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The Dot/Icm Effector SdhA Is Necessary for Virulence of *Legionella pneumophila* in *Galleria mellonella* and A/J Mice

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***Legionella pneumophila* is an intracellular bacterium that resides within amoebae and macrophages in a specialized compartment termed the *Legionella*-containing vacuole (LCV). As well as providing an intracellular niche for replication, the LCV helps to prevent the release of bacterial components into the cytoplasm. Recognition of these components as danger signals by the host activates immune responses leading to clearance of the bacterium. Here, we examined the role of two important virulence factors of *L. pneumophila*, the potent danger signal flagellin and the translocated Dot/Icm type IVB secretion system effector SdhA, which is crucial to maintain LCV integrity, in the *Galleria mellonella* infection model. We demonstrate that flagellin expression does not contribute to virulence, replication, or induction of clearance mechanisms. Conversely, SdhA expression is important for virulence. We found that in the absence of SdhA, the LCV in hemocytes showed signs of instability and leakage. Furthermore, in contrast to wild-type *L. pneumophila*, a Δ*sdhA* mutant caused a transient depletion of hemocytes and reduced mortality. Analysis of the Δ*sdhA* mutant in the A/J mouse model also showed a significant replication defect. Together, our data underline the crucial importance of SdhA in infection across different model organisms.**

Legionella pneumophila is a facultative intracellular bacterial pathogen found in environmental aquatic reservoirs and human-made water systems. Accidental inhalation of contaminated aerosols carries bacteria into the human lung, where they establish a severe, potentially fatal, pneumonia in susceptible populations, called Legionnaires' disease (1). Alveolar macrophages constitute the first line of the airway immune defense (2) and play a key role in *L. pneumophila* infection. Usually these professional phagocytes take up bacteria deposited on the alveolar surface and degrade them in phago-lysosomes as a primary bactericidal mechanism. However, *L. pneumophila* avoids trafficking to the lysosome by creating a replicative vacuole that resembles the rough endoplasmic reticulum, termed the *Legionella*-containing vacuole (LCV) (3). Formation of the LCV is dependent on a type IVB secretion system (T4SS) known as the defective in organelle trafficking/intracellular multiplication (Dot/Icm) secretion system (4, 5). The Dot/Icm T4SS translocates more than 300 effectors into the host cell, where they manipulate a plethora of host cell signaling processes, including vesicular trafficking and innate immune signaling (6–9).

Although wild-type (WT) bacteria remain within a membrane-bound vacuole, pathogen-associated molecular patterns (PAMPs) leaking into the cytoplasm from the LCV activate cytosolic danger signal sensors, such as Naip5/Ipaf, and trigger an inflammatory response (10). In C57BL/6 mice, the Naip5/Ipaf inflammasome detects bacterial flagellin and induces a caspase-1-dependent response (11–13). A/J mice harbor a polymorphism in the Naip5 allele that renders the strain more susceptible to *L. pneumophila* infection (14, 15). Moreover, *L. pneumophila* activates additional inflammatory signaling factors, including NF-κB, RIG-I, and AIM2 (16–18).

One of the few Dot/Icm effectors that are crucial for the replication of *L. pneumophila* in mouse macrophages is SdhA (19). SdhA is a highly conserved effector found in *L. pneumophila*, *Legionella longbeachae*, and *Legionella drancourtii*. SdhA is required for maintaining the integrity of the LCV; deletion of *sdhA* results in destabilization of the LCV and release of the bacterium into the cytoplasm (17, 20). The molecular basis of the LCV-stabilizing function of SdhA is not yet clear; however, there is evidence that SdhA counterbalances the effect of the secreted *L. pneumophila* phospholipase PlaA. A Δ*sdhA* mutant induces type I interferon signaling and caspase-1 activation, culminating in the death of infected host cells (16, 17, 20). Notably, activation of these responses is not dependent on flagellin, but rather is triggered by bacterial nucleic acids (16, 17).

We recently established that the larvae of the wax moth *Galleria mellonella* are an effective model for *L. pneumophila* infection (21). *L. pneumophila* forms LCVs in insect phagocytic cells, known as hemocytes, in a Dot/Icm-dependent manner. Although infection of the larvae induces an immune response, as judged by the activation of the phenoloxidase cascade, nodule formation, and upregulation of antimicrobial peptides, larvae ultimately succumb to *L. pneumophila* infection. It is currently unknown which bacterial PAMPs and Dot/Icm T4SS effectors contribute to *L. pneumophila* pathogenesis in the *G. mellonella* model. Here we sought to determine the role of flagellin and SdhA in *L. pneumophila*-induced larval death and, in the case of SdhA, compare the findings to virulence in A/J mice.

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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 (22). The *L. pneumophila* Δ*dotA* strain is a *dotA* insertion mutant (kanamycin resistance) of *L. pneumophila* strain 130b (23). *G. mellonella* larvae were obtained from Livefood UK and stored at room temperature in the dark.

Plasmid and strain construction. The p4HA-SdhA (pICC1340) complementation plasmid was created using standard molecular biology techniques. Briefly, *sdhA* was amplified by PCR from *L. pneumophila* 130b chromosomal DNA using the primers GNS414 5′-CAG TCC CGG GAT ATT TCA GAA AAG ATC AAG CTT TTA GAA T-3′ and GNS415 5′-CTA TCC CGG GTT ATG CTG ATG GCG CTA ATT GG-3′, digested using XmaI, and ligated into the cleaved pICC562 vector (pMMB207C-HAx4 [24]). The correct orientation and sequence identity of the *sdhA* insert were confirmed by DNA sequencing, and the p4HA-SdhA plasmid was transformed into *L. pneumophila* by electroporation.

To generate an *sdhA* mutant, a 2.3-kb fragment of *L. pneumophila* 130b genomic DNA encoding SdhA was amplified by PCR using the primers 5′-GAC CTG GAG CAT GTC AAA GGG-3′ and 5′-CCG CTA AAG GAT GTA ACA GGC-3′. The amplified product was cloned into pGEM-Teasy and digested with BamHI, removing an internal fragment of *sdhA*, and ligated with a kanamycin resistance gene cassette. This construct was introduced into *L. pneumophila* 130b by natural transformation for homologous recombination (25). Briefly, bacteria were incubated in ACES yeast extract (AYE) broth at 30°C with 10 μg/ml of pGEMTeasy::*sdhA*::*km* until exceeding an optical density at 600 nm greater than 1.5. These cultures were then spread on plates with charcoal-yeast extract (CYE) with kanamycin, and kanamycin-resistant colonies were confirmed to have lost the pGEM-Teasy backbone based on ampicillin sensitivity. The insertion mutation was confirmed by PCR using the primers outside the construct (5′-CCC TAA ATA ATG AAA AGC TGG-3′ and 5′-CAC ATA TCA TTC GAA TAT GTG C-3′) as well as one primer outside the construct and one in the deleted region (5′-ACT ATA AGG GAA TAA AAC CAG-3′).

To generate a deletion mutant of *flaA*, a mutant copy of *flaA* carrying an in-frame deletion was generated by PCR and inserted into a unique SalI restriction enzyme site of the mutagenesis vector pSR47s (26). The mutant copy was generated by overspan PCR using the primers $FlaA_{(1)}$ (5′-AGC TAG GTC GAC AAA ATT ACA AGA TGG GCA AAC C-3′) and $FlaA_{(4)}$ (5′-AGC TAG GTC GAC CTG ACC CTA CGC CTG GTG-3′) and the PCR products produced using the primers $FlaA_{(1)}$/$FlaA_{(2)}$ (5′-ATT GCG TTG GGC TGT AAG-3′) and $FlaA_{(3)}$ (5′-ATG CTT ACA GCC CAA CGC AAT GTA TTA TCG TTG TTA GGT CGA-3′)/$FlaA_{(4)}$ as the templates. The suicide mutagenesis vector pSR47s carries kanamycin resistance, and the *sacB* gene of *Bacillus subtilis* results in sucrose sensitivity. After transformation into *L. pneumophila* 130b via electroporation, isolation of the cointegrates was achieved by selection for the plasmid-carried kanamycin resistance. Loss of the *sacB* gene carried by the cointegrated plasmid was selected by culture on CYE plates containing 5% sucrose. The result of the second homologous recombination event is either gene replacement or reconstitution of the wild type. A PCR spanning the *flaA* gene was used to distinguish mutant and wild-type alleles.

Infection of *G. mellonella*. *L. pneumophila* strains were cultured on CYE plates at 37°C for 4 days, then inoculated into AYE, and *G. mellonella* larvae were infected as previously described (21, 27). Briefly, 10 *G. mellonella* larvae were injected with 10 μl of 10^9 CFU/ml bacteria in Dulbecco's phosphate-buffered saline (D-PBS; unless otherwise indicated) and incubated at 37°C in the dark for up to 72 h, and time of death was recorded. At least three independent replicates of each experiment were performed.

Intracellular growth assay and hemocyte depletion assay. At 0, 5, 18, and 24 h postinfection (p.i.) hemolymph was extracted from three infected larvae and pooled, and the CFU per 0.1 g of hemolymph was determined as previously described (21). Similarly, at the indicated time points, the hemocyte concentration was determined in extracted hemolymph by counting (21).

Indirect immunofluorescence on infected cells. Hemolymph from infected *G. mellonella* was extracted at 5 and 18 h p.i. Cells were fixed and permeabilized as previously described (21) and stained with rabbit anti-*L. pneumophila* antibody (PA1-7227; Affinity BioReagents), donkey anti-rabbit IgG–Alexa Fluor 488–conjugated antibody (711-485-152; Jackson ImmunoResearch), and mouse anti-hemagglutinin (HA) conjugated to tetramethyl rhodamine isothiocyanate (TRITC; H9037; 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 were processed with the Axiovision software (Carl Zeiss).

Transmission electron microscopy (TEM). Hemocytes were extracted from infected and control *G. mellonella* at 5 h p.i. The cells were transferred to 35-mm dishes and centrifuged at 5,000 rpm for 5 min. The cells were washed 3 times with 1× PBS and cooled on ice before fixation with 0.5% glutaraldehyde (Sigma) in 200 mM sodium cacodylate (TAAB) for 5 min on ice, then at room temperature for a further 25 min. The cells were washed with 200 mM sodium cacodylate before postfixation in 1% osmium tetroxide–1.5% potassium ferrocyanide for 1 h. The cells were washed in double-distilled water (ddH_2O) and stained overnight at 4°C with 0.5% uranyl acetate. The cells were washed with ddH_2O before serial dehydration in ethanol and embedded flat in Epon resin. Ultrathin sections (~70 nm) were cut parallel to the surface of the dish by using a Leica ultramicrotome. The sections were collected onto Formvar-coated 50 mesh EM grids and stained for 30 s with Reynold's lead citrate before imaging. Samples were viewed by using an FEI Tecnai G^2 electron microscope with a Soft Imaging System Megaview III charged-coupled-device camera. Images were collected at 1,376 by 1,032 by 16 pixels using AnalySIS version Docu software (Olympus Soft Imaging Solutions).

Infection of mice. All mouse procedures were approved by the University of Melbourne Animals Ethics Committee. The comparative virulence of *L. pneumophila* 130b wild type (WT) and the Δ*sdhA* and Δ*flaA* mutant strains were examined as described previously (28). Briefly, 6- to 8-week-old male or female A/J or C57BL/6 mice were anesthetized and inoculated intranasally with approximately 2.5×10^6 CFU of each *L. pneumophila* strain under investigation. At 72 h after inoculation, mice were killed and their lung tissue isolated. Tissue was homogenized, and complete host cell lysis was achieved by incubation in 0.1% saponin for 15 min at 37°C. Serial dilutions of the homogenate were plated onto both plain and antibiotic selective CYE agar plates to determine the number of viable bacteria and the ratio of WT to mutant bacteria colonizing the lung. For single infections, 2 groups of mice were inoculated separately with approximately 2.5×10^6 CFU each of *L. pneumophila* 130b WT and the Δ*sdhA* mutant strain. Lung CFU were determined 72 h after infection as described above on plain CYE agar.

RESULTS

Flagellin is dispensable for *L. pneumophila* virulence in *G. mellonella*. Given the propensity of *L. pneumophila* flagellin to stimulate inflammatory host cell death, we investigated its role in the *G. mellonella* model. Toward this end, we created an *L. pneumophila* 130b Δ*flaA* mutant. Pulmonary infection of nonpermissive C57BL/6 mice showed that the 130b Δ*flaA* mutant replicated to higher numbers ($P = 0.0003$; Mann-Whitney) than wild-type bacteria in mouse lungs (Fig. 1A), which is consistent with phenotypes reported for the *L. pneumophila* Lp01 and Lp02 Δ*flaA* mutants (12, 29). We then infected *G. mellonella* larvae with 10^7 wild-type *L. pneumophila* 130b or Δ*flaA* mutant and monitored survival over 72 h. No differences between the two strains were observed over the course of the infection (Fig. 1B). In order to determine the viable bacterial load, we extracted hemolymph from infected larvae and determined the CFU per 0.1 g of tissue at 0, 5, 18, and 24 h p.i. This revealed that the Δ*flaA* strain replicated as efficiently as wild-type *L. pneumophila* (Fig. 1C), indi-

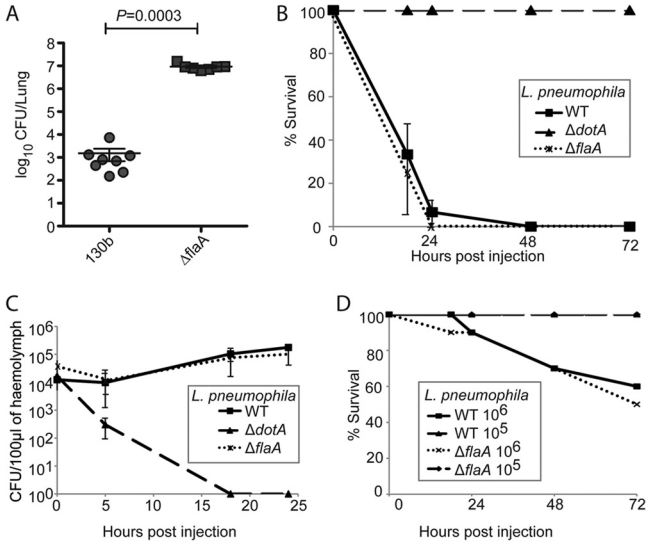

FIG 1 Motility and flagellin expression are dispensable for *L. pneumophila* virulence in *G. mellonella*. (A) In single pulmonary infections of C57BL/6 mice, the *L. pneumophila* 130b Δ*flaA* strain replicated to higher numbers at 72 h p.i. than the parental wild-type strain ($P = 0.0003$; Mann-Whitney). (B) Ten *G. mellonella* larvae were infected with 10^7 CFU of WT, Δ*dotA*, or Δ*flaA* *L. pneumophila* 130b, and mortality was monitored for 72 h. Deletion of the *flaA* gene did not affect mortality compared to WT-infected larvae. (C) Hemolymph from larvae infected as described above was extracted at 0, 5, 18, and 24 h p.i., and the CFU/0.1 g of extracted hemolymph were enumerated. The WT and Δ*flaA* strains replicated similarly, and no significant difference was observed. The Δ*dotA* strain was cleared by 18 h p.i. Results are means of three independent experiments ± standard deviations. (D) *G. mellonella* larvae were infected with 10^5 or 10^6 CFU of the WT or Δ*flaA* strains, and mortality was monitored. For both strains, no mortality was observed when larvae were infected with 10^5 CFU. In larvae infected with 10^6 CFU, some death was seen; however, no significant differences between strains were observed. Results are representative of two independent experiments.

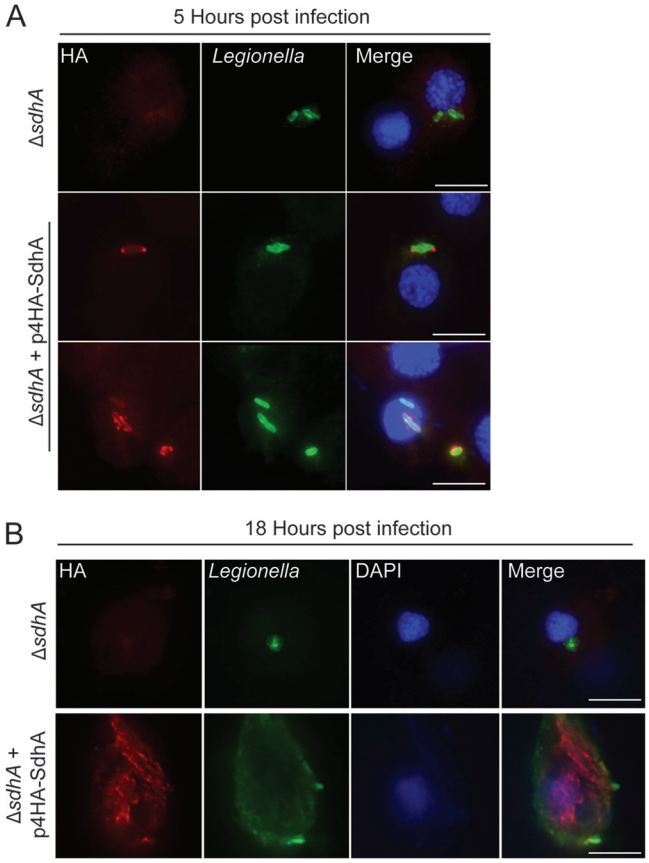

FIG 2 4HA-SdhA is found on LCVs in infected hemocytes. Hemocytes were extracted from *G. mellonella* larvae infected with Δ*sdhA* or Δ*sdhA* plus p4HA-SdhA *L. pneumophila* 130b at 5 or 18 h p.i. and stained using anti-HA (red) and anti-*Legionella* (green) antibodies. 4′,6-Diaimidino-2-phenylindole (DAPI) was used to visualize DNA (blue). (A) At 5 h p.i., 4HA-SdhA localized to the LCV membrane and was concentrated at the poles of the bacteria in approximately 45% of infected cells. (B) At 18 h p.i., 4HA-SdhA could be seen surrounding the bacteria. Results are representative of three independent experiments. Bar, 5 μm.

cating that flagella do not promote *L. pneumophila* virulence in the *G. mellonella* model.

As infection of larvae with 10^7 CFU of *L. pneumophila* causes very rapid death, any increase in virulence of the Δ*flaA* mutant as observed in C57BL/6 mice could have been masked. Therefore, we infected the larvae with infectious doses of 10^5 or 10^6 bacteria, which we have previously shown are less pathogenic (10^6) and nonpathogenic (10^5) for the insect, respectively (21). At these doses, the mortality induced by the *L. pneumophila* Δ*flaA* mutant was indistinguishable from the wild type (Fig. 1D). This suggests that in *G. mellonella* the induction of an antimicrobial response and clearance of low infectious doses of *L. pneumophila* are not dependent on detection of flagellin.

SdhA localizes to and promotes stability of the LCV in infected *G. mellonella* hemocytes. Recently it has emerged that the LCV is an important barrier that prevents the release of PAMPs into the cytoplasm, thereby shielding *L. pneumophila* against cytoplasmic bactericidal immune mediators (17, 20). SdhA plays a key role in maintaining the integrity of the LCV. In the absence of SdhA, macrophages undergo rapid cell death due to the activation of pyroptosis, which prematurely ends intracellular replication of the mutant, explaining the inability of this strain to efficiently replicate in isolated primary mouse macrophages and human macrophage cell lines (17, 19). Our previous work suggested that efficient intracellular replication and the ability to cause hemocyte depletion are important for virulence of *L. pneumophila* in *G. mellonella* larvae (21). Given the importance of SdhA in macrophages, we investigated its role following infection of *G. mellonella* with *L. pneumophila*.

We first generated an *L. pneumophila* 130b Δ*sdhA* deletion mutant and a complementation plasmid that expressed the effector fused to four N-terminal HA tags (4HA-SdhA). Using immunofluorescence microscopy, we confirmed that the 4HA-SdhA protein was translocated into *G. mellonella* hemocytes during infection. At 5 h p.i., SdhA localized to the membrane of the LCV, often displaying strong staining close to the bacterial poles (Fig. 2A). By 18 h p.i., SdhA was found all around the LCV (Fig. 2B), similar to results obtained in mouse macrophages (19).

To determine if SdhA is important to LCV maintenance in *G. mellonella* hemocytes, we infected larvae with wild-type *L. pneumophila* 130b, Δ*sdhA*, or Δ*sdhA* expressing 4HA-SdhA, isolated hemocytes after 5 h of infection, and analyzed their ultrastructure by TEM (Fig. 3). The LCVs of wild-type bacteria were similar in morphology to those described previously (21), by 5 h p.i. intracellular bacteria were contained within large vacuoles to which endoplasmic reticulum (ER)-derived membranes and ribosomes

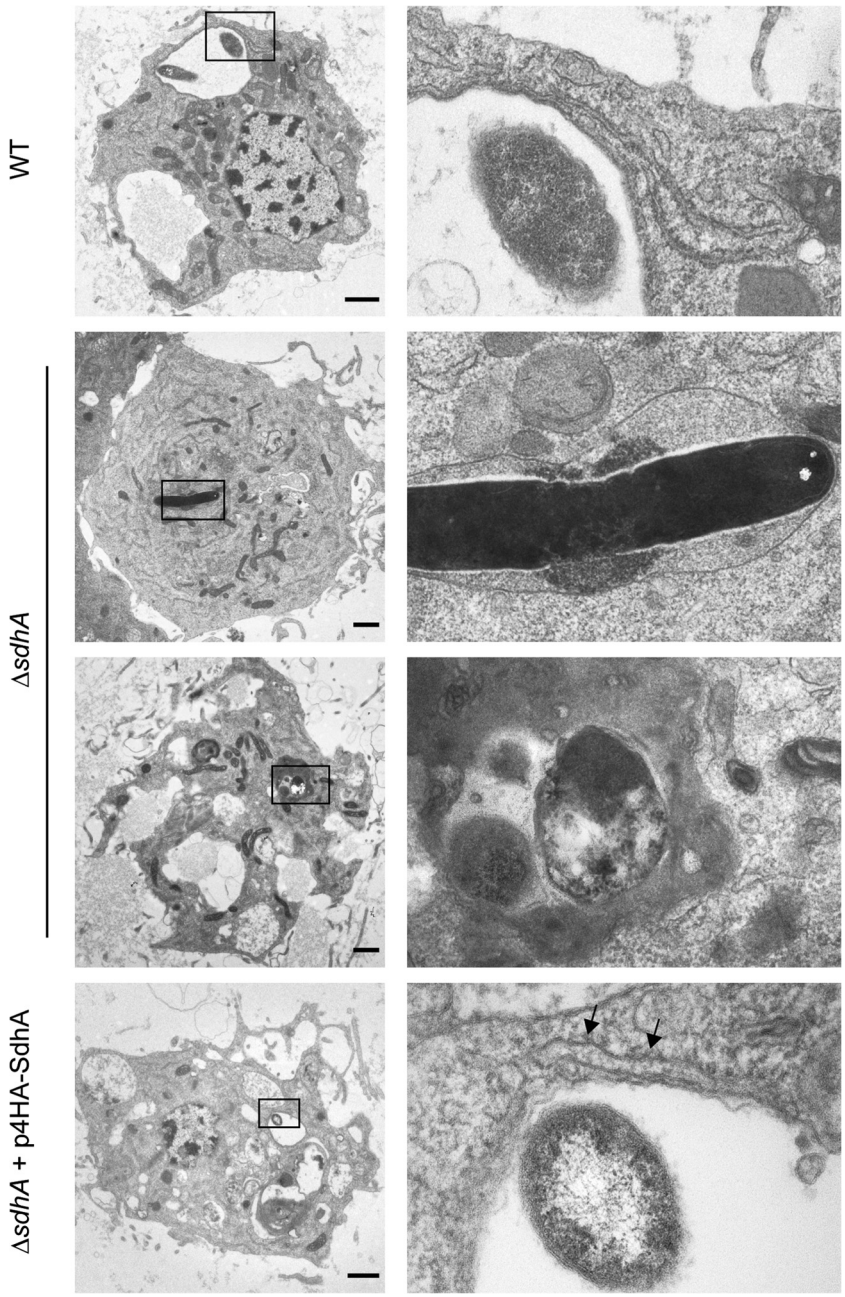

FIG 3 LCVs of *L. pneumophila* Δ*sdhA* in hemocytes, showing signs of instability. *G. mellonella* larvae were infected with WT, Δ*sdhA*, or Δ*sdhA* plus p4HA-SdhA *L. pneumophila* 130b for 5 h. Hemocytes were extracted and processed for transmission electron microscopy. Hemocytes infected with WT bacteria contained LCVs of similar morphology to those previously described. Recruitment of the ER to the LCV is indicated (black arrows). The LCVs of the Δ*sdhA* mutant contained electron-dense material, which may be cytoplasmic or bacterial in origin, indicating loss of stability of the vacuole. The LCVs of the complemented strain were of comparable morphology to those of WT bacteria. Images are representative of two independent experiments. Bar, 1 μm.

were recruited. However, the LCV of the Δ*sdhA* mutant showed signs of instability, with electron-dense material apparent within the vacuole. This may have been due to infiltration of the cytoplasm or release of bacterial contents into the LCV. The LCVs of hemocytes infected with the Δ*sdhA* mutant expressing 4HA-SdhA showed a similar morphology to those infected with wild-type bacteria. These observations indicated that SdhA is important for maintenance of LCV stability within *G. mellonella* hemocytes.

Lack of SdhA results in accelerated hemocyte depletion. Lack of SdhA has been associated with rapid macrophage cell death, with early signs of apoptosis appearing as soon as 30 min p.i. (19, 20). We previously showed that infection of *G. mellonella* with *L. pneumophila* causes hemocyte depletion and high larval mortality at 18 h p.i. (21). In order to determine if deletion of *sdhA* alters hemocyte cytotoxicity, larvae were infected as described above, hemolymph was extracted at 2, 5, 10, 18, and 24 h p.i., and the number of hemocytes was determined by cell counting (Fig. 4). As

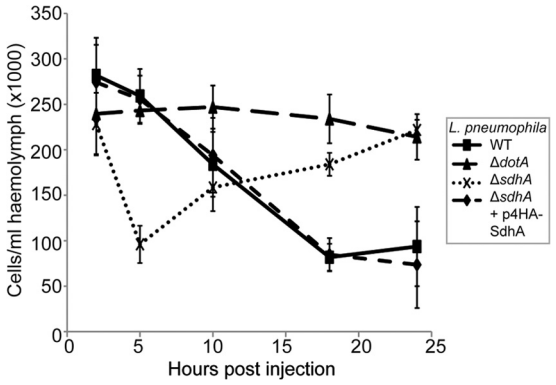

FIG 4 *L. pneumophila* Δ*sdhA* causes rapid, transient hemocyte depletion. Hemocytes from larvae infected with WT, Δ*dotA*, Δ*sdhA*, or Δ*sdhA* plus p4HA-SdhA *L. pneumophila* were extracted at 2, 5, 10, 18, and 24 h p.i., and viable cells were counted. Infection with the Δ*sdhA* mutant, but not with WT or complemented strains, caused significant ($P < 0.0001$) hemocyte depletion at 5 h p.i. By 18 h p.i., the hemocyte levels had almost recovered to the 2-h p.i. levels in larvae infected with the Δ*sdhA* strain, while hemocyte depletion was seen in the larvae infected with WT or Δ*sdhA* plus p4HA-SdhA ($P < 0.0001$ compared to Δ*sdhA* strain). Results are means of at least 4 experiments ± standard deviations.

previously shown, infection with the avirulent Δ*dotA* strain resulted in no change in hemocyte density over the course of this experiment. In contrast, infection with the Δ*sdhA* strain resulted in a significantly higher loss of hemocytes between 2 and 5 h p.i. compared to larvae infected with the WT or the Δ*dotA* mutant strain (Fig. 4) ($P < 0.0001$, unpaired *t* test). In combination with the apparent instability of LCVs containing the Δ*sdhA* strain as observed by TEM, the data indicate that hemocytes, similarly to macrophages, induce rapid cell death in response to cytosolic Δ*sdhA* bacteria.

Interestingly, between 5 and 24 h p.i., hemocyte levels in Δ*sdhA*-infected *G. mellonella* gradually recovered to levels comparable to those in larvae infected with the avirulent Δ*dotA* strain (Fig. 4). In contrast, wild-type *L. pneumophila* and the complemented Δ*sdhA* strain caused significant hemocyte depletion between 10 and 24 h p.i. compared to Δ*sdhA*- or Δ*dotA*-infected larvae ($P < 0.0001$, unpaired, *t* test). These results showed that hemocyte depletion can be transient and suggested that rapid hemocyte depletion can be segregated from larval mortality.

The *L. pneumophila* Δ*sdhA* mutant is attenuated in *G. mellonella*. To further analyze how the observed transient hemocyte depletion correlated with the replication of the Δ*sdhA* strain and to determine the importance of SdhA for replication *in vivo*, we examined the competitive fitness of this strain during mixed infection of *G. mellonella*. Larvae were infected with a 1:1 ratio of wild type:Δ*sdhA* or wild type:Δ*sdhA* plus p4HA-SdhA. Hemolymph was extracted at 24 h p.i., and the ratio between the wild type and mutant was determined and expressed as competitive index (CI) (Fig. 5A). The Δ*sdhA* strain was significantly outcompeted by 24 h p.i., while the complemented strain replicated as well

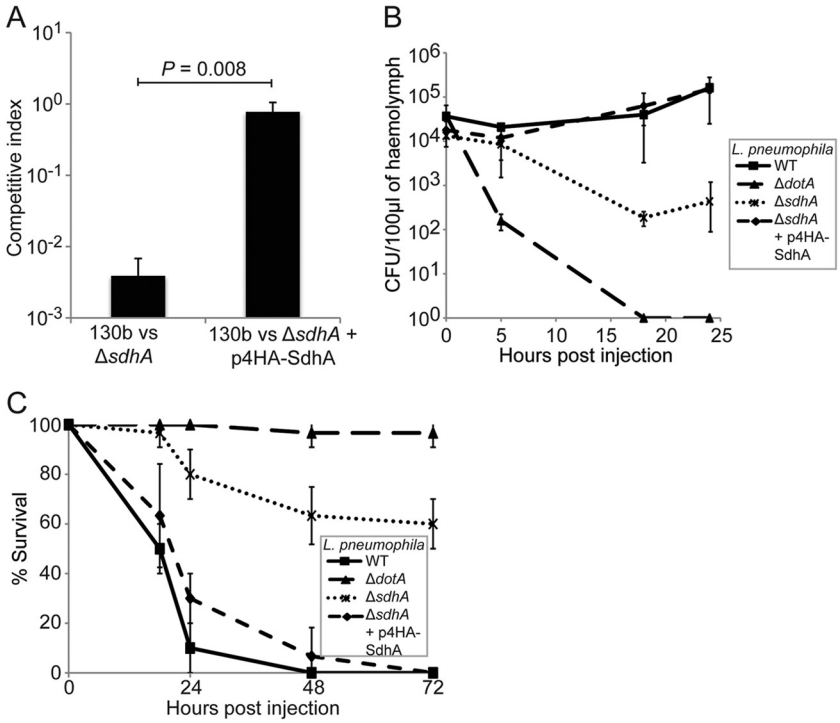

FIG 5 *L. pneumophila* Δ*sdhA* is severely attenuated in infection of *G. mellonella* larvae. (A) *G. mellonella* larvae were infected with a 1:1 ratio of WT:Δ*sdhA* or WT:Δ*sdhA* plus p4HA-SdhA, hemolymph was plated, and CFU were counted at 24 h p.i. to calculate the competitive index. The WT strain significantly outcompeted the Δ*sdhA* strain (CI, 0.002); fitness of the mutant could be restored by expression of 4HA-SdhA (CI, 0.88). (B) Hemolymph from larvae infected with WT, Δ*dotA*, Δ*sdhA*, or Δ*sdhA* plus p4HA-SdhA was extracted at 0, 5, 18, and 24 h p.i., and the CFU/0.1 g of extracted hemolymph were enumerated. The Δ*sdhA* strain did not replicate; however, it persisted over 24 h, unlike the Δ*dotA* strain, which was cleared by 18 h p.i. Results are means of at least three independent experiments ± standard deviations. (C) Larvae were infected with WT, Δ*dotA*, Δ*sdhA*, or Δ*sdhA* plus p4HA-SdhA *L. pneumophila*, and mortality was monitored for 72 h. Infection with WT resulted in 100% mortality within 24 h p.i., while the Δ*sdhA* strain only killed 40% of infected larvae by 72 h p.i.

as the wild type ($P = 0.008$, unpaired t test), showing that SdhA has an important role for *L. pneumophila* fitness in the larvae.

In order to analyze the growth attenuation in *G. mellonella* in more detail, larvae were infected with the individual strains, and the viable CFU per 0.1 g of hemolymph were determined. While the wild-type bacterial load increased steadily over the course of the experiment, the Δ*sdhA* strain did not replicate, and its counts decreased more than 10-fold in the first 18 h p.i. but then remained stable at this level (Fig. 5B). The Δ*dotA* strain was completely cleared by 18 h p.i. Replication of the Δ*sdhA* mutant could be restored by complementation with the 4HA-SdhA expression plasmid.

To characterize how the different hemocyte depletion kinetics and inefficient replication of the Δ*sdhA* strain affect larval mortality, 10 *G. mellonella* larvae were infected with wild-type, Δ*sdhA*, or Δ*sdhA* expressing 4HA-SdhA *L. pneumophila* 130b. Infection with Δ*dotA* was used as a control. The Δ*sdhA* strain was significantly attenuated for induction of larval mortality compared to the wild-type strain; by 72 h p.i., only 40% of infected larvae had succumbed to infection, compared to 100% for the wild-type strain (Fig. 5C). This phenotype was complemented by the addition of 4HA-SdhA on a plasmid. The Δ*sdhA* strain caused increased mortality compared to Δ*dotA*, which was avirulent under these conditions, as reported previously (21). Taken together, these data show that substantial hemocyte depletion alone does not trigger death of *G. mellonella* larvae. In fact, induction of larval death seems to rather be determined by the bacterial load at the time of hemocyte depletion, with increased numbers of wild-type *L. pneumophila* after efficient intracellular replication overwhelming the larvae.

SdhA is required for bacterial replication in the lungs of A/J mice. In order to validate the role of SdhA in *L. pneumophila* virulence, we analyzed the competitive fitness and replication of the Δ*sdhA* mutant in the lungs of A/J mice. In mixed infections, the Δ*sdhA* mutant was strongly outcompeted by the wild-type strain (mean CI of 0.03 for Δ*sdhA* versus wild type) (Fig. 6A), showing that SdhA contributes to the fitness of *L. pneumophila* in murine infection. The determination of bacterial CFU after 72 h of single infections with WTor Δ*sdhA* confirmed that the replication of the Δ*sdhA* strain in mouse lungs was significantly reduced in comparison to the wild-type strain (Fig. 6B) ($P = 0.0001$, Mann-Whitney U). Each data point represents bacterial counts from one animal. Together, these results showed that SdhA is important for the virulence of *L. pneumophila* in the A/J mouse infection model as well as *G. mellonella*.

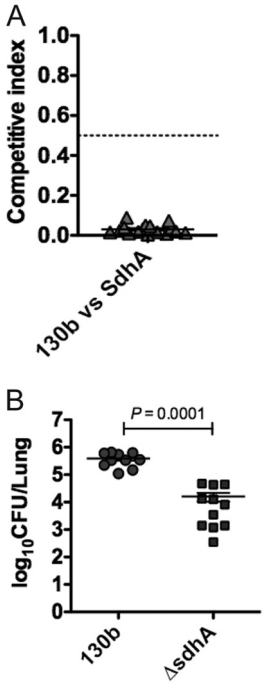

FIG 6 Pulmonary infections of A/J mice with *L. pneumophila*. (A) In mixed infections, wild-type *L. pneumophila* strain 130b and the Δ*sdhA* strain were introduced into the lungs of A/J mice by intranasal inoculation at a 1:1 ratio. At 72 h p.i., the competitive index was calculated (as the ratio of mutant to wild-type bacteria in the lungs divided by the ratio of mutant to wild-type bacteria in the inoculum). The wild-type strain significantly outcompeted the Δ*sdhA* strain by 72 h p.i. (CI, 0.03). (B) In single infections, the Δ*sdhA* strain was significantly attenuated at 72 h p.i. compared to wild-type *L. pneumophila* ($P = 0.0001$, Mann-Whitney U test). Each data point represents the bacterial count from the lungs of one animal; means ± standard errors of the means are shown (although the error bar is not visible for 130b).

DISCUSSION

We recently established the larvae of the wax moth *G. mellonella* as a model for *L. pneumophila* infection (21, 30). After injection into the larvae, *L. pneumophila* replicates in an LCV in insect hemocytes. Infection triggers an antibacterial immune response; however, it ultimately results in drastic depletion of hemocytes and the death of the larvae. Virulence of *L. pneumophila* in *G. mellonella* depends on the Dot/Icm T4SS, and our previous observations suggested that replication, hemocyte depletion, and death of the larvae are linked. However, the contribution to virulence of specific T4SS effectors and other virulence factors remained unknown.

We hypothesized that virulence in the insect might be influenced by the same virulence factors as in macrophages. To test this hypothesis and further compare cell culture and *in vivo* models, we analyzed the role of flagellin and SdhA, which both impact *L. pneumophila*-induced cell death and replication in macrophages.

Infection of the larvae with an *L. pneumophila* Δ*flaA* nonmotile mutant showed that flagellum-based motility or adhesion appear not to be required for bacterial replication or killing of the insects. In contrast, previous studies found that flagella contributed significantly to the virulence of the human pathogens *Listeria monocytogenes* and *Campylobacter jejuni* in *G. mellonella* (42, 43). *In vitro* infection experiments showed that flagella promoted contact and invasion by *L. pneumophila* into tissue culture cells; however, they were dispensable for establishment of the LCV or intracellular replication (44). Our data suggest that in *G. mellonella*, these crucial steps of *L. pneumophila* infection are mediated by other virulence factors.

L. pneumophila flagellin plays a crucial role in the immune response and growth restriction of *L. pneumophila* in mice (12, 29, 31). In murine macrophages, flagellin acts as an important PAMP that triggers inflammasome activation and immune signaling (11, 12). *L. pneumophila* lacking flagellin showed enhanced virulence in mice (31). Conversely, in *G. mellonella* infection, we found that lack of flagellin did not increase the virulence of *L. pneumophila*, demonstrating that flagellin is not an essential trigger for clearance of the bacterium by the insect immune response. This represents an important difference between the *L. pneumophila* mouse and

G. mellonella models. Host specificity of virulence factors has previously been documented in *L. pneumophila* (32), suggesting that the wide host range for *L. pneumophila* has resulted in differential requirements for virulence factors. Our knowledge about specific *G. mellonella* immune-signaling pathways is still limited. PAMP recognition receptors, including Toll-like receptors (33), have been identified in *Lepidopteran* hemocytes (reviewed in reference 34). However, if and how *G. mellonella* responds to flagellin remain to be characterized.

Aside from flagellin, other bacterial components can act as PAMPs and activate immune responses. Creation of a protective intravacuolar niche to avoid detection by danger signal sensors is a strategy employed by several intracellular pathogens (35, 36). Recently, it was shown that SdhA has a crucial role in stabilizing the LCV membrane, ensuring bacterial survival and efficient replication (20). Here we confirmed that in infected hemocytes SdhA localizes around the bacterial vacuole, reminiscent of the localization described previously in macrophages (19). Breakdown of the LCV membrane and degradation of cytoplasmic *L. pneumophila* Δ*sdhA* has previously been inferred by immunofluorescence microscopy with the use of markers for vacuolar lysis (20). The TEM images of the ultrastructure of *L. pneumophila* Δ*sdhA*-infected cells presented here suggest that lack of SdhA leads to release of the bacteria and possibly bacterial contents into the cytoplasm. This verified that SdhA is a major mediator of LCV stability in different host cells, including insect hemocytes.

Lack of SdhA and vacuolar instability have been associated with rapid cell death and defective replication (17, 19, 20). However, these phenotypes and their effects on virulence of *L. pneumophila* Δ*sdhA* had not been investigated in *in vivo* infection models. Infection of *G. mellonella* with the Δ*sdhA* strain resulted in rapid, but transient, hemocyte depletion by 5 h p.i. This correlates with results from primary mouse macrophages in which the Δ*sdhA* strain caused significant cell death by 6 h p.i. (19). As observed in infected macrophages, rapid cell death resulted in inefficient replication of the bacteria in the larvae. Interestingly, between 10 and 24 h p.i., the number of hemocytes increased in larvae infected with the Δ*sdhA* mutant, likely because of the observed lack of bacterial replication. In contrast, hemocyte depletion upon infection with wild-type *L. pneumophila* culminated between 18 and 24 h p.i., after which most larvae had succumbed to the infection.

We and others have previously proposed that hemocyte depletion could cause death of the larvae (21, 37). The transient, nonfatal hemocyte depletion by *L. pneumophila* Δ*sdhA* suggests that different mechanisms of larval death and hemocyte depletion exist. Furthermore, our results suggest that hemocyte depletion *per se* is not sufficient to kill the larvae. Rapid nodule formation by circulating hemocytes to neutralize invading pathogens has been reported as a general response to infection and cause of a transient depletion of hemocytes (38). However, as we did not observe a similar, early decrease in response to infection with wild-type *L. pneumophila*, it is more likely that the drop observed for the Δ*sdhA* strain is not due to a general, nonspecific antibacterial response. Considering the well-demonstrated induction of rapid macrophage death described for *L. pneumophila* Δ*sdhA* (17, 19, 20), a similar rapid death response in hemocytes seems the likely explanation.

Alternatively, the bacterial load in the hemolymph might be decisive for tipping the balance toward either transient or complete, fatal depletion of hemocytes. As in macrophages, rapid hemocyte death might prematurely suppress intracellular replication. This is in line with our observation that the Δ*sdhA* strain did not replicate but did persist in the insect. The numbers of the Δ*sdhA* bacteria after the first incomplete round of replication could be too low to infect all remaining hemocytes, allowing replenishment and control of the infection. Similar to this model, renewal of the epithelium in response to infection was recently described as a major parameter in ensuring the gut homeostasis and host defense of *Drosophila melanogaster* (39). In contrast, after efficient replication, the WT bacteria could overwhelm and destroy all hemocytes in the next rounds of infection. Ultimately, the combination of full hemocyte depletion and additional, adverse effects due to the higher bacterial load result in death of the larvae.

Taken together, our study demonstrates the suitability of the *G. mellonella* model to investigate the role of Dot/Icm T4SS effectors in *L. pneumophila* virulence. Importantly, phenotypes observed in cell culture infection can be reproduced in hemocytes. In addition, we also analyzed replication of the Δ*sdhA* mutant in the lungs of A/J mice. The Δ*sdhA* strain showed reduced bacterial loads in single infections and was outcompeted by WT bacteria in mice, showing a good correlation to the *G. mellonella* model.

Previously, only very few Dot/Icm effectors have been shown to have a significant role in *in vivo* models of infection. AnkB is required for intrapulmonary proliferation in mice (40), while deletion of LubX results in enhanced virulence in *D. melanogaster* (41). We have presented here evidence that an *L. pneumophila* Δ*sdhA* mutant is strongly attenuated in *G. mellonella* and A/J mice, underpinning the fundamental importance of this effector and the maintenance of LCV integrity for the intracellular lifestyle and virulence of *L. pneumophila*.

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