overexpression of *lcsC* strongly increased cytotoxicity of Δ*icmG*, but not of other *icm/dot* mutant strains, on amoebae.

The *L. pneumophila* genome contains two paralogues of the lipid A disaccharide synthase: *lpxB1/lcsC* and *lpxB2*. The corresponding proteins are 34% or 43%, respectively, homologous to the *E. coli* lipid A disaccharide synthase LpxB. The genome of *L. pneumophila* contains not only two paralogues of *lpxB*, but also the acyl transferases *lpxA*, *lpxD* and *lpxL* are present in more than one copy. Presence of multiple copies of lipid A biosynthesis genes was only found in *L. pneumophila* but not in the genomes of any other bacterial species. Genomic localization of the *lpxB* paralogues and their ability to complement a temperature-sensitive *E. coli* *lpxB* mutant indicate that both paralogues function as a lipid A disaccharide synthase.

Expression of the *lpxA* and *lpxB* paralogues under different conditions was examined using reverse transcription and PCR. *lpxA1* and *lpxB1/lcsC* are more strongly expressed compared to their paralogues *lpxA2* and *lpxB2* during early stages of infection of *A. castellanii* and are upregulated upon transfer of a stationary phase culture from nutrient broth into distilled water.

Structural modifications of lipid A affect the protective functions of the outer membrane and the degree of virulence. The presence of multiple acyl transferase genes of the lipid A biosynthesis pathway and the expression of *lpxA* and *lpxB* paralogues depending on different environmental conditions suggest that *L. pneumophila* adjusts the structure of lipid A, most
likely the fatty acid composition, to adapt to the diverse environmental niches inside and outside of host cells.

The screen for virulence genes was performed in the *icmG* mutant strain, and overexpression of *lcsC* rendered Δ*icmG* cytotoxic, but not other partially virulence-defective mutants. Therefore, another project addressed the function of the IcmG protein. IcmG contains a transmembrane domain, and an N-terminal domain was shown to bind to RalF and the Sid effector proteins. A C-terminal coiled-coil region was predicted to function as a SNARE domain. However, we could neither detect secretion of IcmG into the supernatant nor translocation into the host cell. Since translocation is expected to be required for a SNARE protein, it is unlikely that IcmG acts as a SNARE. An *icmG* mutant strain is still able to secrete the type IV effector protein SidC, but shows a decreased recruitment of ER-derived vesicles to the *Legionella* containing vacuole. We therefore propose that IcmG acts as a coupling protein increasing the efficiency of type IV effector translocation into the host rather than acting as a SNARE protein.
Zusammenfassung


Zusammenfassung

Strukturelle Änderungen von Lipid A beeinflussen die protektiven Funktionen der äusseren Membran und das Ausmass der Virulenz. Das Vorhandensein mehrerer Acyltransferasen des Lipid A Biosyntheseweges und die differentielle Expression der lpxA und lpxB Paraloge deuten darauf hin, dass L. pneumophila die Struktur von Lipid A, insbesonders die Fettsäurezusammensetzung, an die verschiedenen Nischen innerhalb und ausserhalb der Wirtszelle anpasst.

# Table of contents

ABSTRACT ........................................................................................................... 2
ZUSAMMENFASSUNG .......................................................................................... 4
TABLE OF CONTENTS ...................................................................................... 6

1 GENERAL INTRODUCTION ........................................................................ 8

1.1 ENVIRONMENTAL GROWTH AND DIFFERENTIATION OF LEGIONELLAE 8
1.2 LEGIONELLA – THE ETIOLOGICAL AGENT OF LEGIONNAIRES' DISEASE 9
1.3 INTRACELLULAR REPLICATION AND VIRULENCE FACTORS OF L. PNEUMOPHILA 10
1.4 THE ICM/DOT TYPE IV SECRETION SYSTEM 11
1.5 LEGIONELLA LPS ................................................................. 14
   1.5.1 Modifications of lipid A .......................................................... 16
   1.5.2 Interaction of lipid A with components of the innate immune system 19
1.6 LITERATURE ...................................................................................... 20

2 THE AMOEBAE PLATE TEST IMPLIES A PARALOG OF LpxB IN THE INTERACTION OF LEGIONELLA PNEUMOPHILA WITH ACANTHAMOEBA CASTELLANII ....... 25

2.1 ABSTRACT ...................................................................................... 26
2.2 INTRODUCTION ............................................................................. 26
2.3 METHODS ...................................................................................... 28
   2.3.1 Bacterial strains, cell culture and reagents ......................................... 28
   2.3.2 Construction of plasmids ..................................................................... 30
   2.3.3 The amoebae plate test ................................................................. 31
   2.3.4 Screening of an L. pneumophila chromosomal library for icm/dot suppressors using the APT ................................................................. 32
   2.3.5 Analysis of suppressor plasmids .......................................................... 33
   2.3.6 Intracellular growth in A. castellanii and RAW264.7 macrophages 33
   2.3.7 Cytotoxicity assay ............................................................................. 33
   2.3.8 Computational and statistical analysis .............................................. 34
2.4 RESULTS ......................................................................................... 34
   2.4.1 Growth of L. pneumophila on CYE agar plates in presence of A. castellanii – the amoeba plate test ................................................................. 34
   2.4.2 Isolation of L. pneumophila icm/dot suppressor strains using the amoeba plate test ................................................................. 36
   2.4.3 Analysis of suppressor plasmid inserts ................................................. 38
   2.4.4 Intracellular growth of icmG suppressor strains in A. castellanii and macrophages ................................................................. 40
   2.4.5 icmG suppressor strains are cytotoxic for A. castellanii ................................................. 42
   2.4.6 Identification of cytotoxic suppressor genes by deletion analysis 44
   2.4.7 Overexpression of suppressor genes .................................................. 46
2.5 DISCUSSION...................................................................................... 49
   2.5.1 Screen for icm suppressors using the amoebae plate test 49
   2.5.2 LcsB, a homolog of NlpD-like membrane-bound metalloproteases 50
   2.5.3 LscC, a paralog of the lipid A disaccharide synthase LpxB 52
   2.5.4 Mechanism of LcsC cytotoxicity ......................................................... 53
2.6 ACKNOWLEDGEMENTS ...................................................................... 54
2.7 REFERENCES .................................................................................. 54

3 THE CYTOTOXIC LEGIONELLA PNEUMOPHILA LpxB PARALOGUE Lcsc IS INVOLVED IN LIPID A BIOSYNTHESIS AND UPREGULATED DURING BACTERIAL GROWTH IN AMOEBAE ............... 60

3.1 ABSTRACT ...................................................................................... 61
3.2 INTRODUCTION ............................................................................. 61
3.3 METHODS ...................................................................................... 65
   3.3.1 Bacteria, amoebae and reagents .......................................................... 63
   3.3.2 Identification of homologues of L. pneumophila lipid A biosynthesis genes 64
   3.3.3 Construction of plasmids ..................................................................... 64
   3.3.4 Complementation of a conditional E. coli lpxB mutant strain with L. pneumophila lpxB paralogues ................................................................. 65
# Table of contents

3.3.5 Cytotoxicity assay .................................................................................................................. 65
3.3.6 Expression analysis of LPS biosynthesis genes by RT-PCR .................................................. 66

3.4 RESULTS .................................................................................................................................. 68
3.4.1 L. pneumophila paralogues of LPS biosynthesis genes ......................................................... 68
3.4.2 Complementation of a conditional E. coli IpxB mutant strain with L. pneumophila
IpxB paralogues ............................................................................................................................. 70
3.4.3 Cytotoxicity of L. pneumophila IpxB paralogues for A. castellanii ............................... 71
3.4.4 Expression of L. pneumophila lpx genes during growth in a complex medium ............. 73
3.4.5 Expression of L. pneumophila lpx genes under osmotic stress and nutrient deprivation.. 75
3.4.6 Expression of L. pneumophila lpx genes during intracellular growth in A. castellanii..... 76

3.5 DISCUSSION ............................................................................................................................. 77
3.5.1 Lipid A biosynthesis paralogues and their functions ......................................................... 77
3.5.2 Differential regulation of L. pneumophila lpx paralogues ............................................... 78
3.5.3 Biological implications of lipid A and LPS modifications ............................................... 79

3.6 ACKNOWLEDGEMENTS .......................................................................................................... 80
3.7 REFERENCES .............................................................................................................................. 81

4 L. PNEUMOPHILA ICMG INCREASES THE EFFICIENCY OF EFFECTOR PROTEIN
SECRETION ...................................................................................................................................... 85

4.1 INTRODUCTION ...................................................................................................................... 85
4.2 MATERIALS AND METHODS ................................................................................................. 87
4.2.1 Cultivation of cells ................................................................................................................ 87
4.2.2 Sequence analysis of IcmG ................................................................................................ 87
4.2.3 Construction of icmG expression vectors .......................................................................... 87
4.2.4 Pull-down assays using GST fusion proteins .................................................................... 88
4.2.5 Co purification of IcmG and binding proteins using His6-tagged IcmG ......................... 90
4.2.6 Generation of antibodies and Western blot ...................................................................... 90
4.2.7 Immunofluorescence microscopy ...................................................................................... 91

4.3 RESULTS ................................................................................................................................ 91
4.3.1 IcmG is present in the bacteria in low amounts ................................................................. 91
4.3.2 IcmG is not translocated during infection ......................................................................... 93
4.3.3 L. pneumophila ΔicmG is able to translocate StaC but is impaired in recruiting ER-derived
vesicles to the LCV ...................................................................................................................... 96
4.3.4 Identification of IcmG binding proteins using a pull down assay with GST fusion proteins.98
4.3.5 Co purification of IcmG binding proteins using His6-tagged IcmG ................................. 101

4.4 DISCUSSION ............................................................................................................................ 103
4.4.1 Expression level and localization of IcmG ......................................................................... 104
4.4.2 Phenotypes of the icmG mutant ....................................................................................... 104
4.4.3 Identification of IcmG binding proteins in pull down assays .......................................... 105
4.4.4 Hypotheses on the function of IcmG .............................................................................. 106

4.5 REFERENCES ............................................................................................................................ 108

5 GENERAL DISCUSSION ......................................................................................................... 111

5.1 Lcsc shows lipid A disaccharide synthase activity ................................................................. 111
5.2 L. PNEUMOPHILA: LIFE IN DIFFERENT NichES AND ADAPTATION OF LIPID A STRUCTURE 112
5.3 Intracellular LPS influences host cell functions ................................................................. 113
5.4 Possible influence of lipid A on outer membrane vesicles ............................................... 114
5.5 Cytoxicity of the lpxB paralogues .......................................................................................... 115
5.6 Putative mechanisms of Lcsc mediated cytotoxicity .......................................................... 116
5.7 Specificity of Lcsc-mediated cytotoxicity for ΔicmG .......................................................... 117
5.8 CONCLUSION ......................................................................................................................... 118
5.9 LITERATURE ............................................................................................................................ 118

CURRICULUM VITAE .................................................................................................................. 120

PUBLICATIONS ............................................................................................................................. 121
1 General introduction

1.1 Environmental growth and differentiation of Legionellae

Legionella spp. are Gram-negative, aerobic, monopolarly flagellated rod-shaped bacteria belonging to the gamma subdivision of proteobacteria. More than 50 Legionella species have been described so far (http://www.bacterio.cict.fr). Legionellae can be found ubiquitously in aquatic habitats and soil. In the environment, L. pneumophila parasitizes within a large variety of amoebae and protozoa rather than multiplying planctonically. However, in the laboratory, L. pneumophila can grow in nutrient rich medium. High numbers of L. pneumophila are often found in biofilms, where they can persist for long times and infect amoebae grazing on bacteria (Fields, 1996; Steinert et al., 2002).

Growth of L. pneumophila can be divided into two phases; replication inside of a host cell after infection, and transmission to a new host, during which the bacteria have to survive in the environment. Both phases expose the bacteria to highly diverse conditions. L. pneumophila adapts to the two phases, and the differentiation state of the bacterium can be distinguished by changes in, e.g., stress resistance, flagellum expression, evasion of lysosome fusion within a host cell, cytotoxicity and sodium sensitivity (Hammer et al., 2002). The switch between these developmental stages is coordinated by a regulatory circuit that is controlled by amino acid concentration and probably also other signals. During the replicative phase, when nutrients are abundant, the post-transcriptional regulator CsrA represses transmission traits. When amino acids become limiting, RelA produces the alarmone ppGpp that leads to expression of transmissive traits via the two component system LetA/S, the enhancer protein LetE, the alternative sigma factor RpoS and probably the master flagellar regulator RpoN (Molofsky and Swanson, 2004). The physiological changes of L. pneumophila during infection of amoebae closely resemble those during growth in broth cultures. During intracellular replication and in exponential growth phase in broth, traits that promote transmission (stress resistance, motility, ability to evade phagosome-lysosome fusion, cytotoxicity) are repressed. As nutrients become limiting either inside the host or in spent nutrient broth during entry in stationary phase, the bacteria switch to the transmissive phase and derepress the traits increasing survival and infection of a new host.

Despite of a generally low number of Legionellae in aquatic habitats and soils, the abundance of bacteria in a habitat is strongly increased by the presence of protozoa, reflecting the requirement for intracellular replication. Furthermore, protozoa can represent a shelter when
environmental conditions become unfavourable. The bacteria can survive high temperature, disinfection and drying if enclosed within an *Acanthamoeba* cyst (Heuner et al., 2002). Replication in amoebae also influences the physiology of the bacteria. Compared to cultures grown in broth, cells released from *A. castellanii* enter human monocytes more efficiently and are more resistant to biocides and antibiotics (Swanson and Hammer, 2000).

### 1.2 Legionella – *the etiological agent of Legionnaires’ disease*

The ubiquitous presence of Legionellae can lead to contamination of man-made technical systems. Elevated temperatures and the presence of biofilms and protozoa play a key role for growth, spreading and persistence of *Legionella* in these devices. Human infection occurs exclusively by inhalation of contaminated aerosols, which can be produced by air conditioning systems, cooling towers, whirlpools, fountains, dental devices or shower heads (Steinert et al., 2002). Inhaled bacteria infect alveolar macrophages in a way very similar to amoebae and may cause the severe pneumonias known as Legionnaires’ disease. 90% of the isolates associated with Legionaire’s disease are identified as *L. pneumophila*, most of them belonging to serogroup 1. In Switzerland, the incidence of Legionaire’s disease since 2000 is 2 cases per 100000 inhabitants with a mortality rate of 6.5% (www.bag.ch)

*L. pneumophila* is relatively resistant to innate and humoral immune responses. The bacteria resist complement-mediated lysis and are not killed by polymorphonuclear cells, even when opsonized with complement or specific antibodies. In a mouse model of Legionnaires’ disease, lung infection is controlled by cell-mediated immunity with interferon-gamma release by natural killer cells being a key element (Sporri et al., 2006; Swanson and Hammer, 2000). A robust immune response is sufficient to clear *L. pneumophila* infections. Main risk factors for legionellosis are immunosuppression and lung diseases.

Despite a high number of reported cases, not a single human to human transmission has been identified. *L. pneumophila* is an opportunistic pathogen whose ability to infect humans likely is a consequence of its coevolution with amoebae, rather than being due to adaptation to humans. The bacteria evolved as parasites of amoebae and acquired capabilities to infect protozoa and also mammalian cells due to the similarity of host cell mechanisms that are manipulated during infection (Swanson and Hammer, 2000).
Upon contact of *L. pneumophila* with a monocyte or an amoeba, the bacterium is taken up by conventional or coiling phagocytosis (Bozue and Johnson, 1996; Horwitz, 1984). The major outer membrane protein MOMP is bound by the complement protein C3 and can mediate uptake via CR1 and CR3 receptors of macrophages. Phagocytosis may also be enhanced by opsonization with immunoglobulins (Swanson and Hammer, 2000). Lectins have been shown to be involved in attachment and invasion of amoebae (Venkataraman *et al.*, 1997). Other proteins likely involved in adherence and invasion are type IV pili, that can mediate a complement-independent attachment, and the 60 kDa heat shock protein Hsp60 (Hoffman *et al.*, 1990; Stone and Abu Kwaik, 1998).

Independent from the mechanism of phagocytosis, *L. pneumophila* is able to inhibit fusion of the Legionella-containing vacuole (LCV) with lysosomes in an Icm/Dot-dependent manner (see below). Instead, the phagosomes recruit early secretory vesicles and ER-derived vesicles to generate a replication-permissive vacuole (Kagan *et al.*, 2004; Roy and Tilney, 2002). After a 6-10 hours lag phase in macrophages, the bacteria start to replicate. Concomitantly, the ability to prevent phagosome-lysosome fusion decreases and the vacuole finally fuses with lysosomes. This maturation into an acidic organelle is necessary for successful multiplication (Sturgill-Koszycki and Swanson, 2000). Finally, amino acid depletion triggers the switch from replicative to transmissive phase, genes necessary for motility and transmission are expressed, and the host cell lyses and releases the bacteria.

A number of factors has been described to be involved in virulence. The presence of a flagellum enhances the invasion capacity but is not required for intracellular growth. The Mip protein (macrophage infectivity potentiator) is exposed on the bacterial surface, contributes to intracellular survival and exhibits peptidyl-prolyl-cis/trans isomerase activity. Since this activity is characteristic for eukaryotic cells, it might be that Mip targets a host protein (Steinert *et al.*, 2002; Swanson and Hammer, 2000). Other factors involved in virulence are phospholipases, proteases and hydrolases, some of which are secreted via a type II secretion system (reviewed in Cianciotto, 2001).
Chapter 1

Figure 1: Intracellular replication of *L. pneumophila*. The bacteria are phagocytosed by the host cell. Phagosomes containing non-pathogenic bacteria or *L. pneumophila icm/dot* mutants fuse with lysosomes, and the bacteria are killed and degraded. Vacuoles containing wild-type *L. pneumophila* evade phagosome-lysosome fusion and associate with smooth vesicles, mitochondria and ER-derived vesicles. Within this specialized vacuole, the bacteria convert to the replicative form and multiply. At later stages of the infection, the vacuole merges with lysosomal compartments and acidifies. Upon depletion of the nutrient supply, the bacteria switch to the transmissive form, lyse the host and are dispersed in the environment where they can infect a new host. (Picture from André Tiaden and Stefan Weber).

1.4 The icm/Dot type IV secretion system

The mechanisms enabling *L. pneumophila* to grow intracellularly are similar both in free-living unicellular eukaryotes like *A. castellanii* and in phagocytic cells of the immune system like macrophages. Intracellular growth requires the Icm/Dot (intracellular multiplication/defective for organelle trafficking) type IV secretion system. Type IV secretion systems are related to conjugation systems and enable bacteria to deliver macromolecules from the bacterial cytosol through the bacterial cell wall and the host cell membrane directly into the host cytosol (Figure 2). The 25 *L. pneumophila icm/dot* genes are located in two clusters in the genome, and most of them are predicted to encode membrane-
spanning proteins (Segal \textit{et al.}, 1998; Vogel \textit{et al.}, 1998). Functional characterization of the single Icm and Dot proteins is still at the beginning, and most information comes from \textit{in silico} analysis and homology searches. DotA is an integral cytoplasmatic membrane protein that can be secreted into the supernatant in an Icm/Dot dependent manner. Secreted DotA polymerizes to hollow rings of unknown function. DotB, IcmO, IcmB and IcmF contain Walker A motifs. For some of these proteins, ATP hydrolyzation was found to be important for efficient intracellular growth. IcmO shows homologies to the TraG-like coupling protein family, but functional studies have yet to be done. IcmG is partially required for intracellular growth and is the only Icm/Dot protein for which binding to effector proteins has been described so far (see Chapter 4). IcmB and IcmK were shown to associate with a fibrous structure in the surface upon incubation of bacteria with macrophages or with macrophage conditioned medium. IcmR possesses chaperon activity on IcmQ and prevents the formation of IcmQ homopolymers. IcmQ is exposed on the bacterial surface and forms pores in lipid membranes in the absence of IcmR. The data known so far about the Icm/Dot secretion system are summarized in a recent review (Segal \textit{et al.}, 2005).

\textbf{Figure 2: Type IV secretion systems enable the bacterium to deliver effector proteins directly into the host cell cytosol.} Schematic representation of a type IV secretion system (© by Gunnar Schroeder, after Christie, 2001).
In *L. pneumophila*, mutation analysis of *icm/dot* genes revealed a requirement for the large majority of Icm/Dot proteins for several steps of the infection. *L. pneumophila* actively upregulates its own phagocytosis by usage of the Icm/Dot system both in macrophages and amoebae (Hilbi *et al.*, 2001; Watarai *et al.*, 2001). Infection of macrophages with a high multiplicity of infection results in increased cytotoxicity caused by osmotic lysis after insertion of pores into the host cell membrane in an Icm/Dot dependent manner (Kirby and Isberg, 1998; Kirby *et al.*, 1998). Inhibition of phagosome-lysosome fusion and recruitment of early secretory vesicles from ER exit sites also depend on a functional Icm/Dot system (Roy *et al.*, 1998; Wiater *et al.*, 1998). After formation of a specialized organelle, bacterial replication proceeds without requiring a functional Icm/Dot system (Coers *et al.*, 1999). It has also been suggested that the Icm/Dot system is responsible for formation of a pore in the host cell after depletion of the host to allow the bacteria to lyse the cell and exit (Molmeret and Abu Kwaik, 2002). Furthermore, due to the relation to conjugal systems, the Icm/Dot system is capable to conjugate plasmids. Mobilizable plasmids inhibit intracellular growth and killing of macrophages probably by competition for the Icm/Dot transporter capacity with other substrates of the Icm/Dot system (Segal and Shuman, 1998).

Only recently, Icm/Dot-transported effector proteins have been identified. RalF is translocated into the host cell and acts as a guanine nucleotide exchange factor that recruits the small GTPase ARF1, a highly conserved regulator of vesicle trafficking, to the *L. pneumophila* phagosome (Nagai *et al.*, 2002). In an elegant screen, the Sid family of secreted effector proteins was identified (Luo and Isberg, 2004). The authors used a bacterial two-hybrid screen with RalF as bait to identify effector protein binding partners. A large portion of the proteins obtained in the screen comprised of IcmG. Assuming that IcmG also binds other effector proteins, it was used as bait in a second bacterial two hybrid screen to identify further effector proteins. Translocation of the proteins identified in the second screen was confirmed, and the newly identified effector proteins were named Sid (substrates of Icm/Dot). Most of the Sid proteins exist as families of paralogues. SidC anchors to the LCV by binding to phosphatidylinositol(4) phosphate, a mediator of membrane dynamics in eukaryotic cells (Weber *et al.*, 2006). LepA and B share sequence similarity with SNARE proteins and seem to mediate the Icm/Dot-dependent release of LCVs from amoebae (Chen *et al.*, 2004). Another protein translocated by the Icm/Dot system is LidA, which is involved in recruitment of early secretory vesicle to the LCV (Derre and Isberg, 2005).
1.5 Legionella LPS

LPS makes up the majority of lipids in the outer monolayer of the outer membrane of Gram-negative bacteria. It consists of three components: the O-chain polysaccharide, the core oligosaccharide and lipid A. Particularly the oligo- and polysaccharide structures can vary between different strains and species and determine to a large part the classification of a strain to a particular serogroup. The O-chain from *L. pneumophila* LPS is a homopolymer of single legionaminic acid residues; a unique, hydrophobic carbohydrate (Knirel *et al.*, 1994). Some *L. pneumophila* strains, among these Philadelphia 1, contain the gene lag-1 that encodes an O-acetyl transferase that modifies the outer legionaminic acid units of the O-chain. However, mutation analysis revealed that a lack of O-acetylation does not influence virulence properties such as serum resistance or uptake and intracellular growth in amoebae and macrophages (Luck *et al.*, 2001). A further, lag-1-independent O-acetylation of core sugars and proximal legionaminic acid units seems to be a common feature of *L. pneumophila* serogroup 1 strains (Kooistra *et al.*, 2001). The legionaminic acid residues proximal to the core oligosaccharide can be N-methylated. Again, this modification did not affect serum resistance or virulence (Kooistra *et al.*, 2002a). The core oligosaccharide is a nonasaccharide that lacks heptose and phosphate, contains mostly 6-deoxy sugars and is highly O-acetylated (see Figure 3).

![Figure 3: Representative structure of *L. pneumophila* serogroup 1 LPS. The number of repeating monosaccharides in the O-chain ranges from about 10 to up to 100 units. GlcN3N: 2,3-diamino-2,3-dideoxy-D-glucose, Kdo: 3-deoxy-D-manno-oct-2-ulosonic acid, Man: D-mannose, GlcNAc: 2-acetamido-2,6-dideoxy-D-glucose (N-acetyl-glucosamine), QuiNAc: 2-acetamido-2,6-dideoxy-D-glucose (N-acetyl-D-quinovosamine), Rha: L-rhamnose, Leg and 4e-Leg: derivatives of legionaminic and 4-epilegionaminic acid, respectively. OAc stands for O-acetyl group. (Picture from Kooistra *et al.*, 2002a).](image)
Recently, it was shown that LPS-containing membrane vesicles released by *L. pneumophila* were able to inhibit phagosome-lysosome fusion temporarily for several hours in an Icm/Dot-independent manner. Inhibition occurred when beads were coinfected with purified outer membrane vesicles, or when beads coated with vesicles were used for infection. The ability to inhibit fusion was observed only for vesicles derived from transmissive phase bacteria but not from replicative phase bacteria. Furthermore, the ability to inhibit fusion correlated with developmentally regulated modifications of the pathogen's surface as judged by LPS profiles and lectin binding (Fernandez-Moreira et al., 2006).

LPS from *Legionella jeonii* was also shown to be involved in inhibition of phagosome-lysosome fusion in *Acanthamoeba proteus*. LPS is released from intracellular bacteria and is inserted into the membrane of the LCV. Masking of LPS in the phagosome membrane by microinjection of an anti-LPS antibody increased the number of phagosomes that fused with lysosomes (Kim et al., 1994). The influence of LPS on vesicle trafficking suggests its classification as a virulence factor.

Lipid A is the membrane anchor of LPS. In contrast to the majority of Gram-negative bacteria, the disaccharide backbone of *L. pneumophila* lipid A is a derivative of 2,3-dideoxy-2,3-diamino-D-glucose rather than glucosamine, yielding a lipid A in which all primary fatty acids are amide linked to the sugar backbone. The fatty acid pattern of lipid A from *L. pneumophila* and other *Legionella* species examined is complex. The acyl chains are unusually long, partially branched and some of them contain additional hydroxyl groups (Moll et al., 1992; Sonesson et al., 1989; Sonesson et al., 1994a; Sonesson et al., 1994b, c; Zahringer et al., 1995). Furthermore, analysis of lipid A from a phase variant strain showed that *L. pneumophila* is able to shift the profile of primary fatty acids towards shorter acyl moieties (Kooistra, 2002b). Phase variation is based on excision and insertion of a 30 kb unstable element that exists as high copy plasmid in the phase variant strain and is inserted in the chromosome in the *L. pneumophila* wild type strain RC1. Existence as extrachromosomal plasmid likely affects regulatory circuits and thus influences several bacterial pathways. The phase variant strain 811 is serum sensitive, unable to replicate intracellularly in the macrophage cell line HL60 and lacks the N-methylation of legionaminic acid mentioned above (Luneberg et al., 2001).

Figure 4 shows the major lipid A structure of *L. pneumophila* RC1 (serogroup 1, subgroup OLDA) and the corresponding phase variant mutant 811.
1.5.1 Modifications of lipid A

The genes and the corresponding enzymes of the lipid A biosynthesis pathway are well characterized (Raetz and Whitfield, 2002; Sweet et al., 2004), and homologues of all of them are present in the genome of *L. pneumophila*.
Figure 5: Biosynthesis pathway of lipid A molecules with four amide-linked acyl chains. UDP-GlcNAc: UDP-N-acetyl-glucosamine, UDP-GlcNAc3N: UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose, Kdo: 3-deoxy-D-manno-oct-2-ulosonic acid. (Picture from Sweet et al., 2004).

However, all acyl transferases and the lipid A disaccharide synthase lpxB are present in more than one copy in the *L. pneumophila* genome, but not in any other bacterial genome examined. At least some paralogues are differentially expressed, suggesting that *L. pneumophila* is able to extensively vary the fatty acid composition of lipid A (see Chapter 4). Besides the phase variation-dependent shortening of primary acyl chains (Kooistra, 2002b), little information is published about modifications of *L. pneumophila* lipid A.

Lipid A is a major component of the outermost bacterial barrier and, therefore, an important structural element for protection against detrimental compounds from the environment. These can be antibiotica, cationic antimicrobial peptides (CAMPs) released from other bacteria in a biofilm, lysosomal contents during infection of host cells, or the arsenal of the innate immune system during infection of a multicellular organism. Sensitivity against these compounds is influenced by the lipid A structure, and adaptation of lipid A helps bacteria to survive in different niches (Miller et al., 2005).

CAMPs are secreted by a wide variety of organisms from animals to unicellular eukaryotes and Gram-positive bacteria. Their cationic charge allows them to interact with the bacterial membrane, which usually, and also in the case of *L. pneumophila*, comprises negatively charged phospholipids. The putative mechanism of CAMP-mediated killing of microorganisms is insertion into and disruption of membranes and thereby loss of barrier function (Guo et al., 1998). Regulation of lipid A acylation has been demonstrated to
promote resistance to CAMPs. The *Salmonella* protein PagP adds an additional palmitate to lipid A, leading to an increased resistance. Thus, *pagP* mutant strains are more susceptible against some CAMPs (Guo *et al.*, 1998). Acylation of lipid A likely alters the fluidity of the outer membrane by strengthening hydrophobic interactions between the increased number or length of acyl residues. The stronger hydrophobic interaction forces could retard or abolish insertion of the CAMP into the membrane, thus keeping the outer barrier intact and preventing CAMPs from reaching the inner membrane, the most important diffusion barrier. Similarly, the formation of membrane pores by complement proteins could be impaired by highly acylated, rigid membranes and lead to increased resistance against complement-mediated killing.

The acylation state of lipid A is adjusted depending on the temperature in *E. coli*. Under cold shock conditions (12 °C), the acyl transferase LpxP is activated that inserts palmitoleate (C16) in place of laurate (C12) as secondary acyl chain, perhaps reflecting the need to adjust outer membrane fluidity at low temperatures. Interestingly, a mutant lacking *lpxP* showed a 10-fold increased sensitivity to rifampicin and vancomycin at 12 °C but not at 30 °C. The increase in acyl chain length might confer a selective advantage upon *E. coli* cells growing at lower temperatures by making the outer membrane a more effective barrier to harmful chemicals (Carty *et al.*, 1999; Vorachek-Warren *et al.*, 2002).

*Yersinia pestis* also regulates its lipid A acylation in response to temperature, reflecting its life in different hosts. At 21 °C or when residing in fleas, the natural host, lipid A is highly acylated and more resistant to CAMPs, the major immune pressure within insects. Contrarily, at 37 °C, lipid A is only tetra-acylated and does not stimulate TLR4 signalling in humans, the other host, thereby hampering recognition by the immune system (Miller *et al.*, 2005). In this case, increased temperature may also serve as environmental signal for being inside a mammalian host, leading to counteractions against the innate immune system.

The importance of the lipid A structure for *L. pneumophila* virulence is demonstrated by the effect of a deletion of *rcp*, a *pagP* like gene. In *S. Typhimurium*, *pagP* is controlled by the PhoP-PhoQ two component regulatory system and expressed under low magnesium concentrations as encountered inside a phagolysosome. PagP is an acyl carrier protein-independent acyl transferase adding palmitate to the primary acyl chain at position 2, thus increasing resistance to some CAMPs (Guo *et al.*, 1998). *L. pneumophila rcp* was identified due to its homology to *pagP*. Corresponding to *pagP*, resistance to CAMPs was induced by growth in low magnesium medium, and an *rcp* mutant showed decreased resistance to different classes of CAMPs. The *rcp* mutant exhibited a decreased recovery in the amoebae
Hartmanella vermiformis and U937 macrophages and was less successful in lung colonization of A/J mice (Robey et al., 2001). Although attachment of an additional acyl chain to lipid A by the rcp gene product has not yet been experimentally proven, it is likely that Rcp modifies lipid A in a way similar to its homologue PagP, and the mutant phenotypes observed are caused by changes in lipid A acylation that impair the functions of the outer membrane as a protective barrier.

1.5.2 Interaction of lipid A with components of the innate immune system

Structural modifications of lipid A fatty acids may also significantly affect the course of an infection in multicellular host organisms. Lipid A is the active part of endotoxin, the only region of LPS to be recognized by the innate immune system and is highly stimulatory even at low concentrations. Structure-function analyses of lipid A signalling indicate that the length and number of acyl side chains are critical for TLR4 signalling in humans. Hexa-acylated lipid A with chain lengths of 12 to 14 carbon atoms is maximally stimulating in human cells, whereas altering the number or length of the fatty acids can reduce the magnitude of the signal (Miller et al., 2005). L. pneumophila lipid A fatty acid chains vary in length from 14 to 20 carbon atoms, and the secondary acyl chains even consist of 16 or 26 carbon atoms. Furthermore, some of the acyl moieties are branched (see Figure 4), probably hampering recognition. Lipid A interacts with LPS binding protein (LBP) in the serum and is delivered by LBP to CD14 receptors on the cell surface, where the complex interacts with TLR4. TLR4 binding induces signal transduction leading to secretion of cytokines and cationic antimicrobial peptides and assists in activating the acquired immune response. L. pneumophila LPS fails to bind the LPS receptor CD14, probably due to its unusual lipid A structure, and therefore, shows a low endotoxic potential in the human monocytic cell line MonoMac6 (Neumeister et al., 1998). The unusual composition of L. pneumophila lipid A fatty acids might help the bacteria to escape recognition by the innate immune system by altering the LPS pathogen associated molecular pattern (PAMP). Further evidence for an influence of the fatty acid composition on endotoxic activity is given by the effect of fatty acid removal. E. coli lpxM mutant strains lacking a lipid A secondary acyl chain are greatly attenuated in their ability to activate human macrophages, and a Salmonella lpxM mutant was much less lethal in an animal shock model than wild type bacteria (Raetz and Whitfield,
2002). The importance of lipid A in detection and clearance of infections is highlighted by the observation that *Tlr4*-null mice lacking endotoxin-dependent activation are highly susceptible to infection with Gram-negative microorganisms (Miller et al., 2005). Therefore, the lipid A structure, particularly the fatty acid composition, is expected to influence significantly the virulence of *L. pneumophila* in multicellular organisms.

### 1.6 Literature


Chapter 1


2 The amoebae plate test implicates a paralog of \textit{lpxB} in the interaction of \textit{Legionella pneumophila} with \textit{Acanthamoeba castellanii}

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Abbreviations: APT, amoebae plate test; BSA, bovine serum albumin; CYE, charcoal-yeast extract; FCS, fetal calf serum; \textit{icml/dot}, intracellular multiplication/defective organelle trafficking; \textit{lcs}, \textit{Legionella} cytotoxic suppressor; MOI, multiplicity of infection; PI, propidium iodide; PYG, proteose-yeast extract-glucose.

Identification of virulence related genes using the amoebae plate test

2.1 ABSTRACT

*Legionella pneumophila* is a bacterial parasite of fresh water amoebae which also grows in alveolar macrophages and thus causes the potentially fatal pneumonia Legionnaires' disease. Intracellular growth within amoebae and macrophages is mechanistically similar and requires the Icm/Dot type IV secretion system. Here, we develop an assay, the amoebae plate test (APT), to analyze growth of *L. pneumophila* wild-type and *icm/dot* mutant strains spotted on agar plates in presence of *Acanthamoeba castellanii*. In the APT, wild-type *L. pneumophila* formed robust colonies even at high dilutions, *icmT, -R, -P*, or *dotB* mutants failed to grow, and *icmS* or *-G* mutants were partially growth defective. The *icmS* or *icmG* mutant strains were used to screen an *L. pneumophila* chromosomal library for genes that suppress the growth defect in presence of the amoebae. An *icmS* suppressor plasmid was isolated that harbored the *icmS* and flanking *icm* genes, indicating that this plasmid complements the intracellular growth defect of the mutant. Contrarily, different *icmG* suppressor plasmids rendered the *icmG* mutant more cytotoxic for *A. castellanii* without enhancing intracellular multiplication in amoebae or RAW264.7 macrophages. Deletion of individual genes in the suppressor plasmids inserts identified *lcs* (*Legionella cytotoxic suppressor*) *A, -B, -C* and *-D* being required for enhanced cytotoxicity of an *icmG* mutant strain. The corresponding proteins show sequence similarity to hydrolases, NlpD-related metalloproteases, lipid A disaccharide synthases, and ABC transporters, respectively. Overexpression of LcsC, a putative paralog of the lipid A disaccharide synthase LpxB, increased cytotoxicity of an *icmG* but not other *icm/dot* or *rpoS* mutant strains against *A. castellanii*. Based on sequence comparison and chromosomal location, *lcsB* and *lcsC* likely encode enzymes involved in cell wall maintenance and peptidoglycan metabolism. The APT established here may prove useful to identify other bacterial factors relevant for interactions with amoeba hosts.

2.2 INTRODUCTION

The Gram-negative bacterium *Legionella pneumophila* is a natural parasite of fresh water amoebae (Rowbotham, 1980; reviewed by Steinert et al., 2002). If inhaled via contaminated aerosols, the bacteria may grow in human alveolar macrophages and cause a severe pneumonia known as Legionnaires' disease. Within phagocytic host cells, *L. pneumophila* establishes a pH-neutral vacuole that does not fuse with lysosomes (Horwitz & Maxfield,
1984; Horwitz, 1983a). This unique, replication-permissive vacuole associates with smooth vesicles, mitochondria and endoplasmic reticulum (ER) (Horwitz, 1983b; Swanson & Isberg, 1995; Tilney et al., 2001) and recruits early secretory vesicles at ER exit sites (Kagan & Roy, 2002; Kagan et al., 2004).

Intracellular growth in macrophages and amoebae, including *Acanthamoeba castellanii* and *Dictyostelium discoideum*, is mechanistically similar and requires the *L. pneumophila* Icm/Dot transporter, a type IV secretion apparatus related to conjugation systems (Hagele et al., 2000; Otto et al., 2004; Segal et al., 1998; Segal & Shuman, 1999b; Solomon et al., 2000; Vogel et al., 1998). The Icm/Dot secretion system determines the initial contact of *L. pneumophila* with host cells and phagosome biogenesis (Hilbi et al., 2001; Watarai et al., 2001), is required to evade immediate endocytic maturation (Roy et al., 1998; Wiater et al., 1998) and governs subsequent formation of the ER-derived, replicative vacuole (reviewed by Nagai & Roy, 2003). Once *L. pneumophila* resides in this nutritionally rich compartment, the vacuole may acidify (Sturgill-Koszycki & Swanson, 2000), and bacterial replication apparently proceeds without requiring a functional Icm/Dot transporter (Coers et al., 1999).

Most of the genes of the *icm/dot* loci are predicted to encode membrane-spanning proteins (Segal & Shuman, 1999a). Interestingly, the IcmG protein contains a t-SNARE domain, and thus this membrane protein might play a direct role in altering host cell vesicle trafficking (Morozova et al., 2004). Biochemical analysis of the soluble Icm proteins revealed that IcmS/IcmW and IcmR/IcmQ directly bind to each other (Coers et al., 2000). IcmR functions as a chaperone of IcmQ, preventing and reversing its aggregation into high-molecular-weight complexes that form pores in lipid membranes (Duménil & Isberg, 2001; Duménil et al., 2004). Intracellular multiplication of and host cell killing by *L. pneumophila* is inhibited by a functional plasmid mobilization system, suggesting that the nucleoprotein conjugal substrate competes with virulence effectors for transport by the Icm/Dot machinery (Segal & Shuman, 1998). Only recently, Icm/Dot-transported proteins have been identified. RalF is translocated into the host cell and acts as a guanine nucleotide exchange factor that recruits the small GTPase ARF1 to the *Legionella* phagosome (Nagai et al., 2002). LepA and LepB share sequence similarity with SNAREs and seem to mediate the Icm/Dot-dependent release of *L. pneumophila*-containing vesicles from amoebae (Chen et al., 2004). Other Icm/Dot-translocated proteins are LidA, which by an unknown mechanism contributes to an efficient formation of the replication vacuole (Conover et al., 2003) and the SidA-H proteins, many of
which comprise families of up to five paralogs (Luo & Isberg, 2004). Secretion into culture supernatants of the polytopic membrane protein DotA requires the Icm/Dot secretion system but neither IcmS nor IcmW, and might lead to the formation of pores in target membranes (Nagai & Roy, 2001).

The Icm/Dot-dependent establishment of the Legionella vacuole eventually leads to host cell death due to intracellular replication and lysis. Additionally, the icm/dot genes mediate cytotoxic events such as (i) contact-dependent immediate cytotoxicity due to pore formation (Kirby et al., 1998; Zuckman et al., 1999), (ii) induction of apoptosis (Zink et al., 2002), and (iii) egress of the bacteria from the vacuole of the spent host cell (Molmeret et al., 2002). Other cytotoxic factors of L. pneumophila include legiolysin (Wintermeyer et al., 1991), the zinc metalloprotease Msp (Quinn & Tompkins, 1989; Szeto & Shuman, 1990) and RtxA, a member of the RTX (repeats in toxin) family of cytotoxic adhesins (Cirillo et al., 2001).

In this report, we describe the amoebae plate test (APT), a novel assay to analyze growth of L. pneumophila wild-type and icm/dot mutants on agar plates in presence of A. castellanii. The APT was used to screen an L. pneumophila chromosomal library for multicopy suppressors of the partial growth defect of icmS or icmG mutant strains. Possible suppressors include genes that i) enhance intracellular bacterial growth and thus kill the amoebae, ii) are otherwise cytotoxic for the amoebae, or iii) interfere with phagocytosis of the bacteria by the amoebae. Among the plasmids isolated, icm/dot region II and cytotoxic genes likely involved in peptidoglycan metabolism were identified.

2.3 METHODS

2.3.1 Bacterial strains, cell culture and reagents

The bacterial strains and plasmids used in this study are listed in Table 1. E. coli was cultured on LB agar plates or in LB broth, supplemented with chloramphenicol (Cm, 30 µg/ml), sodium ampicillin (Ap, 100 µg/ml) or kanamycin sulfate (Km, 50 µg/ml) if required. L. pneumophila was grown on charcoal-yeast extract (CYE) agar plates (Feeley et al., 1979) or in ACES-buffered-yeast extract broth (AYE) containing 0.5% bovine serum albumin (BSA) (Horwitz & Silverstein, 1983). Supplements for L. pneumophila were used at the following concentrations: Cm (5 µg/ml), Km (50 µg/ml), IPTG (0.5 mM). A. castellanii (ATCC 30234) was grown in proteose-yeast extract-glucose medium (PYG) at 30°C (Moffat
& Tompkins, 1992; Segal & Shuman, 1999b) and subcultured once or twice a week. RAW264.7 macrophages (kindly obtained from Dr. David Underhill, University of Washington, Seattle) were cultivated in RPMI1640 medium supplemented with 10% FCS and 2 mM L-glutamine at 37°C in 5% CO₂. High gel strength agar was from Serva, proteose peptone from Becton Dickinson Biosciences and Bacto yeast extract from Difco, respectively. RPMI medium, glutamine and fetal calf serum (FCS) were from Omnilab. All other reagents were from Sigma.

Table 1: Bacterial strains and plasmids used in this study

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<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference</th>
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<td>LW253</td>
<td>F'</td>
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<tr>
<td>TOP10</td>
<td>F' mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL (Sm) endA1 mupG</td>
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<td>Salt-sensitive isolate of AM511</td>
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<td><strong>Plasmids</strong></td>
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Identification of virulence related genes using the amoebae plate test

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<tr>
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<th>pMMB207αb with mobA::Km</th>
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<td>pUA5</td>
<td>pG54-lcsB (ΔNlpD-like protease)</td>
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<td>pUA19</td>
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<td>Amersham</td>
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</table>

Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Sm, Streptomycin resistance.

2.3.2 Construction of plasmids

Plasmids were isolated using commercially available kits from Qiagen or Macherey-Nagel, and DNA manipulations were performed according to standard protocols. In frame deletions of single or several ORFs in the inserts of the suppressor plasmids pG34, pG54, pG65 and pG66 yielded the plasmids pUA3, pUA9, pUA11, pUA17 and pUA27 (Table 1, Fig. 5a) and were generated by PCR (Imai et al., 1991) after subcloning the inserts into pUC19 using the EcoRI restriction sites. To generate the deletion constructs, the oligonucleotides listed in Table 2 were used. The PCR fragments were phosphorylated, circularized, transformed into *E. coli* TOP10, checked by restriction digestion, and the inserts were finally cloned back into pMMB207 using the EcoRI restriction sites, yielding pUA5, pUA13, pUA15, pUA19 and pUA29. Alternatively, ORF1-3 was deleted from pG39 or pG54 by digestion with HindIII or SphI (Fig. 5a), respectively, and re-ligated, thus yielding pUA20 and pUA22. To overexpress *lcsA*, *-B*, *-C* or *-D*, the genes were amplified by PCR using pG34, pG54, pG65 or pG66 as templates and cloned by digestion with EcoRI and BamHI or NdeI into the expression vector.
pMMB207-RBS, yielding pMMB207-RBS\textsubscript{lcsA-D}. pMMB207-RBS harbors the P\textsuperscript{tac} promoter and the ribosome binding site from the T7 gene\textsubscript{10} taken from pGS-GFP-04 (Hilbi et al., 2001). The sequence of all PCR fragments was confirmed by sequencing.

Table 2: Oligonucleotides used in this study

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2.3.3 The amoebae plate test

In the amoebae plate test (APT), A. castellanii is spread on CYE agar plates prior to spotting bacteria in serial dilutions onto these plates. Thus, the APT allows determining growth of bacteria on solid medium in presence of amoebae. A. castellanii cultures were fed with PYG 2 days prior to an experiment. One day before the experiment, the medium was exchanged, and the amoebae were tapped off the tissue culture flask, spun down (5 min, 1500 rpm) and resuspended in PYG (2.67 × 10\textsuperscript{6}/ml). 1.5 ml of suspended amoebae (or PYG only) were applied on a CYE/Cm agar plate, let dry for 1-2 h in a laminar flow hood and left at room
temperature over night. Wild-type and icm/dot mutant L. pneumophila strains used for the APT harbored the plasmid pMMB207ab, pMMB207ab-Km14 or the corresponding complementing plasmids listed in Table 1. For the APT, stationary phase bacterial cultures (OD$_{600} > 4.5$) were adjusted to an identical OD$_{600}$, and series of tenfold dilutions in sterile H$_2$O were prepared. 3 μl of stationary culture (approx. 10$^8$ bacteria) or 3 μl of each dilution were spotted onto the CYE/Cm plates and incubated for 5-7 days at 30°C or 37°C.

2.3.4 Screening of an L. pneumophila chromosomal library for icm/dot suppressors using the APT

To perform a suppressor screen with the APT, the partially growth defective icmS and icmG mutants were chosen as recipients for the L. pneumophila genomic library MW66 (Purcell & Shuman, 1998). MW66 harbors 5-10kb EcoRI fragments in the vector pMMB207. The library was amplified in the E. coli host strain DH5α, isolated and electroporated into the conjugation-competent E. coli strain LW253. Mating of MW66 into the L. pneumophila icm mutant strains was done as described (Mintz & Shuman, 1987; Segal & Shuman, 1998). Briefly, LW253/MW66 grown on LB/Cm plates was suspended in LB, spun down and resuspended in M63 medium. Stationary phase icmS and icmG mutants grown in liquid culture were washed and resuspended in M63 medium. Donor and recipient strains were incubated at a ratio of 1/10 on CYE plates (4 h, 37°C), resuspended in 0.2 ml M63 medium, streaked on CYE/Cm/Km plates and incubated for 4-5 days at 37°C. The icmS and icmG mutants harboring the MW66 library were then suspended in AYE medium, spotted in serial dilutions onto CYE/Cm plates containing 4 x 10$^6$ A. castellanii and incubated at 30°C. JR32 and the icmS and icmG mutants harboring pMMB207ab were used for comparison, respectively. Within 7-14 days several colonies of icm mutants harboring library plasmids appeared at dilutions where no parental icm mutant strains were detected. The APT-selected suppressor strains were grown to stationary phase for 24 h in AYE/Cm broth and spotted again onto CYE/Cm plates containing 4 x 10$^6$ A. castellanii. The MW66 library plasmids of suppressor strains recovered from the second round of selection by APT were analyzed further. In preliminary experiments, the icmS and icmG mutants harboring the MW66 library were spread at an MOI of 1 simultaneously with 5 x 10$^6$ A. castellanii/ml PYG on CYE agar plates and incubated at 30°C. Colonies that appeared using this approach were subjected to a second round of selection as described above.
2.3.5 Analysis of suppressor plasmids

The library plasmids of the suppressor strains were isolated, amplified in *E. coli* and grouped according to their EcoRI restriction pattern. A representative of each group was electroporated into the *icmG* or *icmS* mutant strain which it was isolated from and analyzed again by the APT to exclude effects of chromosomal mutations. The suppressor plasmid inserts were partially sequenced using primers complementary to vector sequences at the 5' and 3' of the insert. The sequences obtained were mapped in the *L. pneumophila* genome (Chien *et al.*, 2004; http://genome3.cpmc.columbia.edu/~legion/) and analyzed for homologs of the ORFs identified.

2.3.6 Intracellular growth in *A. castellanii* and RAW264.7 macrophages

For intracellular growth assays, *A. castellanii* (5 × 10⁴/well) or RAW264.7 macrophages (2 × 10⁴/well) were seeded onto a 96 well plate and let adhere for 3 h or over night, respectively. The phagocytes were infected with *L. pneumophila* grown to stationary phase in AYE medium for 24 h (MOI 1, 880 × g), and incubated at 30°C (*A. castellanii*) or 37°C (RAW264.7). Intracellular growth was quantified by plating appropriately diluted supernatant of the infected host cells on CYE agar plates at the time points indicated. RAW264.7 macrophages are derived from Balb/c mice, which are considered less susceptible to *L. pneumophila* than A/J mice, the established murine model of Legionnaires' disease. *L. pneumophila* does not replicate in peritoneal macrophages elicited from Balb/c mice (Yamamoto *et al.*, 1988). Yet bone marrow-derived macrophages from Balb/c mice permit the intracellular replication of *L. pneumophila* (Wright *et al.*, 2003), which corresponds to the observed permissiveness of RAW264.7 macrophages.

2.3.7 Cytotoxicity assay

To determine cytotoxicity, 4 × 10⁴ *A. castellanii* per well were seeded in PYG onto a 24 well plate the day prior to infection. On the day of infection, the PYG was replaced with Ac buffer (Moffat & Tompkins, 1992). Bacteria from 3 or 4 days old plates were resuspended and diluted in sterile water to infect the amoebae at an MOI of 50 or 500. After spinning down the bacteria (880 × g, 5 min), the plates were incubated at 30°C. Two days post infection,
propidium iodide (PI) solution was added to the wells at a final concentration of 1 μg/ml. After several minutes incubation, the amoebae were viewed in brightfield or by epifluorescence with an inverse microscope (Zeiss Axiovert 200M, 20 × objective). The percentage of dead (PI-positive) amoebae was determined by counting the number of total and fluorescent amoebae.

2.3.8 Computational and statistical analysis

Translated nucleic acid data bases or the conserved domain data base (Marchler-Bauer et al., 2003) were searched using the TBLASTX or RPSBLAST algorithms, respectively (Altschul et al., 1997). The COG data base is maintained by Tatusov et al. (Tatusov et al., 2001). Multiple amino acid sequence alignments were created with the CLUSTALW algorithm. Statistical analysis was done using the Mann-Whitney test taking values of < 0.05 as significant.

2.4 RESULTS

2.4.1 Growth of L. pneumophila on CYE agar plates in presence of A. castellanii – the amoeba plate test.

Intracellular growth of L. pneumophila within A. castellanii requires the icm/dot genes (Segal & Shuman, 1999b). Here, we establish a novel assay, the amoebae plate test (APT) to analyze growth of wild-type and icm/dot mutant L. pneumophila on CYE agar plates in presence of A. castellanii. Stationary phase cultures of L. pneumophila wild-type strain JR32 and icm/dot mutants harboring empty or complementing plasmids were spotted in tenfold serial dilutions onto agar plates in presence or, as a control, absence of amoebae (Fig. 1). Within 5-7 days at 30°C, wild-type L. pneumophila strain JR32 formed robust colonies even at a 10⁵ dilution (MOI approximately 0.2). Under these conditions, the icmT, -R, -O, -P, -C and dotB mutant strains did not grow at all. The icmS and -G mutant strains showed a partial growth defect in presence of A. castellanii and eventually were lysed by the amoebae. An icmN mutant initially formed colonies on amoebae plates similar to wild-type L. pneumophila (data not shown). However, whereas JR32 survived on CYE agar plates in the presence of the amoebae for a prolonged time at room temperature, the icmN mutant was lysed by 14 days. Supplying the corresponding genes on a plasmid restored growth of the icm mutants almost to wild-type
level ($icmT, -S, -P, -O, -R, dotB$) or partially ($icmG, -C$). In absence of amoebae, all strains grew equally well on CYE agar plates.

At 37°C, the $icmS$ mutant grew similar to wild-type and the $icmN$ mutant in the APT. At this temperature, the $icmT$ mutant did not grow at all and the $icmR, -P, -O, -G, -C$ mutants grew only at the highest concentration. The degree of complementation of individual $icm$ mutants was the same as at 30°C (data not shown).

|----------------|------------|-------------|------------|------------|------------|-------------|------------|-------------|------------|------------|------------|------------|

Figure 1: Growth of wild-type *L. pneumophila* and representative *icm/dot* mutants in the amoebae plate test (APT). *L. pneumophila* wild-type strain JR32 and *icmT, -S, -R, -P, -G* and *dotB* mutants harboring empty (pMMB) or corresponding complementing plasmids (picmT, -S, -R, -P, -G, dotB) were spotted in tenfold serial dilutions onto CYE agar plates in presence or absence of *A. castellanii* and incubated for 5 days at 30°C as described in Methods. The experiments were done at least three times and results similar to those shown were obtained.
2.4.2 Isolation of \textit{L. pneumophila icm/dot} suppressor strains using the amoebae plate test.

Among the \textit{icm/dot} mutant strains tested, only the \textit{icmS} and the -\textit{G} mutants showed a partial and not complete growth defect in the APT at 30°C. A partial growth defect for the \textit{icmS} and -\textit{G} mutant strains was shown before using HL-60 macrophages as host cells (Purcell & Shuman, 1998; Segal & Shuman, 1997; Segal & Shuman, 1999b). We reasoned that this phenotype would increase chances to identify in a multicopy suppressor screen genes that increase cytotoxicity or enhance intracellular growth of the mutants. Therefore, the \textit{icmS}, and -\textit{G} mutants were chosen as recipients for the \textit{L. pneumophila} genomic library MW66 (Purcell & Shuman, 1998). The library MW66 was moved by electroporation from \textit{E. coli} DH5α into the conjugation-competent \textit{E. coli} strain LW253 and mated into the \textit{L. pneumophila icmS}, and -\textit{G} mutants. The \textit{icmS} and -\textit{G} mutants harboring the library MW66 were then spotted in serial dilutions onto CYE plates containing chloramphenicol (Cm) and \textit{A. castellanii}. The plates were incubated at 30°C or 37°C, respectively.

At 37°C, the selection was not promising, since at the same dilutions similar numbers of \textit{icm} mutants harboring the library and parental mutants were obtained. At 30°C, however, several colonies of \textit{icm} mutant strains harboring MW66 appeared within 7-14 d at dilutions where no parental \textit{icm} mutants were detected. These APT-selected strains were grown to stationary phase for 24 h in AYE/Cm broth and spotted in serial dilutions onto CYE/Cm/A. castellanii plates again. In the second round of selection, 54 \textit{icmG} and 2 \textit{icmS} suppressor strains were recovered, representatives of which are shown in Fig. 2a. The suppressor strains (\textit{icmS} -/pS37, \textit{icmG} -/pG44-87) grow at 10-100-fold higher dilutions than the corresponding \textit{icm} mutant strains harboring the empty library plasmid pMMB207ab (\textit{icmS} -/pMMB, \textit{icmG} -/pMMB), but not as vigorously as wild-type \textit{L. pneumophila} (JR32/pMMB) or complemented \textit{icm} mutants (\textit{icmS} -/picmS, \textit{icmG} -/picmG). In absence of the amoebae, all strains grew equally well (data not shown).

The library plasmids of the suppressor strains were isolated, amplified in \textit{E. coli} and grouped according to their \textit{EcoRI} restriction pattern into 18 groups (1 \textit{icmS}-, 17 \textit{icmG} - background; data not shown). A representative plasmid of each group was electroporated into the \textit{icmG} or -\textit{S} mutant strain which it was isolated from and analyzed by APT. The strains thus obtained grew better in the APT than the parental \textit{icm} mutant harboring an empty library plasmid, indicating that suppression of the growth defect in presence of amoebae was caused by the
library plasmid and not due to a second site mutation in the bacterial chromosome. Representative strains belonging to different EcoRI restriction groups are shown in Fig. 2b. Retransformation of an icmS mutant strain with plasmid pS37 led to growth in the APT similar to wild-type *L. pneumophila*, consistent with the finding that pS37 complements the icmS mutant strain (see below).

Figure 2: Suppressor strains isolated by APT. (a) *L. pneumophila icmS* and *icmG* suppressor strains harboring *Legionella* chromosomal library plasmids (pS37, pG44 - pG87) were isolated by APT, grown to stationary phase and re-tested by APT. Representative suppressor strains are shown in comparison with wild-type, *icmG* and *icmS* mutant strains harboring the empty library plasmid (pMMB) and corresponding complemented strains (picmG, picmS). The APT was repeated twice with strains isolated in a first round of selection, and similar results were obtained. (b) Growth defect suppression in an APT by *L. pneumophila icmS* or *icmG* mutants transformed freshly with isolated suppressor plasmids (pS37, pG34, pG54, pG65).
2.4.3 Analysis of suppressor plasmid inserts.

The inserts from representative suppressor plasmids were partially sequenced and mapped in the *L. pneumophila* strain Philadelphia genome (http://genome3.cpmc.columbia.edu/~legion/). Some of the inserts were identical (pS36/pS37; pG54/pG58/pG63; pG65/pG72; pG71/pG79/pG83), and the inserts of the suppressor plasmids pG47 and pG61 were independently selected twice, since they covered the same loci in the *L. pneumophila* genome but were of different length (1.8 kb or 2.5 kb, respectively). The inserts of the suppressor plasmids were analyzed by BLASTX searches for homologs to open reading frames (ORFs), and thus, 12 different inserts were identified (pS37, pG34, pG39, pG41, pG47, pG53, pG54, pG65, pG66, pG67, pG71, and pG78), each harboring 1-10 ORFs (Table 3).

The insert of the *icmS* suppressor plasmid pS37 contains a 9.0 kb fragment of *icm/dot* region II. The fragment spans a region between 1.7 kb upstream of the *icmT* and the *icmL* gene and includes the *icmS* gene. This result indicates that pS37 complements the *icmS* mutant and thus, increased growth of the IcmS/pS37 strain in the APT is due to intracellular growth within and killing of the amoebae. Contrarily, the ORFs identified in the inserts of the 11 different *icmG* suppressor plasmids did not immediately suggest a mechanism accounting for their growth defect suppression.

Table 3: Suppressor plasmids identified in this study

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The suppressor strains might grow better on agar plates in presence of amoebae because of more efficient intracellular replication and host cell killing or due to a cytotoxic activity not related to intracellular replication. To test these possibilities, intracellular growth of the icmG suppressor strains within A. castellanii or murine RAW264.7 macrophages and cytotoxicity towards amoebae was determined.
2.4.4 Intracellular growth of icmG suppressor strains in A. castellanii and macrophages

Intracellular growth of the icmG suppressor strains was assayed by quantifying bacteria released into the supernatant from A. castellanii infected with bacteria at an MOI of 1. To match the conditions used for the APT, the infected amoebae were incubated for 4 days at 30°C, rather than at 37°C described previously (Segal & Shuman, 1999b). At 30°C, the number of wild-type L. pneumophila or icmG mutant bacteria harboring the empty library plasmid increased within 3 days about 4 or 1 orders of magnitude, respectively (Fig. 3a). Supplying the icmG gene on a plasmid only partially complemented the intracellular growth defect, as was observed previously (Segal & Shuman, 1999b). None of the 11 icmG suppressor strains (harboring pG34 - pG78) grew better than the icmG mutant, ruling out that the growth defect suppression observed in the APT was due to enhanced intracellular growth. Rather, we noted that the suppressor strains tended to grow even worse than the icmG mutant strain. We also performed an intracellular growth assay with a high MOI of 500. Under these conditions, wild-type L. pneumophila grew 2 orders of magnitude within 3 days (data not shown). In this experiment, the relative growth of the wild-type and icmG mutant strains was the same as above, and the 11 icmG suppressor strains tested also grew worse than the icmG mutant. At 37°C, the suppressor strains icmG-/-pG54 or icmG-/-pG65 did also not grow better in amoebae (MOI of 1) than an icmG mutant strain harboring the empty library plasmid (data not shown).

To analyze intracellular multiplication of the 11 icmG suppressor strains in macrophages, the murine macrophage cell line RAW264.7 was used. Within 4 days, the number of wild-type or icmG mutant bacteria released into the supernatant increased 4 or 3 orders of magnitude, respectively (Fig. 3b). Supplying the icmG gene on a plasmid complemented growth of the icmG mutant almost to wild-type level. In RAW264.7 macrophages, the icmG suppressor strains (icmG-/-pG34 - pG78) grew worse than the icmG mutant strain, similar to what was observed for growth of these strains in A. castellanii. Taken together, growth defect suppression of the icmG suppressor strains is not due to enhanced intracellular growth within phagocytes.
Figure 3: Intracellular multiplication of icmG suppressor strains within A. castellanii and macrophages. Intracellular growth of the icmG suppressor strains was assayed using (a) A. castellanii or (b) murine RAW264.7 macrophages as host cells. The phagocytes were infected at an MOI of 1, and bacteria released into the supernatant were quantified at the time indicated. Growth of suppressor strains icmG /pG34 - pG78 (○), an icmG mutant harboring the empty library plasmid (△), or plasmid-encoded icmG (▲) and wild-type strain JR32 (■) is shown. The experiments were repeated at least two times at two different MOIs each, and similar results were obtained.
2.4.5 icmG suppressor strains are cytotoxic for A. castellanii

Since compared to the parental icmG mutant strain, the icmG suppressor strains did not grow better in amoebae or macrophages, we tested whether the suppressor plasmids pG34 - pG78 confer increased cytotoxicity to icmG mutants. A. castellanii was infected with wild-type L. pneumophila, an icmG mutant harboring the empty library plasmid, a complemented icmG mutant, or the icmG suppressor strains (harboring pG34 - G78). Cytotoxicity was determined by propidium iodide (PI) uptake 2 days post infection. Under the conditions described, the amoebae infected with either wild-type L. pneumophila or the complemented icmG mutant rounded up and were all dead as judged by their complete disintegration or by PI uptake (Fig. 4a). In contrast, amoebae infected with an icmT mutant harboring the vector control all survived and remained spread out and firmly attached. Amoebae infected with an icmG mutant harboring the vector control rounded up, but only about 15% stained with PI. Most of the suppressor strains were found to be significantly more cytotoxic than the icmG mutant strain, thus providing a rationale for their isolation in the suppressor screen (Fig. 4b). Notably, icmG mutants harboring pG34, pG39, pG41, pG54, or pG66 showed an increased cytotoxicity in 4-5 out of 5 independent experiments. The suppressor strains icmG /pG47, /pG53, /pG65 and /pG71 were more cytotoxic than the icmG mutant in some experiments, and icmG /pG67 and /pG78 did not show increased cytotoxicity in any of the experiments performed. At 37°C, the suppressor strains icmG /pG54 or icmG /pG65 were cytotoxic for amoebae to the same extent as at 30°C (data not shown).
(a)

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Identification of virulence related genes using the amoebae plate test

Fig. 4. Suppressor strains are cytotoxic for *A. castellanii*. (a) *A. castellanii* was infected with wild-type *L. pneumophila*, *icmT* or *icmG* mutant strains harboring the empty library plasmid (pMMB) or with suppressor strains (*icmG* /pG34 - pG78). Cytotoxicity was determined by uptake of propidium iodide (PI) 2 days post infection as described in Methods. (b) The data shown are the means and standard deviations of the percentage of PI-positive *A. castellanii* counted in 3 different wells on a 24-well plate and are representative of at least five independent experiments.

2.4.6 Identification of cytotoxic suppressor genes by deletion analysis

To identify cytotoxic suppressor genes, individual or several ORFs were deleted in the suppressor plasmid inserts, and cytotoxicity was assessed by the PI uptake assay. For the deletion analysis, we focused on the cytotoxic suppressor plasmids pG34, pG39, pG41, pG47, pG54, pG65 and pG66, and only complete ORFs were considered. The suppressor plasmid pG41 was not studied further since the encoded DNA-interacting proteins (integration host factor, histone methyltransferase) presumably increase cytotoxicity unspecifically. Deletion of the LuxR-type transcription factor in pG47 did not decrease cytotoxicity (data not shown), and thus, this plasmid was not analyzed further. Deletion of all the complete ORFs in the insert of pG39 did also not decrease cytotoxicity, but left the fragment of an ORF encoding the C-terminus of a putative lytic murein transglycosylase...
These findings and considerations left the cytotoxic suppressor plasmids pG34, pG54, pG65 and pG66 to be analyzed in detail. The inserts of the plasmids pG34, pG54, pG65 and pG66 harbor homologs of hydrolases, NlpD-like metalloproteases, lipid A disaccharide synthases, and ABC transporters, respectively (Table 3, Fig. 5a). Deletion of the genes encoding the hydrolase, NlpD-like metalloprotease, lipid A disaccharide synthase, or ABC transporter by a PCR-based method or by restriction enzyme digestion reproducibly reduced cytotoxicity, indicating that the corresponding proteins are required for the enhanced cytotoxicity of the suppressor plasmids (Fig. 5b). The genes were termed lcs (Legionella cytotoxic suppressors) A (hydrolase), -B (NlpD-like metalloprotease), -C (lipid A disaccharide synthase homolog), and -D (ABC transporter). Deletion of ORF1-3 in the insert of pG54 also decreased cytotoxicity, presumably because lcsB forms an operon with ORF3 and consequently will not be expressed in the pG54-ORF1-3 deletion mutant. Deletion of the DNA-binding regulator protein Fis (factor of inversion stimulation) substantially increased cytotoxicity of plasmid pG65-fis carrying lcsC as the only remaining complete ORF (Fig. 5b) and, as expected, deletion of lcsC and fis in the insert of suppressor plasmid pG65 abolished cytotoxicity (data not shown).
Identification of virulence related genes using the amoebae plate test

2.4.7 Overexpression of suppressor genes

Prompted by the finding that strain \( \text{icmG}^+ / \text{pG65-fis} \) showed enhanced cytotoxicity compared to \( \text{icmG}^- / \text{pG65} \), we cloned \( \text{lcsA}, \text{-B}, \text{-C}, \) and \( \text{-D} \) into the expression vector pMMB207-RBS, overexpressed the proteins under the control of the \( P_{\text{lac}} \) promoter, and quantified cytotoxicity of \( \text{icmG} \) mutant strains harboring these plasmids by PI uptake. Interestingly, the strain \( \text{icmG}^- / \text{pLcsC} \), overexpressing the \( \text{LpxB} \) homolog, was 9 or 4 times more cytotoxic than \( \text{icmG}^- / \text{pMMB} \) or \( \text{icmG}^- / \text{pG65} \), respectively (Fig. 6a, b), suggesting that \( \text{LcsC} \)-induced cytotoxicity might be dose-dependent. The \( \text{icmG}^- / \text{pLcsC} \) strain did not grow more efficiently in \( \text{A. castellanii} \) (30°C, 37°C) or in RAW264.7 macrophages compared to \( \text{icmG}^- / \text{pMMB} \) (data not shown). Contrarily, \( \text{icmG} \) mutants expressing the putative hydrolase \( \text{LcsA} \), NlpD-like metalloprotease \( \text{LcsB} \) or the ABC transporter \( \text{LcsD} \) were less cytotoxic than the \( \text{icmG} \) mutant.
strain. We noted, however, that induction of LcsB prevented growth of the bacteria, and induction of LcsC and LcsD resulted in fewer and smaller bacterial colonies, indicating that overexpression of these proteins is toxic for *L. pneumophila* (data not shown). In agreement with this assumption, the strains icmG*/pLcsA-D grew well on agar plates, if IPTG was omitted, and therefore, the genes were expressed only at low levels from the P_{lac} promoter. Under these conditions, strain icmG*/pLcsC was consistently more cytotoxic than the parental suppressor strain icmG*/pG65 in three independent experiments (data not shown). Strain icmG*/pLcsB was more cytotoxic than the negative control icmG*/pMMB207 but not as cytotoxic as the parental suppressor strain icmG*/pG54. Finally, the strains icmG*/pLcsA and icmG*/pLcsD were not cytotoxic for the amoebae (data not shown).

In presence of IPTG LcsC was cytotoxic for *A. castellanii* only if overexpressed in an icmG but not in an icmS, -F or rpoS mutant background (Fig. 6c), even though the icmF as well as the rpoS mutant strains harboring the empty plasmid pMMB207 were cytotoxic for the amoebae to a similar extent as the icmG mutant. Equal to the icmS mutant strain, the icmC, -E, -O, -R, -T, -W, or dotB mutants overexpressing LcsC were not cytotoxic for amoebae (data not shown). Moreover, about 30% of *A. castellanii* infected with wild-type *L. pneumophila* (MOI of 5) harboring either an empty plasmid or pLcsC were killed within 2 days, and thus, LcsC apparently did not increase cytotoxicity of wild-type *L. pneumophila* against the amoebae (data not shown).
Identification of virulence related genes using the amoebae plate test

(a)

*icmG−/pMMB*

*icmG−/pLcsC*

(b)

Pl - positive A. castellani (%)
Figure 6: Overexpression of LcsC is cytotoxic for *A. castellanii* in an *icmG* but not in other *icm* or *rpoS* mutants. *A. castellanii* was infected with the *icm* (a-c) or *rpoS* (c) mutant strains indicated harboring the empty library plasmid (pMMB), complementing plasmid (pICM), suppressor plasmids (pG34, pG54, pG65, pG66), or expression plasmids (pLcsA-D). Cytotoxicity was determined by uptake of propidium iodide (PI) 2 days post infection. The data shown are the means and standard deviations of the percentage of PI-positive *A. castellanii* counted in 3 different wells on a 24-well plate and representative of at least three independent experiments.

### 2.5 DISCUSSION

#### 2.5.1 Screen for *icm* suppressors using the amoebae plate test

In this manuscript we describe a novel assay, the amoebae plate test (APT), to determine growth of bacteria on CYE agar plates in presence of amoebae. Wild-type *L. pneumophila* replicates within and kills amoebae, and thus grows on solid medium. Contrarily, *icm/dot* mutant strains are defective for intracellular growth, killed by the amoebae and do not form colonies on agar plates (Fig. 1). The APT was routinely done at 30°C, allowing the amoebae...
to feed on the icm/dot mutant strains, as judged from the observation that most mutants did not form colonies under these conditions. At 37°C, the icmS and other icm/dot mutants grew better than at 30°C in presence of amoebae. Possible explanations for this finding include that (i) the bacteria grow faster on agar plates at 37°C and thus gain an advantage over the amoebae in the putative competition between growth on solid medium and being phagocytosed, or (ii) the amoebae are stressed at a temperature far from environmental conditions, and therefore, are less phagocytic or less bactericidal.

In screens using the partially growth defective icmS or icmG mutants, we identified icm/dot region II complementing the icmS mutant and isolated several plasmids that conferred increased cytotoxicity to an icmG mutant, without alleviating its intracellular growth defect (Fig. 3, 4). Some cytotoxic icmG suppressor genes were identified by deletion analysis (lcsA-D, Legionella cytotoxic suppressor) and encode a putative hydrolase, an NlpD-like metalloprotease, a lipid A disaccharide synthase homolog and an ABC transporter, respectively (Fig. 5, Table 3). To obtain clues about the cellular pathways which these genes participate in, we inspected their vicinity in the L. pneumophila genome and searched the conserved domain and translated nucleic acid data bases, respectively. The lcsB and lcsC genes were found to be of particular interest, since both genes are possibly involved in peptidoglycan metabolism. Another gene possibly involved in cell wall degradation is a lytic murein transglycosylase (COG2821), a fragment of which is encoded by the cytotoxic suppressor plasmid pG39 (Table 3). Deletion of ORF1-3 of plasmid pG39 left only the truncated lytic murein transglycosylase downstream of the $P_{tac}$ promoter. This construct was still cytotoxic in an icmG mutant background, possibly due to expression of a cytotoxic 242 amino acid C-terminal enzyme fragment by using the $P_{tac}$ promoter and the first internal ATG as a start codon.

2.5.2 LcsB, a homolog of NlpD-like membrane-bound metalloproteases

LcsB is 26% identical to the E. coli YibP protease and 17-18% identical to the NlpD orthologs from L. pneumophila and E. coli (Fig. 7). The C-terminal domains of the 4 proteins belong to COG4942 (membrane-bound metallopeptidases, involved in cell division and chromosome partitioning) and are characteristic for members of the M23/M37 family (Pfam01551) of putative zinc metallo-endopeptidases from Gram-negative and Gram-positive
bacteria (http://www.sanger.ac.uk/Software/Pfam/). The M37 protease family includes staphylococcal glycy1glycine endopeptidases that hydrolyze the polyglycine interpeptide bridges of peptidoglycan of Gram-positive bacteria (Ramadurai & Jayaswal, 1997; Ramadurai et al., 1999; Recsei et al., 1987; Sugai et al., 1997) and the \( E.\ coli \) lipoprotein NlpD that probably functions in cell wall formation and maintenance of Gram-negative bacteria (Ichikawa et al., 1994; Lange & Hengge-Aronis, 1994). In \( E.\ coli \) as well as in \( L.\ pneumophila \), the \( nlpD \) gene is located immediately upstream of the \( rpoS \) gene encoding the stationary growth phase transcription factor RpoS (Hales & Shuman, 1999). Among the proteins found in the data bases, \( L.\ pneumophila \) LcsB is most closely related to \( E.\ coli \) YibP. Purified YibP has endoprotease activity which is inhibited by EDTA, and a fraction of the protein is membrane-bound (Ichimura et al., 2002). A chromosomal \( yibP \) deletion mutant is defective for cell division, FtsZ ring formation and growth at 42°C but not at 37°C. At the non-permissive temperature, the \( yibP \) mutant forms filamentous, multi-nucleoided cells that tend to lyse, indicating that YibP is required for cell wall degradation and proper cell division.

Figure 7: Alignment of \( L.\ pneumophila \) LcsB, \( E.\ coli \) YibP and the membrane-bound metalloproteases NlpD from \( L.\ pneumophila \) and \( E.\ coli \).
2.5.3 LcsC, a paralog of the lipid A disaccharide synthase LpxB

LcsC is homologous to lipid A disaccharide synthases (LpxB) and, as determined by searching the conserved domain data base, distantly to MurG glycosyltransferases involved in peptidoglycan synthesis (COG0707). Interestingly, the genome of *L. pneumophila* Philadelphia-1 harbors 2 lipid A disaccharide synthase paralogs which are 42% or 30% identical to *E. coli* LpxB, respectively (Fig. 8). The gene more closely related to *E. coli* lpxB is most likely the *L. pneumophila* lpxB ortholog since it is located within a cluster encoding components of the lipopolysaccharide (LPS) biosynthesis pathway, including a neuraminidase, lauroyl/myristoyl acyltransferase, LpxA, LpxD, LpxB, and the ABC transporter WlaB. The arrangement of the lpx gene cluster is similar in *E. coli*, where lpxB is located downstream of lpxA, fabZ and lpxD (Raetz, 1996).

![Figure 8: Alignment of L. pneumophila LcsC with lipid A disaccharide synthase (LpxB) homologs from L. pneumophila and E. coli.](image)

In the *L. pneumophila* genome, the lcsC gene appears to form an operon with an oxidoreductase/dehydrogenase gene and is separated from the putative rodA-ftsI operon by only one ORF. The latter genes encode the rod shape-determining protein RodA and the peptidoglycan transglycosylase/peptidase FtsI. RodA and FtsI are membrane-bound, interact with each other and are required for cell envelope biogenesis and cell division, respectively (Begg et al., 1986). Analogously, in the *E. coli* genome, the murG glycosyltransferase gene is flanked by genes involved in peptidoglycan biosynthesis and cell division (Heijenoort, 1996). At present, we can not exclude that LcsC participates in lipid A biosynthesis. However, the vicinity of lcsC to rodA-ftsI, its lower homology to lpxB and its similarity to murG suggest...
that LcsC catalyzes a disaccharide synthase/glycosyltransferase reaction in peptidoglycan biosynthesis.

The remote structural similarity of LcsC/LpxB and MurG corresponds to the fact that both glycosyltransferases catalyze the synthesis of a β-linked lipodisaccharide moiety from an acylated uridine 5'-diphosphate (UDP)-D-glucosamine (GlcN) and a lipid, and both enzymes liberate UDP during the reaction. LpxB catalyzes the β,1'-6 condensation of UDP-2,3-diacyl-GlcN with lipid X (2,2-diacyl-GlcN-1-phosphate), and MurG couples N-acetyl-GlcN via an β,1'-4 linkage to lipid I (N-acetyl-muramyl-pentapeptide-phosphoryl-undecaprenol) to form the lipodisaccharide lipid II that is the minimal subunit of peptidoglycan (Heijenoort, 1996).

### 2.5.4 Mechanism of LcsC cytotoxicity

Cytotoxicity of an icmG mutant strain harboring the suppressor plasmid pG65 was not only decreased upon deletion of lcsC (Fig. 5), but overexpression of the gene in an icmG background enhanced cell death of A. castellanii (Fig. 6), suggesting that the product(s) of the LcsC enzyme are cytotoxic. While the structure of the toxic product of LcsC remains to be identified, it might either associate with bacteria or be released into the supernatant. Soluble cytotoxic peptidoglycan derivatives have been described for Bordetella pertussis, Neisseria gonorrhoeae, Haemophilus influenzae and other bacteria (Burroughs et al., 1993; Cloud & Dillard, 2002; Cookson et al., 1989; Luker et al., 1993; Luker et al., 1995). The murein-derived tracheal cytotoxin (TCT; N-acetyl-GlcN-1,6-anhydro-N-acetyl-muramyl-L-Ala-γ-D-Glu-meso-diaminopimelyl-D-Ala) from B. pertussis, e.g., is released by growing bacteria and is sufficient to reproduce the cytopathology observed during whooping cough. The murein-derived toxins from N. gonorrhoeae or H. influenzae are also disaccharide muramyl tetrapeptides, i.e., consist of the monomeric subunit of Gram-negative bacterial peptidoglycan.

Overexpression of LcsC apparently is cytotoxic for the amoebae specifically in an icmG mutant but not in other icm/dot or an rpoS mutant background (Fig. 6c). One possibility to account for this observation would be that LcsC requires a functional Icm/Dot secretion system to exert cytotoxicity. Among the strains tested, the icmG, icmS, icmF, icmW, icmR and rpoS mutants are expected to form at least partially functional Icm/Dot secretion systems,
since these mutants can grow intracellularly in macrophage cell lines (Coers et al., 2000; Hales & Shuman, 1999; Segal & Shuman, 1999b). Moreover, the icmG mutant is only partially defective for intracellular growth within *A. castellanii* at 30°C (Fig. 4), the *icmG* and *icmF* mutants persist in *Dictyostelium* (Otto et al., 2004), and the *icmS* and *icmW* mutants are not impaired in Icm/Dot-dependent immediate cytotoxicity (Coers et al., 2000; Zuckman et al., 1999). However, LcsC was cytotoxic specifically in an *icmG* mutant but neither in other mutant strains only partially defective for intracellular growth, nor in wild-type *L. pneumophila* (data not shown). Therefore, a (limited) functional Icm/Dot transporter is apparently not sufficient for LcsC cytotoxicity. In contrast to other *icm/dot* mutants, the *icmG* mutant grows to some extent in *A. castellanii* and persists in *D. discoideum*, suggesting that entry and/or intracellular trafficking of the *icmG* mutant differs from other *icm/dot* mutants as well as from wild-type *L. pneumophila*. It is feasible that overexpression of LcsC exerts cytotoxic effects only in certain cellular compartments. We currently investigate entry and intracellular trafficking of the *icmG* mutant and effects of LcsC on these processes to establish a link between loss of IcmG (possibly involved in intracellular trafficking) and cytotoxicity of LcsC (a putative enzyme of peptidoglycan metabolism).

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### 2.7 REFERENCES


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3 The cytotoxic Legionella pneumophila lpxB paralogue lcsC is involved in lipid A biosynthesis and upregulated during bacterial growth in amoebae

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Abbreviations: ACES, N-(2-acetamido)-2-aminoethanesulfonic acid; CYE, charcoal-yeast extract; icm/dot, intracellular multiplication/defective organelle trafficking; GlcN3N, 2,3-diamino-2,3-dideoxy-α-D-glucopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucose (N-acetyl-glucosamine); GlcNAc3N, 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose; lcs, Legionella cytotoxic suppressor; MOI, multiplicity of infection; RT-PCR, reverse transcription polymerase chain reaction; PI, propidium iodide; PYG, proteose-yeast extract-glucose; T4SS, type IV secretion system.

Manuscript in preparation
3.1 ABSTRACT

The facultative intracellular pathogen *Legionella pneumophila* replicates within amoebae and macrophages. Intracellular growth takes place within a replicative vacuole and requires the bacterial Icm/Dot type IV secretion system. Using *Acanthamoeba castellanii* as a host cell, we previously identified *lcsC* (*Legionella* cytotoxic suppressor), a paralogue of the lipid A disaccharide synthase *lpxB*, as a cytotoxic factor of *L. pneumophila*. Among bacteria, *L. pneumophila* is unique in harboring two paralogues of *lpxB* and two or three paralogues of the UDP-GlcNAc acyltransferases *lpxA* or *lpxD*, respectively. In the genome of *L. pneumophila*, *lcsC* (*lpxB1*) lies adjacent to *gnaA*. GnaA is an NAD⁺-dependent oxidase, which catalyzes the first step in the conversion of UDP-GlcNAc to the 3-amino derivative UDP-GlcNAcN3, the precursor of GlcN3N analogues of lipid A. *lpxB2* clusters with *lpxD2*, *lpxA2* and other genes involved in lipid A biosynthesis. We show here that *lpxB1* and *gnaA* form a transcriptional unit. Moreover, *lpxB1* or *lpxB2* complemented the growth defect of the temperature sensitive *E. coli* *lpxB* mutant strain MN7 at the non-permissive temperature to a similar extent, indicating that both corresponding enzymes possess lipid A disaccharide synthase activity. However, the two *L. pneumophila* *lpxB* paralogues are functionally not equivalent, since expression of *lpxB1* but not *lpxB2* in an *icmG* mutant was cytotoxic for *A. castellanii*. Using an RT-PCR approach, we demonstrate that in AYE broth *lpxB1, lpxB2, lpxA1*, and *lpxA2* are expressed in exponential as well as in early stationary growth phase. Interestingly, in *A. castellanii*, *lpxB1* and *lpxA1* are expressed at early time points (4 h post infection), while *lpxB2* and *lpxA2* are expressed only at later time points (24 h post infection). These results are in agreement with the hypothesis that *L. pneumophila* evolved and, depending on environmental conditions, differentially regulates paralogues of UDP-GlcNAc acyl transferases and disaccharide synthases involved in LPS biosynthesis.

3.2 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; Steinert et al., 2002), but also colonizes and persists in biofilms (Mampel et al., 2006; Murga et al., 2001). Moreover, *L. pneumophila* is an opportunistic human pathogen that causes the life-threatening pneumonia Legionnaires’ disease and grows within
The cytotoxic lipid A disaccharide synthase paralogue LcsC

macrophages from different sources, including human alveolar macrophages (Nash et al., 1984), primary mouse macrophages from certain mouse strains (e.g. A/J; Sporri et al., 2006; Yamamoto et al., 1988) and several macrophage-like cell lines (Fields, 1996).

Phagocytosis and intracellular replication of *L. pneumophila* depends on the Icm/Dot type IV secretion system (T4SS), a conjugation apparatus which transports more than 30 putative “effector” proteins (Hilbi et al., 2001; Segal et al., 1998; Vogel et al., 1998). The Icm/Dot T4SS is also required for *L. pneumophila* to survive and grow on agar plates in presence of *Acanthamoeba castellanii*. This “amoebae plate test” was recently used to screen an *L. pneumophila* chromosomal library for multicopy suppressors of the partial growth defect of an *icmG* mutant strain (Albers et al., 2005). 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 for *A. castellanii*, but did not enhance intracellular replication of the bacteria.

The genomes of the *L. pneumophila* strains Philadelphia, Paris and Lens (Cazalet et al., 2004; Chien et al., 2004) encode orthologues of the *E. coli* lipid A biosynthetic genes, indicating that *L. pneumophila* employs a lipid A biosynthesis pathway similar to the one described for *E. coli* (Raetz and Whitfield, 2002). *L. pneumophila* synthesizes a lipid A analogue containing 2,3-diamino-2,3-dideoxyglucose (Zahringer et al., 1995). The initial step in the biosynthesis of this lipid A analogue is the conversion of UDP-GlcNAc to its 3-amino derivative UDP-GlcNAc3N, which in *Acidithiobacillus ferrooxidans* is catalyzed by the NAD⁺-dependent oxidase GnnA and the L-glutamate-dependent aminotransferase GnnB (Sweet et al., 2004). Lipid A of *L. pneumophila* is substituted with unusual long-chain, branched and dihydroxylated fatty acids (Moll et al., 1992). The O-antigen of *L. pneumophila* LPS is a homo-polymer of an unique carbohydrate unit, legionaminic acid, which can be further O-acetylated and N-methylated (Knirel et al., 1994; Kooistra et al., 2002a).

Structural variations of lipid A alter the physical properties of the outer membrane and thereby influence endotoxic activity and resistance against antibiotic compounds (Raetz and Whitfield, 2002). *L. pneumophila* LPS has low endotoxic activity, possibly due to the unusually long and branched fatty acid residues (Neumeister et al., 1998). Moreover, *L. pneumophila* harbors a gene similar to the *Salmonella* lipid A acyl transferase *pagP* that confers resistance to cationic antimicrobial peptides and promotes intracellular replication (Robey et al., 2001).

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 variation of *L. pneumophila* serogroup 1 subgroup OLDA strain RC1 was found to decrease virulence in a guinea pig infection model, diminish intracellular replication within macrophage-like HL60 cells or *A. castellanii*, and reduce serum resistance (Luneberg *et al.*, 1998). Phase variation in *L. pneumophila* was shown to be due to the reversible chromosomal excision of a 30 kb genetic element (Luneberg *et al.*, 2001). In the virulent wild-type strain RC1 the 30 kb element is integrated into a specific site in the chromosome. Contrarily, in the phase variant strain 811, the element is excised and replicates as a high copy number plasmid. The 30 kb element does not harbor genes related to LPS biosynthesis, yet phase variation abolished N-linked methylation of legionaminic acid in the O-chain polysaccharide (Kooistra *et al.*, 2002b) and shifted the profile of fatty acids attached to lipid A towards shorter chain moieties (Kooistra *et al.*, 2002a), thus demonstrating the capability of *L. pneumophila* to alter its LPS and lipid A structure.

In this report, we analyze in more detail the previously identified cytotoxic *L. pneumophila* lipid A disaccharide synthase paralogue *lcsC*. We demonstrate that both *L. pneumophila lpxB* paralogues are involved in lipid A biosynthesis, but only one (*lcsC*) is cytotoxic for amoebae. Analysis of the lipid A biosynthesis genes present in the *L. pneumophila* genome reveals that, in contrast to other bacteria, the acyltransferases are present in multiple copies. Expression analysis of *lpxA* and *lpxB* shows differential regulation of paralogous genes upon intracellular replication of *L. pneumophila* within *A. castellanii* and upon transfer from nutrient broth into distilled water.

### 3.3 METHODS

#### 3.3.1 Bacteria, amoebae and reagents

The *L. pneumophila* strains used were wild-type strain JR32 (Philadelphia-1, serogroup 1; (Sadosky *et al.*, 1993), and an isogenic deletion mutant containing a kanamycin (Km) resistance cassette in *icmG* (MW635; Purcell and Shuman, 1998). *L. pneumophila* was grown on charcoal-yeast extract (CYE) agar plates (Feeley *et al.*, 1979) or in ACES-buffered-yeast extract broth (AYE). Supplements were used at the following concentrations: chloramphenicol (Cm; 5 μg/ml), Km (50 μg/ml), IPTG (0.25 mM or 0.05 mM to induce expression of *L. pneumophila lcsC* or *lpxB*, respectively). *Escherichia coli* strain TOP10 (Invitrogen) or the conditional *E. coli lpxB* mutant strain MN7 (Nishijima *et al.*, 1981) were
The cytotoxic lipid A disaccharide synthase paralogue LcsC cultured in LB medium, supplemented with Cm (30 \mu g/ml), ampicillin (Ap, 100 \mu g/ml), or 0.2% L-(+)-arabinose, if required. 

*A. castellanii* (ATCC 30234) was grown in proteose-yeast extract-glucose medium (PYG) at 30 °C (Moffat and Tompkins, 1992; Segal and 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, respectively. All other reagents were from Sigma.

### 3.3.2 Identification of homologues of *L. pneumophila* lipid A biosynthesis genes

To identify proteins involved in lipid A biosynthesis, a blastp search (Altschul *et al.*, 1997) was performed in the genomes of *L. pneumophila* strains Philadelphia-1, Lens and Paris (Cazalet *et al.*, 2004; Chien *et al.*, 2004) and other bacterial genomes. The proteins from *E. coli* K12 were used as query (LpxA, accession number P0A722; LpxB, P10441; LpxC, P0A725; LpxD, P21645; LpxH, P43341). The *A. ferrooxidans* proteins GnnA (AAS48421) and GnnB (AAS48422), catalyzing the conversion of UDP-GlcNAc to its 3-amino derivative UDP-GlcNAc3N, were used as query to search for the corresponding *L. pneumophila* enzymes.

### 3.3.3 Construction of plasmids

DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits from Qiagen or Macherey-Nagel. To construct plasmid pMMB207C-RBS-lcsC, the lcsC gene (lpg1371) including a ribosome binding site (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*, fill in with Klenow polymerase and religation with T4 DNA polymerase. To construct plasmid pMMB207C-RBS-lpxB the corresponding open reading frame (lpg2945) was amplified by PCR using chromosomal DNA as template and the oligos oLpxBfo and oLpxBre (Table 1), respectively. The PCR fragment was cut with *NdeI* and *PstI* and inserted
into pMMB207C-RBS-lcsC cut with the same enzymes, thus replacing lcsC by lpxB. All constructs containing PCR fragments were sequenced.

3.3.4 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 lpxB mutant was examined using the temperature sensitive *E. coli* lpxB mutant MN7 (kindly provided by C.R.H. Raetz, Duke University, NC; Nishijima et al., 1981). The mutant was transformed by electroporation with empty vectors (pUC18, pMMB207C-RBS), with pSR8 (expressing *E. coli* lpxB), or with vectors expressing the *L. pneumophila* lpxB paralogues (pMMB207C-RBS-lcsC, pMMB207C-RBS-lpxB).

Cultures grown over night were adjusted to an OD600 of 1.0, and dilutions were plated on LB agar with 80 µg/ml Ap (pUC18, pSR8) or 20 µg/ml Cm (pMMB207C and derivatives). Expression of lpxB in pSR8 was induced with 0.2% arabinose. Since induction of lcsC or lpxB with IPTG is toxic for the bacteria, expression of these two genes was not induced and, therefore, remained at a basal level due to leakiness of the Ptac promotor. The plates were incubated at 30 °C or 42 °C, respectively, and the number of colonies was determined after 24 hours.

3.3.5 Cytotoxicity assay

4 × 10⁴ *A. castellanii* per well were seeded in PYG onto a 24 well plate and let grow over night. Before an infection, the PYG medium was replaced with Ac buffer (Moffat and Tompkins, 1992). Bacteria from 3 or 4 days old plates grown in the presence of 0.2 mM IPTG to induce expression of *L. pneumophila* lcsC or lpxB, respectively, were resuspended and diluted in sterile water to infect the amoebae at an MOI of 50. The infection was synchronized by spinning down the bacteria (880 × g, 5 min), and the plates were incubated at 30 °C. Two days post infection, propidium iodide (PI) solution was added to the wells at a final concentration of 1 µg/ml. After several minutes incubation, the amoebae were viewed in brightfield or by epifluorescence microscopy with an inverse microscope (Zeiss Axiovert
The cytotoxic lipid A disaccharide synthase paralogue LcsC

200M, 20 × objective). The percentage of dead (PI-positive) amoebae was determined by counting the number of total and fluorescent amoebae.

### 3.3.6 Expression analysis of LPS biosynthesis genes by RT-PCR

Expression of LPS biosynthesis genes was quantified in *L. pneumophila* strain JR32 grown for 3-4 d on CYE agar plates followed by growth in AYE medium and exposure to different conditions. Cotranscription of the *gnaA* and *IcsC/lpxB1* genes was analyzed in *L. pneumophila* grown in AYE to exponential growth phase (OD₆₀₀ 0.8). To compare gene expression in exponential and post-exponential growth phase, 3 ml AYE medium was inoculated (OD₆₀₀ 0.1) with bacteria grown on agar plates, and at the time points indicated, samples were processed for RNA isolation as described below. Gene expression under osmotic stress and nutrient deprivation was determined by splitting a bacterial culture in stationary growth phase (23 h, OD₆₀₀ 3.3). Half of the culture was washed once with distilled water and resuspended in distilled water, the other half remained in the growth medium as control. Both cultures were kept at room temperature for 4 h before RNA was extracted.

To analyze RNA from bacteria growing intracellularly in amoebae, 2 × 10⁶ *A. castellanii* were seeded in a 10 cm dish the day before the experiment, infected with *L. pneumophila* in early stationary growth phase at an MOI of 40 and incubated at 30 °C. 2.5 h post infection, 50 µg/ml gentamicin was added to kill extracellular bacteria. After additional 1.5 or 21.5 h, the supernatant was aspirated, the plates were chilled on ice, and the amoebae were scraped into 1.75 ml RNeasy protect bacteria reagent (Qiagen). To disrupt the host cells, the suspension was passed four times through a ball homogenizer (Isobiotec, Heidelberg) with a clearance of 6 µm. Host cell debris were removed by centrifugation (2 min, 250 × g). To pellet bacteria the supernatant was spun again (10 min, 5200 × g), and RNA was extracted.

RNA was extracted with the RNeasy Midi Prep kit according to the manufacturer’s instructions (Qiagen) and quantified by OD₂₆₀. Contaminating genomic DNA was digested using the DNAses RQ1 (Promega). To synthesize cDNA, M-MLV reverse transcriptase (Promega) and the 3’ primers listed in Table 1 were used. Cotranscription of *gnaA* and *IcsC/lpxB1* was analyzed using primer oUA50 to synthesize cDNA and primers oUA50 and oUA55 for the PCR.

The primers were annealed to the RNA for 10 min at 70 °C and cooled down 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. For the PCR reaction, 2.5 μl reverse transcription mixture was used as template, and 50 pmol each of 3' and 5' primer (Table 1), 0.2 mM of each dNTP and 1 μl Taq polymerase were added. The primers were designed to have a melting temperature of 58-60 °C and to yield a 480-520 bp product with 40-60% GC content. The PCR conditions were: 95 °C (30 s), 55 °C (30 s), 72 °C (40 s) during the first 10 cycles, and afterwards 72 °C (45 s). Samples were taken after the number of cycles indicated to determine the amount of the PCR products on an agarose gel. Sample size and settings for photographic documentation were kept identical in all assays.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGS-Lc-63-14</td>
<td><em>icmGCD</em> in pMMB207-Km14</td>
<td>(Purcell and Shuman, 1998)</td>
</tr>
<tr>
<td>pMMB207C</td>
<td>RSF1010 derivative, <em>incQ, oriT</em>, lacF, Pacc, ΔmobA, CmR</td>
<td>(Chen et al., 2004)</td>
</tr>
<tr>
<td>pMMB207C-RBS-lcsC</td>
<td>Expression of <em>L. pneumophila lcsC/lpxB1</em></td>
<td>This work</td>
</tr>
<tr>
<td>pMMB207C-RBS-lxB</td>
<td>Expression of <em>L. pneumophila lpxB2</em></td>
<td>This work</td>
</tr>
<tr>
<td>pSR8</td>
<td>Expression of <em>E. coli lpxB</em>, P_{araB}, ApR</td>
<td>(Crowell et al., 1986)</td>
</tr>
<tr>
<td>pTS-10</td>
<td>pMMB207C-RBS, ΔmobA, RBS</td>
<td>This work</td>
</tr>
<tr>
<td>pUA26</td>
<td>pMMB207-RBS-lcsC, MobA pos.</td>
<td>(Albers et al., 2005)</td>
</tr>
<tr>
<td>pUC19</td>
<td></td>
<td>(Yanisch-Perron et al., 1985)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Oligos</th>
<th>Sequence</th>
<th>Notes</th>
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<tr>
<td>oLpxB2fo</td>
<td>CGGAATTC CATATG ACTATG AA 5' of lpxB2 (lpg 2945)</td>
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<tr>
<td></td>
<td>AAGACCG ACC</td>
<td></td>
</tr>
<tr>
<td>oLpxB2re</td>
<td>GTACA ACTG GTTGTTACGTTAGAAGG 3' of lpxB2 (lpg 2945)</td>
<td></td>
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<tr>
<td></td>
<td>GAAGTCCC AAAATC</td>
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<td>oUA39</td>
<td>CCGCAT GTGTAATTGGAAGG 5' of lpxA1 (lpg 0511)</td>
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<td>oUA40</td>
<td>CACGT CGCAAACAAATCCAATC 3' of lpxA1 (lpg 0511)</td>
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</tr>
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<td>oUA41</td>
<td>CCGTCA TGGTTCTCTATGTTG 5' of lpxA2 (lpg 2943)</td>
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</tr>
<tr>
<td>oUA42</td>
<td>CTTGCAGACAGA CTTGATGCC 3' of lpxA2 (lpg 2943)</td>
<td></td>
</tr>
<tr>
<td>oUA49</td>
<td>CTGGAAATT AGTGCGCATTGG 5' of lcsC/lpxB1 (lpg 1371)</td>
<td></td>
</tr>
<tr>
<td>oUA50</td>
<td>GGCAGT AAGGCAATA TTTG 3' of lcsC/lpxB1 (lpg 1371)</td>
<td></td>
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<td>oUA51</td>
<td>GTG GTCCTCAATGCTAAGG 5' of lpxB2 (lpg 2945)</td>
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<tr>
<td>oUA52</td>
<td>GATT ACCTACCTACCCGA CACTAC 3' of lpxB2 (lpg 2945)</td>
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<td>oUA53</td>
<td>TAAGGACTCCGGTCTGTAAC 5' of lpxC (lpg 2608)</td>
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<td>oUA54</td>
<td>GCCCTACA AGACTTCTGAGC 3' of lpxC (lpg 2608)</td>
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<td>oUA55</td>
<td>CACT CGTGTAAGC AATGTAAC 5' of glnA (lpg 1372)</td>
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<tr>
<td>oUA56</td>
<td>AGGACCAT GTAA GAGGTTACAG 5' of rpoA (lpg 0354)</td>
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The cytotoxic lipid A disaccharide synthase paralogue LcsC

<table>
<thead>
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<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Function</th>
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</thead>
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<tr>
<td>oUA57</td>
<td>CGAACAGTCAACTCCAAATC</td>
<td>3' of rpoA (lpg 0354)</td>
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<tr>
<td>oUA60</td>
<td>GCAGCAACCAGTATTAACCTC</td>
<td>5' of flaA (lpg 1340)</td>
</tr>
<tr>
<td>oUA61</td>
<td>TAGCAACAGTACCCACCAACTC</td>
<td>3' of flaA (lpg 1340)</td>
</tr>
<tr>
<td>oUA64</td>
<td>ATACCGCATAATGTCTGAGG</td>
<td>5' of 16S rDNA (lpg 0302)</td>
</tr>
<tr>
<td>oUA65</td>
<td>TACACCGGAATCCTCACCTAC</td>
<td>3' of 16S rDNA (lpg 0302)</td>
</tr>
</tbody>
</table>

Table 1: Plasmids and oligonucleotides used. *Restriction sites are underlined.

3.4 RESULTS

3.4.1 *L. pneumophila* paralogues of LPS biosynthesis genes

The *L. pneumophila* lcsC gene was recently identified as a gene which is cytotoxic for amoebae upon overexpression (Albers et al., 2005). LcsC is 34% identical to *E. coli* LpxB, the lipid A disaccharide synthase, which catalyzes the glycosyl transferase reaction between UDP-2,3-diacylglucosamine and 2,3-diacylglucosamine-1-phosphate to form the tetra-acylated lipid IV<sub>A</sub> in the course of lipid A biosynthesis (Figure 1). The *L. pneumophila* genome harbors another *lpxB* gene (*lpxB2*), which is 43% identical to *E. coli* *lpxB* on an amino acid level. The apparent paralogues LcsC/LpxB1 and LpxB2 share 31% identity.

In order to gain more insight into lipid A biosynthesis in *L. pneumophila*, we examined the genomes of *L. pneumophila* Philadelphia-I, Paris and Lens (Cazalet et al., 2004; Chien et al., 2004) for the presence of lipid A biosynthesis genes. The genome of *L. pneumophila* was found to harbor orthologues of all the *E. coli* genes required for GlcNAc-based lipid A biosynthesis (*lpxA, -B, -C, -D, -H, -K, -L, and WaaA (KdtA)). Moreover, in agreement with the finding that *L. pneumophila* synthesizes a GlcNAc3N-derivative of lipid A (Zahringer et al., 1995), *L. pneumophila* also harbors orthologues of *A. ferooxidans gnnA* and *gnnB*, which are required for the conversion of UDP-GlcNAc into its 3-amino derivative UDP-GlcNAc3N (Sweet et al., 2004). The proposed pathway of lipid IV<sub>A</sub> biosynthesis in *L. pneumophila* based on the presence of these genes is shown in Figure 1. Interestingly, all three *L. pneumophila* strains harbor two copies of *lpxA* and *lpxB*, and three copies of *lpxD*. LpxA and LpxD are acyl transferases attaching primary acyl residues to the carbohydrate backbone.

68
Kdo2-lipid IV$_A$ is converted into lipid A in *E. coli* by addition of lauroyl and myristoyl residues to the primary acyl chains of the Kdo2-linked sugar catalyzed by the acyl-carrier protein-dependent acyl transferases LpxL and LpxM. The *L. pneumophila* genome contains three *E. coli* lpxL homologues (see Figure 1). These genes also show similar degrees of homology to the *E. coli* acyl transferase LpxP that inserts palmitoleate instead of laurate as secondary acyl chain under cold shock conditions. These genes do not show significant homology to *E. coli* lpxM. Furthermore, the *L. pneumophila* genome contains the pagP homologue rcp (lpgo025) (Robey *et al.*, 2001), that seems to be activated by low magnesium concentrations and most likely adds an additional third secondary acyl chain to lipid A.

The paralogues of lpxA, lpxB, and lpxD are clustered similarly in the *L. pneumophila* strains, yet the genomic organization of the genes varies to some extent (Table 2). The *L. pneumophila* genome is unique among 12 bacterial genomes analyzed in containing multiple paralogues of lpxA, lpxB, and lpxD. The lpxB gene was found to be present only as a
single copy in 25 other bacterial genomes analyzed. Among the bacteria examined were *Coxiella burnetii*, the closest relative of *L. pneumophilia*, and other pathogenic or symbiotic species from different taxonomic groups.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Philadelphia-1 (lpg)</th>
<th>Paris (lpp)</th>
<th>Lens (lpl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lpxB1</em>/<em>lcsC-gmA</em></td>
<td>1371-1372</td>
<td>1325-1326</td>
<td>1322-1323</td>
</tr>
<tr>
<td><em>lpxL-A2-D2-B2</em></td>
<td>2940-ORF-ORF-2943-2944-2945 (L--ADB)</td>
<td>3012-ORF-3014-3015-3016 (L-BDA)</td>
<td>2870-ORF-2872-2873-2874 (L-BDA)</td>
</tr>
<tr>
<td><em>lpxD1-A1</em></td>
<td>0508-ORF-ORF-0511</td>
<td>0571-ORF-0573</td>
<td>0547-ORF-0549</td>
</tr>
<tr>
<td><em>lpxK-msbA</em></td>
<td>1818-1819</td>
<td>1781-1782</td>
<td>1782-1783</td>
</tr>
</tbody>
</table>

Table 2: Genomic arrangement of selected lipid A biosynthesis genes in *L. pneumophilia* strains.

1 Gene number as annotated in the Philadelphia-1 (lpg), Paris (lpp) or Lens (lpl) genome. ORF indicates an open reading frame, which is not involved in lipid A biosynthesis or whose function is not clear. The genes lpg2941, lpp3013 and lpl2871 encode proteins with weak homologies to acyl transferases, and lpg2942 shows no homology to known proteins. The genes lpg0510, lpp0572 and lpl0548 are annotated as hydroxymyristoyl[ACP]dehydratase FabZ, and lpg0509 is annotated as ORF belonging to a sugar transporter family.

2 MsbA is an ABC transporter involved in lipid A translocation through the inner membrane.

In summary, *L. pneumophilia* seems to be unique in harboring multiple copies of lipid A disaccharide synthases (*LpxB*) and acyl transferases (*LpxA*, -D, -L). A common feature of these enzymes is that they modify the acylation state of lipid A or its precursors in the LPS biosynthesis pathway. The paralogues might have different acyl substrate specificities and thus determine the variable length of the lipid A acyl chains identified in the *L. pneumophilia* phase variant. Contrarily, in other bacterial genomes, the genes necessary for lipid A biosynthesis are present in only one copy (Raetz and Whitfield, 2002).

### 3.4.2 Complementation of a conditional *E. coli* *lpxB* mutant strain with *L. pneumophilia* *lpxB* paralogues

The genomic organization of the *L. pneumophilia* *lpxB* genes suggests that both paralogues are involved in lipid A biosynthesis. To test for their function as lipid A disaccharide synthases, a complementation assay was performed. Since deletion of *lpxB* is lethal for *E. coli*, the temperature sensitive *E. coli* *lpxB* mutant strain MN7 was used (Nishijima et al., 1981). The strain was transformed with *L. pneumophilia* *lcsC*/*lpxB1* or *lpxB2* expression vectors or control vectors and grown at 30 °C (permissive temperature) or 42 °C (non permissive temperature) for 24 h. Under these conditions, expression of *E. coli* *lpxB* (pSR8)
restored growth at 42 °C to a level similar to that at 30 °C. Expression of *L. pneumophila lcsC/lpxB1* or *lpxB2* complemented growth at the non- permissive temperature (Table 3), indicating that both *L. pneumophila lpxB* paralogues function as lipid A disaccharide synthases during biosynthesis of lipid A. The *L. pneumophila* enzymes only partially complement *E. coli lpxB*. This is likely due to the structural difference between *L. pneumophila* and *E. coli* lipid A, causing a reduced efficiency of lipid A synthesis. The sugar backbone of *L. pneumophila* lipid A consists of diamino-dideoxy-glucopyranose instead of glucosamine, and the primary fatty acid chains differ considerably in length (C20 or C14, respectively). Furthermore, expression of *E. coli lpxB* was fully induced, whereas expression of the *L. pneumophila lpxB* paralogues remained restricted to the basal level of the *P_{lac}* promoter to avoid toxic overexpression effects. It might be that the amount of the *L. pneumophila* LpxB enzymes was not sufficient.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Dilution</th>
<th>Number of colonies after 24 hrs incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>pUC18</td>
<td>$10^{-6}$</td>
<td>411</td>
</tr>
<tr>
<td>pSR8</td>
<td>$10^{-6}$</td>
<td>416</td>
</tr>
<tr>
<td>pMMB207C</td>
<td>$10^{-1}$</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>pMMB207C-lcsC</td>
<td>$10^{-1}$</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>pMMB207C-lpxB</td>
<td>$10^{-1}$</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

Table 3: Complementation of the temperature sensitive *E. coli lpxB* mutant MN7 with *L. pneumophila lcsC* and *lpxB*. MN7 transformed with the plasmids indicated was plated in different dilutions and grown at 30 °C (permissive temperature) and 42 °C (non permissive temperature). After 24 hours incubation were the number of cfu determined. pUC18: empty vector, pSR8: expression vector for *E. coli lpxB*, pMMB207C: empty vector, pMMB207C-lcsC; -lpxB: expression vectors for *L. pneumophila lcsC* or *lpxB*, respectively.

### 3.4.3 Cytotoxicity of *L. pneumophila lpxB* paralogues for *A. castellanii*

Since *lcsC/lpxB1* was identified due to its ability to render bacteria cytotoxic for *A. castellanii*, we tested whether the expression of *lpxB2* is also toxic for amoebae. *A. castellanii* was infected with an *L. pneumophila icmG* mutant strain expressing either of the *lpxB* paralogues, and cytotoxicity was determined by propidium iodide (PI) uptake 2 d
post infection. Under these conditions, overexpression of \( lcsC \), but not \( lpxB \), was cytotoxic for \( A. \) castellanii, without enhancing intracellular replication of the bacteria within the amoebae (data not shown; Albers et al., 2005). While the expression of \( lcsC/lpxB1 \) in an \( icmG \) mutant background killed approximately 35% of the amoebae within 2 d, expression of \( lpxB2 \) did not increase cytotoxicity above background level (Figure 2). This finding suggests that the two \( L. \) pneumophila \( lpxB \) paralogues are functionally not equivalent.

Upon induction in exponential growth phase with IPTG, \( lcsC/lpxB1 \) but not \( lpxB2 \) expression reduced the growth rate of liquid cultures. On agar plates, overexpression of \( lpxB2 \) was apparently more cytotoxic than \( lcsC/lpxB1 \), as lower concentrations of IPTG were required to inhibit bacterial growth on plates (data not shown). The distinct effects of overexpression give further evidence for functional differences between LcsC/LpxB1 and LpxB2.
3.4.4 Expression of *L. pneumophila* lpx genes during growth in a complex medium

Both *L. pneumophila* lpxB paralogues are located adjacent to other lipid A biosynthesis genes in the genome: lpxB2 (lpg2945) lies next to lpxD2 (lpg2944) and lpxA2 (lpg2943), but is encoded on the opposite strand and, therefore, does not form an operon with other genes. lcsC/lpxB1 (lpg1371) is located immediately downstream of gnnA (lpg1372), encoding the putative first enzyme of the pathway. To examine whether the two genes are cotranscribed, RNA was isolated, and a RT-PCR was performed using primers hybridizing to gnnA and to lcsC/lpxB1, respectively. The presence of a PCR product demonstrates that gnnA and lcsC/lpxB1 are cotranscribed (Figure 3), supporting the idea that both enzymes belong to the same biosynthetic pathway.

Figure 3: PCR analysis of cotranscription of lcsC and gnnA. RNA from bacteria in exponential growth phase was assayed by reverse transcription and PCR with primers binding in gnnA and lcsC. Genomic: control with genomic DNA as template, MW: molecular weight marker, -/+ RT: absence or presence of reverse transcriptase during cDNA synthesis.

The existence of multiple paralogues of genes that are predicted to modulate the acylation state of lipid A suggests that *L. pneumophila* has the potential to synthesize structurally different lipid A molecules in response to different environmental conditions. To test whether *L. pneumophila* lipid A biosynthesis genes are differentially transcribed, we examined the expression levels of the genes by RT-PCR under different conditions. Samples of the PCR reaction were taken after different cycle numbers and the amount of product was assessed on an agarose gel. Thereby, the time point of appearance and the strength of a band correspond to the amount of mRNA in a sample.

The method was validated using genes with known expression profiles during different growth phases in liquid medium: rpoA encodes the α-subunit of RNA polymerase, which is maximally expressed in logarithmic growth phase and slightly downregulated in stationary phase (Bruggemann et al., 2006). 16S ribosomal RNA was used as a control for RNA present constitutively at high levels. flaA encodes the major flagellum component (flagellin), which is strongly upregulated during stationary growth phase, corresponding to the observation that
*L. pneumophila* becomes motile upon entry into stationary phase (Byrne and Swanson, 1998; Heuner *et al.*, 1999). As expected, *rpoA* was expressed constitutively in all growth phases, but slightly downregulated in late stationary phase, 16S rRNA was abundant and expressed constitutively, and the expression level of *flaA* was lower than that of *rpoA*, but upregulated at late stationary phase (Figure 4). The expected regulation patterns demonstrate that the method is suitable for gene expression analysis in *L. pneumophila*. In absence of reverse transcriptase, faint bands of the target genes were visible only after 32 PCR cycles, indicating that the amount of contaminating DNA in the assay was low.

![Figure 4: RT-PCR analysis of expression in different growth phases in liquid cultures.](image)

The expression levels of the *lpxB* and *lpxA* paralogues were examined in exponential, early stationary and late stationary growth phase, respectively (Figure 5). The amount of *lcsC/lpxB1* and *lpxB2* RNA was low in all growth phases, and expression of *lcsC/lpxB1* was further reduced in late stationary growth phase. The two *lpxA* paralogues were expressed at comparable levels, preferentially in exponential growth phase and more strongly than the *lpxB* paralogues. 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.
Figure 5: Gene expression of \textit{lpxB} and \textit{lpxA} paralogues in different growth phases in liquid medium. RNA was extracted from bacteria grown in liquid medium to different growth phases and expression levels were assayed by reverse transcription and PCR. Exp: exponential growth phase, (OD$_{600}$ 0.8, 6 hours incubation), ES: early stationary phase (OD$_{600}$ 2.4, 16 hours), LS: late stationary phase (OD$_{600}$ 3.4, 26 hours), G: PCR from genomic DNA.

3.4.5 Expression of \textit{L. pneumophila lpx} genes under osmotic stress and nutrient deprivation

\textit{L. pneumophila} might adapt to osmotic stress or nutrient deprivation by modulating the expression of lipid A biosynthesis genes, and consequently, altering its lipid A structure. To test this hypothesis, an \textit{L. pneumophila} liquid culture in stationary growth phase (OD$_{600}$ 3.3) was split and either suspended in distilled water or kept in AYE medium. \textit{L. pneumophila} tolerates exposure to distilled water, and differences in gene expression were examined four hours after incubation at room temperature. To assay effects of osmotic stress or nutrient deprivation on the expression of \textit{lpx} genes, bacteria in stationary growth phase were chosen, since incubation of exponentially growing bacteria in water induces a transition from exponential to stationary phase, leading to downregulation of \textit{lpx} genes (Figure 5). The effects of growth phase transition would likely mask effects caused by osmotic stress or nutrient deprivation.

While expression of \textit{flaA} was not affected upon transition of \textit{L. pneumophila} from rich medium to distilled water, \textit{lcsC/lpxB1} and \textit{lpxA1} were upregulated and \textit{lpxB2} was slightly downregulated under these conditions (Figure 6). The expression of \textit{lpxA2} and \textit{lpxC} in stationary growth phase was very low upon incubation of \textit{L. pneumophila} in distilled water. The inverse regulation of \textit{lcsC/lpxB1} and \textit{lpxB2} and the upregulation of \textit{lpxA1} suggest that under osmotic stress or nutrient deprivation \textit{L. pneumophila} might employ \textit{lcsC/lpxB1} and \textit{lpxA1} to modulate its lipid A structure.
The cytotoxic lipid A disaccharide synthase paralogue LcsC

Figure 6: Gene expression under osmotic stress and nutrient deprivation. *L. pneumophila* from stationary phase liquid cultures were split and either incubated in AYE medium or in distilled water for 4 hours at room temperature. RNA was extracted and expression levels were assayed by reverse transcription and PCR. G: PCR from genomic DNA.

3.4.6 Expression of *L. pneumophila lpx* genes during intracellular growth in *A. castellanii*

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 analyzed shortly after infection (4 h) and after 24 h. As RNA was extracted from infected amoeba, it is from both bacteria and amoeba. Due to intracellular bacterial replication, the proportion of bacterial RNA is higher in the 24 h sample compared to 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 of sampling.

Interestingly, at 4 h post infection, *lpxA1* and *lcsC/lpxB1* were much stronger expressed than *lpxA2* and *lpxB2* (Figure 7). The expression level of *lpxA1* was similar to *lpxC*, a gene with no paralogues in the *L. pneumophila* genome. 24 hours after infection, all *lpx* paralogues tested were expressed abundantly and at comparable levels. This finding suggests that while not detectable at 4 h post infection, *lpxA2* and *lpxB2* become more important at later stages of an infection. Taken together, the data suggest that during early stages of an infection *lpxA1* and *lcsC/lpxB1* are predominantly expressed, whereas in late stages of infection *lpxA2* and
IpxB2 expression increases. The IpxA1 and lcsC/lpxB1 genes were also expressed with a similar pattern upon incubation of the bacteria in distilled water (Figure 6), suggesting that the genes might be co-regulated.

Figure 7: Expression of lipid A biosynthesis genes during intracellular growth in A. castellanii. RNA from uninfected A. castellanii amoebae (0) or from amoebae 4 or 24 hours post infection (4h, 24h) was extracted and expression levels were assayed by reverse transkription and PCR. RNA preparations from different time points contain different proportions of RNA from amoebae and bacteria, and therefore, expression levels can be directly compared only within identical RNA preparations prepared at the same time post infection. G: PCR from genomic DNA.

3.5 DISCUSSION

3.5.1 Lipid A biosynthesis paralogues and their functions

The identification of the cytotoxic lpxB paralogue lcsC (Albers et al., 2005) prompted us to analyze the lipid A biosynthesis pathway in L. pneumophila. A search for genes homologous to E. coli and A. ferrooxidans lipid A biosynthesis genes revealed that L. pneumophila harbors genes required for the conversion of GlcNAc to GlcNAcN3, and multiple copies of the acyl transferases IpxA, IpxD, and IpxL, as well as the lipid A disaccharide synthase lpxB. Both lpxB paralogues partially complement an E. coli conditional lpxB mutant, indicating that the L. pneumophila lpxB genes indeed function as lipid A disaccharide synthases (Table 3). However, the enzymes are distinct, as overexpression of lcsC/lpxB1 but not lpxB2 is cytotoxic for amoebae (Figure 2). Interestingly, all of the genes existing in more than one
copy in the *L. pneumophila* genome are presumably involved in lipid A acylation, or in case of *lpxB*, might process differentially acylated substrates.

In *E. coli*, LpxA functions as an accurate hydrocarbon ruler that incorporates 14-carbon acyl chains two orders of magnitude faster than 12- or 16-carbon acyl chains, and LpxA from *P. aeruginosa* specifically adds 10-carbon acyl chains (Raetz and Whitfield, 2002). The *L. pneumophila* LpxA and LpxD paralogues likely also have different substrate specificities and might preferentially use as substrates fatty acids with distinct chain length, branches or other modifications. The two lipid A disaccharide synthases then presumably condense the different substrates generated by the LpxA and LpxD paralogues, resulting in lipid IV \( \text{A} \) moieties that vary in primary fatty acid composition. The three *L. pneumophila* LpxL paralogues possibly catalyze the transfer of different acyl chains. *E. coli* LpxL and the cold shock-induced acyl transferase LpxP both catalyze the addition of secondary fatty acids to the \( \beta \)-hydroxyl groups of the primary acyl chains (Raetz and Whitfield, 2002). Lipid A of *L. pneumophila* contains C\(_{16}\) and C\(_{28}\) secondary fatty acids attached to position 2' and 3' (Kooistra et al., 2002a) and it is conceivable that the three LpxL homologues and the *pagP* like gene *rcp* enable *L. pneumophila* to vary also the composition of the secondary fatty acids. In summary, the presence of multiple *lpx* paralogues suggests that *L. pneumophila* synthesizes lipid A molecules modified by a variety of different primary and secondary acyl chains.

Synthesis of lipid A with acyl chains of different length has been shown for *L. pneumophila* serogroup 1 subgroup OLDA. This strain undergoes phase variation based on excision and insertion of a 30 kb unstable element (Luneberg et al., 2001). In the phase variant strain, the length of the primary 3-hydroxylated fatty acids attached to position 2 and 2' of the GlucN3N disaccharide is shifted from C\(_{20}\) to shorter chains of C\(_{16}\) or C\(_{18}\), while the lipid A backbone and the phosphorylation pattern remain unaffected (Kooistra et al., 2002a). The three different LpxD paralogues are candidate enzymes to catalyze these modifications.

### 3.5.2 Differential regulation of *L. pneumophila lpx* paralogues

As an adaption to different niches, *L. pneumophila* might produce distinct lipid A structures under different environmental conditions. Therefore, we examined whether transcription of *lpx* paralogues is differentially regulated. During growth in nutrient broth, there was no large difference between the expression of *lpxA1* and *lpxA2* or between expression of *lcsC/lpxB1*.
and \( lpxB2 \) (Figure 5). However, upon transfer of \( L. \) \( pneumophila \) from nutrient broth into distilled water, expression of \( lcsCl/lpxB1 \) and \( lpxA1 \) was upregulated, while expression of \( lpxB2 \) was downregulated (Figure 6). 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 extracellular environment.

Shortly after infection of \( A. \) \( castellanii \) by \( L. \) \( pneumophila \), \( lpxA1 \) and \( lcsCl/lpxB1 \) are stronger expressed than their paralogues \( lpxA2 \) and \( lpxB2 \), respectively (Figure 7). As expression of \( lcsCl/lpxB1 \) and \( lpxA1 \) was also upregulated upon transfer of \( L. \) \( pneumophila \) to water, these paralogues might represent lipid A biosynthesis genes required during the transmissive/infective phase of \( L. \) \( pneumophila \) (Molofsky and Swanson, 2004). Contrarily, 24 h post infection, \( lpxA1 \) and \( lpxA2 \), as well as \( lcsCl/lpxB1 \) and \( lpxB2 \) were expressed at a similar level, indicating a role in lipid A biosynthesis for all \( lpx \) paralogues at later stages of the infection in the replicative phase of \( L. \) \( pneumophila \). In agreement with this notion, the \( lpx \) paralogues were also expressed at similar levels during growth in nutrient broth (Figure 5). In summary, \( L. \) \( pneumophila \) expresses distinct \( lpx \) paralogues under specific growth and environmental conditions, suggesting that the lipid A structure is modified as an adaption to a changing environment.

### 3.5.3 Biological implications of lipid A and LPS modifications

Lipid A modifications profoundly effect various interactions between pathogenic bacteria and their environment, including host cells and organisms. A prominent example is \( Salmonella \) \( enterica \) serovar Typhimurium, which, dependent on PhoP-PhoQ and other two-component regulatory systems, regulates a number of lipid A-modifying enzymes (Miller et al., 2005). The acyl transferase PagP is synthesized under low magnesium concentrations encountered inside a phagosome and adds an additional palmitate to the primary acyl chain at position 2 of lipid A, thus increasing the resistance of the bacteria to cationic antimicrobial peptides (CAMPs) (Bishop et al., 2000; Guo et al., 1998). Interestingly, \( L. \) \( pneumophila \) harbors the \( pagP \)-like gene \( rcp \), conferring resistance to CAMPs in low magnesium medium (Robey et al., 2001). Similar to a \( pagP \) mutant, an \( rcp \) mutant showed decreased resistance to CAMPs. Moreover, the \( rcp \) mutant was defective for growth in \( Hartmanella \) \( vermiformis \) amoebae and \( U937 \) macrophages, as well as for lung colonization of \( A/J \) mice.
The cytotoxic lipid A disaccharide synthase paralogue LcsC

Structural modifications of lipid A may also significantly affect the course of an infection in multicellular host organisms. Lipid A 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 and Whitfield, 2002). *L. pneumophila* LPS fails to bind CD14, probably due to the unusually long and branched fatty acid residues of its lipid A (Neumeister et al., 1998). The unusual composition of *L. pneumophila* lipid A fatty acids, possibly attached by the different *lpx* paralogues, might help the bacteria to escape recognition by the innate immune system.

Recently, LPS-containing vesicles released from the outer membrane of growing *L. pneumophila* were 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 but not the replicative phase inhibited phagosome-lysosome fusion. The structure of LPS was regulated in a growth phase dependent manner, indicating that LPS modifications might be involved in altering vesicle trafficking. Intracellular distribution of LPS during infection and an impact of intracellular LPS on vesicle trafficking were also shown for *Legionella jeonii* infecting *Acanthamoeba proteus*. LPS released from the bacteria inserted into the LCV membrane and assisted in preventing fusion of the LCV with lysosomes (Kim et al., 1994). It is conceivable that modifications of the membrane anchor lipid A affect the interactions of LPS with host cell functions and thus modulate the course of infection.

In summary, due to the involvement of lipid A in protection, immune recognition and probably also in intracellular life, modification of its structure contributes to the adaption of *L. pneumophila* to its diverse niches. It will be of great interest to identify the biochemical functions of the different *lpx* paralogues, the structure of lipid A molecules synthesized by the paralogues and the mechanisms by which the different lipid A variants interact with host organisms.

### 3.6 ACKNOWLEDGEMENTS

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3.7 REFERENCES


The cytotoxic lipid A disaccharide synthase paralogue LcsC


4 **L. pneumophila** IcmG increases the efficiency of effector protein secretion

4.1 Introduction

*L. pneumophila* is a Gram-negative bacterium that can be found ubiquitously in the environment. It survives and multiplies mainly intracellularly in various protozoa, where it manipulates the host cell vesicular machinery to prevent phagosome-lysosome fusion and to establish a replication permissive vacuole (Fields, 1996; Roy and Tilney, 2002). Upon transmission into the human lung, the bacteria are phagocytosed by alveolar macrophages, replicate within the cells and finally kill the macrophages, thereby causing the life-threatening pneumonia known as Legionnaire's disease (Nash et al., 1984).

Efficient phagocytosis and intracellular replication of *L. pneumophila* depend on a type IV secretion system encoded by the *icm/dot* (intracellular multiplication/defective in organelle trafficking) genes (Hilbi et al., 2001; Segal et al., 1998; Vogel et al., 1998). The *icm/dot* genes are located within the *Legionella* genome in two clusters: Region I contains the *dotDCB* and *dotA-icmVWX* genes, and Region II comprises 18 genes, including *icmG*. The gene clusters were identified by transposon mutagenesis (Purcell and Shuman, 1998; Segal and Shuman, 1999), and most of the *icm/dot* genes are absolutely required for intracellular growth and killing of human macrophages and the protozoan host *Acanthamoeba castellanii*. Mutation of the *icmG, icmS* or *icmF* gene led to only slight defects in the ability to grow within and kill macrophage-like HL-60 cells but abolished growth in amoebae (Segal and Shuman, 1999). In contrast to the majority of *icm/dot* mutants, the *icmG* and *icmS* mutant are capable to survive and grow on agar plates in presence of amoebae, albeit with a strong growth defect compared to wild type bacteria. The *icmG* mutant also shows a partial phenotype with respect to cytotoxicity for *A. castellanii* (Albers et al., 2005).

The IcmG protein contains a predicted transmembrane and a coiled-coil domain (Figure 1). A bacterial two-hybrid screen revealed that the part of IcmG spanning amino acids 28 to 123 specifically interacts with *L. pneumophila* effector proteins, namely RalF and the Sid effector proteins (Luo and Isberg, 2004).

Morozova and coworkers performed a comparative sequence analysis of *icm/dot* genes and predicted a putative SNARE domain in IcmG at amino acids 142 to 210 (Morozova et al., 2004). SNARE proteins (soluble NSF attachment receptor; NSF: N-ethyl-maleimide-
sensitive fusion protein) form a family of proteins that mediate intracellular membrane fusion events in eukaryotic cells (Chen and Scheller, 2001). Modification of vesicular trafficking is a prerequisite for *L. pneumophila* to generate a replication permissive vacuole, and interfering with SNARE-mediated vesicle fusion could be a method to alter host cell vesicle trafficking. A SNARE domain was also predicted in the corresponding region of *Coxiella burnetii* IcmG, the only known protein showing sequence similarities to *Legionella* IcmG.

**Figure 1: In silico analysis of IcmG.** The protein sequence was analyzed as described in Material and Methods. The binding region for effector proteins was described in Luo and Isberg, 2004. The presence of a putative SNARE domain was stated by Morozova *et al.*, 2004. TM: Transmembrane domain.

The *icmG* mutant was recently used in a screen to identify genes involved in virulence of *L. pneumophila* (Albers *et al.*, 2005). In the “amoebae plate test”, the ability of bacteria was examined to grow and survive on agar plates in the presence of the phagocytic amoeba *A. castellanii*. An *L. pneumophila* chromosomal library was introduced into ΔicmG and screened in the amoebae plate test for plasmids suppressing the partial growth defect of the *icmG* mutant. In this screen, a paralogue of the *E. coli* lipid A disaccharide synthase *lpxB* was identified and termed *lcsC* (*Legionella* cytotoxic suppressor), since overexpression of *lcsC* in ΔicmG rendered the bacteria more cytotoxic for the amoebae. Surprisingly, overexpression of *lcsC* in other *L. pneumophila* mutants showing partial growth defects in the amoebae plate test or in intracellular growth assays (e.g., ΔicmS, ΔicmF, ΔrpoS) did not render the bacteria more cytotoxic (see Chapter 2, Albers *et al.*, 2005).

The restriction of *lcsC* mediated cytotoxicity to the *icmG* mutant and the possibility of IcmG acting as a SNARE protein prompted us to examine the function of IcmG in more detail to better understand its role in infection and LcsC-mediated cytotoxicity. We show that IcmG is expressed at low levels in *L. pneumophila*, is most likely not translocated during infection and that an *icmG* mutant strain retains the capability to translocate effector proteins but is impaired in the ability to recruit ER derived vesicles. The data suggest that IcmG is part of...
the Icm/Dot secretion system and acts as a coupling protein supporting the release of effector proteins.

4.2 Materials and methods

4.2.1 Cultivation of cells

The *L. pneumophila* wild type strain used was Philadelphia-1 serogroup 1 JR32 (Sadosky *et al.*, 1993). The *icmG* mutant strain MW635 (Purcell and Shuman, 1998) is an isogenic mutant containing a kanamycin resistance gene inserted in the *icmG* gene, and GS3011 is an avirulent mutant containing a kanamycin resistance gene insertion in the *icm/dot* transporter gene *icmT* (Segal and Shuman, 1998). *L. pneumophila* was grown on charcoal yeast extract (CYE) plates (Feeley *et al.*, 1979) and in AYE liquid medium (Horwitz and Silverstein, 1983). Plasmids were maintained by addition of 5 μg/ml chloramphenicol. *E. coli* TOP10 (Invitrogen) was used for cloning and *E. coli* BL21 (DE3) (Novagen) was used for expression of GST and GST fusion proteins. *D. discoideum* amoebae (Hagele *et al.*, 2000; Solomon *et al.*, 2000) were grown axenically at 23 °C in culture flasks in HL5 medium (Weber *et al.*, 2006). RAW246.7 macrophages were cultivated in RPMI1640 supplemented with 10% FCS and 2 mM L-glutamine at 37 °C in 5% CO₂.

4.2.2 Sequence analysis of IcmG

Hydropathicity was analyzed with the ProtScale software (www.expasy.org). The transmembrane region was identified by TMHMM 2.0 (www.cbs.dtu.dk), coiled coil structures were searched using Coils 2.1 (www.embl.de). Protein family and domain search was performed with InterProScan (www.ebi.ac.uk) and Pfam (pfam.wustl.edu) algorithms.

4.2.3 Construction of *icmG* expression vectors

To generate an N-terminal fusion of GST to IcmG, the *icmG* gene was amplified using genomic DNA from JR32 as template and the primers oUA32 (GATCGTACGGATCCATGATGGCAGAGCACG) and oUA33 (GATCGTACGTCGACTCTAACTATCTTCTTGACTAAAC), digested with BamHI and Sall and ligated into the
Characterization of IcmG

BamHI-SalI-digested pGEX-4T-1 (Amersham Biosciences) to generate pUA47 (pGEX-IcmG).

The same cloning strategy was employed to generate N-terminal fusions of GST to the N-terminal part of IcmG (amino acids 1-123) and the C-terminal part (amino acids 124-269). For PCR amplification of the N-terminal part of IcmG, oUA32 and oUA37 (GTACGATCGTGACTCAATCGGGGTCATCTTCAATAATAG) were used. The C-terminal part was amplified using oUA38 (GATCGTACGGATCCCTAAAGAAGAAAGTTTCAGCAATAGAAATG) and oUA33. BamHI-SalI digest and ligation into pGEX-4T-1 yielded pUA52 (pGEX-IcmG-N123) and pUA53 (pGEX-IcmG-C146).

An N-terminal His$_6$ tag was attached to IcmG by amplifying the icmG gene from genomic DNA from JR32 with oUA34 (GTACGTACATATGGCGAGAGCACGATC) and oUA33, digestion of the PCR product with NdeI-SalI and ligation into the NdeI-SalI-digested pET28a(+) (Novagen), yielding pUA48 (pET-icmG-HisN).

For inducible expression of icmG and N-terminally His$_6$-tagged icmG in L. pneumophila, the genes were transferred into the broad host plasmid pMMB207C (Chen et al., 2004). The NdeI-HindIII insert from pUA48 was cloned into the NdeI-HindIII-digested pMMB207C to yield pUA50 (pMMB207C-icmG). The XbaI-HindIII insert from pUA48 was cloned into the XbaI-HindIII-digested pMMB207C to yield pUA51 (pMMB207C-IcmG-HisN).

For inducible expression of N-terminally M45-tagged icmG in L. pneumophila, the insert from the BamHI-SalI digested pUA47 (pGEX-IcmG) was ligated into the BamHI-SalI-digested pCR33 (pMMB207C-RBS-M45-Gly$_6$; Weber et al., 2006) to yield pUA54 (pMMB207C-M45-IcmG).

4.2.4 Pull-down assays using GST fusion proteins

N-terminal GST-IcmG fusion proteins were used to search for IcmG binding proteins in pull down assays. GST-IcmG, GST-IcmG-N123 (GST fused N-terminally to amino acids 1-123 of IcmG) and GST-IcmG-C146 (GST fused N-terminally to amino acids 124-169) and GST alone as a control were expressed in E. coli BL21 (DE3) (Novagen). The BL21 (DE3) strains were diluted to an OD$_{600}$ of 0.1 and grown at 37°C until the OD$_{600}$ reached 0.6-0.8. Expression was induced with 0.5 mM IPTG, cells were grown for additional 4 hours at 30°C and pelleted (6000 × g, 10 min, 4 °C). 25 ml of the bacterial culture expressing GST were
used per batch. All three GST-IcmG derivatives are poorly soluble and required a volume of 150 ml culture per batch to obtain sufficient soluble protein. The bacterial pellets were resuspended in 0.5 ml (GST-expressing bacteria) or 1.2 ml (GST-IcmG-fusions-expressing bacteria) lysis buffer A (50 mM Tris, 150 mM NaCl, 0.5 % Triton X100, 1 mM CaCl₂, 100 μM PMSF, 1 mg DNAse per 100 ml, pH 7.4). GST-expressing bacteria were lysed by three times 10 seconds sonication on ice. GST-IcmG fusion proteins were lysed by three passages through a French pressure cell and supplemented with 2 mM EDTA. Cell debris was removed by two centrifugation steps (21000 x g, 15 min, 4°C).

Glutathione sepharose 4B (Amersham Biosciences) was equilibrated in lysis buffer A. GST or the GST-IcmG derivatives were bound to 100 μl glutathione sepharose at 4°C for 4 hours, washed four times on a rotating wheel at 4°C with 5 ml of the lysis buffer A and two times with lysis buffer B (50 mM Tris, 50 mM NaCl, 0.1 % Triton X100, 1 mM CaCl₂, 100 μM PMSF, 1 spatula tip DNAse per 100 ml, pH 7.4).

To search for *Legionella* proteins binding to IcmG, 200 ml AYE medium per batch were inoculated with JR32 at an OD₆₀₀ of 0.15 and grown for 22 hours at 37°C. The bacteria in early stationary phase were pelleted, resuspended in 2 ml lysis buffer B, lysed by three passages in a French pressure cell and supplemented with 2 mM EDTA. Cell debris was removed by two centrifugations (21000 x g, 15 min, 4°C) to obtain the JR32 lysate.

To search for host cell proteins binding to IcmG, seven T150 flasks (1050 cm²) were inoculated with RAW264.7 macrophages. The cells were grown to 90% confluency, washed with ice cold PBS and scraped off in 4 ml lysis buffer B. The macrophages were lysed by 10 passages through a 26 GA needle, and the lysate was supplemented with 2 mM EDTA. Cell debris was removed by two centrifugation steps (21000 x g, 15 min, 4°C) to obtain the clear macrophage lysate.

The glutathione sepharose beads loaded with GST or the GST-IcmG derivatives were incubated over night at 4°C with the JR32 lysate, the RAW264.7 lysate or lysis buffer as a control. The beads were washed extensively five times with 5 ml lysis buffer B at 4°C and boiled for 5 minutes after addition of Laemmli sample buffer to release proteins. Proteins bound to 100 μl beads were separated on a 8%-18% gradient SDS gel, transferred onto PVDF membranes, stained on the membrane with Coomassie brilliant blue, and bands were cut out and analyzed by Edman degradation at the ETH protein service laboratory. In some cases, the protein bands were stained with Coomassie brilliant blue within the gel, cut out and analyzed by tryptic digest and MALDI-MS at the protein service laboratory.
4.2.5 Copurification of IcmG and binding proteins using His$_6$-tagged IcmG

To copurify IcmG and binding partners, icmG with an N-terminal His$_6$ tag was overexpressed in *L. pneumophila* ΔicmG. An over night culture of ΔicmG/pMMB207C-IcmG-HisN was diluted to an OD$_{600}$ of 0.4, incubated at 37 °C until the OD$_{600}$ reached 1.0, induced with 0.5 mM IPTG and kept growing for four hours. Bacteria from 200 ml culture were used per batch. Cells were spun down (6000 x g, 15 min, 4 °C), resuspended in 3.5 ml lysis buffer H1 (10 mM imidazol, 150 mM NaCl, 50 mM NaH$_2$PO$_4$, 0.2% Triton X100, 100 μM PMSF, pH 8.0) or lysis buffer H2 (2.5 mM imidazol, 200 mM NaCl, 50 mM NaH$_2$PO$_4$, 0.5% Triton X100, 100 μM PMSF, pH 8.0), and lysed by three passages through a French pressure cell. Cell debris was removed by two centrifugation steps (21000 x g, 20 min, 4 °C) and added to 200 μl Ni-NTA beads (Qiagen) that had been preequilibrated in lysis buffer. After 2 hours of incubation at 4 °C, the beads were washed five times with 5 ml lysis buffer H1 containing 10 mM imidazol, respectively, and eluted with increasing imidazol concentrations or further washed with increasing NaCl concentrations as described in the figure legends. Proteins were separated on an SDS gel and stained with Coomassie brilliant blue or blotted onto nitrocellulose, and His-tagged proteins were detected with an anti-His$_6$ antibody (Qiagen).

4.2.6 Generation of antibodies and Western blot

An antibody against a partial amino acid sequence of IcmG (amino acids 189 to 203) was derived by immunization of rabbits with the chemically synthesized peptide CARTTPKVKVKSRI coupled to the carrier protein KLH (keyhole limpet hemocyanin) (NeoMPS, Strasbourg, France). The antibody was used in Western blots diluted 1:2000 in blocking buffer (PBS with 5% fat free milk powder and 0.05% Tween 20) and visualized by a goat anti rabbit-HRP conjugate (Becton Dickinson, diluted 1:4000 in blocking buffer) and luminescence detection (ECL kit, Amersham Biosciences). The presence of 20% or 30% methanol and the lack of SDS in the Tris-glycine blotting buffer turned out to be crucial for strong signals.

The anti-SidC and anti-M45 antibodies are described elsewhere (Weber *et al.*, 2006).
4.2.7 Immunofluorescence microscopy

Intracellular localization of proteins was examined by immunofluorescence of infected Dictyostelium discoideum AX3 expressing a calnexin-gfp fusion protein as described in Weber et al., 2006. In brief, $2 \times 10^4$ amoebae were seeded onto glass cover slips in a 24-well plate the day prior to infection. Bacteria for infection were inoculated at an OD$_{600}$ of 0.1 and grown for 21 hours at 37 °C on a rotating wheel. Expression of $M45$-sidC was induced upon inoculation, expression of $M45$-icmG was induced after 17 hours. On the day of infection, bacteria were added at a multiplicity of infection of 100, spun onto the amoebae ($880 \times g$, 5 min), and the amoebae were incubated for one hour at 25 °C. After a wash with SorC (Malchow et al., 1972), the cells were fixed for 30 minutes at 4 °C with 4% PFA in PBS, permeabilized for 10 min at 25 °C with 0.1% Triton X100 in SorC, and blocked over night with 2% normal human AB serum in SorC.

M45-tagged proteins were detected by a monoclonal anti-M45 hybridoma supernatant (Obert et al., 1994, diluted 1:4 in blocking solution). SidC was detected with an affinity-purified rabbit anti-SidC antibody (Weber et al., 2006, diluted 1:1000 in blocking solution). Cy5-conjugates from Jackson ImmunoResearch were used as secondary antibodies (1:200 in blocking solution). Bacteria were either detected with a rhodamine-labelled anti-Legionella pneumophila Philadelphia-1 Serogroup 1 antibody (m-Tech, 1:100 in blocking solution) or by constitutive expression of the red fluorescent protein DSRedExpress from plasmid pSW001 (Mampel et al., 2006). The cover slips were mounted and analyzed using a Zeiss Axiovert 200 microscope and a Perkin-Elmer Ultraview confocal imaging system.

To control for similar expression levels of M45-tagged proteins, whole cell cultures were adjusted to an OD$_{600}$ of 1.0, boiled in Laemmli buffer, separated by SDS gel electrophoresis and analyzed by Western blot using the anti-M45 antibody described above.

4.3 Results

4.3.1 IcmG is present in the bacteria in low amounts

The intracellular concentration of a protein and the regulation of its expression can give hints about its function, and, therefore, synthesis of IcmG was analyzed by Western blot. A polyclonal rabbit antibody against an IcmG-derived peptide was generated, and the amount of protein in early stationary growth phase cultures, during which L. pneumophila is most
Characterization of IcmG

virulent, was determined by Western blot (Figure 2). IcmG expression under control of the 
P_tae promoter for 4 or 8 hours yielded strong bands even after 30 seconds exposure time, whereas IcmG from wild type (JR 32) or expressed from a plasmid containing the icmGCD genomic region (∆G/pGCD) became only visible as a weak band after 25 minutes exposure time, indicating that IcmG is present in the bacteria only at low concentrations.

![Figure 2: Expression levels of icmG under native conditions and after 4 and 8 hours overexpression.](image)

To examine whether icmG might be stronger expressed at growth phases between logarithmic and late stationary phase, 14 to 27 hours old cultures were examined for the amount of IcmG protein by Western blot. All of the samples showed a low IcmG expression comparable to that one shown in Figure 2 (data not shown).

Expression of icmG could also be induced by environmental conditions inside of a host cell. This possibility was examined using A. castellanii as host cells. The amoebae were infected with JR32 and ∆icmG, and the presence of IcmG was assayed in TCA-precipitated culture medium supernatant and in amoeba lysates after 24 and 48 hours post infection by Western blot. IcmG was not detected in the supernatants or in amoebae infected with ∆icmG. The concentration of IcmG in amoebae infected with JR32 was compared with the concentration in the early stationary culture used for infection. Bacterial numbers were estimated by taking the intensity of the unspecific band above IcmG as a standard. There was no difference between IcmG levels in JR32 in early stationary cultures and in amoebae infected for one or two days, indicating that intracellular conditions do not increase expression of icmG significantly (data not shown).
Acting as a SNARE protein would possibly require secretion of IcmG. Secretion would also decrease the intracellular concentration and could lead to the low protein levels detected in the bacteria. No substances are known that induce secretion of Legionella effector proteins into the culture medium. Congo Red has been shown to induce secretion of effector proteins in Shigella flexneri (Bahrani et al., 1997), and contact with cholesterol rich liposomes was able to activate S. flexneri type III secretion (van der Goot et al., 2004). Therefore, Congo Red and cholesterol were tested for their ability to induce secretion of effector proteins also in Legionella. SidC has been shown to be translocated during infection (Luo and Isberg, 2004; Weber et al., 2006) and was used to determine whether secretion of effector proteins into the culture supernatant occurs under the experimental conditions chosen. TCA precipitated supernatant (20% TCA, over night) from 20 ml untreated or Congo Red (20 μM) or cholesterol (50 μM) supplemented cultures and bacterial cells (JR32 and IcmT) were examined by Western blot. SidC was strongly expressed intracellularly in all cultures but could not be detected in the untreated or induced supernatants, indicating that secretion does not take place during growth in liquid medium and neither Congo Red nor cholesterol could induce secretion into the supernatant (data not shown). IcmG was weakly expressed in the bacterial pellets and was not detected in the supernatants, indicating that the low intracellular IcmG concentration is not due to release into the supernatant.

4.3.2 IcmG is not translocated during infection

Acting as a SNARE protein probably requires translocation of IcmG during infection into the host cytosol and insertion of the protein into the membrane of the Legionella containing vacuole. Thus, the SNARE domain facing the cytosol can interact with SNARE proteins of other vesicles to direct vesicle fusion. Immunofluorescence microscopy was used to localize IcmG during infection and to find out whether it is translocated to the LCV membrane. Since SidC has been shown to localize to the LCV membrane (Weber et al., 2006), it was used as positive control for vesicular staining. M45-tagged icmG and sidC were overexpressed, D. discoideum amoebae were infected with early stationary growth phase bacteria, and proteins were localized one hour post infection by immunofluorescence. Bacterial protein expression levels were assessed by Western blot (Figure 3).
Figure 3: Expression of M45-tagged proteins for immunofluorescence analysis. Whole cell cultures used for infection were adjusted to an identical OD_600 and analysed by anti-M45 Western blot to control for similar protein expression.

The left pictures in Figure 4 show localization of M45-SidC in amoebae infected with wild type bacteria. During the staining procedure, most bacteria get permeabilized, and intrabacterial M45-SidC gets stained. Comparable intrabacterial staining is also visible in bacteria that are not located inside of or attached to host cells and thus did not induce translocation. However, a large part of intracellular wild type bacteria show M45-SidC signals that localize clearly outside of the bacteria and often colocalize with calnexin-GFP, a marker for the LCV membrane. In contrast, M45-IcmG never co-localized with calnexin (Figure 4, right images). In these preliminary experiments, we could not discriminate whether IcmG solely localized inside the bacteria, or whether a portion of the protein was also secreted to the bacterial surface. Similar results were obtained for infections with ΔicmG expressing M45-IcmG (data not shown). Intrabacterial concentrations of M45-IcmG in the different strains were similar or even higher than those of M45-SidC (Figure 3), and M45-IcmG and M45-SidC signals inside of lysed bacteria had a comparable intensity indicating that staining intensities for both proteins are comparable. A possible interference of the M45-tag with translocation of IcmG was not analysed in detail and can not be excluded. However, since M45-tagged SidC was translocated efficiently, it is likely that the tag does not inhibit translocation of IcmG either. Therefore, translocation of IcmG during infection in amounts comparable to other effector proteins like SidC is unlikely to occur.
Figure 4: Immunofluorescence analysis of translocation of M45-tagged proteins during infection with *L. pneumophila* JR32/pMMB207C-M45-sidC (left pictures) and JR32/pMMB207C-M45-icmG (right pictures). *D. discoideum* Ax3 were infected with an m.o.i. of 100 for one hour and stained using an anti-M45 antibody (blue). *L. pneumophila* were stained with an anti-*L. pneumophila* antibody (red). The amoebae express a calnexin-GFP fusion protein which localizes to the ER. GFP signals are shown in green. Arrows indicate bacteria in which translocation of M45-SidC can easily be seen (left pictures). Scale bars denote 5 μm.
4.3.3 \textit{L. pneumophila} \textit{ΔicmG} is able to translocate SidC but is impaired in recruiting ER-derived vesicles to the LCV

The Icm/Dot transporter translocates effector proteins during infection of a host cell. In order to get a better understanding of the role of IcmG, its requirement for effector protein translocation was examined. The Icm/Dot-secreted protein SidC was used as a model effector protein and was expressed in \textit{L. pneumophila} wild type JR32, the \textit{icmG} mutant and the Icm/Dot transport deficient \textit{icmT} mutant. \textit{D. discoideum} was infected, and localization of proteins was determined one hour post infection by immunofluorescence using an affinity purified polyclonal anti SidC antibody.

Figure 5 shows that SidC localizes to the LCV membrane in amoebae infected with wild type bacteria and is visible only intracellularly in lysed bacteria in amoebae infected with the \textit{icmT} mutant. Translocation of SidC was also detected in infections with the \textit{icmG} mutant, and the signals localized to the membrane of the LCV in the same pattern as seen in infections with wild type bacteria, thus proving that translocation of at least the effector protein SidC is still possible in an \textit{icmG} mutant. However, a large difference was observed between wild type and \textit{ΔicmG} infections in the ratio of LCVs stained with SidC and calnexin-GFP. Calnexin is an ER marker and indicates the recruitment of ER-derived vesicles to the LCV membrane (Derre and Isberg, 2004). Whereas about two-thirds of the LCV membranes in an infection with wild type bacteria co-localized with calnexin-GFP, less than ten percent of the LCVs containing the \textit{icmG} mutant showed calnexin-GFP signals. Only calnexin-GFP positive vacuoles were further examined for the presence of SidC in their membranes. 75% of the wild type containing vesicles (27 out of 36) but only 39% of the \textit{ΔicmG} containing vesicles (7 out of 18) stained also positive for SidC. No calnexin-GFP positive LCVs and no SidC translocation were found in infections with the \textit{icmT} mutant.

This data show that the \textit{icmG} mutant is impaired for the recruitment of ER derived vesicles and the translocation of effector proteins. However, IcmG is not absolutely required for translocation of at least the effector protein SidC, as SidC is still translocated to some LCV membranes.
Figure 5: Immunofluorescence analysis of translocation of SidC during infection with *L. pneumophila* strains. *D. discoideum* Ax3 were infected with an m.o.i. of 100 for one hour and stained using an anti-SidC antibody. SidC signals are shown in the left picture and localize to the membrane of the LCV with exception of ΔicmT. Bacteria are visualized by fluorescence of DsRedExpress and are shown in red. The amoebae express a calnexin-GFP fusion protein which localizes to the ER. GFP signals are shown in green. Scale bars denote 5 μm.
4.3.4 Identification of IcmG binding proteins using a pull down assay with GST fusion proteins

The C-terminal part of IcmG contains a coiled-coil domain. These domains are often involved in protein-protein interactions, such as assembly of subunits (Delahay and Frankel, 2002). A SNARE domain was predicted in IcmG at amino acids 140 to 210 (Morozova et al., 2004). SNARE domains promote vesicle fusion by binding SNAREs on target vesicle membranes. Furthermore, a bacterial two hybrid screen revealed that a number of effector proteins interact with an IcmG fragment spanning amino acids 28-123 (Luo and Isberg, 2004). The identification of proteins interacting with these or other domains of IcmG could give clues about its function. Thus, we examined protein binding to IcmG using pull down assays.

GST was fused to full length IcmG and the N- and C-terminal part of IcmG, respectively. The fusion proteins were loaded onto glutathione beads and used as baits to bind proteins either from *L. pneumophila* JR32 lysate to identify bacterial binding partners, or to bind proteins from host cell lysate to identify host cell binding partners.

Figure 6 shows a pull down assay designed to identify bacterial binding partners. Solubility of the three GST-IcmG fusion proteins was poor, and despite of a sixfold increased culture volume, more GST (26kDa) bound to the glutathione beads than GST-IcmG (56kDa), GST-IcmG-N123 (apparent MW 45 kDa, calculated MW 40kDa) and GST-IcmG-C146 (apparent MW 38 kDa, calculated MW 42 kDa). Also, much less unspecific binding was observed for GST-loaded beads, compared to beads loaded with GST-IcmG fusion proteins (Figure 7 and Figure 8, see lanes in which beads were incubated with buffer only).

Several proteins were identified that bound specifically to the GST-IcmG fusion proteins. The numbered bands were analyzed by Edman sequencing. However, only band 5 gave a reliable sequence. BLAST search against the sequenced *L. pneumophila* genomes (strains Philadelphia, Lens and Paris) did not yield a protein sequence fully matching the Edman result. The best hit was 75% identical (6 out of 8 amino acids) with the N-terminus of *L. pneumophila* proteins annotated as Hsp60 60 kDa chaperonin HtpB (strain Philadelphia) or as 60 kDa chaperonin Cpn60 (GroEL) (strains Lens and Paris). To examine whether this protein might originate from *E. coli* BL21, a BLAST search against *E. coli* proteins was done. Again, no fully matching protein could be identified and the best hit yielded 77% (7 out of 9 amino acids) identity to *E. coli* hsp60 (GroEL).
In another pull down experiment (Figure 7), band 3 was clearly identified as the 40 kDa heat shock protein DnaJ from *L. pneumophila* with 10 out of 10 amino acids matching. The remaining numbered bands shown in Figure 6 and Figure 7 were also analyzed by Edman sequencing, but no reliable results could be obtained. Some bands gave no signals in the amino acid detection, and in one case a band contained two proteins in similar amounts, thus eliminating the possibility to assemble sequences.

Figure 6: Purification of IcmG binding proteins from *L. pneumophila* wild type lysate using a pull down assay. GST-fusion proteins of IcmG or the N- and C-terminal part of IcmG, respectively, were immobilized on glutathione sepharose beads and incubated with lysate from JR32. Unbound proteins were washed off, bound proteins were released by boiling the beads in Laemmli buffer, separated on an SDS gel, transferred onto PVDF membrane and visualized by staining with Coomassie brilliant blue. The marked bands were analyzed by Edman sequencing. Abbreviations: Ly: lysate from JR32. B: lysis buffer, GST-IcmG: GST fused to full length IcmG, GST-G-N123: GST fused to the N-terminal 123 amino acids of IcmG, GST-G-C146: GST fused to the 146 C-terminal amino acids of IcmG.
Characterization of IcmG

Figure 7: Purification of IcmG binding proteins from *L. pneumophila* wild type JR32 and from murine RAW264.7 macrophage lysate using a pull down assay. GST and a GST-IcmG fusion protein were immobilized on glutathione sepharose beads and incubated with bacterial or eukaryotic lysate. Unbound proteins were washed off, bound proteins were released by boiling the beads in Laemmli buffer, separated on an SDS gel and visualized by staining with Coomassie brilliant blue. The marked bands were cut out and analyzed by MALDI-MS. Abbreviations: GST-G: GST-IcmG fusion, MW: molecular weight marker.

Bands obtained from pull down assays using lysate from RAW264.7 macrophages as prey were identified using MALDI-MS. The protein fragments from analyzed bands matched with a RIO2 kinase like protein (UniProt identifier Q8BH74), a nuclear export factor CRM1 (Q80U96) and a protein similar to GCN1, a transcriptional regulator involved in amino acid synthesis in yeast (Q8CHH7).
4.3.5 Copurification of IcmG binding proteins using His$_6$-tagged IcmG

Pull down of IcmG binding proteins using GST fusion proteins requires reconstitution of protein complexes \textit{in vitro}. An alternative approach to identify IcmG binding proteins that avoids reconstitution in artificial buffer is copurification of IcmG together with its binding partners. His$_6$-tagged IcmG was overexpressed in \textit{ΔicmG}, the lysate was incubated with Ni-NTA beads, unbound proteins were washed off, and His-IcmG and binding proteins were eluted and analyzed. Since initial experiments yielded a high number of unspecifically bound proteins, the effect of the major parameters imidazol concentration and ion strength on the enrichment of His-IcmG was examined. Ni-NTA beads were loaded with bacterial lysate, washed with lysis buffer H1 (10mM imidazol, 150 mM NaCl) and treated either with increasing imidazol or NaCl concentration to determine the highest concentrations which could be used to wash off proteins binding unspecifically without releasing His-IcmG. Figure 8 shows that after 5 washes with 5 ml lysis buffer H1 large amounts of many different proteins remain bound to Ni-NTA and are released by an increase in imidazol concentration (left image). Only a small part of these proteins can be detached by increasing ion strength (right image). Bound His-IcmG appears to be firmly attached to the beads, since it can not be eluted with 500 mM imidazol but only by boiling with EDTA (see anti-His Western blot, left picture). Unfortunately, the portion of His-IcmG binding to the beads seems to be marginal. 1.25 µl lysate (100 µg protein) were applied directly to the first two lanes in the left gel and yielded strong bands in the Western blot. His-IcmG from 4500 µl lysate was enriched on the Ni-NTA beads but yielded only a signal not stronger than that from 1.25 µl lysate after releasing bound His-IcmG by boiling the beads in 100 µl 500 mM EDTA in water. The presence of His-IcmG in the lysate after incubation with Ni-NTA beads shows that the protein was available in the lysate in excess and therefore, binding of His-IcmG to Ni-NTA was poor.
Characterization of IcmG

Figure 8: Binding and release of His₆-tagged IcmG and other proteins bound to Ni-NTA beads. Proteins from 200 ml culture from ΔicmG/pMMB207C-IcmG-HisN were extracted using lysis buffer H1, applied to 200 μl Ni-NTA beads, and unbound proteins were washed off with lysis buffer H1 as described in Material and Methods. Proteins were separated on SDS gels and stained with Coomassie brilliant blue (top panel) or blotted onto nitrocellulose membrane and analyzed by an anti-His Western blot (bottom panel). The left gel shows the protein pattern of the lysates and the proteins eluted with 200 μl lysis buffer containing increasing concentrations of imidazol. The right gel shows the proteins washed off after loading the beads (wash 1 to wash 5) and the proteins subsequently eluted by addition of 200 μl lysis buffer containing 10 mM imidazol and increasing NaCl concentrations. In both cases, the beads were boiled in Laemmli buffer containing 500 mM EDTA after the last elution or wash step to quantitatively release all bound proteins. Abbreviations: Lys+ before b.: lysate from ΔicmG/pMMB207C-icmG-HisN induced with IPTG before incubation with Ni-NTA beads, Lys+ after beads: the same lysate after incubation with Ni-NTA beads, Lys-IPTG: lysate from ΔicmG/pMMB207C-icmG-HisN without induction.

Possible reasons for poor binding of His-IcmG could be a tertiary structure which embeds the His tag inside the protein, or aggregation of His-IcmG with itself or other proteins via the hydrophobic transmembrane or the coiled-coil domain. This could render the His tag inaccessible and prevent binding of the tag to the nickel ions.

To discriminate whether biochemical properties of IcmG or experimental conditions led to poor binding of His-IcmG, the purification was repeated including His₆-tagged SidC as a control protein. One purification was performed using denaturing conditions (8 M urea) during lysis, binding and washing to disrupt tertiary structures. Figure 9 shows a strong enrichment of His-SidC under native conditions, whereas not enough His-IcmG has bound to be visualized by Coomassie staining. An anti-His Western blot of the lysate proteins confirmed that His-SidC and His-IcmG were present at similar concentrations (data not shown). Western blot of the released proteins (right image) also showed that under denaturing conditions binding of His-IcmG was increased.
Figure 9: Enrichment of His6-tagged IcmG and SidC by binding to Ni-NTA beads. Proteins from 200 ml culture were extracted using lysis buffer H2 and were applied to 200 µl Ni-NTA beads as described in Material and Methods. For G/pG denat, both lysis and wash buffer were supplemented with 8 M urea to examine binding under denaturing conditions. The beads were washed with lysis buffer H2 adjusted to 50 mM imidazol and boiled in Laemmli buffer supplemented with 500 mM EDTA to release all bound proteins. Proteins were separated on an SDS gel and stained with Coomassie brilliant blue (left picture) or analyzed by Western blot with an anti-His antibody (right picture). The position of His-tagged IcmG and His-tagged SidC is indicated with black triangles. Abbreviations: G/pG: \Delta icmG/pMMB207C-icmG-HisN, T/Sid: \Delta icmTlpMMB207C-sidC-HisN, Coli/pG: E. coli TOP10/pMMB207C-icmG-HisN, nat: lysis buffer H2 without 8 M urea, denat: lysis buffer H2 with 8 M urea.

The successful enrichment of His-SidC shows that not the experimental conditions but the biochemical properties of His-icmG cause poor binding to the beads. Denaturing conditions increase binding of His-IcmG but disrupt protein bindings and can therefore not be used in a copurification assay. Thus, a successful copurification of IcmG binding proteins using His-tagged IcmG is unlikely, and this approach was not further pursued.

4.4 Discussion

The presence of a SNARE domain in IcmG was predicted by in silico analysis, and acting as a SNARE protein would render IcmG an important player in the modification of vesicle trafficking during infection. Furthermore, the cytotoxic effect of the lpxB paralogue lcsC was only observed when lcsC was expressed in the icmG mutant, but not in other mutants with partial virulence phenotypes. This prompted us to characterize IcmG in more detail to get a better understanding of its role in infection and in the mechanism of lcsC-mediated cytotoxicity.
4.4.1 Expression level and localization of IcmG

The intracellular concentration of IcmG was low in all growth phases examined and also during intracellular growth in *A. castellanii*. Furthermore, secretion of IcmG could not be detected, suggesting that the *icmG* expression is constantly kept low. Secretion and SNARE function would probably require expression of larger amounts of protein to ensure that sufficient protein is available to be inserted in all parts of the LCV membrane and to come in contact with host SNARE proteins. Neither secretion nor translocation could be detected by Western blot of TCA-precipitated supernatants (see 4.3.1) and by immunofluorescence localization of N-terminally M45-tagged IcmG in infected *D. discoideum* (see 4.3.2). If IcmG would act as a SNARE, it is expected to be translocated and its transmembrane domain to be inserted into the LCV membrane with the C-terminal SNARE domain facing the cytosol. This localization would result in a staining pattern similar to SidC, which localizes on the LCV membrane. Using the relatively insensitive immunofluorescence microscopy as detection method, it can not be excluded that small amounts of IcmG are translocated. Both the low expression level and the fact that translocation does not occur or only at levels below the detection limit argue against a SNARE function.

The prediction of Morozova and coworkers is based upon PFAM domain searches and an alignment of IcmG and three bacterial protein domains showing similarities to SNARE domains from eukaryotic proteins. Our own domain search using Pfam 15.0 (pfam.wustl.edu) yielded a SNARE domain with an expectation value of 0.36. E-values above 0.05 should not be considered as significant. Together with the data presented here, it is likely that the predicted SNARE domain does not function as such.

4.4.2 Phenotypes of the *icmG* mutant

We examined the effect of loss of IcmG on the ability to translocate effector proteins during infection. By immunofluorescence, it could be shown that ΔicmG is able to translocate SidC to the LCV membrane and, therefore, the Icm/Dot secretion system is at least partially functional. However, recruitment of ER derived vesicles and the portion of SidC-positive LCVs were clearly reduced in ΔicmG, indicating that IcmG is required for optimal function of the transporter. Thus, IcmG could be part of the transporter complex rather than functioning as a SNARE protein. If IcmG is a part of the transporter complex, one would
expect that it binds to other Icm/Dot proteins, and it remains unclear why none of these were identified in the pull down assays. IcmG was poorly soluble even when fused to GST and dissolved in a detergent-containing lysis buffer. 15 out of 21 Icm/Dot proteins are predicted to localize in the inner membrane due to the presence of one or more transmembrane domains. It might be that some of these Icm/Dot proteins do form a complex together with IcmG in vivo, but they suffered from poor solubility and were not available in sufficient amounts in the pull down assays to give a clear band in the Coomassie staining or a clear sequence in Edman analysis.

4.4.3 Identification of IcmG binding proteins in pull down assays

Pull down assays with GST fusion proteins were performed to identify IcmG binding proteins from L. pneumophila wild type lysate or from murine RAW264.7 macrophages. The pull down assays with JR32 lysate clearly identified DnaJ and, less confidently, a Hsp60 like protein. DnaJ bound to full length IcmG and Hsp60 to amino acids 124-269 of IcmG. Both DnaJ and Hsp60 are chaperons involved in folding of a large portion of bacterial proteins after their synthesis. Since binding to many different proteins is part of their chaperone function, copurification with IcmG or binding to the immobilized GST-fusion proteins does not necessarily mean that binding of these chaperones to IcmG is functionally relevant. This is supported by the observation that DnaJ is frequently copurified with overexpressed proteins. It might be that IcmG altered its structure during bacterial lysis or pull down, was recognised as misfolded and bound by the chaperones, thus leading to purification of chaperones.

However, Hsp60 function is not restricted to folding proteins intracellularly and it may bind IcmG specifically. Hsp60 has been described to localize on the bacterial surface and to mediate invasion of L. pneumophila in HeLa cells (Garduno et al., 1998). Hsp60 also interferes with signalling cascades, actin modification and vesicle trafficking (Garduno, 2005). Secretion of Hsp60 was dependent on an intact Icm/Dot secretion apparatus, since several icm/dot mutants were defective in localizing Hsp60 onto their surface and accumulated Hsp60 intracellularly (Hoffman and Garduno, 1999). This Icm/Dot dependency shows a connection between Hsp60 and Icm/Dot proteins, and it might be that IcmG is involved in Hsp60 secretion. However, the sequence obtained by Edman analysis of the isolated protein matches only 75% with L. pneumophila Hsp60. It will be necessary to
confirm that it actually is Hsp60 that binds IcmG before further experiments regarding the relation between the two proteins are done.

Luo and Isberg described binding of the RalF and the Sid effector proteins to IcmG (Luo and Isberg, 2004). In this work, no IcmG-binding effector proteins were identified. Reason for this might be the different approaches used for identification of binding partners. Luo and Isberg used a bacterial two hybrid assay, which identifies also temporally or weakly binding proteins. In a pull down assay, as used in this work, the binding needs to be established \textit{in vitro} and maintained during several wash steps, and thus probably only strongly binding proteins can be identified. Both RalF and SidC are secreted effector proteins that, since IcmG itself is not secreted, interact only temporarily with IcmG. This is a possible reason why these interactions were not detected in the pull down assays.

Pull down of proteins from RAW264.7 macrophages with GST fusion proteins identified a kinase-like protein, a nuclear export factor and a putative regulatory protein similar to GCN1. Specific binding of these proteins to IcmG \textit{in vivo} requires secretion of IcmG into the host cell. Since neither secretion nor translocation of IcmG during infection could be shown (see 4.3.1 and 4.3.2) is it likely that the binding observed here is an artefact and does not have a biological meaning.

\textbf{4.4.4 Hypotheses on the function of IcmG}

The data presented here argue against the hypothesis that IcmG acts as a SNARE and suggest that IcmG is part of the Icm/Dot transporter. The weak expression level of \textit{icmG} argues for an involvement in the transporter apparatus rather than for a SNARE function. Presumed that every transporter complex within a bacterium contains only one or a few copies of IcmG, only a low number of molecules would be necessary within a cell.

The binding to the translocated effectors RalF and at least eight Sid proteins shows that IcmG interacts with a number of different effectors. It might act as a coupling protein directing different effectors towards the translocation channel and assist in their secretion, or it could be involved in regulation of effector translocation. Loss of a coupling protein or misregulation of translocation in \textit{ΔicmG} would either reduce the translocation efficiency of all or a subset of proteins, or it could lead to an uncontrolled release of effectors before or during infection, hence causing the impaired recruitment of ER derived vesicles. However, SidC was not detected in TCA precipitated supernatants of a \textit{ΔicmG} culture (see 4.3.1),
indicating that uncontrolled release of effectors does at least not happen during growth in liquid medium.

It might be possible that IcmG is required for translocation only of a subset of effector proteins. A lack of translocation of these effectors or a reduced amount of translocated proteins would then cause the partial phenotype of the \textit{icmG} mutant. The \textit{Yersinia} protein TyeA is an example for a translocation control protein required for translocation of only a subset of Yop effector proteins (Iriarte \textit{et al.}, 1998).

To learn more about the function of IcmG, it would be interesting to compare the ability of \textit{ΔicmG} to secrete not only SidC, but also other known effector proteins. This could reveal whether IcmG acts as coupling protein for all or only for a subset of effectors. A quantitative time course of effector translocation would give information whether translocation in \textit{ΔicmG} is delayed or the amount of translocated proteins is reduced.

\textit{lcsC} encodes a lipid A disaccharide synthase (see Chapter 3) and is toxic only when overexpressed in \textit{ΔicmG}, but not in other mutants with partial phenotypes like \textit{ΔicmS}, \textit{ΔicmF} and \textit{ΔrpoS}. The proposed functions of IcmG need to be in accordance with these data and, ideally, can give an explanation for the restriction of cytotoxicity to \textit{ΔicmG}. Lipid A disaccharide synthases are cytosolic enzymes that form lipid A, the active moiety of endotoxin. Overexpression of \textit{lcsC} might lead to accumulation of lipid A or other LPS precursors. If \textit{ΔicmG} is impaired in regulation of translocation, it might be that toxic products of \textit{lcsC} overexpression are translocated through the Icm/Dot apparatus in \textit{ΔicmG}, but not in other mutants with partial phenotypes. These molecules would be released into the host cell and could interfere with host cell mechanisms leading to increased cell death. Type IV dependent secretion of molecules other than proteins has been described for protein bound DNA in \textit{Legionella} (Segal and Shuman, 1998) and peptidoglycan in \textit{Helicobacter} (Viala \textit{et al.}, 2004).

Another hypothesis not presuming Icm/Dot-dependent secretion of an LcsC product is that a toxic compound can exert its cytotoxic effect only in certain intracellular compartments. It might be that trafficking of the \textit{icmG} mutant differs compared to other mutants and only \textit{ΔicmG} enters a compartment enabling the bacteria to become cytotoxic.

However, explanations for the mechanism of LcsC-mediated cytotoxicity will be speculative unless more information are available about the translocation capabilities and the intracellular behavior of the different mutants. It will also be necessary to more closely examine whether
products from the LPS biosynthesis pathway accumulate during lcsC overexpression, whether these products are secreted by the Icm/Dot secretion system and how and where they interact with host cell pathways to cause cell death.

4.5 References


Characterization of IcmG


5 General discussion

5.1 LcsC shows lipid A disaccharide synthase activity

In the amoebae plate test, an *L. pneumophila* genomic library was screened for genes increasing the survival of *L. pneumophila* on plates in presence of *A. castellanii*. The screen identified *lcsC*, a gene conferring cytotoxicity to the bacteria (see Chapter 2, Albers et al., 2005). Search for homologues revealed that *LcsC* (LpxB1) shows 34% identity on protein level to the *E. coli* lipid A disaccharide synthase LpxB. Interestingly, the *L. pneumophila* genome contains a second LpxB homologue (LpxB2) with 43% identity to the *E. coli* enzyme. In almost all cases, the genes necessary for lipid A biosynthesis are present in only one copy (Raetz and Whitfield, 2002). Therefore, we examined the genomic vicinity of both *lpxB* paralogues to assess whether one of the genes might have another function than a lipid A disaccharide synthase. *LpxB2* is located within a cluster of other genes involved in lipid A biosynthesis. In contrast, *lpxB1/lcsC* is not located in the vicinity of genes belonging to the classical lipid A biosynthesis pathway. Instead, genes involved in peptidoglycan metabolism were found, and a domain in LcsC is distantly related to MurG, a glycosyl transferase involved in peptidoglycan biosynthesis. Therefore, we hypothesized that LcsC might be involved in peptidoglycan metabolism and cytotoxicity might be caused by the release of toxic peptidoglycan fragments, as described for a number of other bacteria (Burroughs et al., 1993; Cloud and Dillard, 2002; Luker et al., 1993).

However, a closer inspection of lipid A biosynthesis and the genes involved revealed that the gene immediately upstream of *lcsC* encodes a homologue of the *Acidithiobacillus ferrooxidans* oxidase GnnA. The first step of lipid A biosynthesis in *A. ferrooxidans* and most likely also in *L. pneumophila* is the conversion of UDP-GlcNAc to UDP-GlcNAc3N, which is catalyzed by GnnA and GnnB (Sweet et al., 2004). Reverse transcription and PCR proved that *gmnA* and *lcsC* are cotranscribed, suggesting that the corresponding two enzymes are employed in the same biosynthetic pathway. Furthermore, both *lpxB1/lcsC* and *lpxB2* complemented an *E. coli lpxB* mutant, demonstrating their activity as lipid A disaccharide synthases. Therefore, it can be concluded that *lcsC* encodes a lipid A disaccharide synthase rather than an enzyme involved in peptidoglycan synthesis.
5.2 L. pneumophila: life in different niches and adaptation of lipid A structure

*L. pneumophila* is able to survive under highly diverse conditions. In the environment, the bacterium colonizes and persists in biofilms and multiplies within various protozoa (Fields, 1996). After lysing a spent host, the bacteria have to survive in the aquatic environment or in soils, until they are phagocytosed by another host cell. In macrophages, *L. pneumophila* resides in a vacuole that finally fuses with lysosomes and matures into an acidic compartment (Sturgill-Koszycki and Swanson, 2000). The drastic differences in the conditions within these different niches require adaptation of the bacterium. To adjust their physiology, the *L. pneumophila* life cycle comprises of bacterial differentiation into a replicative state, designated to multiply within a host cell, and in a transmissive state, in which the bacteria adjust their physiology to survive in the environment and to promote infection of a new host cell (Molofsky and Swanson, 2004). Differentiation is directed by global regulatory circuits, and it is tempting to speculate that also the outer membrane, the outermost protective barrier, undergoes changes to adapt to the highly diverse environments. Lipid A is a major part of the outer membrane. All genes in *L. pneumophila* encoding the lipid A acyl transferases and the lipid A disaccharide synthase are present in more than one copy, and at least the *lpxA* and the *lpxB* paralogues are differentially expressed (see Chapter 3). Examination of a phase variant strain showed that *L. pneumophila* is able to alter the composition of the primary fatty acids of lipid A. It is therefore conceivable, that the lipid A biosynthesis paralogues are used to adjust the lipid A structure, most probably the fatty acid composition, by differential expression of paralogous genes with the purpose to adapt the properties of the outer membrane to the diverse environmental conditions.

The amoeba plate test was applied to identify genes increasing the survival of the partially growth defective *icmG* mutant on agar plates in presence of amoeba (see Chapter 2, Albers et al., 2005). The identification of the lipid A disaccharide synthase *lpxB1/lcsC* in this screen is an evidence for the importance of lipid A structure for the interaction of *L. pneumophila* with *A. castellanii*, and it is likely that a lipid A structure synthesized by LcsC is required for a successful infection of amoebae.

The impact of lipid A structure, particularly the fatty acid composition, on virulence is further supported by literature describing the effect of mutations of genes involved in lipid A acylation, e.g., *pagP, lpxP* or *lpxM* in different pathogenic bacteria (Guo et al., 1998; Raetz and Whitfield, 2002; see also Chapter 1). Deletion of these genes alters the fatty acid
composition of lipid A, and thus influences sensitivity to antimicrobial peptides or antibiotics or alters TLR4 dependent activation of the immune response. Therefore, a well regulated adaptation of lipid A structure is expected to be a valuable tool for *L. pneumophila* to survive under diverse conditions and to multiply in a variety of host cells. Differential expression of paralogous enzymes is an adequate means to perform such structural modifications.

### 5.3 Intracellular LPS influences host cell functions

Interference of *Legionella* LPS and material derived from the outer membrane with host cell functions has been described in the literature, and it is conceivable that modifying the structure of the membrane anchor lipid A will have an influence on the effect of LPS on host cell functions.

For LPS from *Legionella jeonii* (Park et al., 2004), the ability to alter vesicular trafficking in *Acanthamoeba proteus* has been suggested. Microinjection of an anti-LPS antibody into the host cell increased the number of phagosomes that fused with lysosomes, indicating that LPS assists in the prevention of phagosome-lysosome fusion (Kim et al., 1994).

Membrane-derived vesicles from *L. pneumophila* have recently been shown to inhibit phagosome-lysosome fusion in an Icm/Dot-independent manner. The ability of these outer membrane-derived vesicles to interfere with host vesicle fusion correlated with developmentally regulated modifications of the pathogen’s surface. Bacterial surface properties were assayed by LPS profile and binding to a lectin, suggesting that the sugar structure of LPS is altered during the switch from replicative to transmissive phase (Fernandez-Moreira et al., 2006). The correlation of the ability to inhibit vesicle fusion and LPS properties and the influence of an anti-LPS antibody on vesicle fusion in amoebae infected with *L. jeonii* render LPS a candidate molecule for interference with host vesicle trafficking.

Intracellular LPS distribution and interference with host cell mechanisms was examined for *Brucella*, a persistent intracellular pathogen leading to chronic diseases in a wide variety of mammals (Lapaque et al., 2005). Similarly to *L. pneumophila* LPS, *Brucella* LPS has a GlucN3N-based disaccharide backbone with unusually long acyl chains and a low endotoxic activity. *Brucella* LPS impairs phagosome-lysosome fusion during the first hours of infection (comparable to the effect of *L. pneumophila* membrane vesicles, Fernandez-Moreira et al., 2006), travels through the endocytic route and can later be found on the cell surface in
detergent-resistant membrane regions called macrodomains. These domains have a reduced membrane fluidity and remain stable in macrophages for several months (Lapaque et al., 2006). There, LPS interferes with MHC-II presentation to specific CD4\(^+\) T cells, probably to control host immune response by prevention of Brucella-specific T cell activation.

Release and delivery of LPS to target compartments of the cell is a prerequisite for acting as a virulence factor. LPS release and distribution within numerous host cell vesicles was examined in detail for the infection of eukaryotic cell lines with S. Typhimurium and was found to occur exclusively when bacteria localize inside of a cell (Garcia-del Portillo et al., 1997). Salmonella LPS was detectable 30 minutes after infection in areas surrounding internalized bacteria and at 6 hours \(p.i.\) in numerous vesicles throughout the cytosol, including vesicles not containing intracellular bacteria. The occurrence of LPS depended on intracellular presence of bacteria, since addition of purified LPS to the medium did not produce LPS-positive vacuoles even after induction of ruffling and macropinocytosis. Also, uninfected cells neighbouring cells containing bacteria remained unstained. Interestingly, the ability to spread within the cell was determined by the structure of LPS, since neither \(E.\ coli\) LPS added simultaneously with infecting Salmonella nor LPS from Salmonella mutants with altered LPS structures were released to host vesicles as efficiently as wild type LPS.

Trafficking and localization of \(L.\ pneumophila\) LPS has not yet been examined throughout the whole course of infection. However, data from the literature suggest an involvement of intracellular LPS in virulence. It is not unlikely that the structure of the membrane anchor lipid A influences the intracellular trafficking of LPS to a large extent. Modification of lipid A could determine localization and interactions of LPS with intracellular targets and thus direct the effects of LPS on virulence.

### 5.4 Possible influence of lipid A on outer membrane vesicles

Membrane-derived vesicles from \(L.\ pneumophila\) inhibit phagosome-lysosome fusion, demonstrating their role in pathogenicity (Fernandez-Moreira et al., 2006). Outer membrane vesicles (OMV) are naturally produced during growth of most Gram-negative bacteria. \(E.\ coli\) packages 0.2-0.5% of outer membrane and periplasmic proteins in vesicles (Kuehn and Kesty, 2005). The OMVs are produced by outer membrane budding during growth and are composed of proteins and lipids of the outer membrane and periplasmic material, but not of inner membrane or cytoplasmic components. The vesicles are capable to fuse with other
bacterial and eukaryotic membranes and deliver their content, which can range from enzymes and toxins to antibiotic compounds and DNA, to the recipient. Vesicle surface molecules can mediate adhesion to host cells and subsequent internalization of vesicle material (Kuehn and Kesty, 2005).

Acylation of lipid A affects the physical properties of the outer membrane bilayer. It might be that changing lipid A fatty acid composition by differential use of distinct acyl transferase paralogues will have an impact on number, size or properties of released outer membrane vesicles. For example, altered membrane fluidity might change the frequency of membrane budding, the fusogenic properties of the vesicles, or affect the size of vesicles formed and thereby the amount of lipids and vesicle content transported. Therefore, modifying outer membrane properties by adjusting lipid A acylation might control the formation and release of OMVs and affect their role in virulence.

5.5 Cytotoxicity of the lpxB paralogues

Overexpression of lpxB1/lcsC increases the ability of L. pneumophila to kill amoebae. We were interested to know whether lpxB2 overexpression also shows an effect but could not find an increase in cytotoxicity. However, this result may be compromised by the toxic effect of lpxB2 overexpression on the bacteria. Overexpression of lpxB2 impairs growth of the icmG mutant on plates more strongly than overexpression of lcsC. Overexpression of lcsC on plates with 0.5 mM IPTG yields colonies with an only slightly reduced diameter, whereas induction of lpxB2 expression with the same concentration inhibits growth of ΔicmG. The cytotoxicity assay was performed with bacteria from plates. It might be that the ability of lpxB2-expressing bacteria to infect and kill amoebae was impaired due to the larger toxicity of lpxB2, and thus a possible cytotoxic effect was diminished. To exclude this possibility, it will be necessary to control the plating efficiency of the bacteria used for infection, and also to determine whether lpxB2-overexpressing bacteria are impaired in intracellular growth compared to lcsC/lpxB1-expressing strains.
5.6 **Putative mechanisms of LcsC mediated cytotoxicity**

The lipid A disaccharide synthase paralogue LcsC increases bacterial survival in presence of *A. castellanii* due to its cytotoxic effect on amoebae (see Chapter 2, Albers *et al.*, 2005). The mechanism of cytotoxicity remained unclear. Likely, overexpression of lcsC alters the composition of lipid IV<sub>A</sub> by favoring synthesis of an LcsC-derived molecule. This lipid IV<sub>A</sub> molecule might be used to synthesize the full LPS molecule, and then exert toxic effects during infection. However, it is not clear whether LPS with an LcsC-derived lipid A moiety is the cytotoxic agent, or whether overexpression of lcsC leads to accumulation of lipid IV<sub>A</sub> or other products of the LPS biosynthesis pathway which exert the cytotoxic effect. Furthermore, it can not be excluded that indirect effects of lcsC overexpression increase cytotoxicity. As a last speculative possibility, the enzyme itself could enter the eukaryotic host cell and disturb cellular saccharide or lipid metabolism.

LPS can be released and distributed within the cell by intracellular bacteria and interfere with host cell mechanisms, e.g., inhibit vesicle fusion. If *Legionella* LPS is distributed throughout many different host cell vesicle membranes, it might be that LPS or LPS derivatives released from intracellularly growing bacteria lead to a severe disturbance of host vesicle trafficking, which could finally cause cell death. The cytotoxicity assays with amoebae infected with lcsC-overexpressing strains were performed two days after infection, and it is reasonable to speculate that this period of time will be sufficient to kill an infected amoebae by interruption of vesicular trafficking.

The question remains why lcsC needs to be overexpressed to increase cell death. It might be that lcsC overexpression leads to an increase in lipid A or LPS release, damaging the host by elevated LPS concentrations in the membranes. Alternatively, lcsC overexpression could enlarge the proportion of LcsC/LpxB1-derived lipid A compared to LpxB2-derived lipid A. The putative differences in fatty acid composition of LcsC- or LpxB2-derived lipid A might lead to different interactions with the host, e.g., the ability of LPS to persist in specific membrane domains or to associate with host membrane factors is distinct. LcsC-derived lipid A molecules might therefore act at other interaction sites or have a greater stability, thus altering the place or duration of putative LPS interactions and damaging the host more severely. Differences in release or distinct interaction sites of LcsC- and LpxB2-derived molecules could also explain the difference in cytotoxicity between the lcsC/lpxB1 and lpxB2 overexpressing strains.
The cytotoxic effect of \( lcsC \) overexpression could also be connected to the formation of outer membrane vesicles. Release of LPS from intracellular bacteria could occur in form of OMVs. Fusion of OMVs with the membrane of the LCV could be a mechanism by which LPS is inserted into cellular membranes, from where it is distributed throughout the eukaryotic cell. Increasing OMV release could increase the amount of LPS (or other outer membrane factors) that is inserted into host cell membranes and is available to interfere with host cell functions. Since during fusion of an OMV with the LCV membrane the contents of the OMV are released into the host cell cytosol, an increase in OMV formation could also lead to a more massive disposal of putative toxic vesicle contents into the cytosol. Both increased LPS transfer into host membranes and increased release of toxic molecules from OMV content could cause the \( lcsC \)-mediated cytotoxicity observed.

### 5.7 Specificity of LcsC-mediated cytotoxicity for \( \Delta icmG \)

Overexpression of \( lcsC \) rendered the \( icmG \) mutant strain cytotoxic, but not other mutants with partial phenotypes. A reliable explanation why \( lcsC \) cytotoxicity is restricted to \( \Delta icmG \) remains difficult unless the toxic compound itself is identified and the function of IcmG is known in more detail. However, the data collected so far allow some speculations. If an \( icmG \) mutant releases only a subset or an insufficient amount of effector proteins, the trafficking of the \( icmG \) mutant might be different from that of other partially virulence-defective mutants. The \( icmG \) mutant-containing vacuole could interact with other vesicles, allowing putative toxic LcsC-derived molecules to travel from the LCV membrane to host organelles that are not affected in strains other than \( \Delta icmG \). There, they could interfere with host processes in a way detrimental for the cell. An alternative explanation presumes that IcmG regulates the selective transfer of molecules through the Icm/Dot secretion system. If IcmG acts as a regulatory protein, it might be that lack of the protein leads to leakiness and uncontrolled release of molecules. Thus, toxic LcsC products could enter the host cell via the type IV secretion system and kill the amoebae.
### 5.8 Conclusion

Using the amoebae plate test, we screened for genes involved in interactions of *L. pneumophila* with *A. castellanii*. Genes were identified whose overexpression lead to an increased killing of amoebae. Among the genes identified, *lesC* showed the strongest cytotoxic effect. It could be demonstrated that *lesC* encodes a lipid A disaccharide synthase. *LesC* and the acyl transferases necessary for lipid A biosynthesis are present in more than a single copy in the genome of *L. pneumophila*, and at least the *lpxA* and *lpXB* paralogues are differentially regulated. These findings suggest that *L. pneumophila* modifies its lipid A structure by use of distinct enzymes to adapt to different environmental conditions. Many examples in the literature show that the lipid A structure affects different aspects of bacterial survival and bacteria-host interactions in *L. pneumophila* and other pathogenic and nonpathogenic species.

Currently available data only allow speculations about the role and effects of lipid A modifications in *L. pneumophila*. Detailed analysis of lipid A biosynthesis gene regulation, biochemical functions of the enzymes, the structures of lipid A synthesized by different paralogues and the localization of lipid A and LPS during infection will be necessary to get a better understanding of the biological meaning and the effects of *L. pneumophila* lipid A variations.

### 5.9 Literature


Curriculum Vitae

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