**Legionella pneumophila Pathogenesis in the Galleria mellonella Infection Model**

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*Legionella pneumophila* is a facultative intracellular human pathogen and the etiological 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 that 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 postinfection without inducing mortality. Immunofluorescence and transmission electron microscopy revealed that *L. pneumophila* resided within insect hemocytes 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 damage to the insect organs and triggered immune responses, including activation of the phenoloxidase cascade leading to melanization, 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.

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*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 the intracellular survival and pathogenicity of *Legionella* (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 multiprotein 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 and inhibit apoptosis and immune signaling, the function of the majority of T4SS effectors during infection is still unknown (17a).

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 the virulence factors that enable *Legionella* 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 amoebae 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. (3). However, one caveat to the use of *C. elegans* is that the bacteria replicate in the intestinal lumen and do not invade intestinal epithelial cells, limiting the usefulness of this model to study the virulence determinants required for the intracellular lifestyle of *Legionella*.

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 hemocytes that phagocytose pathogens and form aggregates which encapsulate and neutralize foreign microorganisms (30). Moreover, activated hemocytes can trigger a phenoloxidase (PO) melanization cascade, leading to physical restriction of intruders and the production of antimicrobial compounds (8). Hemocyte-mediated responses are complemented by the production and secretion of antimicrobial peptides by the insect fat body, an organ similar to the mammalian liver (29, 31).

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

### MATERIALS 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/H9004 DotA* 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 *H9004 IcmT* strain is an icmT isogenic mutant in the JR32

#### Table 1 Primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Gallerimycin FW</td>
<td>GAAGATCGCTTTCATAGTCGC</td>
</tr>
<tr>
<td>Gallerimycin RV</td>
<td>TACTCCCTGACATTAGCAATG</td>
</tr>
<tr>
<td>Prophenoloxidase FW</td>
<td>CCGCGAACACCGATCATCATTCAAG</td>
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<td>Prophenoloxidase RV</td>
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<tr>
<td>Gloverin FW</td>
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<td>Gloverin RV</td>
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</tr>
<tr>
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<td>PRPB RV</td>
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<tr>
<td>Actin RV</td>
<td>CACGCTCTGTGAGGATCT</td>
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</tbody>
</table>

* FW, forward; RV, reverse.
* All sequences are from reference 23.

**FIG 1** *L. pneumophila* infection of *G. mellonella* induces dose- and Dot/Icm T4SS-dependent lethality. *G. mellonella* larvae were injected with PBS or *L. pneumophila* strain 130b, Paris, or JR32 (10^7 CFU per larvae, if not otherwise indicated), and survival was monitored over 72 h p.i. (A) All three strains caused time-dependent death of the infected larvae, with strain 130b inducing significantly higher (P < 0.005) mortality at 18 h p.i. (B) Mortality of the larvae upon infection with *L. pneumophila* strain 130b was dose dependent. (C) Larval survival was dependent on the growth phase of *L. pneumophila*. Larvae were inoculated with *L. pneumophila* 130b cultured to exponential (E), post exponential (PE), or stationary (S) phase. Bacteria in postexponential phase demonstrated significantly (P < 0.005) higher toxicity than bacteria in other growth phases at 18 h p.i. (D) *L. pneumophila*-induced mortality in *G. mellonella* was dependent on the Dot/Icm T4SS. *G. mellonella* insects were injected with *L. pneumophila* 130b wild-type (WT) or the T4SS-deficient ΔDotA strain. The T4SS mutant did not induce any mortality in the larvae at 72 h p.i. Results represent the mean of at least three independent experiments ± standard deviations with 10 larvae per condition.
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 Livefood UK, Ltd., and stored at room temperature in the dark.

**Infection of G. mellonella.** L. pneumophila strains were cultured on charcoal-yeast extract (CYE) plates for 4 days and then inoculated into ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-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 optical density at 600 nm (OD₆₀₀) of 1, which corresponds to 10⁹ CFU/ml unless otherwise indicated. Gene expression in strains containing the p4HA plasmid (which carries four copies of the hemagglutinin epitope [4HA]) was additionally induced during infection with 1 mM isopropyl-β-D-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, 10 larvae were injected with PBS alone, and 10 untreated insects were included with every experiment. Larvae were individually examined for pigmentation, and time of death was recorded. Assays were allowed to proceed for only 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 postinfection (p.i.), hemolymph was extracted from three infected larvae and pooled as previously described (23). Cells were lysed by incubation of the hemolymph with 1 μl of 5 mg/ml digitonin for 5 min at room temperature. Extracted hemolymph was serially diluted in AYE medium and plated onto CYE plates. To prevent contamination, the extracted hemolymph was plated on CYE plates supplemented with spectinomycin (50 μg/ml) for L. pneumophila strain 130b or streptomycin (100 μg/ml) for the Philadelphia-1-derived strains. Plates were incubated at 37°C for 3 days, viable bacteria were enumerated, and the number of CFU was normalized to the weight of hemolymph extracted.

**Plasmids.** A fragment of the SidC homologue from L. pneumophila 130b containing the phosphatidylinositol-4 phosphate (PI4P) binding domain (amino acids 41 to 918 [SidC⁴₁–₉₁₈]) was cloned into the XbaI and BamHI sites of the p4HA plasmid (13) to yield the IPTG-inducible 4HA-SidC⁴₁–₉₁₈ expression plasmid pICCS62 using the forward primer 5'-CG TATTCTAGATAAACCCTGCAAAACAGGTGGAG-3' and the reverse primer 5'-GGCTAGATCCCTATTTCTTTATAACTCCGTGTA C-3' and standard molecular biology techniques.

**Indirect immunofluorescence on extracted hemocytes.** Hemolymph from infected G. mellonella larvae was extracted at 5 and 24 h postinfection. The extracted hemolymph was dispensed onto poly-l-lysine-coated glass coverslips and centrifuged at 500 × g for 10 min to allow sedimentation and attachment of hemocytes. 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 bacteria were stained with a mouse anti-L. pneumophila lipopolysaccharide (LPS) antibody (Virostat) and a donkey anti-mouse rhodamine-red-X-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grafton, CT).

**FIG 2.** L. pneumophila is able to persist and replicate in G. mellonella. Hemolymph from three L. pneumophila-infected G. mellonella insects was extracted, and the numbers of CFU/100 μl were quantified. (A) Wild-type L. pneumophila 130b replicated within the larvae over the infection course, while the ΔDotA mutant was cleared from G. mellonella by 24 h p.i. (B) L. pneumophila 130b invades and replicates within hemocytes. External and total bacteria were immunostained. By 5 h p.i., both wild-type and ΔDotA bacteria were found inside cells. By 24 h p.i., wild-type-infected hemocytes had high loads of intracellular bacteria. (C and D) G. mellonella mortality depends on L. pneumophila persistence. G. mellonella was inoculated with L. pneumophila strain JR32, JR32 ΔIcmT, or LP02. (C) 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 a higher level throughout the course of infection. (D) Only the wild-type JR32 strain, but not the ΔIcmT or Lp02 strain, induced mortality in G. mellonella by 72 h p.i. Results are representative of at least two independent experiments.
inc.) After permeabilization of the cells with 0.1% Triton in PBS and blocking with 2% (wt/vol) bovine serum albumin (BSA) in PBS, total bacteria were stained with a rabbit anti-<i>L. pneumophila</i> antibody (Affinity BioReagents) and a donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch).

To visualize 4HA-SidC41–918 in hemocytes, fixed cells were permeabilized and blocked for 1 h in PBS containing 2% (wt/vol) BSA. Samples were stained with rabbit anti-<i>L. pneumophila</i> antibody (Affinity BioReagents), donkey anti-rabbit Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch), mouse anti-HA conjugated to tetramethyl rhodamine isothiocyanate (TRITC) (Sigma), and 5 μg ml<sup>−1</sup> of 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Samples were analyzed using an Axio M1 Imager microscope, and images were processed with the AxioVision software (Carl Zeiss).

**Staining of formalin-fixed sections of <i>G. mellonella</i>.** <i>G. mellonella</i> larvae were fixed in formalin for 1 week at room temperature, paraffin embedded, sectioned, and stained either with hematoxylin and eosin (H&E) or by indirect immunofluorescence as described previously (18). <i>L. pneumophila</i> was stained with rabbit anti-<i>L. pneumophila</i> 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 (TEM).** Hemolymph was extracted from 10 infected <i>G. mellonella</i> larvae per condition and time point. Cells were spun down onto six-well plates, washed once with PBS, and fixed in 2% glutaraldehyde. Samples were processed as described previously (26) and examined using a Tecnai 12 (FEI) electron microscope. Images were taken with a charge-coupled-device (CCD) camera (TVIPS, Gauting, Germany).

**Hemocyte quantification and viability assay.** Infected hemolymph was extracted at 3 and 18 h p.i. Trypan blue (0.02% [vol/vol] in PBS) was added to cells and incubated at room temperature for 10 min. Viable cells were enumerated using a hemocytometer, and each sample was analyzed in triplicate. The average of three independent experiments was plotted graphically.

**PO activity assay.** At 5 and 18 h p.i. hemolymph from three infected insects per condition was extracted and pooled. Cells and debris were removed by centrifugation at 20,000 ×<sub>g</sub> for 10 min at 4°C. The phenoloxidase (PO) activity in the plasma was quantified using a microplate enzyme assay as described previously (15). The change in absorbance at 490 nm was read for 1 h at room temperature with a reading taken every minute using a Fluostar Optima plate reader (BMG Labtech, Germany). The experiment was performed in triplicate and independently repeated at least three times. Phenoloxidase activity was expressed as the mean OD<sub>490</sub>min<sup>−1</sup>/minute.

**RNA extraction and RT-PCR.** At time points indicated in the figure, fat bodies from three larvae were collected and stored in RNAlater (Qiagen) at 4°C until processing. Tissue was homogenized by a gentleMACS homogenizer (Miltenyi Biotech) using M tubes and the 90-s RNA setting. RNA was extracted using an RNeasy kit (Qiagen), and contaminating DNA was digested using a Turbo DNA-free kit (Ambion) following the manufacturer’s instructions. Two-step reverse transcription-PCR (RT-PCR) was performed using Superscript reverse transcriptase (Invitrogen) using 2 μg of RNA as a template and random hexamers (Invitrogen). Genes were amplified using RedTaq Ready Mix (Sigma) and 0.6 PM concentrations of gene-specific primers (Table 1), as described previously (23). DNA was analyzed on a 1% agarose gel with SYBR Safe (Invitrogen) and quantified using ImageJ software (NIH).

**RESULTS**

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

**Mortality in *L. pneumophila*-infected *G. mellonella* is dose dependent.** To determine if the mortality caused by *L. pneumophila* infection was dependent on the number of injected bacteria, *G. mellonella* larvae were injected with 10⁴, 10⁵, or 10⁶ CFU of *L. pneumophila* strain 130b. While injection with 10⁷ CFU resulted in 100% *G. mellonella* mortality within 24 h of infection, mortality was reduced to less than 40% in larvae injected with 10⁵ CFU, and no mortality was observed in *G. mellonella* larvae injected with any of the lower doses (10⁴ and 10⁵ CFU) (Fig. 1B). These results show that *L. pneumophila* induces dose-dependent *G. mellonella* mortality.

**The growth phase of *L. pneumophila* influences the kinetics of *G. mellonella* mortality.** During its life cycle, *L. pneumophila* alternates between replicative and transmissive forms (19). A number of virulence factors that promote infection of new host cells are downregulated in the replicative phase and upregulated in the transmissive phase. In broth culture, the transmissive traits cells are downregulated in the replicative phase and upregulated in number of virulence factors that promote infection of new host alternates between replicative and transmissive forms (19). A bacteria enter the postexponential phase (19). In order to assess are repressed in the exponential phase and expressed as the growth phase of *G. mellonella* strain 130b. While infection with 10⁷ CFU resulted in 100% *L. pneumophila* mortality, whereas no bacteria could be found in hemocytes from *G. mellonella* infected with 130b bacteria were found inside (green bacteria) and attached to *G. mellonella* larvae, whereas no bacteria could be found in hemocytes from *G. mellonella* infected with 130b or JR32 were full of bacteria surrounded by 4HA-SidC⁴¹–⁹₁₈. In contrast, far fewer bacteria were observed in hemocytes from *Galleria* infected with strain Lp02. Scale bar, 5 μm.

**FIG 4** SidC is localized to the LCV in hemocytes of infected *G. mellonella*. *G. mellonella* larvae were injected with *L. pneumophila* strain 130b, JR32, or Lp02 overexpressing 4HA-SidC⁴¹–⁹₁₈. At 5 and 24 h p.i., hemocytes were extracted, fixed, and stained with anti-HA antibody. By 5 h p.i. anti-HA staining revealed that SidC⁴¹–⁹₁₈ was localized on the LCV surface in hemocytes extracted from *G. mellonella* infected with wild-type *L. pneumophila* strains but not the 130b ΔDotA strain. By 24 h p.i., hemocytes from *G. mellonella* infected with 130b or JR32 were full of bacteria surrounded by 4HA-SidC⁴¹–⁹₁₈. In contrast, far fewer bacteria were observed in hemocytes from *Galleria* infected with strain Lp02. Scale bar, 5 μm.

**For intracellular survival and the establishment of a replicative vacuole** (4, 45). The 130b ΔDotA strain has a kanamycin resistance cassette inserted in the dotA gene, resulting in a nonfunctional T4SS (43). Infection of *G. mellonella* with 10⁷ CFU of 130b ΔDotA did not cause any mortality of the larvae over the 3 days of the experiment, whereas the parental wild-type strain killed all larvae within 24 h (Fig. 1D). This demonstrated that *L. pneumophila*-induced mortality of *G. mellonella* is dependent on the presence of a functional Dot/Icm T4SS.

**G. mellonella mortality depends on *L. pneumophila* persistence.** In order to determine the viable bacterial load within the hemolymph of *G. mellonella* larvae infected with *L. pneumophila*, larvae were injected with 10⁷ CFU of wild-type or ΔDotA 130b. At selected time points, hemolymph from three living larvae was extracted and pooled, and the number of CFU/100 μl of extracted hemolymph was determined (Fig. 2A). The 130b ΔDotA mutant was cleared from the injected larvae by 24 h p.i. and did not exhibit any replication. In contrast, infection of *G. mellonella* with wild-type 130b resulted in an initial 10-fold reduction of CFU at 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 hemolymph, hemocytes were extracted from infected *G. mellonella* larvae at 5 and 24 h p.i. and immunostained for external and total bacteria (Fig. 2B). By 5 h p.i. both wild-type and ΔDotA 130b bacteria were found inside (green bacteria) and attached to (yellow bacteria) hemocytes. By 24 h p.i. hemocytes extracted from *G. mellonella* infected with wild-type bacteria were full of intracellular *L. pneumophila*, whereas no bacteria could be found in hemocytes of *G. mellonella* infected with the 130b ΔDotA strain (data not shown). This result indicates that *L. pneumophila* replicates in *G. mellonella* hemocytes.

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 number of CFU extracted from the hemolymph over 24 h (Fig. 2C) showed that the JR32 bacteria 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 a higher CFU count than the JR32 ΔIcmT strain at 18 h p.i. before ultimately being 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 3 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 (see 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 than 18 h.

L. pneumophila resides in an LCV in hemocytes. In order to assess if L. pneumophila forms an LCV in hemocytes, we analyzed hemocytes from infected G. mellonella larvae 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 hemocytes were filled with bacteria. By 24 h p.i., the LCV was studded with ribosomes. L. pneumophila therefore appears to reside in the hemocytes of infected G. mellonella larvae in LCVs, which are similar to those seen in human monocytes (21).

To further characterize the LCVs formed in hemocytes, 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 SidCΔ11-918 was expressed in L. pneumophila, and the localization of the protein was analyzed by immunofluorescence (Fig. 4). To ensure that the protein was expressed, larvae were injected with a bacterial suspension containing 1 mM IPTG. The presence of IPTG alone did not affect survival (data not shown).
shown). Similarly to human A549 cells (data not shown), anti-HA staining of SidC$_{41-918}$ surrounded intracellular bacteria in hemocytes. No anti-HA staining was observed in the control hemocytes extracted from larvae infected with $L. pneumophila$ 130b/DotA expressing 4HA-SidC$_{41-918}$. At 24 h p.i. hemocytes from $G. mellonella$ larvae 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 at 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 $G. mellonella$ hemocytes.

**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 hematoxylin and eosin (H&E) and evaluated for histological changes (Fig. 5). Mock-infected controls appeared healthy, with no bacteria observed in the hemocoel and with individually distributed hemocytes occasion-ally forming loose aggregations. However, in both wild-type- and ΔDotA strain-infected insects, vigorous host defenses appeared to be mounted. At 16 h p.i. with 130b ΔDotA, fewer individual hemocytes were observed than in the mock-infected control, with the majority of hemocytes present in tightly packed aggregation nodules and some evidence of melanization. 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. hemocytes were observed in nodules attached to organ structures, with clearly visible nodule melanization. By 24 h p.i. nodules were still observed; however, septicemia was found in much of the hemocoel, and organ structures, including the gut, appeared severely damaged.

In order to confirm that the bacteria observed in formalin-fixed sections of the infected $G. mellonella$ insects were $L. pneumophila$, sections were stained using a specific anti-$L. pneumophila$ (α-Legionella) antibody; DAPI was used to visualize bacterial and eukaryotic cell DNA, and phalloidin was used to counter-stain the tissue. No $L. pneumophila$ staining was observed in the uninfected or ΔDotA controls. $G. mellonella$ infected with wild-type $L. pneumophila$ demonstrated a systemic infection with large numbers of bacteria in the hemolymph. Bacteria were usually associated with cells (arrowheads), a proportion of which displayed apoptotic nuclei (arrows). Scale bar, 20 μm.
Bacteria stained with the anti- L. pneumophila antibody were found throughout the hemolymph (Fig. 6) and occasionally in cells within the fat bodies (data not shown). Bacteria were exclusively associated with cells and were usually found in aggregates of hemocytes.

Altogether, these data indicate that L. pneumophila triggers an immune response in G. mellonella that successfully clears the ΔDotA mutant from the larvae, whereas wild-type L. pneumophila bacteria are resistant to host defenses.

The G. mellonella immune responses to L. pneumophila infection. Progression of L. pneumophila infection resulted in an increase in G. mellonella pigmentation (Fig. 7A), which is usually indicative of activation of the PO cascade. Upon recognition of pathogen-associated molecular patterns (PAMPs), the pro-PO system components are released from hemocytes into the hemolymph, leading to activation of PO. The activity of this enzyme subsequently induces the formation of quinones and melanin, which are involved in defense reactions against pathogens invading the hemocoel, such as nodule formation and encapsulation (8).

In order to quantify this innate immune response, we assayed at selected time points the level of PO activity in the hemolymph of G. mellonella insects infected with strain 130b (Fig. 7B). By 5 h p.i. insects injected with wild-type L. pneumophila exhibited dramatically increased PO activity compared to larvae injected with PBS (P < 0.005). Larvae inoculated with L. pneumophila ΔDotA presented an intermediate level of PO activity. At 18 h p.i. the level of PO activity had not significantly changed in the PBS- and 130b ΔDotA-injected G. mellonella insects. However, in G. mellonella insects injected with wild-type bacteria, levels of PO activity significantly dropped compared to levels at 5 h p.i. (P < 0.005), reaching levels similar to the level of the PBS control. These results indicate that L. pneumophila infection initiates an immune defense in G. mellonella through PO activation, a response which is nonetheless abrogated by 18 h p.i.

In order to test if the absence of PO activity at 18 h p.i. could be due to hemocyte depletion, insects were infected with the wild-type or ΔDotA 130b strain or injected with PBS as a control, and hemocytes were counted by light microscopy at 5 and 18 h p.i. (Fig. 7C). At 5 h p.i. the concentrations of hemocytes per ml of hemolymph were comparable in the different groups. However, by 18 h p.i. the number of hemocytes was reduced by almost 90% in G. mellonella inoculated with wild-type 130b compared to the level at 5 h p.i. or in the controls, suggesting that L. pneumophila infection induces hemocyte destruction. The reduction in hemocyte number observed is likely to contribute to the decreased PO activation observed 18 h p.i.

A major component of the defense response of insects is the production of antimicrobial peptides (AMPs) (29, 31). In order to assess if G. mellonella produced AMPs following L. pneumophila infection, their expression was tested in fat bodies, where they are mainly produced. Semiquantitative RT-PCR on extracted mRNA showed that infection with wild-type L. pneumophila resulted in an upregulation of most of the immune-related peptides tested compared to the levels in a PBS-injected control (Fig. 8), with a significantly increased expression of gallerin and pro-PO (PPO) as early as 2 h p.i. (P < 0.006). In contrast, gallerimycin, galiomyacin, and the iron binding protein transferrin were significantly upregulated only after 18 h of infection (P < 0.0005 for gallerimycin and transferrin and P < 0.005 for galiomyacin). The expression of the peptidoglycan recognition protein B (PRPB) did not significantly increase upon inoculation with wild-type L. pneumophila. Injection with the ΔDotA strain did not cause significant change from the baseline level with the exception of the AMP gallerin, the mRNA level of which increased after 24 h (P < 0.01). These results show that G. mellonella mounts an immune response to L. pneumophila infection that, nonetheless, is not effective in clearing the wild-type bacteria.

**DISCUSSION**

Adequate infection models that approximate human disease are the key to analyzing 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 larva of the greater wax moth Galleria mellonella has recently been reported as an 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 a new infection model for L. pneumophila.

Using three prototypic L. pneumophila strains, we found that
G. mellonella insects could withstand a low infectious dose, but the larvae succumbed to infection at 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 intratracheal infection of A/J mice (40). In a comparative assessment of the virulence traits of 27 *L. pneumophila* and non-*pneumophila* *Legionella* strains, 130b was the third-most cytopathogenic strain (2). Taking these observations together, this indicates that the *G. mellonella* model can reproduce the 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 amoebae, 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 *G. mellonella* also depended on a functional Dot/Icm T4SS. A T4SS-deficient mutant did not show any virulence even at the highest (10^7 CFU) inoculum injected. This contrasts observations described for the *G. mellonella* model of *Listeria* infection, in which nonpathogenic strains with increasing doses up to 10^7 CFU per larva also induced mortality (23, 34). It was proposed that this could be attributed to a form of sepsis, and subsequent death was caused by bacterial overload and was not due to specific virulence factors. Our data indicate that the threshold at which bacterial load triggers sepsis and death may vary from pathogen to pathogen.

Although the *Drosophila* model was used to determine virulence phenotypes of *L. pneumophila* mutants in the fly, further aspects underlying *L. pneumophila* pathogenesis in the insect have not been characterized (27). We show for the first time that *L. pneumophila* resides in a vacuole in hemocytes isolated from infected insects. This vacuole ultrastructurally resembled the LCV observed in human macrophages and amoebae, including association of mitochondria, acquisition of a rough ER-like structure (1, 21), and recruitment of SidC, which was previously shown to be tethered to the LCV via a phosphatidylinositol-4 phosphate anchor (51). The recruitment of ribosomes and the T4SS substrate SidC to the hemocyte LCV suggests that *L. pneumophila* uses at least some of the fundamental strategies which are employed to establish a replicative vacuole in mammalian cells and amoebae also to infect insect hemocytes.

Analysis of *L. pneumophila* replication in *G. mellonella* by direct bacterial enumeration demonstrated that, following an initial 10-fold reduction in the number of CFU of wild-type bacteria at 5 h p.i., bacterial CFU counts quickly recovered and increased by 100-fold from the inoculum by 24 h p.i. The ΔDotA mutant was cleared by 24 h p.i. The level of *L. pneumophila* replication appears to be higher than in the mouse model, in which strain 130b could exhibit up to a 20-fold increase in the CFU count within 48 h (6, 40) or in the *Drosophila* model, in which an increase in the number 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 an LCV in hemocytes. 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* bacteria at early stages of infection. However, enough wild-type bacteria evade destruction by phagocytes and start replicating. Release from hemocytes following replication is most likely accomplished by destruction of the hemocytes. This model is supported by the fact that 90% of the hemocytes are lost by 18 h postinfection and that this model reproduces virulence.

**Depletion of hemocytes**,

Bacterial replication in hemocytes 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 hemocytes or the sequestration of hemocytes in nodules or to a combination of both. However, nodules were observed in wild-type- and ΔDotA strain-infected *G. mellonella*, and there was no significant loss of hemocytes in the ΔDotA-infected larvae, suggesting that replication and T4SS-dependent toxicity are the most likely causes of the loss of cells.

**Depletion of hemocytes**, the major source of prophenoloxidase (pro-PO), which triggers the melanization response upon infection, would also explain why we observed an initial activation of PO activity at 18 h p.i. following wild-type *L. pneumophila* infection. Depletion of circulating hemocytes 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 hemocytes or the sequestration of hemocytes in nodules or to a combination of both. However, nodules were observed in wild-type- and ΔDotA strain-infected *G. mellonella*, and there was no significant loss of hemocytes in the ΔDotA-infected larvae, suggesting that replication and T4SS-dependent toxicity are the most likely causes of the loss of cells.

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