Legionella pneumophila pathogenesis in the *Galleria mellonella* infection model

Running title: *L. pneumophila* pathogenesis in *Galleria mellonella*

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Abstract

Legionella pneumophila is a facultative intracellular human pathogen and the aetiological agent of severe pneumonia known as Legionnaires' disease. Its virulence depends on protein secretion systems, in particular the Dot/Icm type IV secretion system (T4SS), which is essential to establish a replication permissive vacuole in macrophages. The analysis of the role of these systems and their substrates for pathogenesis requires easy-to-use models which approximate human infection. We examined the effectiveness of the larvae of the wax moth Galleria mellonella as a new model for L. pneumophila infection. We found that the L. pneumophila strains 130b, Paris and JR32 caused mortality of the G. mellonella larvae, which was strain-, infectious dose-, growth-phase- and T4SS-dependent. Wild type L. pneumophila persisted and replicated within the larvae whereas T4SS mutants were rapidly cleared. L. pneumophila strain Lp02, which is attenuated in the absence of thymidine, but has a functional T4SS, resisted clearance in G. mellonella up to 18 h post infection without inducing mortality. Immunofluorescence and transmission electron microscopy revealed that L. pneumophila resided within insect haemocytes in a vacuole that ultrastructurally resembled the Legionella containing vacuole (LCV) observed in macrophages. The vacuole was decorated with the T4SS effector and LCV marker SidC. Infection caused severe damages to the insect organs and triggered immune responses including activation of the phenoloxidase cascade leading to melanisation, nodule formation and upregulation of antimicrobial peptides. Taken together, these results suggest that G. mellonella provides an effective model to investigate the interaction between L. pneumophila and the host.

Introduction

Legionella pneumophila is a Gram-negative bacterium found ubiquitously in environmental water reservoirs where it replicates in free-living protozoa (38). Following inhalation of contaminated aerosols, *L. pneumophila* is capable of infecting human alveolar macrophages and causing disease ranging from mild flu-like symptoms to Legionnaires' disease, a severe, life-threatening pneumonia (16). *L. pneumophila* thrives in professional phagocytes by avoiding killing by the phago-lysosomal pathway (21). Instead it establishes a specialized *Legionella* containing vacuole (LCV), which shows characteristics of the rough endoplasmic reticulum (ER) (48).

L. pneumophila employs several specialized protein secretion systems, e.g. the twin-arginine translocation (Tat) pathway and a type II secretion system (T2SS), to secrete virulence factors, some of which have been shown to contribute to *Legionella*'s intracellular survival and pathogenicity (11, 12). However, the essential virulence determinant of *L. pneumophila* is the Dot (defective in organelle trafficking)/Icm (intracellular multiplication) type IV secretion system (T4SS), which is indispensable for intracellular survival and establishment of the replication-permissive LCV in both amoebae and macrophages (4, 45). The Dot/Icm T4SS is a multi-protein complex able to translocate at least 275 effector proteins directly into host cells (35, 53). Although it has been demonstrated that several T4SS effectors manipulate host cell vesicular trafficking, inhibit apoptosis and immune signaling, the function of the majority of T4SS effectors during infection is still unknown (5).

Free-living freshwater amoebae such as *Acanthamoeba castellanii* or *Hartmannella vermiformis* routinely serve as model hosts to study molecular aspects of *Legionella* pathogenesis (1, 20). As natural hosts, these professional phagocytes are believed to have exerted evolutionary pressure for the selection of *Legionella's* virulence factors that enable the bacteria to overcome the antimicrobial activities of human macrophages (32). In addition,

Dictyostelium discoideum has become a prevalent protozoan model organism as it can readily be genetically modified (47). Although, protozoan *Legionella* infection models have proven successful, they do not fully reflect the infection of macrophages as amoeba employ less complex antimicrobial mechanisms than mammalian cells.

The nematode *Caenorhabditis elegans* possesses an innate immune system and is a well-established model for several bacterial pathogens including *Legionella* spp. (5). However, one caveat to the use of *C. elegans* is that bacteria replicate in the intestinal lumen and do not invade intestinal epithelial cells, limiting the usefulness of this model to study virulence determinants required for *Legionella's* intracellular lifestyle.

Typically, human *Legionella* infection is modeled using mammalian hosts (3, 6). Disease progression in the guinea pig resembles Legionellosis in humans and pathology includes lymphocyte infiltration, goblet cell metaplasia, mild fibrosis and emphysema (3). In contrast, the majority of mouse strains are resistant to *Legionella* infection (52) with the exception of the inbred albino A/J mouse, which develops a self-limiting infection (6).

Due to the high cost and ethical considerations associated with the use of mammalian hosts, the search for alternative models is ongoing. Insect model organisms, in particular *Drosophila melanogaster*, have been introduced to study bacterial pathogenesis (44). *L. pneumophila* replicates in *D. melanogaster* and kills the flies in a Dot/Icm T4SS-dependent manner (27). The human and insect innate immune systems demonstrate many similarities (24, 29) with most insect species containing specialized cells known as haemocytes that phagocytose pathogens and form aggregates which encapsulate and neutralize foreign microorganisms (30). Moreover, activated haemocytes can trigger a phenoloxidase (PO) melanisation cascade leading to physical restriction of intruders and the production of antimicrobial compounds (8). Haemocyte-mediated responses are complemented by the

89 production and secretion of anti-microbial peptides by the insect fat body, an organ similar to
90 the mammalian liver (29, 31).

91 Besides *D. melanogaster,* the larva of the greater wax moth *Galleria mellonella* has become a
92 widely adopted insect model to study a wide range of human pathogens including *Listeria*
93 spp. (23), *Streptococcus pyogenes* (36), *Campylobacter jejuni* (10), *Yersinia*
94 *pseudotuberculosis* (9) and several pathogenic fungi (17, 33). *G. mellonella* larvae can be
95 easily maintained and infected by injection without anesthesia and sustain incubation at 37˚C
96 (33). A good correlation between the pathogenicity of several microorganisms in *G.*
97 *mellonella* and other mammalian models of infection has been established (22, 23). The aim
98 of this study was to determine if *G. mellonella* could be used as a model to study *L.*
99 *pneumophila* pathogenesis.

Material and Methods

Bacterial strains and *G. mellonella* larvae. *L. pneumophila* serogroup 1 strain 130b is a spectinomycin-resistant clinical isolate from the Wadsworth Veterans Administration Hospital, Los Angeles, CA (14). The *L. pneumophila* ΔDotA strain is a *dotA* insertion mutant (kanamycin resistance) of *L. pneumophila* strain 130b (41). *L. pneumophila* strain JR32 is a salt sensitive streptomycin-resistant *L. pneumophila* strain Philadelphia-1 isolate (39) and the ΔIcmT strain is an *icmT* isogenic mutant in the JR32 strain (46). *L. pneumophila* strain Lp02 is a thymine auxotroph streptomycin-resistant derivative of the Philadelphia-1 strain (4). *L. pneumophila* strain Paris is a worldwide epidemic strain (7) *G. mellonella* larvae were obtained from Livefoods, UK and stored at room temperature in the dark.

Infection of *G. mellonella*. *L. pneumophila* strains were cultured on charcoal-yeast extract (CYE) plates for four days then inoculated into ACES yeast extract (AYE) as described previously (43). For the Lp02 strain, thymidine (100 μg/ml) was added. After 21 h of growth, bacteria were diluted in Dulbecco's phosphate buffered saline (PBS) to an OD_{600} of 1 which corresponds to 10^9 CFU/ml unless otherwise indicated. Gene expression in strains containing the p4HA plasmid was additionally induced during infection with 1mM isopropyl β-d-1-thiogalactopyranoside (IPTG). Ten *G. mellonella* larvae were injected with 10 μl of bacterial suspension as previously described (37) and were incubated at 37˚C in the dark. As a control ten larvae were injected with PBS alone and ten untreated insects were included with every experiment. Larvae were individually examined for pigmentation and time of death was recorded. Assays were only allowed to proceed for 3 days as pupa formation could occasionally be seen by day 4. At least three independent replicates of each experiment were performed.

Intracellular growth assay. At 0, 2, 5, 18 and 24 h post infection (p. i.) haemolymph was extracted from three infected larvae and pooled as previously described (23). Cells were lysed by incubation of the haemolymph with 1 µl of 5 mg/ml digitonin for 5 min at room temperature. Extracted haemolymph was serially diluted in AYE media and plated onto CYE plates. To prevent contamination, the extracted haemolymph was plated on CYE plates supplemented with spectinomycin (50 µg/ml) for the *L. pneumophila* strain 130b or streptomycin (100 µg/ml) for the Philadelphia-1-derived strains. Plates were incubated at 37 ˚C for three days, viable bacteria were enumerated and the number of CFU was normalized to the weight of haemolymph extracted.

Plasmids. A fragment of the SidC homologue from *L. pneumophila* 130b containing the phosphatidylinositol-4 phosphate binding domain (amino acids 41 to 918) was cloned into the *Xba*I and *BamH*I sites of the p4HA plasmid (13) to yield the IPTG-inducible 4HA-SidC$_{41-918}$ expression plasmid pICC562 using the forward primer 5'-cgtattctagataacacctgccaaacagcagttgag-3' and the reverse primer 5'-ggctaggatccctatttctttataactcccgtgtac-3' and standard molecular biology techniques.

Indirect immunofluorescence on extracted haemocytes. Haemolymph from infected *G. mellonella* was extracted at 5 and 24 h post infection. The extracted haemolymph was dispensed onto poly-L-lysine coated glass coverslips and centrifuged at 500 x g for 10 min to allow sedimentation and attachment of haemocytes. Coverslips were washed twice with PBS and fixed using 4% paraformaldehyde for 20 min followed by quenching with 50 mM ammonium chloride. Extracellular *L. pneumophila* were stained with a mouse anti-*L. pneumophila* LPS antibody (ViroStat) and a donkey anti-mouse Rhodamine Red-X-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc.). After permeabilization of the cells with 0.1% Triton in PBS and blocking with 2% (w/v) bovine serum albumin (BSA) in PBS, total bacteria were stained with a rabbit anti-*L. pneumophila* antibody (Affinity

BioReagents) and a donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch).

To visualize 4HA-SidC$_{41\text{-}918}$ in haemocytes, fixed cells were permeabilised and blocked for 1 h in PBS containing 2% (w/v) BSA. Samples were stained with rabbit anti-*L. pneumophila* antibody (Affinity BioReagents), donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch), mouse anti-HA conjugated to Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) (Sigma) and 5 μg ml^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Samples were analyzed using an Axio M1 Imager microscope and images processed with the AxioVision software (Carl Zeiss).

Staining of formalin fixed sections of *G. mellonella*. *G. mellonella* were fixed in formalin for one week at room temperature, paraffin embedded, sectioned and stained either with haematoxylin and eosin (H&E) or by indirect immunofluorescence as described previously (18). *L. pneumophila* was stained with rabbit anti-*L. pneumophila* antibody (Affinity BioReagents) and donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch). Cellular and bacterial DNA was stained with DAPI and the shape of the tissues was visualized using Rhodamine Phalloidin (Invitrogen).

Transmission electron microscopy. Haemolymph was extracted from ten infected *G. mellonella* per condition and time point. Cells were spun down onto 6 well plates, washed once with PBS and fixed in 2% glutaraldehyde. Samples were processed as described previously (26) and examined using a Tecnai12 (FEI) electron microscope. Images were taken with a CCD camera (TVIPS, Gauting, Germany).

Haemocyte quantification and viability assay. Infected haemolymph was extracted at 5 and 18 h p.i., Trypan blue (0.02% (v/v) in PBS) was added to cells and incubated at room temperature for 10 min. Viable cells were enumerated using a haemocytometer and each

172 sample was analysed in triplicate. The average of three independent experiments was plotted
173 graphically.
174 **Phenoloxidase (PO) activity assay.** At 5 and 18 h p.i. haemolymph from three infected
175 insects per condition was extracted and pooled. Cells and debris were removed by
176 centrifugation at 20000 x *g* for 10 min at 4 ˚C. The phenoloxidase activity in the plasma was
177 quantified using a microplate enzyme assay as described previously (15). The change in
178 absorbance at 490 nm was read for 1 h at room temperature with a reading taken every
179 minute using a Fluostar Optima plate reader (BMG labtech, Germany). The experiment was
180 performed in triplicate and independently repeated at least three times. Phenoloxidase activity
181 was expressed as the mean OD_{490}/minute.
182 **RNA extraction and RT-PCR.** At indicated time points fat bodies from three larvae were
183 collected and stored in RNAlater (Qiagen) at 4 ˚C until processing. Tissue was homogenized
184 by a gentleMACS homogeniser (Miltenyi Biotech) using M tubes and the 90 s RNA setting.
185 RNA was extracted using a RNAeasy kit (Qiagen) and contaminating DNA was digested
186 using Turbo DNA-free kit (Ambion) following the manufacturer's instructions. Two-step RT-
187 PCR was performed using Superscript reverse transcriptase (Invitrogen) using 2 µg of RNA
188 as a template and random hexamers (Invitrogen). Genes were amplified using RedTaq
189 readymix (Sigma) and 0.6 pM of gene specific primers (Table 1) as described previously (23).
190 DNA was analyzed on a 1% agarose gel with SYBRSafe (Invitrogen) and quantified using
191 ImageJ software (NIH).

192 **Results**

193

194 ***L. pneumophila* infection causes death of *Galleria mellonella* larvae**

195 In order to investigate the pathogenicity of *L. pneumophila* in *G. mellonella* larvae, we used
196 three serogroup 1 *L. pneumophila* strains: 130b, Paris and JR32, which are commonly used
197 for molecular pathogenesis studies. The bacteria were injected into the larvae and their
198 survival monitored over 72 h (Fig. 1A). All three *L. pneumophila* strains caused time-
199 dependent death of at least 70% of the *G. mellonella;* strain 130b caused significantly
200 ($P<0.005$) higher mortality than the JR32 or Paris strains at 18 h p.i. No mortality was
201 observed in the control buffer-injected *G. mellonella*. These results demonstrate that *G.*
202 *mellonella* is susceptible to *L. pneumophila* infection.

203

204 **Mortality in *L. pneumophila*-infected *G. mellonella* is dose-dependent**

205 To determine if the mortality caused by *L. pneumophila* infection was dependent on the
206 number of injected bacteria, *G. mellonella* were injected with 10^4, 10^5 10^6 or 10^7 CFU of *L.*
207 *pneumophila* strain 130b. While infection with 10^7 CFU resulted in 100% *G. mellonella*
208 mortality within 24 h of infection, mortality was reduced to less than 40% in larvae injected
209 with 10^6 CFU, and no mortality was observed in *G. mellonella* injected with any of the lower
210 doses (10^4 and 10^5 CFU) (Fig. 1B). These results show that *L. pneumophila* induces dose
211 dependent *G. mellonella* mortality.

212

213 **The growth phase of *L. pneumophila* influences the kinetics of *G. mellonella* mortality**

214 During its lifecycle, *L. pneumophila* alternates between replicative and transmissive forms
215 (19). A number of virulence factors that promote infection of new host cells are down-
216 regulated in the replicative phase and up-regulated in the transmissive phase. In broth culture,

217 the transmissive traits are repressed in the exponential phase and expressed as the bacteria
218 enter the post-exponential phase (19). In order to assess if expression of the transmissive
219 traits were important to induce *G. mellonella* mortality, insects were inoculated with 10^7 CFU
220 *L. pneumophila* 130b cultured to exponential (OD_{600}~0.4), post-exponential (OD_{600}~3) or
221 stationary (OD_{600}>4) phases. Over the entire time course, significantly more (P<0.0005 at 18
222 h p.i.) larvae injected with exponential and stationary phase bacteria survived than the ones
223 inoculated with post-exponential phase bacteria (Fig. 1C). This indicates that the growth
224 phase of *L. pneumophila* influences virulence in the *G. mellonella* model.
225
226 **The *L. pneumophila* Dot/Icm T4SS is essential for *G. mellonella* infection**
227 The Dot/Icm T4SS of *L. pneumophila* is essential for intracellular survival and the
228 establishment of a replicative vacuole (4, 45). The 130b ΔDotA strain has a kanamycin
229 resistance cassette inserted in the *dotA* gene resulting in a non-functional T4SS (43).
230 Infection of *G. mellonella* with 10^7 CFU 130b ΔDotA did not cause any mortality of the
231 larvae over the three days of the experiment, whereas the parental wild type strain killed all
232 larvae within 24 h (Fig. 1D). This demonstrated that *L. pneumophila*-induced mortality of *G.*
233 *mellonella* is dependent on the presence of a functional Dot/Icm T4SS.
234
235 ***G. mellonella* mortality depends on *L. pneumophila* persistence**
236 In order to determine the viable bacterial load within the haemolymph of *G. mellonella*
237 infected with *L. pneumophila,* larvae were injected with 10^7 CFU wild type or ΔDotA 130b.
238 At selected time points, haemolymph from three living larvae was extracted, pooled and the
239 number of CFU/100 µl of extracted haemolymph was determined (Fig. 2A). The 130b
240 ΔDotA mutant was cleared from the injected larvae by 24 h p.i. and did not exhibit any
241 replication. On the contrary, infection of *G. mellonella* with wild type 130b resulted in an

initial 10-fold reduction of CFU 5 h p.i., but the bacterial numbers then increased up to 100-fold from the inoculum until 24 h p.i., demonstrating that *L. pneumophila* is able to replicate in *G. mellonella*.

To analyze if *L. pneumophila* was replicating intracellularly in the haemolymph, haemocytes were extracted from infected *G. mellonella* at 5 and 24 h p.i. and immuno-stained for external and total bacteria (Fig. 2B). By 5 h p.i. both wild type and ΔDotA 130b were found inside (green bacteria) and attached to (yellow bacteria) haemocytes. By 24 h p.i. haemocytes extracted from *G. mellonella* infected with wild type bacteria were full of intracellular *L. pneumophila*, whereas no bacteria could be found in haemocytes of *G. mellonella* infected with the 130b ΔDotA strain (data not shown). This result indicates that *L. pneumophila* replicates in *G. mellonella* haemocytes.

In order to determine the impact of *L. pneumophila* persistence and intracellular replication on *G. mellonella* mortality, we tested two closely related strains derived from the *L. pneumophila* strain Philadelphia-1, JR32 and Lp02 with JR32 ΔIcmT as a T4SS-deficient control. While both JR32 and Lp02 encode a functional Dot/Icm T4SS, the latter is a thymine auxotroph showing reduced intracellular survival and replication in cultured cells in the absence of added thymine or thymidine (4). Quantification of the CFU extracted from the haemolymph over 24 h (Fig. 2C) showed that the JR32 persisted in injected *G. mellonella* throughout the infection, while the JR32 ΔIcmT strain, which does not have a functional T4SS, was cleared within 18 h. The Lp02 strain persisted to higher CFU than the JR32 ΔIcmT strain 18 h p.i., before ultimately cleared by 24 h p.i. While JR32 killed all the infected insects, both the Lp02 and JR32 ΔIcmT strains were unable to cause death in injected *G. mellonella* over three days p.i., (Fig. 2D). These data indicate that a functional T4SS which enables the Lp02 strain to translocate effectors during the first hours of infection (Fig. 4) is not sufficient to induce death of the larvae. The mortality of *L. pneumophila*-

injected *G. mellonella* depends therefore on both the T4SS and the ability of the bacteria to persist within the larvae for more then 18 h.

L. pneumophila resides in a LCV in haemocytes

In order to assess if *L. pneumophila* forms a LCV in haemocytes, we analyzed haemocytes from infected *G. mellonella* by transmission electron microscopy (TEM) (Fig. 3). By 5 h p.i *L. pneumophila* 130b was observed within distinct vacuoles, which were associated with mitochondria and ribosomes. As the infection progressed, more bacteria could be seen within the LCV until the majority of haemocytes were filled with bacteria. By 24 h p.i. the LCV was studded with ribosomes. *L. pneumophila* therefore appears to reside in haemocytes of infected *G. mellonella* in LCVs, which are similar to those seen in human monocytes (21).

To further characterize the LCVs formed in haemocytes, we evaluated the recruitment of SidC, a T4SS *L. pneumophila* effector previously shown to bind the LCV membrane through interaction with phosphatidylinositol-4 phosphate (PI4P) (51). A 4HA epitope-tagged $SidC_{41-918}$ was expressed in *L. pneumophila* and the localization of the protein was analyzed by immunofluorescence (Fig. 4). To ensure the protein was expressed, larvae were injected with bacterial suspension containing 1 μM IPTG. The presence of IPTG alone did not affect survival (data not shown). Similarly to human A549 cells (data not shown), anti-HA staining of $SidC_{41-918}$ surrounded intracellular bacteria in haemocytes. No anti-HA staining was observed in the control haemocytes extracted from larvae infected with *L. pneumophila* 130b ΔDotA expressing 4HA-$SidC_{41-918}$. At 24 h p.i. haemocytes from *G. mellonella* infected with wild type 130b were full of bacteria surrounded by 4HA-$SidC_{41-918}$-stained LCVs. Similar results were obtained with the thymine prototroph strain JR32. In accordance with the results presented in Fig. 2C, the thymine auxotroph strain Lp02 did not show evidence of replication 24 h p.i. yet it displayed recruitment of 4HA-$SidC_{41-918}$ to the LCV membrane at both 5 and

24 h p.i. These results indicate that similar to infection of protozoan or mammalian host cells, *L. pneumophila* is able to translocate a T4SS-substrate and to form an LCV in *G. mellonella* haemocytes.

G. mellonella **pathology in response to** ***L. pneumophila*** **infection**

In order to examine the effect of *L. pneumophila* infection on *G. mellonella* physiology, the infected larvae were fixed and paraffin embedded sections were stained with haematoxylin and eosin (H&E) and evaluated for histological changes (Fig. 5). Mock-infected controls appeared healthy with no bacteria observed in the haemocoel and individually distributed haemocytes occasionally forming loose aggregations. However, in both wild type and ΔDotA-infected insects, vigorous host defenses appeared to be mounted. At 16 h p.i. with 130b ΔDotA, fewer individual haemocytes were observed compared to the mock infected control, with the majority of haemocytes present in tightly packed aggregation nodules and some evidence of melanisation. By 24 h p.i. we observed similar features, but the majority of the tissue looked healthy. In larvae infected with wild type bacteria at 16 h p.i. haemocytes were observed in nodules attached to organ structures, with clearly visible nodule melanisation. By 24 h p.i. nodules were still observed however septicemia was found in much of the haemocoel and organ structures including the gut appeared severely damaged.

In order to confirm that the bacteria observed in formalin fixed sections of the infected *G. mellonella* were *L. pneumophila,* sections were stained using a specific anti-*L. pneumophila* antibody. DNA was visualized by DAPI staining and the tissue structure was counter strained using rhodamine-conjugated phalloidin (Fig. 6). Anti-*L. pneumophila* antibodies did not stain any bacteria in the uninfected or 130b ΔDotA infected insects at 18 h p.i. In the *G. mellonella* infected with wild type 130b, bacteria stained with the anti-*L. pneumophila* antibody were found throughout the haemolymph (Fig. 6) and occasionally in cells within the fat bodies (not

317 shown). Bacteria were exclusively associated with cells and were usually found in aggregates
318 of haemocytes.
319 Altogether, these data indicate that *L. pneumophila* triggers an immune response in *G.*
320 *mellonella* that successfully clears the ΔDotA mutant from the larvae, whereas wild type *L.*
321 *pneumophila* are resistant to host defenses.
322
323 **The *G. mellonella* immune responses to *L. pneumophila* infection**
324 Progression of *L. pneumophila* infection resulted in an increase in *G. mellonella* pigmentation
325 (Fig. 7A), which is usually indicative of activation of the PO. Upon recognition of pathogen
326 associated molecular patterns (PAMPs), the pro-PO system components are released from
327 haemocytes into the haemolymph, leading to activation of PO. The activity of this enzyme
328 subsequently induces the formation of quinones and melanin, which are involved in defense
329 reactions against pathogens invading the haemocoel, such as nodule formation and
330 encapsulation (8). In order to quantify this innate immune response, we assayed at selected
331 time points the level of PO activity in the haemolymph of *G. mellonella* infected with 130b
332 (Fig. 7B). By 5 h p.i. insects injected with wild type *L. pneumophila* exhibited dramatically
333 increased PO activity compared to larvae injected with PBS ($P<0.005$). Larvae inoculated
334 with *L. pneumophila* ΔDotA presented an intermediate level of PO activity. By 18 h p.i. the
335 level of PO activity did not significantly change in the PBS and 130b ΔDotA injected *G.*
336 *mellonella*. However in *G. mellonella* injected with wild type bacteria, levels of PO activity
337 significantly dropped compared to 5 h p.i. ($P<0.005$), reaching levels similar to the PBS
338 control. These results indicate that *L. pneumophila* infection initiates an immune defense in *G.*
339 *mellonella* through PO activation, a response which is nonetheless abrogated by 18 h p.i.
340 In order to test if the absence of PO activity at 18 h p.i. could be due to haemocyte depletion,
341 insects were infected with wild type or ΔDotA 130b, or injected with PBS as a control and

342 haemocytes were counted by light microscopy at 5 and 18 h p.i. (Fig. 7C). At 5 h p.i. the
343 concentration of haemocytes per ml of haemoplymph was comparable in the different groups.
344 However, by 18 h p.i. the number of haemocytes was reduced by almost 90% in *G.*
345 *mellonella* inoculated with wild type 130b as compared to 5 h p.i. or the controls, suggesting
346 that *L. pneumophila* infection induces haemocyte destruction. The reduction in haemocyte
347 number observed is likely to contribute to the decreased PO activation observed 18 h p.i.
348 A major component of the defense response of insects is the production of antimicrobial
349 peptides (AMP) (29, 31). In order to assess if *G. mellonella* produced AMPs following *L.*
350 *pneumophila* infection, their expression was tested in fat bodies, where they are mainly
351 produced. Semi-quantitative RT-PCR on extracted mRNA showed that infection with wild
352 type *L. pneumophila* resulted in an up-regulation of most of the immune-related peptides
353 tested compared to a PBS-injected control (Fig. 8), with a significantly increased expression
354 of gloverin and pro-PO (PPO) as soon as 2 h p.i. ($P<0.006$). In contrast, gallerimycin,
355 galliomycin and the iron binding protein transferrin were significantly up-regulated only after
356 18 h of infection ($P<0.0005$ for gallerimycin and transferrin and $P<0.005$ for galliomycin).
357 The expression of the peptidoglycan recognition protein B (PRPB) did not significantly
358 increase upon inoculation with wild type *L. pneumophila*. Injection with ΔDotA did not cause
359 significant change from the baseline level with the exception of the AMP gloverin, the
360 mRNA level of which increased after 24 h ($P<0.01$). These results show that *G. mellonella*
361 mounts an immune response to *L. pneumophila* infection that nonetheless is not effective in
362 clearing the wild type bacteria.

Discussion

Adequate infection models that approximate human disease are the key to analyze the molecular basis of bacterial pathogenesis. Substantial advances in our knowledge about their genetics and immune responses have led to the increased use of insects as surrogate hosts. In particular, the larvae of the greater wax moth *Galleria mellonella* have recently been reported as easy-to-use model organism for several pathogenic Gram-positive and Gram-negative bacteria (23, 36). These studies demonstrated a good correlation between the *G. mellonella* and mammalian infection models (10, 23, 36). In this study we characterized *G. mellonella* as new infection model for *L. pneumophila*.

Using three prototypic *L. pneumophila* strains, we found that *G. mellonella* could withstand a low infectious dose but the larvae succumbed to infection with higher doses. At the highest dose all three tested strains caused substantial death of the larvae; however the kinetics of lethality differed with *L. pneumophila* strain 130b being more virulent than strains JR32 and Paris. Although a systematic comparison of the virulence phenotypes of all the three strains in amoeba or mammalian models has not been reported, strain 130b was previously shown to replicate more efficiently than JR32 following intra-tracheal infection of A/J mice (40). In a comparative assessment of the virulence traits of 27 *L. pneumophila* and *non-pneumophila* strains, 130b was the third-most cytopathogenic strain (2). Taken together this indicates that the *G. mellonella* model can reproduce strain-to-strain variations in virulence observed in mammalian cell culture and animal models, which makes it a quick and inexpensive tool to compare the virulence of different *L. pneumophila* isolates or *Legionella* species.

The Dot/Icm T4SS of *L. pneumophila* is essential for infection of amoeba, human macrophages, mice, and *D. melanogaster* (4, 27, 42, 45). The *D. melanogaster* model has been successfully used to demonstrate the contribution of the Dot/Icm effector LubX to *L. pneumophila* replication and fly lethality. We found that *L. pneumophila*-induced mortality of

388 *G. mellonella* also depended on a functional Dot/Icm T4SS. A T4SS-deficient mutant did not
389 show any virulence even at the highest (10^7 CFU) inoculum injected. This contrasts
390 observations described for the *G. mellonella* model of *Listeria* infection, in which
391 nonpathogenic strains with increasing doses up to 10^7 CFU per larvae also induced mortality
392 (23, 34). It was proposed that this could be attributed to a form of sepsis, and subsequent
393 death was caused by bacterial overload and was not due to specific virulence factors. Our
394 data indicates that the threshold at which bacterial load triggers sepsis and death may vary
395 from pathogen to pathogen.
396 Although the *Drosophila* model was used to determine virulence phenotypes of *L.*
397 *pneumophila* mutants in the fly, further aspects underlying *L. pneumophila* pathogenesis in
398 the insect have not been characterized (27). We show for the first time that *L. pneumophila*
399 resides in a vacuole in haemocytes isolated from infected insects. This vacuole
400 ultrastructurally resembled the LCV observed in human macrophages and amoeba, including
401 association of mitochondria, acquisition of a rough ER-like structure (1, 21) and recruitment
402 of SidC, which was previously shown to be tethered to the LCV via a phosphatidylinositol-4
403 phosphate anchor (51). The recruitment of ribosomes and the T4SS-substrate SidC to the
404 haemocyte LCV suggests that *L. pneumophila* uses at least some of the fundamental
405 strategies which are employed to establish a replicative vacuole in mammalian cells and
406 amoeba also to infect insect haemocytes.
407 Analysis of *L. pneumophila* replication *in G. mellonella* by direct bacterial enumeration
408 demonstrated that, following an initial 10-fold reduction in CFU of wild type bacteria at 5 h
409 p.i., bacterial CFU quickly recovered and increased by 100-fold from the inoculum by 24 h
410 p.i. The ΔDotA mutant was cleared by 24 h p.i. The level of *L. pneumophila* replication
411 appears to be higher than in the mouse model, in which the strain 130b could exhibit up to 20
412 fold increase of CFU within 48 h (6, 40) or in the *Drosophila* model in which an increase of

CFU up to 20 fold within 10 days was reported (27). The importance of bacterial persistence for *L. pneumophila* virulence in the *G. mellonella* model is demonstrated by the fact that *L. pneumophila* strain Lp02, which did not persist after 18 h p.i. was unable to kill *G. mellonella* despite having a functional T4SS and forming a LCV in haemocytes. Moreover, the 130b strain, which replicated better in the larvae than the JR32 strain, induced death more rapidly than the JR32 strain, suggesting that in addition to persistence, bacterial replication also contributes to *L. pneumophila* virulence in the *G. mellonella* model.

These data suggest a scenario in which immune cells successfully clear a fraction of the inoculated *L. pneumophila* at early stages of infection. However, enough wild type bacteria evade destruction by phagocytes and start replicating. Release from haemocytes following replication is most likely accompanied with destruction of the haemocytes. This model is supported by the fact that 90% of the haemocytes are lost by 18 h p.i. following wild type *L. pneumophila* infection. Depletion of circulating haemocytes upon bacterial infection has previously been reported and correlated with *G. mellonella* mortality caused by pathogenic fungi and Gram-negative bacteria (9, 34). This loss may be due to the death of infected haemocytes or the sequestration of haemocytes in nodules or a combination of both. However, nodules were observed in wild type and ΔDotA infected *G. mellonella* and there was no significant loss of haemocytes in ΔDotA infected larvae, suggesting that replication and T4SS-dependent toxicity are the most likely cause of the loss of cells.

Depletion of haemocytes, the major source of pro-phenoloxidase (pro-PO) which triggers the melanisation response upon infection, would also explain why we observed an initial activation of PO which was followed by a sharp drop at 18 h post infection. An alternative hypothesis is that *L. pneumophila* may also specifically reduce PO activity; indeed, the insect pathogen *Photorhabdus luminescens* can inhibit PO activity at 18 h post infection (15). In conclusion, we demonstrate that *G. mellonella* is susceptible to *L. pneumophila* infection and

that this model reproduces virulence phenotypes observed in amoeba and mammalian infection models. Virulence depends on the Dot/Icm T4SS and bacteria seem to reside and replicate in a typical LCV. Future advances in our knowledge about the *G. mellonella* immune gene repertoire (50) cell death pathways (25) and haemocyte biology (28, 30) together with initiatives to advance RNA interference systems in *Lepidoptera* spp. (49) will further increase the value of *G. mellonella* as an infection model which could potentially be used to study the role of Dot/Icm T4SS effectors, the T2SS and other factors in virulence of *Legionella* spp.

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454

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622 **Table 1**

Primer	**Sequence**
Gallerimycin FW	GAAGATCGCTTTCATAGTCGC
Gallerimycin RV	TACTCCCTGCAGTTAGCAATGC
Prophenoloxidase FW	CCGCGAACACCGATCATCATTCCAAG
Prophenoloxidase RV	GTGCACGCTTCCGTAGAGTTCCCGG
Gloverin FW	CGGTAGTCGGGTGTTGAGCCCGTATG
Gloverin RV	CGTCTGATACGATCGTAGGTGCC
Peptidoglycan recognition protein B FW	GGTCATCATCCAGCATACAGTGACG
Peptidoglycan recognition protein B RV	CCATCCAGTTGGGCCAGCTTCTTAT
Transferrin FW	CCCGAAGATGAACGATCAC
Transferrin RV	CGAAAGGCCTAGAACGTTTG
Galliomicin FW	CCTCTGA TTGCAA TGCTGAGTG
Galliomicin RV	GCTGCCAAGTTAGTCAACAGG
Actin FW	GGGACGATATGGAGAAG
Actin RV	CACGCTCTGTGAGGATCT
All sequences from (24)	

623 **Figure Legends**

624 **Figure 1. *L. pneumophila* infection of *Galleria mellonella* induces dose- and Dot/Icm**
625 **T4SS-dependent lethality.** *G. mellonella* larvae were injected with PBS or *L. pneumophila*
626 *strain* 130b, Paris or JR32 (10^7 CFU per larvae, if not otherwise indicated) and survival was
627 monitored over 72 h p.i. **(A)** All three strains caused time-dependent death of the infected
628 larvae, with strain 130b inducing significantly higher ($P<0.005$) mortality at 18 h p.i. **(B)**
629 Mortality of the larvae upon infection with *L. pneumophila* strain 130b was dose-dependent.
630 **(C)** Larvae survival was dependent on the growth phase of *L. pneumophila*. Larvae were
631 inoculated with *L. pneumophila* 130b cultured to exponential (E), post exponential (PE) or
632 stationary (S) phase. Bacteria in post exponential phase demonstrated significantly ($P<0.005$)
633 higher toxicity than bacteria in other growth phases at 18h p.i. **(D)** *L. pneumophila*-induced
634 mortality in *G. mellonella* was dependent on the Dot/Icm T4SS. *G. mellonella* were injected
635 with *L. pneumophila* 130b wild type or T4SS-deficient strain ΔDotA. The T4SS mutant did
636 not induce any mortality in the larvae 72 h p.i. Results represent the mean of at least three
637 independent experiments ± standard deviations with 10 larvae per condition.
638
639 **Figure 2. *L. pneumophila* is able to persist and replicate in *G. mellonella*.** Haemolymph
640 from three *L. pneumophila*-infected *G. mellonella* was extracted and the CFU/100µl were
641 quantified. **(A)** Wild type *L. pneumophila* 130b replicated within the larvae over the infection
642 course, while the ΔDotA mutant was cleared from *G. mellonella* by 24 h p.i. **(B)** *L.*
643 *pneumophila* 130b invades and replicates within haemocytes. External and total bacteria were
644 immuno-stained. By 5 h p.i. both wild type and ΔDotA bacteria were found inside cells. By
645 24 h p.i., wild type infected haemocytes had high loads of intracellular bacteria. **(C and D)** *G.*
646 *mellonella* mortality depends on *L. pneumophila* persistence. *G. mellonella* was inoculated
647 with *L. pneumophila* strains JR32, JR32 ΔIcmT or Lp02. **(C)** While the JR32 ΔIcmT strain

was rapidly killed, the thymine auxotroph strain Lp02 declined slowly until 18 h p.i., before being cleared until 24 h p.i. JR32 persisted at higher level throughout the course of infection. **(D)** Only the wild type JR32 strain, but neither the ΔIcmT nor Lp02 strain induced mortality in *G. mellonella* by 72 h p.i. Results are representative of at least two independent experiments.

Figure 3. *L. pneumophila* forms a LCV in *G. mellonella* haemocytes. Haemocytes from *G. mellonella* infected with *L. pneumophila* 130b were extracted 5, 12 and 24 h p.i. and imaged by transmission electron microscopy. **(A)** At 5 h p.i., a few bacteria could be observed in distinct vacuoles within haemocytes. As the infection progressed, more bacteria per vacuole were found, until cells appeared filled with bacteria by 24 h p.i. Scale bar represents 2 µm **(B)** At 5 h p.i., mitochondria (arrowheads), ribosomes (arrows) and ribosome-associated vesicles were observed on the surface of the LCV. By 24 h p.i. the LCV was studded with ribosomes (arrows). Scale bar represents 500 nm.

Figure 4. SidC is localized to the LCV in haemocytes of infected *G. mellonella*. *G. mellonella* larvae were injected with *L. pneumophila* strains 130b, JR32 or Lp02 overexpressing 4HA-SidC$_{41\text{-}918}$. At 5 and 24 h p.i., haemocytes were extracted, fixed and stained with anti-HA antibody. By 5 h p.i. anti-HA staining revealed that SidC$_{41\text{-}918}$ was localized on the LCV surface in haemocytes extracted from *G. mellonella* infected with wild type *L. pneumophila* strains but not 130b ΔDotA. By 24 h p.i., haemocytes from *G. mellonella* infected with 130b or JR32 were full of bacteria surrounded by 4HA-SidC$_{41\text{-}918}$. In contrast, far fewer bacteria were observed in haemocytes from *Galleria* infected with strain Lp02. Scale bar represents 5 µm.

673 **Figure 5. *L. pneumophila* infection of *G. mellonella* initiates a robust innate immune**
674 **response.** *G. mellonella* larvae were injected with *L. pneumophila* 130b, fixed and paraffin
675 embedded sections were stained with H&E. Uninfected *G. mellonella* appeared healthy with
676 some occasional loose aggregations of haemocytes (A). At 16 h p.i. with wild type bacteria a
677 number of nodules (N) could be observed with evidence of melanisation (arrows). At 16 h p.i.
678 with ΔDotA some nodules were visible but by 24 h p.i., the larvae appeared similar to the
679 uninfected control. At 24 h p.i. with the wild type bacteria, some nodules were still visible
680 but a large number of *L. pneumophila* were visible in the haemcoel (B).
681
682 **Figure 6. Indirect immunofluorescence microscopy of formalin fixed sections of *L.***
683 ***pneumophila* infected *G. mellonella*.** *G. mellonella* larvae were infected with *L.*
684 *pneumophila* 130b for 18 h, fixed and paraffin embedded sections were stained using a
685 specific anti-*L. pneumophila* antibody, DAPI was used to visualize bacterial and eukaryotic
686 cell DNA and phalloidin to counter stain the tissue. No *L. pneumophila* staining was
687 observed in the uninfected or ΔDotA controls. *G. mellonella* infected with wild type *L.*
688 *pneumophila* demonstrated a systemic infection with large numbers of bacteria in the
689 haemolymph. Bacteria were usually associated with cells (arrowheads), a proportion of which
690 displayed apoptotic nuclei (arrows). Scale bar represents 20 µm.
691
692 **Figure 7. Characterization of the *G. mellonella* innate immune response to *L.***
693 ***pneumophila* infection.** *G. mellonella* larvae were infected with *L. pneumophila* 130b. **(A)**
694 Larvae and extracted haemolymph became progressively darker over the course of the
695 infection, indicative of melanin production by phenoloxidase (PO). **(B)** PO activity was
696 quantified in the plasma of infected *Galleria* at 5 and 18 h p.i. In larvae infected with wild
697 type *L pneumophila*, PO activity increased dramatically at 5 h p.i. and was almost abolished

698 at 18 h p.i. **(C)** Haemocyte concentration was recorded at 5 and 18 h p.i. with *L. pneumophila*
699 130b. Infection with wild type bacteria resulted in ~ 90% reduction in haemocyte
700 concentration after 18 h of infection. Results represent the mean of three independent
701 experiments ± standard deviations with three larvae per condition. *** P<0.005.
702
703 **Figure 8. *L. pneumophila* infection of *G. mellonella* up-regulates the expression of**
704 **antimicrobial peptides**. Larvae were injected with PBS, wild type *L. pneumophila* 130b, or
705 ΔDotA. Fat bodies of three infected *G. mellonella* were harvested and pooled at indicated
706 time points. Semi-quantitative RT-PCR was performed and the results were normalized to
707 actin mRNA expression. Larvae infected with wild type bacteria demonstrated increased
708 expression of antimicrobial peptides. Results are the mean of three independent experiments
709 ± standard deviation.
710

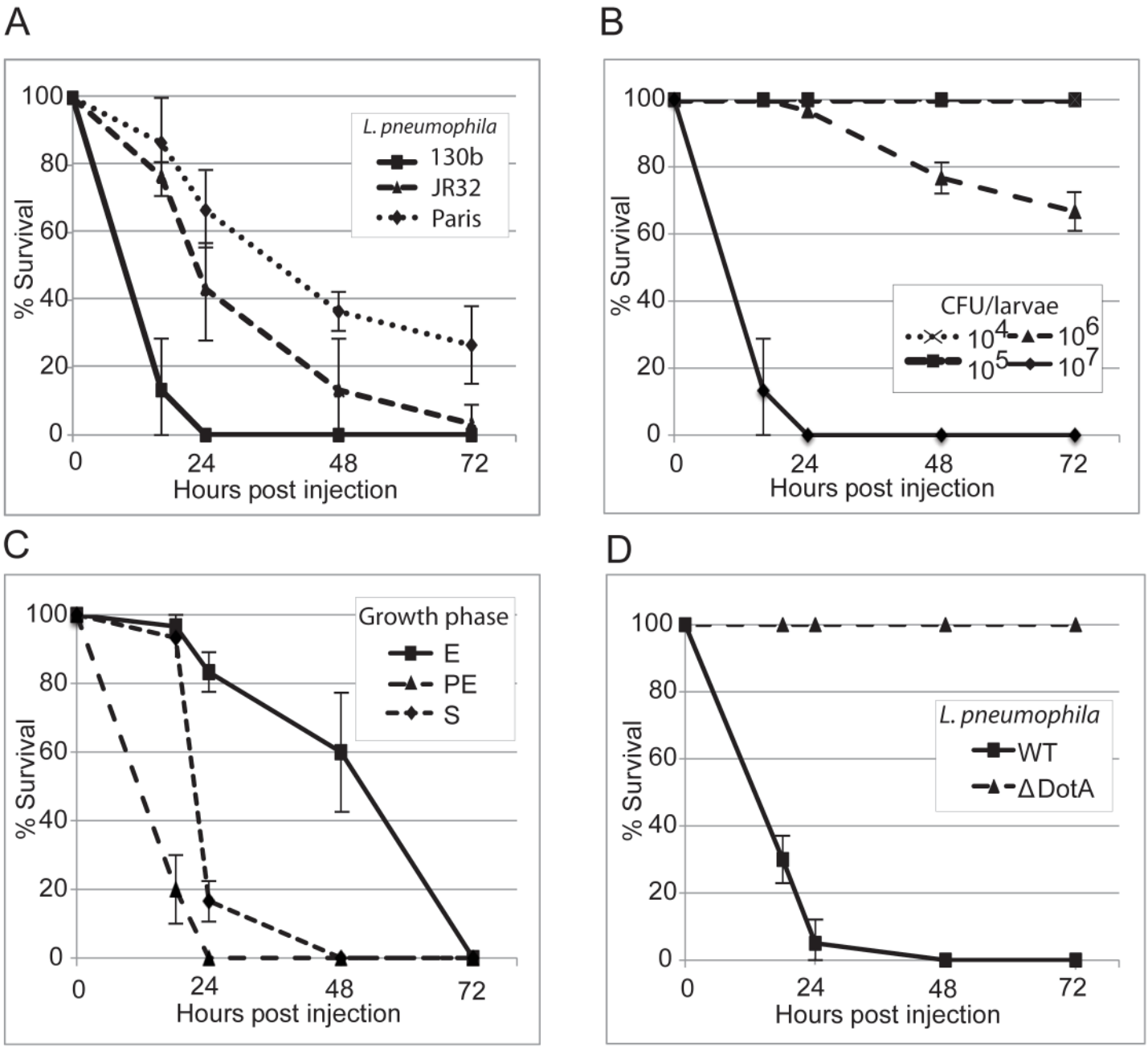
A
100
80
60
40
20
0
% Survival
0
24
48
72
Hours post injection
L. pneumophila
130b
JR32
Paris
B
100
80
60
40
20
0
% Survival
0
24
48
72
Hours post injection
CFU/larvae
10^4
10^6
10^5
10^7
C
100
80
60
40
20
0
% Survival
0
24
48
72
Hours post injection
Growth phase
E
PE
S
D
100
80
60
40
20
0
% Survival
0
24
48
72
Hours post injection
L. pneumophila
WT
ΔDotA

Figure 1

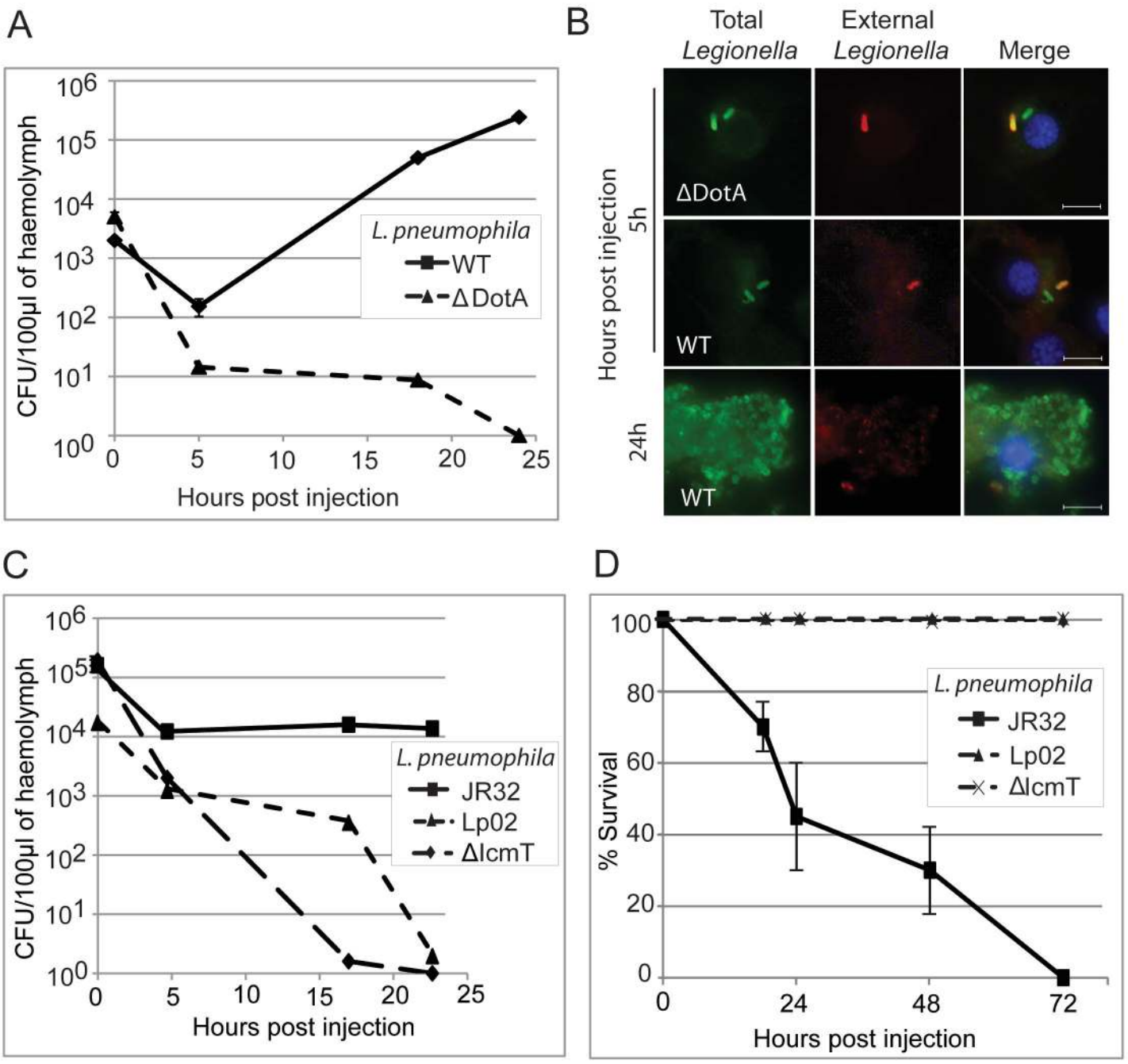
A
CFU/100µl of haemolymph
Hours post injection
L. pneumophila
WT
ΔDotA
B
Total Legionella
External Legionella
Merge
Hours post injection
5h
24h
ΔDotA
WT
WT
C
CFU/100µl of haemolymph
Hours post injection
L. pneumophila
JR32
Lp02
ΔIcmT
D
% Survival
Hours post injection
L. pneumophila
JR32
Lp02
ΔIcmT

Figure 2

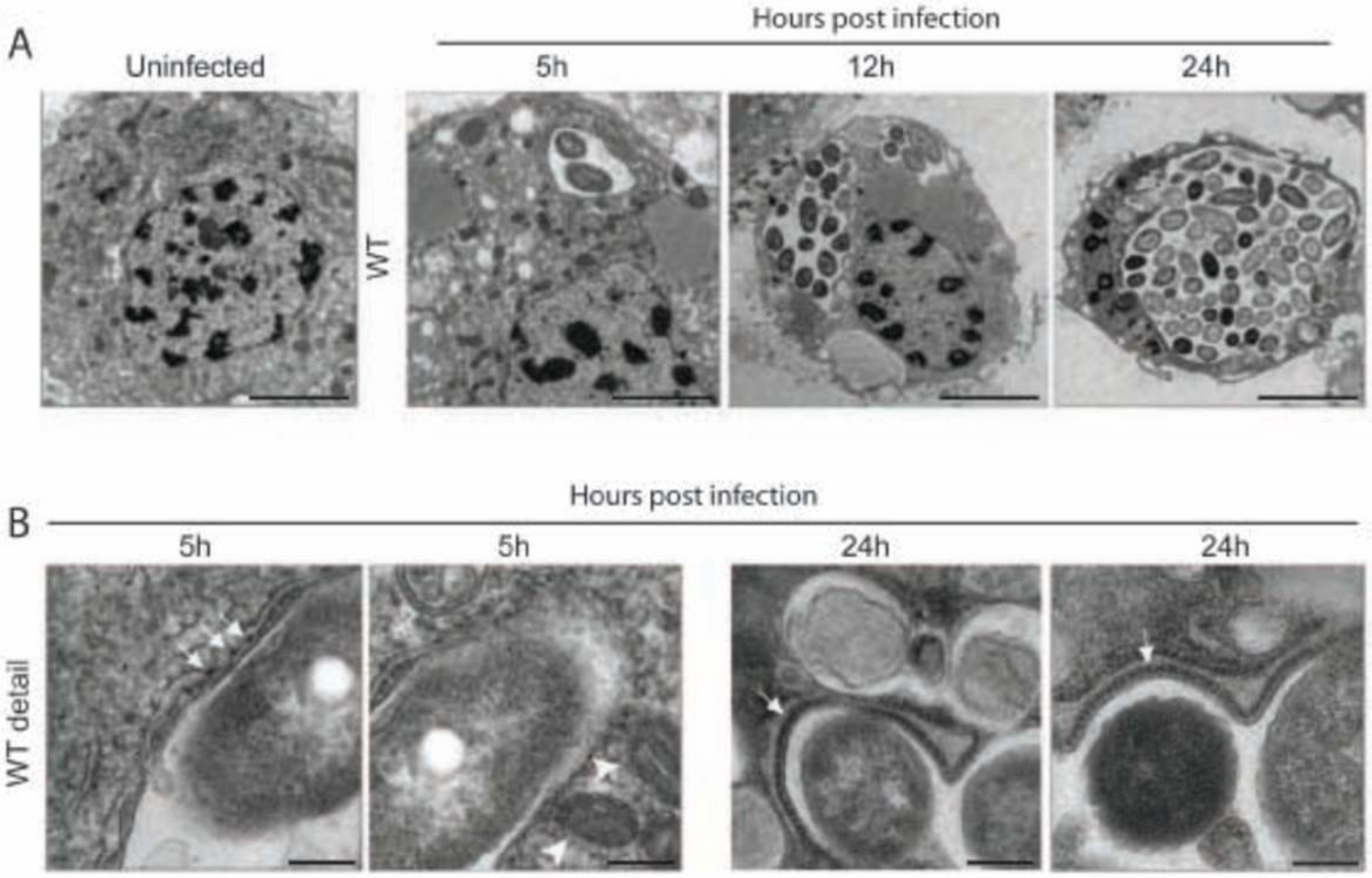
A
Hours post infection
Uninfected
5h
12h
24h
WT
B
Hours post infection
5h
5h
24h
24h
WT detail

Figure 3

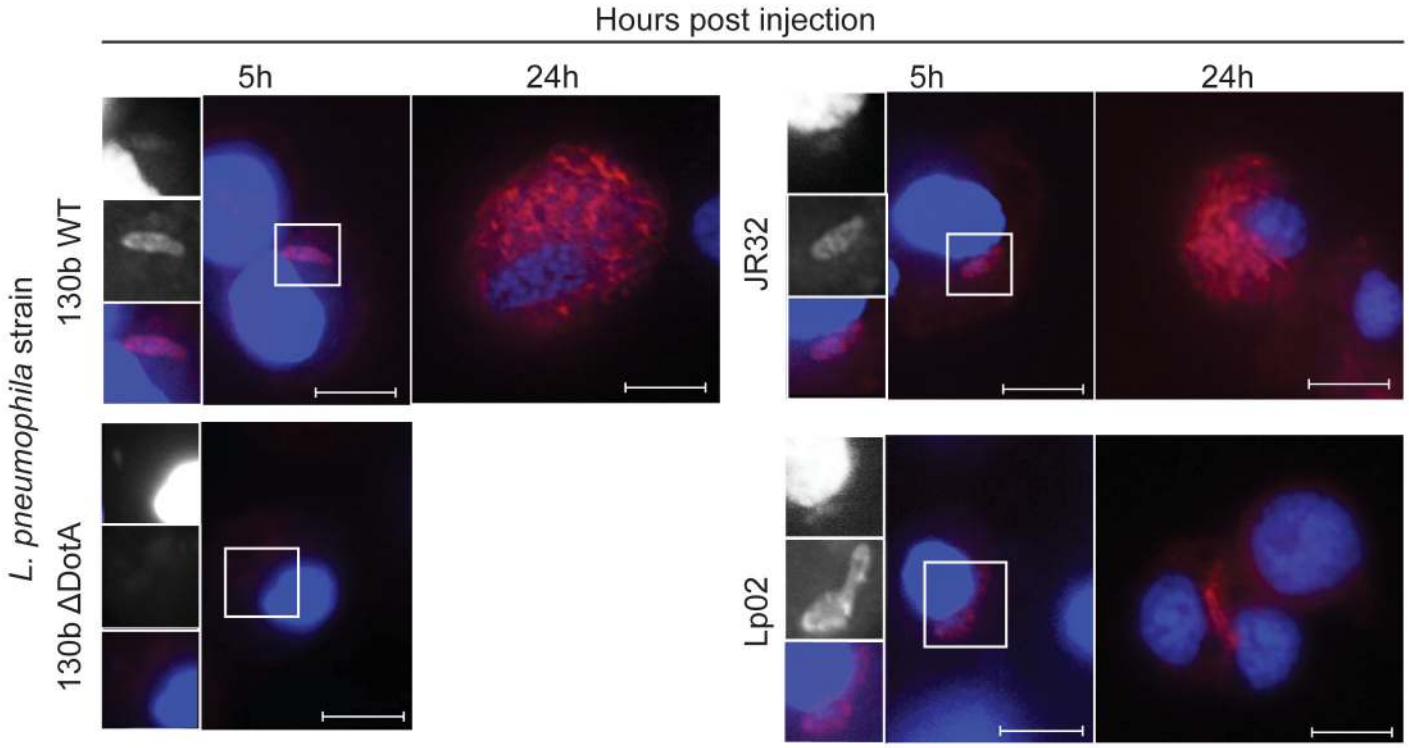
Hours post injection
5h
24h
5h
24h
L. pneumophila strain
130b WT
130b ΔDotA
JR32
Lp02

Figure 4

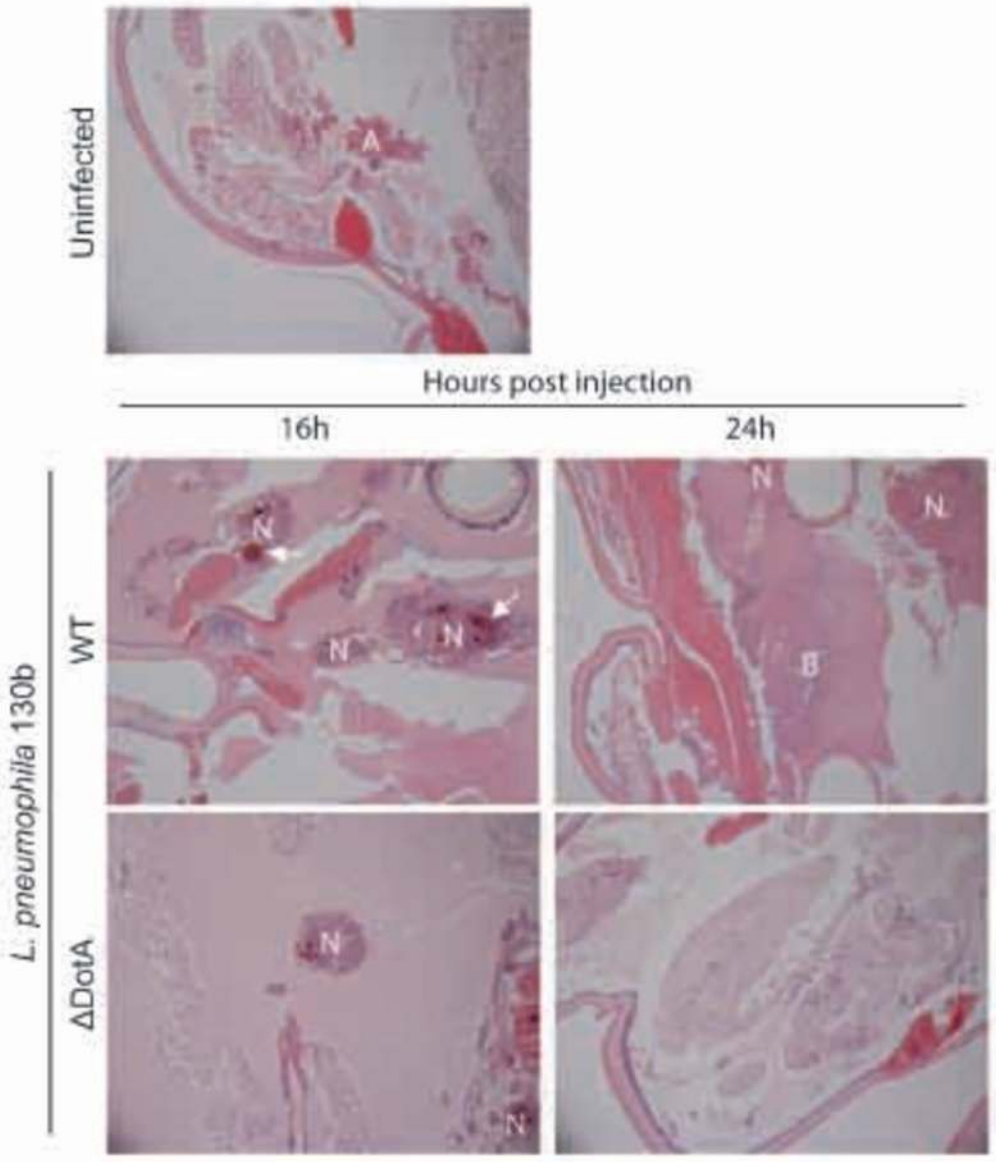
Uninfected
A
Hours post injection
16h
24h
L. pneumophila 130b
WT
ΔDotA
N
B

Figure 5

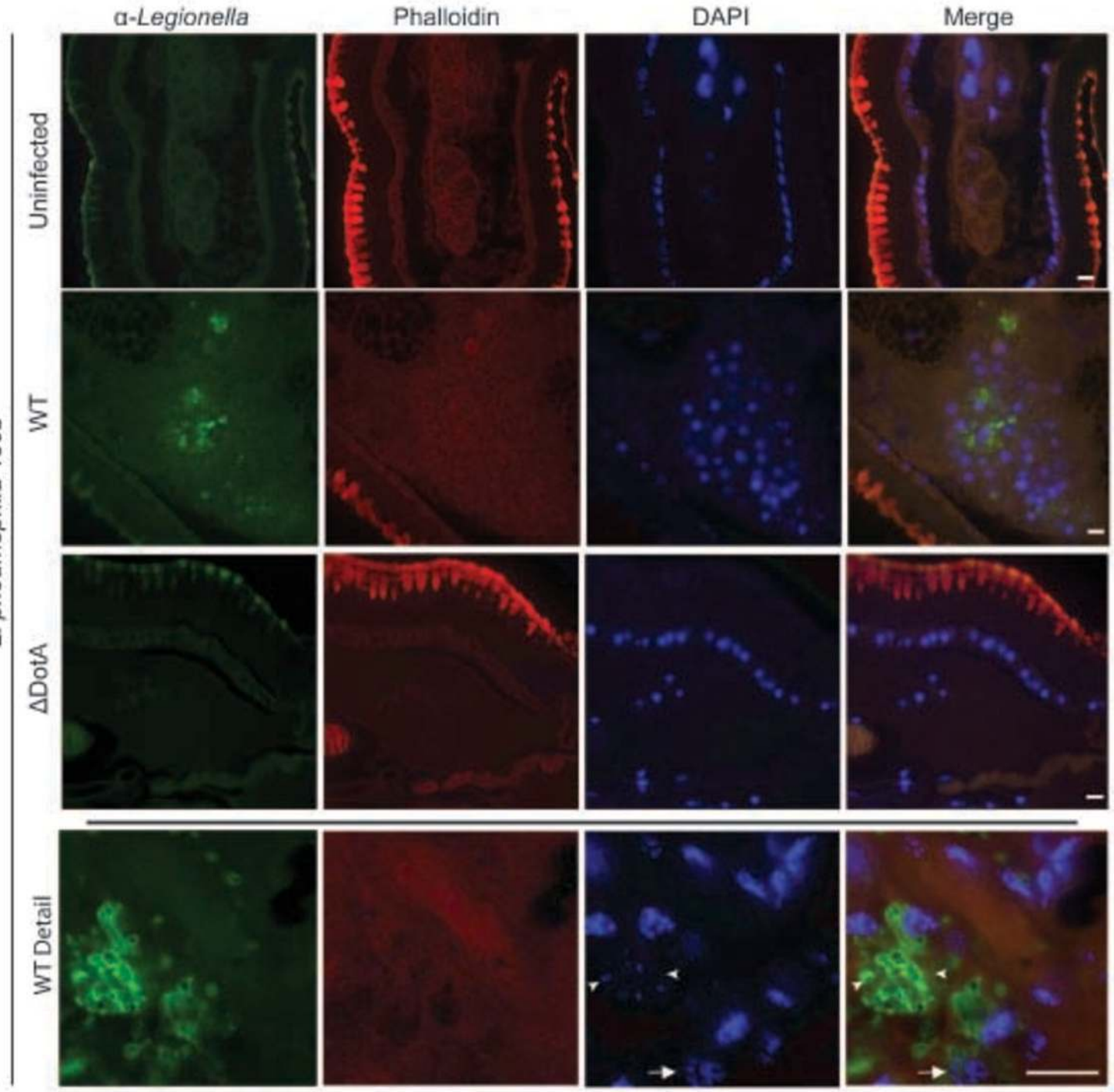
α-Legionella
Phalloidin
DAPI
Merge
Uninfected
WT
ΔDotA
WT Detail
L. pneumophila 130b

Figure 6

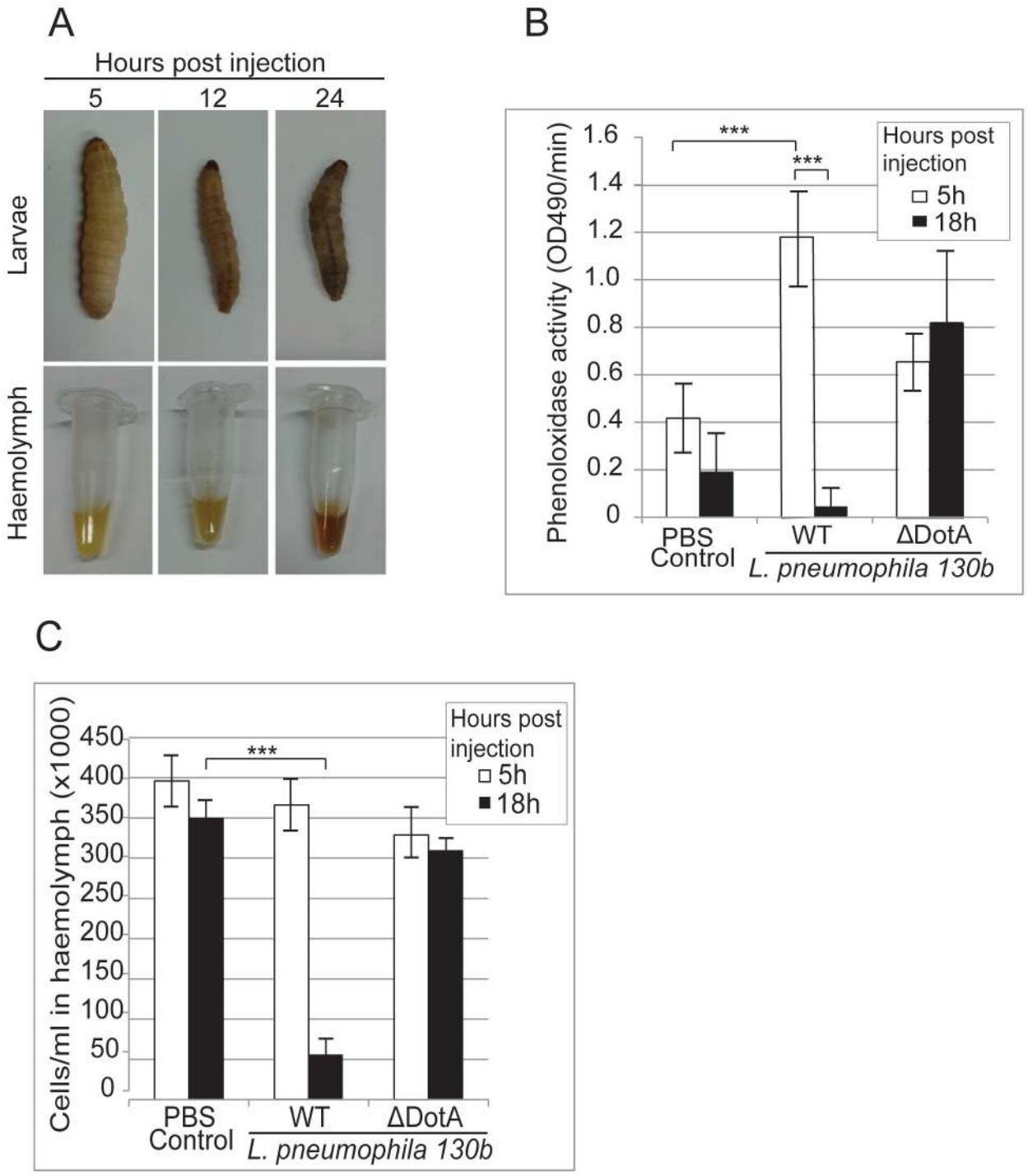
A
Hours post injection
5
12
24
Larvae
Haemolymph
B
Phenoloxidase activity (OD490/min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
***
Hours post injection
5h
18h
PBS Control
WT
ΔDotA
L. pneumophila 130b
C
Cells/ml in haemolymph (x1000)
450
400
350
300
250
200
150
100
50
0
***
Hours post injection
5h
18h
PBS Control
WT
ΔDotA
L. pneumophila 130b

Figure 7

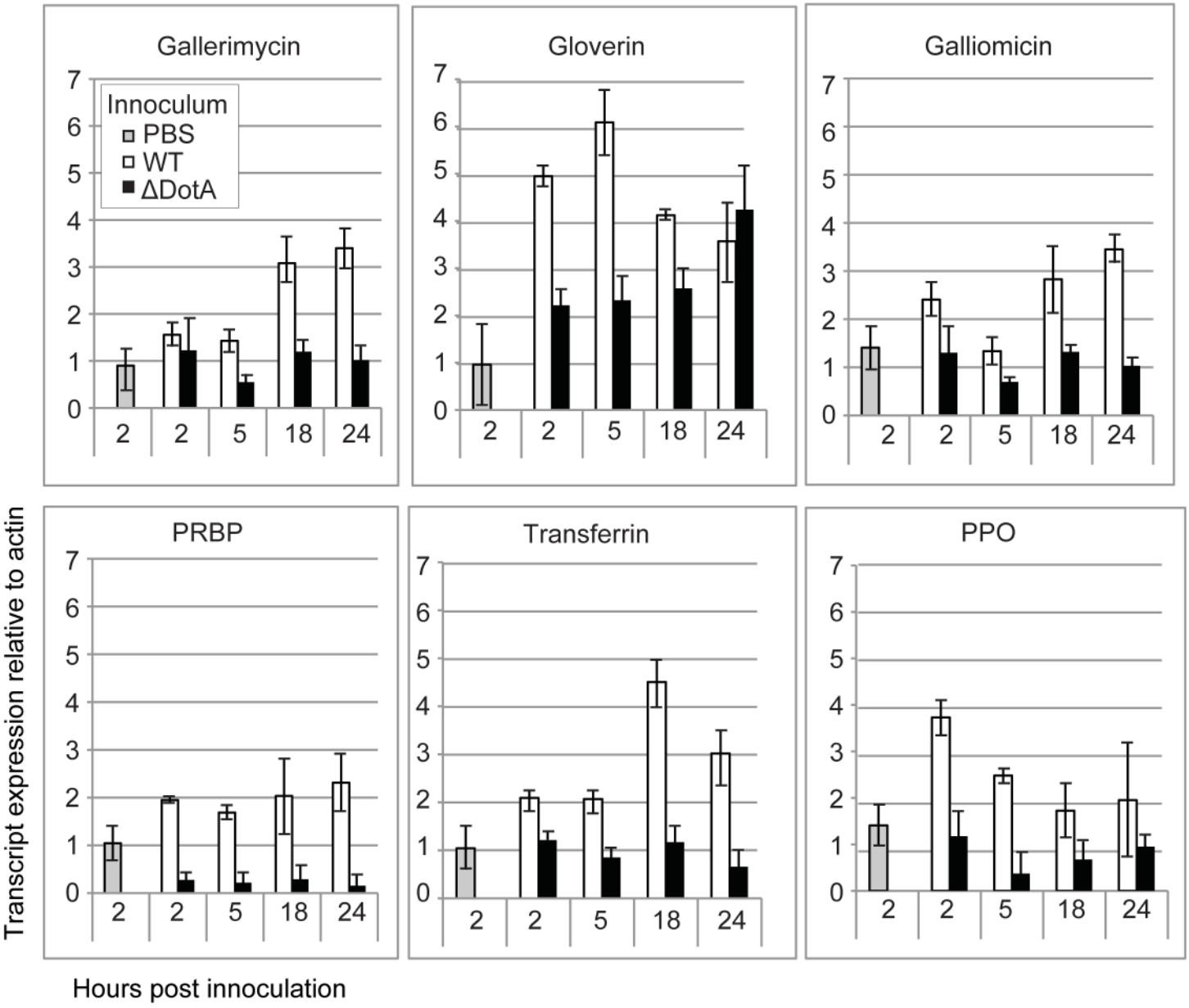
Gallerimycin
Gloverin
Galliomicin
PRBP
Transferrin
PPO
Innoculum
PBS
WT
ΔDotA
0
1
2
3
4
5
6
7
2
2
5
18
24
Transcript expression relative to actin
Hours post innoculation

Figure 8