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A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa

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Summary

The Dot/Icm type IV secretion system of *L. pneumophila* translocates numerous bacterial effectors into the host cell and is essential for bacterial proliferation within macrophages and protozoa. We have recently shown that *L. pneumophila* strain AA100/130b harbors 11 genes encoding eukaryotic-like ankyrin (Ank) proteins, a family of proteins involved in various essential eukaryotic cellular processes. In contrast to most Dot/Icm-exported substrates, which have little or no detectable role in intracellular proliferation, a mutation in *ankB* results in a severe growth defect in intracellular replication within human monocyte-derived macrophages (hMDMs), U937 macrophages, and *Acanthamoeba polyphaga*. Single cell analyses of co-infections of hMDMs have shown that the intracellular growth defect of the *ankB* mutant is totally rescued *in-cis* within communal phagosomes harboring the wild type strain. Interestingly, distinct from *dot/icm* structural mutants, the *ankB* mutant is also rescued *in-trans* within cells harboring the wild type strain in a different phagosome, indicating that AnkB is a transacting secreted effector. Using adenylate cyclase fusions to AnkB, we show that AnkB is translocated into the host cell via the Dot/Icm secretion system in an IcmSW-dependent manner, and that the last 3 C-terminal amino acid residues are essential for translocation. Distinct from the *dot/icm* structural mutants, the *ankB* mutant-containing phagosomes exclude late endosomal and lysosomal markers and their phagosomes are remodeled by the RER. We show that at the post exponential phase of growth, the LetA/S and PmrA/B two component systems confer a positive regulation on expression of the *ankB* gene, whereas RpoS, LetE, and RelA suppress its expression. Our data show that the eukaryotic-like AnkB protein is a Dot/Icm-exported effector that plays a major role in intracellular replication of *L. pneumophila* within macrophages and protozoa, and its expression is temporally controlled by regulators of the post-exponential phase of growth.

Keywords

Legionnaires' disease; regulation; infection; trafficking; dot/icm

Introduction

The Gram-negative bacterium, *Legionella pneumophila*, is ubiquitous in aquatic environments in both biofilm and planktonic forms, and is able to survive and replicate within protozoa (Abu Kwaik, 1998; Abu Kwaik *et al.*, 1998; Hilbi *et al.*, 2007; Molmeret *et*

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al., 2005). At least 13 species of amoebae and 2 species of ciliated protozoa support intracellular replication of *L. pneumophila* (Fields, 1996). Protozoa are the primary natural hosts of *L. pneumophila*, and this interaction is considered to be at the crux of pathogenesis and ecology of *L. pneumophila* (Barbaree et al., 1986; Molmeret et al., 2005; Rowbotham, 1980). When water aerosol containing *L. pneumophila* is inhaled or contaminated water is aspirated, *L. pneumophila* infect alveolar macrophages and epithelial cells, causing a severe atypical pneumonia designated Legionnaire's disease (Kaufmann et al., 1981; Winn, 1988). Once in the host cell, *L. pneumophila* evades the default endocytic pathway, and remodels its vacuole by the rough endoplasmic reticulum (RER) (Horwitz, 1983; Kagan and Roy, 2002). Evasion of the endocytic pathway is exhibited in the two evolutionarily distant hosts, protozoa and human cells, and is mediated by the Dot/Icm type IV secretion system (Segal and Shuman, 1999).

The Dot/Icm type IV secretion system is required for *L. pneumophila* to modulate various cellular processes and to maintain integrity of the *Legionella*-containing phagosome (LCP), and these modulations are believed to be mediated through numerous secreted effectors (Abu-Zant et al., 2007; Dorer et al., 2006; Marra et al., 1992; Molmeret et al., 2007; Vogel et al., 1998). A large number of Dot/Icm-exported substrates have been identified by a variety of techniques, but the role of most of these proteins in the intracellular infection is not yet known (Campodonico et al., 2005; Chien et al., 2004; Shohdy et al., 2005). Unlike mutations in the *dot/icm* genes encoding components of the structural secretion apparatus that result in a severe defect in intracellular growth, mutations in most of the characterized effectors have little or no detectable effect on intracellular growth (Campodonico et al., 2005; Chen et al., 2004; Luo and Isberg, 2004). A mutation in *sidJ*, which encodes a Dot/Icm-exported effector, results in only ~15 fold reduction in intracellular growth within macrophages (Liu and Luo, 2007). A mutant defective in the Dot/Icm substrate SdhA and its two paralogs is severely impaired in intracellular growth within macrophages, but the defect is modest within *Dictyostelium discoideum* (Laguna et al., 2006).

A biphasic developmental cycle associated with bacterial differentiation governs the life cycle of *L. pneumophila* (Faulkner and Garduno, 2002). Upon growth transition from the exponential (E) to the post-exponential (PE) phase, many virulence and transmission traits are regulated by regulatory cascades that include the stationary-phase sigma factor RpoS, the LetA/S Two Component System (TCS), the flagellar sigma factor FliA, and LetE (Bachman and Swanson, 2001; Bruggemann et al., 2006; Hales and Shuman, 1999; Hammer and Swanson, 1999; Hammer et al., 2002). The transmission phenotypes that are triggered upon growth transition at the PE phase include bacterial motility, evasion of the endocytic pathway, and intracellular growth (Hammer and Swanson, 1999).

The genome of *L. pneumophila* reflects its co-evolution with its eukaryotic hosts, as it codes for a high number of eukaryotic-like proteins, which are thought to interfere with host processes by mimicking eukaryotic protein functions (Cazalet et al., 2004; de Felipe et al., 2005; Steinert et al., 2007). Eleven genes encoding eukaryotic-like ankyrin repeats-containing proteins (Ank) are present in the clinical isolate of *L. pneumophila* strain AA100/130b (Habyarimana et al., 2008) and are common to the other clinical isolates Corby, Paris, Lens and Philadelphia-1 (Cazalet and Buchrieser, 2005; Cazalet et al., 2008; Glockner et al., 2007; Steinert et al., 2007). The *ank* genes have been suggested to be acquired through horizontal gene transfer (Cazalet et al., 2004; de Felipe et al., 2005; Glockner et al., 2007). No homologies are detected in genetic databases for any of the *L. pneumophila ank* genes (Cazalet and Buchrieser, 2005), indicating that they are novel. The ANK domain is the most common protein domain in the eukaryotic kingdom and is composed of a 33-residue motif (Amstutz et al., 2005; Howell et al., 2000; Kitazawa et al., 2005; Mosavi et al., 2002a; Mosavi et al., 2002b; Mosavi et al., 2004). The ANK domain

mediates protein-protein interaction involved in various eukaryotic cellular processes such as cell motility, cell signaling, cell cycle, transcriptional regulation, and oncogenesis (Amstutz *et al.*, 2005; Batchelor *et al.*, 1998; Michel *et al.*, 2001). *In silico* analysis of the ANK domain has revealed six non-conserved residues that determine the specific target protein with which an ankyrin protein interacts (Magliery and Regan, 2005). In addition to the absence of homologues to the Ank proteins of *L. pneumophila* (Habyarimana *et al.*, 2008), no homologies are detected in genetic databases for the six specificity-determining residues for any of the *L. pneumophila* ANK domains (Habyarimana *et al.*, 2008), suggesting that they may have unique targets in the host cell (Cazalet and Buchrieser, 2005). The ANK domains were first identified in the yeast cell cycle regulator, Swi6/Cdc10, and the *Drosophila* signaling protein, Notch (Howell *et al.*, 2000; Kitazawa *et al.*, 2005; Mosavi *et al.*, 2004), but have been recently found in various microbial pathogens. The AnkA protein of the intracellular bacterium *Anaplasma phagocytophilum* is thought to be translocated by the type IV secretion system into the host cell, where it interacts with the host cell protein, SHP-1 (Ijdo *et al.*, 2007), and alters gene transcription (Park *et al.*, 2004). Among human pathogens, *L. pneumophila* harbors the highest number of Ank proteins (Cazalet *et al.*, 2004; de Felipe *et al.*, 2005; Steinert *et al.*, 2007).

Ten of the 11 *ank* genes in strain AA100 have been recently characterized (Habyarimana *et al.*, 2008). Eight of the 10 Ank proteins are dispensable for intracellular proliferation, whereas the *ankH* and *ankJ* mutants of *L. pneumophila* exhibit a mild defect in robust intracellular proliferation in macrophages and protozoa (Habyarimana *et al.*, 2008). The role of the 11th *ank* gene (*ankB*) in the intracellular infection is not known. In this study we show that despite its severe intracellular growth defect within human macrophages and *A. polyphaga*, the *ankB* mutant evades the endocytic pathway and remodels its phagosome into an ER-derived vacuole, similar to the parental strain. The intracellular growth defect of the *ankB* mutant is rescued *in-cis* within communal phagosomes harboring the wild type (WT) strain, and also *in-trans* within cells harboring the wild type strain in a different phagosome, indicating that AnkB is a trans-acting effector. Using adenylate cyclase fusions to AnkB, we show that AnkB is translocated into the host cell via the Dot/Icm secretion system in an IcmSW-dependent manner, and that the last 3 C-terminal amino acid residues are essential for translocation. Similar to other Dot/Icm effectors, expression of AnkB is triggered at the PE phase of growth and many PE phase regulators play roles in temporal regulation of *ankB* expression.

Results

The role of *L. pneumophila* AnkB in the intracellular infection of protozoa

The *ankB* gene (*lpg2144*, *legAU13*, *lpl2072*, *lpp2082*, *lpc 1593*) is a monocistronic gene that encodes a 172 amino acid hydrophilic protein that harbors two ANK domains and one N-terminus F-box domain, which is involved in eukaryotic protein ubiquitination (Fig. 1). To characterize the role of AnkB in the intracellular infection, we generated an isogenic *ankB* mutant of *L. pneumophila* strain AA100 by allelic exchange. The growth rate of the *ankB* mutant during *in vitro* BYE broth culture was similar to the wild type strain, and no difference in the length of the lag phase was observed (data not shown). Next, we assessed the role of AnkB in intracellular replication within *A. polyphaga*. Compared to the wild type strain, the *ankB* mutant had a defect in intracellular growth within *A. polyphaga* with ~400-fold fewer CFUs recovered at 48h post-infection (Fig. 2A). The defect was fully complemented by the wild type gene (Fig. 2A). As expected, the *dotA* mutant control did not replicate.

The role of AnkB in the intracellular infection of human macrophages

We determined the role of *ankB* in intracellular proliferation in human monocyte derived macrophages (hMDMs) and the U937 macrophage cell line. As expected, the wild type strain AA100 grew normally in both cells, whereas the *dotA* mutant control did not grow. In both macrophage types, the *ankB* mutant showed a severe growth defect with ~2000-fold fewer CFUs recovered at 48h post infection, compared to the wild type strain (Fig. 3). There was no change in the CFUs of the *ankB* mutant at 72h from that at 48h, while the WT strain CFUs started to decline due to loss of viability of the released bacteria into the tissue culture medium after lysis of the host cell. Complementation of the *ankB* mutant with the respective wild type gene on a plasmid fully restored the wild type phenotype (Fig. 3). The minor differences in the growth kinetics of the *ankB* mutant within macrophages and amoeba suggest minor differences in the temporal requirement of AnkB by *L. pneumophila* to proliferate robustly within the two evolutionarily distant hosts.

The transposon insertion in the *ankB* mutant is located at 330 bp from the start of the 516 bp ORF. To exclude the possibility that the insertion in *ankB* results in expression of a truncated AnkB protein with potential deleterious effects on the host cell, we utilized two approaches. First, we complemented both the wild type strain AA100 and the *ankB* mutant with an *ankB* gene that had a deletion of the last 3 codons (*ankB*₁₋₁₆₉). This truncated AnkB was expressed similarly to the full length AnkB (see below) but did not affect the growth of the wild type strain and did not complement the growth defect of the *ankB* mutant in either *A. polyphaga* or U937 cells (Fig. 2B and Fig. 3C). Second, RealTime qRT-PCR using primers to amplify the first 300bp of *ankB* upstream of the kan insertion showed no detectable mRNA in the *ankB* insertion mutant compared to the wild type strain at both the E and PE phases (data not shown). Therefore, the defect of the *ankB* insertion mutant in intracellular proliferation is due to the lack of expression of AnkB and is fully complemented by the wild type *ankB*.

To determine whether the *ankB* mutant growth defect was due to a defect in a subset of the bacterial population or to a balanced bacterial division and death, we performed single cell analysis by confocal microscopy (Fig. 4). At 2 hours after infection, approximately 90% of the cells infected with the different strains harbored one organism (Fig. 4A). After 10 hours of infection, approximately 75% of the cells harboring the wild type strain contained 6 to 15 bacteria. In contrast, the *ankB* mutant showed no detectable replication, similar to the *dotA* mutant (Fig. 4B). Thus, the growth defect of the *ankB* mutant was homogenous, and was not due to a balanced bacterial division and death.

Intracellular trafficking of the *ankB* mutant within hMDMs

Evasion of the endocytic pathway and recruitment of early secretory vesicles by the *Legionella* containing phagosome are both controlled by the Dot/Icm type IV secretion system (Derre and Isberg, 2004; Kagan *et al.*, 2004). Since the *ankB* mutant exhibited a severe defect in intracellular replication, we examined intracellular trafficking of the *ankB* mutant within hMDMs. Confocal laser scanning microscopy was used to assess co-localization of the late endosomal/lysosomal marker, (LAMP-2), and the luminal lysosomal enzyme, cathepsin D, with phagosomes harboring the wild type strain AA100 and the *ankB* mutant (Fig. 5). Formalin-killed bacteria, which traffic to the phagolysosomes, were used as a positive control for co-localization with LAMP-2 and cathepsin D. The data showed that at 2h post-infection, phagosomes harboring the wild type strain and the *ankB* mutant co-localized with LAMP-2 at a frequency of approximately 30 and 44% respectively, whereas phagosomes containing formalin-killed bacteria showed 78% co-localization (Fig. 5A). Approximately 80% of the formalin-killed bacteria co-localized with the lysosomal marker cathepsin D, whereas the wild type strain AA100 and the *ankB* mutant showed 31 to 40%

co-localization, respectively (Fig. 5B). The difference in LAMP2 and cathepsin D co-localization between the wild type strain and the *ankB* mutant was not significant (Student *t*-test, $p > 0.1$).

We examined by confocal microscopy the presence of RER-associated proteins in the phagosomes at 4h post-infection, using an antibody that recognizes the KDEL amino acid sequence, which is the signal for ER retention. The data showed that at 4h post-infection of hMDMs, ~70–80% of the phagosomes harboring the wild type strain and the *ankB* mutant co-localized with the KDEL marker, whereas phagosomes harboring the formalin-killed bacteria did not acquire the KDEL marker (20%) (Fig. 5C). These results were confirmed by transmission electron microscopy, which showed the presence of ribosome-studded ER membrane around the organism (Supplementary Fig. S1). No significant difference (Student *t*-test $p > 0.1$) was observed in recruitment of the RER to the vacuole containing the wild type strain and the *ankB* mutant (Fig. 5D). We conclude that despite its severe intracellular growth defect, trafficking of the *ankB* mutant-containing phagosomes is not distinguishable from the parental strain.

***In-cis* and *in-trans* rescue of the *ankB* mutant growth defect within macrophages harboring the wild type strain**

Mutants of the *dot/icm* genes encoding structural components of the type IV secretion apparatus are defective in modulating phagosomal biogenesis and in intracellular replication. The *dot/icm* mutants are rescued for their intracellular defect within communal phagosomes harboring the wild type strain of *L. pneumophila*, which is able to modulate phagosomal biogenesis into a niche suitable for bacterial replication (Coers *et al.*, 1999). However, *L. pneumophila* mutants defective in intracellular replication due to a defect in stress response genes, such as *htrA* or *rpoS*, that are required for adaptation to the phagosomal microenvironment, are not rescued within communal phagosomes harboring the wild type strain (Abu-Zant *et al.*, 2006; Coers *et al.*, 1999; Pedersen *et al.*, 2001). To examine whether the AnkB protein is required for formation of replicative phagosomes or for adaptation to the phagosomal microenvironment, we co-infected macrophages with the wild type strain and the *ankB* mutant and determined whether the mutant replicated in communal phagosomes harboring the wild type strain. We used co-infection of wild type *L. pneumophila* and the two isogenic mutants, *dotA* and *htrA*, as positive and negative controls, respectively. In all co-infections, only ~10 % of the phagosomes were communal phagosomes harboring the two different strains. Our data showed that in ~85% of the cells harboring the *ankB* mutant within communal phagosomes containing the wild type strain, the mutant replicated robustly (Fig. 6). There were equivalent numbers of bacteria for both strains in the communal phagosomes harboring the two strains. There was no detectable rescue of the *ankB* mutant between cells, since there was no detectable proliferation of the *ankB* mutant without the presence of the wild type strain within the same cell. In the control co-infection of *L. pneumophila* and its *dotA* mutant, replication of the *dotA* mutant was rescued in 80% of the communal phagosomes containing the wild type strain (Fig. 6A–C). Control co-infection with *L. pneumophila* and its *htrA* mutant showed failure of the wild type strain to rescue the *htrA* mutant in communal phagosomes, since rescue was detected only in ~15 of the communal phagosomes harboring the wild type strain (Fig. 6A and C) (Pedersen *et al.*, 2001).

Our data above showed that synchronous uptake of the *ankB* mutant and the wild-type bacteria into a communal phagosome resulted in an *in-cis* rescue of the growth defect of the *ankB* mutant within the communal phagosomes, which was similar to the rescue of the *dot/icm* mutants defective in structural components of the secretion apparatus. To determine whether the *ankB* mutant can be rescued *in-trans* by the wild type strain located in a different phagosome within the same cell, we did sequential infections by the two strains.

First, we infected hMDMs for 30 min with one strain, followed by extensive washing to remove the extracellular bacteria, and then super-infected the macrophages with the second strain. In control super-infections by the wild type strain and the *dotA* mutant, there was no detectable rescue of the defect of the *dotA* mutant in intracellular proliferation, regardless of the order of the infection by the two strains (Fig. 6). This confirmed entry of the two strains into different phagosomes in our sequential infection protocol. When the wild type strain-infected hMDMs were super-infected by the *ankB* mutant, 76% of the dually infected cells showed replication of the *ankB* mutant by 10h post infection (Fig. 6). When the order of the infection was reversed and the *ankB* mutant-infected hMDMs were super-infected by the WT strain, 72% of the dually infected cells showed replication of the *ankB* mutant by 10h post infection (Fig. 6A,C). There was no detectable rescue of the *ankB* mutant between cells, since there was no detectable proliferation of the *ankB* mutant without the presence of the wild type strain within the same cell. To ensure reliability of our analyses of the *in-cis* and *in-trans* rescue of the *ankB* mutant by the wild type strain, we switched GFP expression from the wild type strain into the *ankB* mutant and used non-fluorescent wild type strain. The GFP-expressing *dotA* and *htrA* mutants were used as controls. The data showed that switching GFP expression from the wild type strain into the *ankB* mutant had no effect on the *in-cis* or *in-trans* rescue of the *ankB* mutant by the wild type strain, since the results were similar to those when GFP-expressing wild type strain was used (Fig. 6 and data not shown). These results showed that the wild type strain can rescue the intracellular growth defect of the *ankB* mutant *in-cis* within communal phagosomes and *in-trans* within different phagosomes within the same cell. This is distinct from the *dot/icm* structural mutants (such as *dotA*), where the mutants are only rescued *in-cis*, but not *in-trans*, by the wild type strain (Coers *et al.*, 1999). Our data suggest that AnkB is a trans-acting effector that is likely exported by the wild type strain into the host cell to exhibit the *in-trans* rescue of the *ankB* mutant.

Dot/Icm-mediated translocation of AnkB

The ability of the wild type strain to rescue the *ankB* mutant *in-trans* in a different phagosome within the same cell indicated that AnkB is likely to be a trans-acting Dot/Icm-translocated effector. To assess translocation of AnkB, we constructed fusions of this protein to the C-terminus of the catalytic domain of *Bordetella pertussis* adenylate cyclase (Cya) (Sory and Cornelis, 1994). Adenylate cyclase activity has been extensively used as a reporter to measure translocation of *L. pneumophila* T4SS substrates in a variety of host cells (Cambronne and Roy, 2007; Chen *et al.*, 2004; Nagai *et al.*, 2005). Translocation of AnkB was examined using the wild type strain AA100 and an isogenic strain defective in the T4SS apparatus ($\Delta dotA$). Levels of cAMP were measured at 1 hour post-infection. U937 cells that were mock infected, or infected with the wild type strain AA100 harboring a plasmid encoding only the Cya domain (empty vector) exhibited low levels of cAMP (Fig. 7A). In contrast, cells infected with a strain harboring a Cya-RalF fusion, as a positive control, demonstrated a high level of cAMP after 1 hour, as shown plasmid encoding a Cya-AnkB fusion also exhibited high levels of cAMP, indicating that AnkB is translocated into host cells (Fig. 7A). In contrast, levels of cAMP in U937 cells infected by the *dotA* mutant harboring a plasmid encoding Cya-AnkB were similar to mock-infected or empty vector controls (Fig. 7A).

Since IcmS and IcmW have been shown to act as a bimolecular chaperone complex for many Dot/Icm-exported effectors, we examined translocation of AnkB in the *icmS* and *icmW* isogenic mutants. The data showed that U937 cells infected with $\Delta icmS$ or $\Delta icmW$ mutants harboring plasmids encoding Cya-AnkB generated levels of cAMP that were similar to mock-infected or empty vector controls (Fig. 7A). Taken together, our data

indicate that translocation of AnkB is dependent on the Dot/Icm T4SS and that the IcmSW chaperone complex is essential for its translocation.

The *L. pneumophila* T4SS translocation signal has been shown to reside within the C-terminus of some but not all Dot/Icm substrates (Laguna *et al.*, 2006; Nagai *et al.*, 2005). Similar to RalF, which is a Dot/Icm-exported effector, AnkB has hydrophobic residues at the -2 and -3 positions relative to the C-terminus, and a leucine residue at the -3 position, which is crucial for translocation of the RalF effector (Nagai *et al.*, 2005) (Fig. 1). To assess the requirement for the C-terminus and determine which residues were critical for translocation, truncated mutants of AnkB fused to the Cya domain were generated and assayed for translocation (Fig. 1). Removal of the C-terminal cysteine residue (AnkB 1–171) had no effect on translocation of AnkB into the host cell (Fig. 7B). However, removal of the last two residues (VC) of AnkB (AnkB 1–170) reduced translocation by 50% relative to full-length AnkB (Fig. 7B). Furthermore, removal of the last three C-terminal residues (LVC) of AnkB (AnkB 1–169) and further truncations of the C-terminus completely abolished translocation of AnkB (Fig. 7B). Immunoblot analyses of the cell lysates, probed with an antibody that recognizes the M45 tag peptide portion of the Cya fusion proteins, showed that expression of Cya-AnkB and the derived truncation mutants in strain AA100 and in the isogenic mutants was similar among the strains. The blots were re-probed with anti-CAT antibodies, which showed equivalent expression of another protein encoded on the same reporter plasmid (data not shown). The data indicated that differences in cAMP levels were not due to differences in cellular concentration or protein stability of the fusion constructs in the wild type strain or the mutants (Fig. 7C). We conclude that the -2 and -3 residues (V and L respectively) are required for translocation of AnkB into the host cell.

Regulation of expression of *ankB*

Entry into the PE phase is triggered by the activation of the stringent response regulator RelA and accumulation of its product, ppGpp (Hammer and Swanson, 1999). Together with RelA and LetE, the two-component regulatory system LetA/S, (Bachman and Swanson, 2004; Hammer *et al.*, 2002), the sigma factors RpoS, RpoN, and FlhA (Bachman and Swanson, 2001; Hales and Shuman, 1999) play critical roles in the expression and regulation of many virulence factors. In addition, the PmrA response regulator controls the expression of several Dot/Icm substrates (Zusman *et al.*, 2007). Most *L. pneumophila* genes required for intracellular replication are triggered at the PE phase (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Hammer *et al.*, 2002). To determine whether expression of *ankB* is governed by the regulatory cascade at the PE phase (Fig. 8A) (Hammer *et al.*, 2002), we examined expression of *ankB* by RealTime qRT-PCR in the *rpoS*, *relA*, *rpoS-relA*, *letE*, *letA/S*, and *pmrA/B* isogenic mutants and compared it to the wild type strain. The *flaA* gene encoding the flagellum subunit protein was used as a positive control for a gene that is highly induced at the PE phase (Bosshardt *et al.*, 1997).

The data showed a seven-fold increase in expression of *ankB* in the *relA* mutant at both the E and PE phases (Fig. 8A and B), while in the *relA-rpoS* double mutant a 26-fold increase in expression of *ankB* was detected at the PE phase. In the *letA* mutant, expression of *ankB* was decreased by 50-fold upon transition into the PE phase (Fig. 8B). In contrast, the *letE* mutant exhibited a 123 to 156 fold increase in expression of *ankB* at the E and PE phases, respectively (Fig. 8). Expression of *ankB* was 22-fold less in the *pmrA* mutant at the PE phase relative to the E phase, consistent with previous findings that the promoter region of the *ankB* gene contains a PmrA-binding consensus motif (Zusman *et al.*, 2007). We conclude that during the developmental life cycle of *L. pneumophila*, many PE phase regulators are responsible for the temporal regulation of *ankB* expression.

Discussion

The *L. pneumophila* genome codes for an unexpectedly high number of eukaryotic-like proteins, which are thought to interfere with various cellular processes by mimicking functions of eukaryotic proteins (Cazalet *et al.*, 2004; de Felipe *et al.*, 2005; Steinert *et al.*, 2007). The large number and wide variety of eukaryotic-like proteins encoded in the *L. pneumophila* genome is unique for a prokaryotic genome (Habyarimana *et al.*, 2008). These proteins include ankyrin (Ank), Sel-1 (TPR), U-box and F-box motifs (Kubori *et al.*, 2008; Steinert *et al.*, 2007). In *L. pneumophila*, the Ank proteins are considered the most prominent family of proteins with eukaryotic-like domains and may play an important role in the intracellular life cycle (Habyarimana *et al.*, 2008; Steinert *et al.*, 2007). The Ank proteins have also been identified in other pathogens including *Coxiella burnetii* and *Anaplasma phagocytophilum* (Caturegli *et al.*, 2000; Ogata *et al.*, 2005; Park *et al.*, 2004; Seshadri *et al.*, 2003; Wu *et al.*, 2004), where they have been shown to be multifunctional and are involved in modulation of various cellular processes in the host.

To generate a replicative niche within the host cell, *L. pneumophila* translocates more than 100 effector proteins via the Dot/Icm T4SS apparatus into the host cell (Campodonico *et al.*, 2005; Chen *et al.*, 2004; Conover *et al.*, 2003; Murata *et al.*, 2006; Shohdy *et al.*, 2005; Zusman *et al.*, 2007). Some but not all the Dot/Icm substrates contain specific translocation motifs that target them for translocation. There is a correlation between the presence of hydrophobic residues at the C-terminus of some but not all *L. pneumophila* Dot/Icm-translocated effectors (Nagai *et al.*, 2005). The AnkB protein is translocated into the host cell by the Dot/Icm T4SS apparatus in an IcmSW-dependent manner, and the last 3 C-terminal amino acids are essential for translocation into the host cell (Nagai *et al.*, 2005). The lack of complementation of the intracellular growth defect of the *ankB* insertion mutant by the *ankB* allele with deletion of only the last three residues excludes the possibility that the insertion mutant expresses a truncated AnkB with potential deleterious effects on the host cell. In addition, there is no detectable mRNA for the region of *ankB* upstream of the kanamycin cassette insertion in the *ankB* mutant. Taken together, we conclude that the insertion mutation is fully complemented by the wild type *ankB* and the insertion has not resulted in expression of any detectable truncated AnkB protein. Importantly, our data show that Dot/Icm-mediated translocation of AnkB is essential for intracellular proliferation.

Consistent with translocation of AnkB into the host cell, sequential infections by the wild type strain and the *ankB* mutant indicated that the mutant is rescued *in-trans* within macrophages harboring the wild type strain in a separate vacuole. This is consistent regardless of the order of the infection by the two strains. In contrast, in sequential infections with the wild type strain and the *dotA* mutant control, there is not detectable rescue of the *dotA* mutant within cells harboring the wild type strain in separate vacuoles. This is also consistent regardless of the order of the infection by the two strains. These observations indicate that our super-infections do not involve any detectable contamination of the first strain during the super-infection by the second strain, which allows entry of the second strain into a separate vacuole. However, when co-infected, the *dotA* mutant is only rescued within vacuoles occupied by the wild type strain. We conclude that the *ankB* mutant is rescued *in-trans* by the wild type strain.

Although the Dot/Icm system plays an important role in the modulation of eukaryotic cellular processes, most mutants lacking single Dot/Icm-exported effectors, such as SidJ, do not exhibit any defect or are only modestly defective in intracellular proliferation (Campodonico *et al.*, 2005; Luo and Isberg, 2004; Ninio *et al.*, 2005). The *ankB* mutant is severely defective in intracellular proliferation within hMDMs. The *sdhA* mutant is severely defective within macrophages (Laguna *et al.*, 2006), but it has not been tested in

Acanthamoeba. Therefore, AnkB and SdhA are the only two known Dot/Icm-exported effectors that play a major role in the intracellular growth of *L. pneumophila* within macrophages, which makes these two effectors unique among the Dot/Icm-exported substrates.

Despite its severe defect in intracellular replication, the *ankB* mutant evades the endocytic pathway, and remodels its phagosome into an RER-derived compartment, similar to the WT strain. This may suggest a role for AnkB in the formation of a replicative phagosome, independent of evasion of endocytic fusion and remodeling of the phagosome by the RER. The lack of homology of the AnkB protein and the 6 specificity-determining residues within the two ANK domains to other *L. pneumophila* Ank proteins or other proteins in databases and the presence of a eukaryotic F-box domain indicates a novel function for AnkB in modulating biology of the host cell to remodel it into a suitable proliferation niche. Future studies to elucidate the function of AnkB, and the role of the two ANK domains and the F-box domain in its function will enhance our knowledge of the cell biology of this intracellular pathogen.

Similar to many Dot/Icm secreted effectors and virulence factors, the expression levels of *ankB* are higher at the PE phase of growth in an RpoS dependant mechanism (Habyarimana *et al.*, 2008). Examination of the *ankB* promoter region did not identify any regulatory sequence recognized by the RpoS transcription factor, which suggests a role for other PE phase regulators that may act through RpoS in a cascade manner. At both the E and PE phases, the expression profiles of *ankB* are similar for all regulatory mutants *relA*, *rpoS*, and *letE* (Bachman and Swanson, 2001; Feldman and Segal, 2007; Hammer *et al.*, 2002; Zusman *et al.*, 2007), with the exception of the *letA/S* and *pmrA/B* mutants. At both growth phases, the three regulators LetE, RelA, and RpoS suppress expression of *ankB*, while the PmrA/B TCS induces its expression (Zusman *et al.*, 2007). Recent data derived from genome-wide microarray analyses of *pmrA/B* mutants are consistent with these findings and show a more global role for this two component regulator in expression of numerous genes encoding Dot/Icm effectors, including the *ank* genes (Al-Khodor *et al.*, unpublished data).

On the other hand, both LetA and LetS suppress the expression of *ankB* at the E phase, whereas growth transition into the PE phase relieves this suppression. Therefore, the LetA/S TCS plays a major role in temporal regulation of *ankB* in a growth phase-dependent manner. Upon depletion of amino acids, the RelA product (ppGpp) triggers two parallel signaling cascades in *L. pneumophila*. One of the cascades is controlled by RpoS, while the other is controlled by LetE (Molofsky and Swanson, 2003). Both regulators confer a negative regulation on *ankB*. We show that all known regulatory pathways are involved in temporal regulation of the expression of *ankB* and we propose another signaling pathway through PmrA/B. In other pathogens, PmrA/B has been shown to be activated through many signals, such as pH change or iron levels, where both can occur during the stringent response (Wosten *et al.*, 2000). The signals that trigger PmrA/B in *L. pneumophila* within the phagosome are still to be elucidated. Interestingly, although AnkB is required during early stages of infection of macrophages and protozoa, its expression is triggered at the PE phase, which is consistent with phenotypic modulation of the bacteria after they terminate proliferation in the host cell and prepare to infect a new susceptible cell in the human lung or in the aquatic environment.

Experimental Procedures

DNA manipulations

DNA manipulations and restriction enzyme digestions were performed using standard procedures (Stone and Abu Kwaik, 1998). Restriction enzymes and T4 DNA ligase were

purchased from Promega (Madison, WI). *L. pneumophila* chromosomal DNA was prepared using the Puregene DNA isolation kit from Gentra Systems (Minneapolis, MN). Plasmid preparations were performed with the Bio-Rad Quantum miniprep kit. Purification of DNA fragments from agarose gels for subcloning was carried out with a QIAquick gel purification kit (Qiagen Inc, Valencia, CA). Fragments containing the *L. pneumophila ankB* gene were cloned into the plasmid vector pBC-SK⁺ from Stratagene, and the resulting clone was mutagenized using the EZ-Tn5TM <KAN-2> *in vitro* transposome insertion kit from Epicentre. Transformation of *E. coli* strain DH5 α by electroporation was performed with a BTX ECM 630, as recommended by Invitrogen Corp. (Carlsbad, California). Mutations of the parental strain AA100 were carried out by allelic exchange with the mutagenized *ankB* clone after natural transformation, as previously described (Stone and Abu Kwaik, 1999; Stone *et al.*, 1999). The isogenic *ankB* mutant was trans-complemented with the plasmid vector pBC-SK⁺ harboring the corresponding gene. Primers used to amplify the *L. pneumophila ankB* gene by PCR were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and are as follows: for cloning of *ankB* (*lpg2144*, *legAU13*, *lpl2072*, *lpp2082*, *lpc1593*) genomic region (F:GCCGGACTTCATTCATA and R:CCGCTGATCCTGGTAGTA), to amplify the coding sequence of *ankB* for Kanamycin insertion verification (F:TTGCTGCTATGAAAAGA and R:CTTGCTCTGTGCTATTTT). To generate Cya-AnkB fusions, the *ankB* orf was PCR amplified from strain AA100, using primers listed in Table S1, to generate either full length AnkB or C-terminal truncations of the protein. PCR products were cloned into pCR2.1 via topoisomerization as described by the manufacturer (Invitrogen). *ankB* inserts were sub-cloned into the BamHI-PstI sites of pCYA-*ralF* (Nagai *et al.*, 2005), resulting in replacement of the *ralF* gene with *ankB* or its truncated mutants in frame with *cya*. Recombinant plasmids were electroporated into AA100 and isogenic mutant derivatives $\Delta dotA$, $\Delta icmS$ and $\Delta icmW$. To verify that the *ankB* insertion mutant is not expressing truncated fragments of AnkB, we complemented both the WT strain and the *ankB* mutant using a clone of *ankB* with a deletion of the last 3 codons (AnkB₁₋₁₆₉).

Bacterial strains and media

L. pneumophila serogroup I parental strain AA100/130b (ATCC BAA-74) and the mutants *dotA*, *ankB*, *htrA*, *rpoS*, *relA*, $\Delta rpoS$ - $\Delta relA$, *letA*, *letS*, *letE*, *pmrA*, and *pmrB* (Hammer *et al.*, 2002; Pedersen *et al.*, 2001) were grown from frozen stocks on buffered charcoal-yeast extract (BCYE) agar at 37°C or in buffered yeast extract (BYE) broth at 37°C with shaking. The plates and broth used for the cultivation of the mutant strains listed above were supplemented with thymidine (100 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹) or chloramphenicol (5 μ g ml⁻¹) as needed. *Escherichia coli* strain DH5 α was used as surrogate to clone the *ankB* gene. *E. coli* strains were cultured with the appropriate antibiotic on Luria-Bertani (LB) agar plates at 37°C or in LB broth at 37°C with shaking.

Cell cultures

Isolation and preparation of the human monocyte-derived macrophages (hMDMs) from peripheral blood of volunteers and macrophage-like U937 cells was carried out as previously described (Doyle *et al.*, 2001; Santic *et al.*, 2005; Tilney *et al.*, 2001). Cells were maintained in RPMI-1640 tissue culture medium (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum FBS (Atlas Biologicals). Axenic *Acanthamoeba polyphaga* was cultured as adherent cells in PYG medium as previously described (Gao *et al.*, 1997). All cells were grown at 37°C in presence of 5% CO₂.

Intracellular growth kinetics

The bacterial strains were grown in BYE medium to an optical density (OD₅₅₀) of 2.0 to 2.2 (PE phase). The mammalian or protozoan cells were infected with the bacteria at a

multiplicity of infection (MOI) of 10. To synchronize the infection, the plates were centrifuged for 5 min at 500Xg using a Centra GP8R Thermo IEC centrifuge. After 1 h of incubation in 5% CO₂ at 37°C, the infected cells were washed 3 times with the culture medium to remove extracellular bacteria, and incubated with 50 µg ml⁻¹ gentamicin for 1 h to kill the remaining extracellular bacteria. The infected cells were washed 3 times to remove the gentamicin. This step was considered the zero (T₀) time point and the infected cells were subsequently incubated for several time intervals. At the end of each time interval, the culture supernatant was removed and the macrophages were lysed hypotonically by the addition of 200 µl of sterile water for 10 min or with 0.04% Triton X-100 for protozoan cells. The supernatant and the lysates were combined, and serial dilutions were prepared and aliquots plated on BCYE plates for counting. The number of bacteria was expressed as the number of CFU ml⁻¹. Three independent experiments, in triplicate, were performed.

Confocal laser scanning microscopy

Approximately 5 × 10⁵ hMDMs were grown on circular glass coverslips (Fisher) in 24-well culture plates. After infection, cells were washed three times with warm RPMI containing 10% FBS, and processed for confocal microscopy as we described previously (Santic *et al.*, 2005). To study the role of AnkB in intracellular replication, both wild type and the isogenic mutant were labeled with polyclonal rabbit anti-*L. pneumophila* antiserum (Pedersen *et al.*, 2001) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG purchased from Molecular Probes (Invitrogen, Carlsbad, CA). The anti-LAMP-2 (H4B4) monoclonal antibody (developed by J. T. August and J. E. K. Hildreth) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). To label the lysosomes, macrophages were incubated with mouse monoclonal anti-cathepsin D antibodies (BD Transduction, Franklin Lakes, NJ). Mouse anti-KDEL monoclonal antibodies purchased from StressGen Biotechnologies (Ann Arbor, Michigan) were used to label the ER proteins, followed by Alexa Fluor 555-conjugated donkey anti-mouse IgG (Molecular Probes). For the coinfection experiments, cells were coinfecting simultaneously with the wild type strain of *L. pneumophila* expressing GFP (Coers *et al.*, 1999; Pedersen *et al.*, 2001) and isogenic mutants at a MOI of 10, with the exception of the *dotA* mutant using a MOI of 20. To synchronize the infection, the plates were centrifuged for 5 min at 500Xg using a Centra GP8R Thermo IEC centrifuge. After 1 h of incubation in CO₂ at 37°C, the infected cells were washed 3 times with the culture medium to remove extracellular bacteria and incubated with 50 µg ml⁻¹ gentamicin for 1 h to kill the remaining extracellular bacteria. Infected cells were further incubated for 10 h and the cells were processed for confocal microscopy as described below. All bacteria were labeled with polyclonal rabbit anti-*L. pneumophila* antiserum and Alexa Fluor 555-conjugated donkey anti-rabbit IgG antibody, and therefore, the GFP-expressing bacteria become yellow when the two colors are combined, while the bacterial strain that did not have GFP is detected by red fluorescence. The *dotA* and *htrA* mutants were used as positive and negative controls, respectively. For the superinfection, the cells were infected with one strain for 30 min, washed 3 times, incubated for 30 min with gentamicin to remove extracellular bacteria, and then infected with the second strain for another 30 min. The cells were then incubated for 30 min with gentamicin. The *dotA* mutant was used as negative control for *in-trans* rescue (Coers *et al.*, 1999). The cells were examined using an Olympus Fv500 laser scanning confocal microscope as described previously (Santic *et al.*, 2005). On average, 8–15 0.2 µm serial Z sections of each image were captured and stored for further analysis, using Adobe photoshop 6.0.

Transmission electron microscopy

For transmission electron microscopy, monolayers in six-well plates were infected with *L. pneumophila* strains at a MOI of 10 for 1 h, followed by 1 h of gentamicin treatment. At 6

hpost infection, the infected monolayers were washed and the cells were fixed in 3.5% glutaraldehyde, dehydrated in alcohol, processed and stained for TEM as described previously (Abu Kwaik, 1998). Sections were examined with a Hitachi H-7000/STEM electron microscope (Hitachi, Ltd.) at 80 kV (Gao *et al.*, 1998; Molmeret *et al.*, 2004).

Cya translocation assays

L. pneumophila strains used in this assay were plated on BCYE agar with antibiotic selection as required and incubated at 37°C for 3 days. U937 cells were plated into 24 well plates at a density of 5×10^5 per well and chemically differentiated using PMA (final concentration 50 ng ml⁻¹), and incubated for 2 days at 37°C and 5% CO₂ prior to infection. Bacteria were added to U937 monolayers (MOI of 50) and centrifuged at 200 g for 5 min. Infected monolayers were incubated at 37°C for 1 h, after which they were washed 3 times with PBS to remove extracellular bacteria, and subsequently lysed in 200 µl of 0.1M HCl + 0.5 % v/v Triton X-100. Cell lysates were centrifuged at 600 g for 5 min and supernatants were retained. cAMP levels in the supernatants were measured using the Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs) following the acetylation protocol as described by the manufacturer. Immunoblots of bacterial cell lysates probed with the anti-M45 peptide antibody and re-probed with the anti-CAT antibodies were performed according to standard procedures.

Quantitative Real Time PCR

For analysis of expression of the *ankB* gene *in vitro*, samples of bacterial cultures from the wild type strain AA100, the mutants *letE*, *rpoS*, *relA*, *pmrA*, *pmrB*, *ArpoS-ArelA* double mutant, *letA*, and *letS*, were grown in BYE medium to an optical density (OD₅₅₀) of 0.8–1 (E phase) or 2.0–2.2 (PE phase). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. RNA integrity was assessed by visualizing ethidium bromide-stained 0.8% agarose gel. Total RNA was treated with DNase I (Ambion, Austin, TX) at 37°C for 30 min. Equal amounts of RNA from both the wild type and the *ankB* mutant were used for cDNA synthesis with Superscript III Plus RNase H⁻ reverse transcriptase (RT) (Invitrogen, California) and random primers. The generated cDNA was diluted five-fold with RNase-free water. Real-time qPCR was done using the DNA Engine Opticon System (MJ Research), and carried out in triplicate using the DyNAmo SYBR Green qPCR Kit in a 20 µl reaction volume, as recommended by the manufacturer (New England Biolabs, Ipswich, MA). The *ankB* gene was amplified using the following primers (F: TATGGGGAAATCTTATGGTG and R: TCCAGAGGTAATTTGCAGTT), and the primers (F: GTTACCCACAGAAGAAGCAC and R: CCACTACCCTCTCCCACT) were used to amplify the 16sRNA. The *flaA* gene was amplified by the two primers (F: CGATGGTTCTTTCTCTGG and R: GCTACTTCTGTTCTGTTG). To verify the absence of detectable *ankB* mRNA in the *ankB* insertion mutant, we used the following primers (F: TTTTCTGATCTTCCTGAG and R: AGTTTGATATGCTTTTCCTT) to amplify the region (1–300 bp) upstream of the transposon insertion in *ankB*. PCR conditions were 5 min at 94°C, 15 s at 96°C, and 15 s at 72°C for 30 cycles. The concentration was determined by the comparative C_T method (threshold cycle number at the cross-point between amplification plot and threshold) and values were normalized to the 16sRNA. Relative quantitation by quantitative reverse transcriptase PCR was validated by equivalent and linear amplification of 16sRNA and *ankB* gene at the assay concentrations. Negative or positive values were considered as down-regulation or up-regulation of expression of *ankB*, respectively, represented by a minimum of two-fold difference.

Statistical analysis

All experiments were performed at least three times and the data shown are representatives of one experiment. To analyze for statistically significant differences between different sets of data, the two-tail Student's *t*-test was used and the *p* value was obtained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Genetic organization of *ankB*, the relevant protein domains, and the C-terminal amino acid sequence of the protein and the deletion mutants. The top illustration shows the organization of the *ankB* gene in the *L. pneumophila* chromosome, illustrating the upstream and downstream adjacent genes. The middle illustration represents the *ankB* gene with the corresponding domains (ankyrin and F-box), and location of the *kan^r* insertion in the *ankB* mutant. The lower two illustrations represent the AnkB protein with the domain locations and the C terminal sequence of the deletion mutants.

Fig. 2. Intracellular growth kinetics within *A. polyphaga*

(A) Intracellular growth kinetics of the wild type strain AA100, *ankB* mutant and its complemented strain in *A. polyphaga*. (B) Intracellular growth kinetics of the WT strain and *ankB* mutant both complemented with an *ankB* gene that had a deletion of the last 3 codons (*ankB*₁₋₁₆₉). The infection was carried out in triplicate with an MOI of 10 for 1 h, followed by 1 h of gentamicin treatment to kill extracellular bacteria. The infected monolayers were lysed at different time intervals and plated onto agar plates for colony enumeration. The experiment was done 3 times, and the data are representative of one independent experiment. Error bars represent standard deviations, but some were too small to appear in the figure.

Fig. 3. Defect in intracellular growth of the *ankB* mutant within macrophages
 Intracellular growth of the *ankB* mutant in hMDMs (A) and U937 cells (B). (C) Intracellular growth of the wild type strain AA100 and the *ankB* mutant both complemented with an *ankB* allele that had a deletion of the last 3 codons (*ankB*₁₋₁₆₉). The infection was carried out in triplicate with an MOI of 10 for 1 h, followed by 1 h of gentamicin treatment to kill extracellular bacteria. The infected monolayers were lysed at different time intervals and plated onto agar plates for colony enumeration. The experiment was done 3 times, and the data are representative of one independent experiment. Error bars represent standard deviations, but some were too small to appear in the figure.

Fig. 4. Single cell analysis of replicative phagosomes

After 2 and 10h post-infection of hMDMs, 100 infected cells were analyzed by laser scanning confocal microscopy for formation of replicative phagosomes. *L. pneumophila* was stained by a polyclonal anti *L. pneumophila* antibody and Alexa Fluor 555-conjugated anti-rabbit IgG. Representative quantitation of the number of bacteria/cell at 2 (A) and 10 hours (B) is shown. The *dotA* mutant was used as negative control. Infected cells from multiple cover slips were examined in each experiment. The results are representative of three independent experiments performed in triplicate. Error bars represent standard deviations. The difference between the WT strain and the *ankB* mutant was statistically significant (Student *t*-test, $p < 0.1$).

Fig. 5. Quantitative analysis of intracellular trafficking of the *ankB* mutant

Quantitative analyses of confocal microscopy images of infected hMDMs of co-localization of the bacterial phagosomes with the late endosomal marker LAMP2 (A), the lysosomal enzyme cathepsin D (B), and the KDEL marker (C) at 2 h post-infection. Formalin killed bacteria (FK) were used as a positive control for LAMP2 and cathepsin D co-localization and as a negative control for KDEL and ER recruitment. At least 100 infected cells from multiple cover slips were examined in each experiment. (D) Quantitative analysis by TEM of recruitment of the RER. At least 100 infected cells from multiple sections obtained from different blocks were analyzed. Results shown are representative of three independent experiments performed in triplicate. Data represents means $\pm$ standard deviation. No

significant difference was observed between the WT strain and the *ankB* mutant (Student *t*-test, $p>0.1$). Asterisks represent significant difference ($p<0.05$) between the WT and FK bacteria.

Fig. 6. In-cis and in-trans rescue of intracellular growth of the *ankB* mutant by the wild type strain within hMDMs

Wild-type *L. pneumophila* strain AA100 expressing GFP and the *ankB* mutant strain were phagocytosed by hMDMs simultaneously (co-infection with AA100+*ankB*) or sequentially spaced 30 min apart by super-infection of strain AA100 followed by the *ankB* mutant, (AA100/*ankB*) or infection by the *ankB* mutant followed by strain AA100, (*ankB*/AA100). Infections were carried out using an equivalent amount of total CFUs for the different strains based on the optical density, which was further confirmed later by enumerating the CFUs after growth on agar plates. Infected cells were gentamicin treated, washed and fixed 10 h after infection. Bacterial phagosomes were scored for all macrophages in randomly selected

fields by fluorescence microscopy at 10h post-infection. Macrophages harboring phagosomes containing both strains (GFP-WT and mutants) were scored. The *dotA* mutant was used as positive control for the co-infection illustrating the *in-cis* rescue, whereas the *htrA* mutant was used as negative control (A). Representative confocal images and quantitation are shown in panel A for *in-cis* and *in-trans* rescue. Panel B represents a single infection by the wild type strain AA100 and the *ankB* mutant after 10h. Rescue was calculated by quantifying the percentage of the cells harboring the *ankB* mutant replicating within communal phagosomes with the WT strain (*in cis*) or within cells super-infected with the WT strain (*in trans*). Quantitation is shown in panel C. The results are representative of three independent experiments performed in triplicate. Error bars represent standard deviation.

Fig. 7. Translocation of AnkB into host cells is Dot/Icm-dependent and the IcmS and IcmW chaperones are essential for translocation

(A) Requirement of Dot/Icm components for AnkB translocation. U937 cells were infected with the wild type strain or the isogenic mutants, $\Delta dotA$, $\Delta icmS$ or $\Delta icmW$ harboring the indicated plasmids (MOI 50) for 1 h. Data points are the average cAMP concentration per well $\pm$ SD for an experiment performed in triplicate, and are representative of three independent experiments. (B) Requirement of C-terminal residues for AnkB translocation. U937 cells were infected with AA100 harboring the indicated plasmids at an MOI of 50 for 1 h. Data points are the average cAMP concentration per well $\pm$ SD for an experiment performed in triplicate. (C) Immunoblots of Cya-AnkB fusions expressed in *L. pneumophila*. Proteins derived from equivalent numbers of bacteria (1×10^8 bacteria) were loaded onto an SDS-PAGE gel and Cya fusion proteins were detected using a $\alpha M45$ antibody, recognizing the N-terminal M45 epitope present on all Cya fusions used.

Fig. 8. Regulation of expression of *ankB* by the *L. pneumophila* wild type strain and different regulatory mutants

Real Time qRT-PCR was used to determine regulation of expression of *ankB* by the wild type strain and the *pmrA/B*, *relA*, *rpoS*, $\Delta rpoS$ - $\Delta relA$, *letA/S*, and *letE* mutants. (A) and (B) represent the *in vitro* qRT-PCR expression of *ankB* by the wild type strain and its isogenic regulatory mutants at the E and PE phases of growth, respectively. The *ankB* mRNA was normalized internally to the 16S rRNA. The results of the qRT-PCR are represented as the ratios of ratios, i.e., (*ankB*/16S) mutant over (*ankB*/16S) wild type. Individual experiments were performed three times, and the results of one experiment are shown. Error bars represent standard deviations of triplicate samples.