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Peptidoglycan deacetylation controls type IV secretion and the intracellular survival of the bacterial pathogen *Legionella pneumophila*

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Peptidoglycan is a critical component of the bacteria cell envelope. Remodeling of the peptidoglycan is required for numerous essential cellular processes and has been linked to bacterial pathogenesis. Peptidoglycan deacetylases that remove the acetyl group of the *N*-acetylglucosamine (NAG) subunit protect bacterial pathogens from immune recognition and digestive enzymes secreted at the site of infection. However, the full extent of this modification on bacterial physiology and pathogenesis is not known. Here, we identify a polysaccharide deacetylase of the intracellular bacterial pathogen *Legionella pneumophila* and define a two-tiered role for this enzyme in *Legionella* pathogenesis. First, NAG deacetylation is important for the proper localization and function of the Type IVb secretion system, linking peptidoglycan editing to the modulation of host cellular processes through the action of secreted virulence factors. As a consequence, the *Legionella* vacuole mis-traffics along the endocytic pathway to the lysosome, preventing the formation of a replication permissive compartment. Second, within the lysosome, the inability to deacetylate the peptidoglycan renders the bacteria more sensitive to lysozyme-mediated degradation, resulting in increased bacterial death. Thus, the ability to deacetylate NAG is important for bacteria to persist within host cells and in turn, *Legionella* virulence. Collectively, these results expand the function of peptidoglycan deacetylases in bacteria, linking peptidoglycan editing, Type IV secretion, and the intracellular fate of a bacterial pathogen.

Legionella | polysaccharide deacetylase | peptidoglycan | type IV secretion system | DotK

Significance

Peptidoglycan is an essential component of the bacterial cell wall. At the same time, it is a barrier to the assembly and function of critical macromolecular machines in the cell envelope, and avoiding detection and eradication by the immune system. The ability of bacterial pathogens to resolve these conflicting roles is a key determinant of virulence. Herein, we demonstrate that peptidoglycan deacetylation by *Legionella* drives the proper localization and function of the Dot/Icm Type IV secretion system, allowing *Legionella* to establish a replication vacuole. In parallel, the same modification protects against lysozyme when bacteria mis-traffic to the lysosome, allowing bacterial survival in this otherwise degradative compartment. Our findings reveal a multitiered role for a polysaccharide deacetylase in bacterial pathogenesis.

Peptidoglycan is a critical component of the bacteria cell wall, consisting of glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid disaccharides cross-linked by short peptides. In gram-negative bacteria, the peptidoglycan is located between the bacterial inner and outer membranes, providing shape, structure, rigidity against turgor, and a scaffold for anchoring other cell envelope components (1, 2).

Bacteria remodel their peptidoglycan in a variety of cellular processes that require cell wall reorganization and turnover including germination, cell elongation, cell division, and sporulation (3–6). Peptidoglycan restructuring also plays a central role in bacterial pathogenesis. For example, lytic transglycosylases that hydrolyze the peptidoglycan polymer are central to toxin secretion and the assembly of large macromolecular complexes in the cell envelope, including Type III, Type IV, and Type VI secretion systems (7–10). Bacteria also alter their peptidoglycan through covalent modifications. For example, polysaccharide deacetylases of the carbohydrate esterase family 4 (CE-4)/Nodulation protein B (NodB) superfamily (11) mediate the deacetylation of cell wall glycans, including *N*-linked acetyl groups of the peptidoglycan subunit NAG (12–14). The best characterized effects of NAG deacetylation are in bacterial pathogenesis, whereby this modification impairs peptidoglycan recognition by host immune cells and confers resistance to lysozyme released by immune cells at the site of infection (6, 14–20). Due to the importance of peptidoglycan, it is possible that modifications of this polymer play additional roles in the virulence of many bacterial pathogens.

Legionella pneumophila is an intracellular bacterial pathogen (21) that inhabits both fresh and potable water distribution systems (22, 23). In the environment, *L. pneumophila* is a parasite of a broad assortment of free-living amebae (24). When contaminated water aerosols are inhaled by humans, *L. pneumophila* enter the lungs (25) where they replicate within alveolar macrophages (26), causing pneumonia (27).

Upon phagocytosis by host cells, *L. pneumophila* are encased in a membrane-bound compartment called the *Legionella*-containing vacuole (LCV). *L. pneumophila* prevent trafficking of this vacuole along the endocytic pathway to the lysosome (28–30) and instead, remodel the LCV into a replication-permissive compartment (31, 32). While

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The authors declare no competing interest.

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endocytic trafficking to a lysosomal compartment can occur as early as 5 min post infection, bacterial replication does not begin until 4 to 6 h after entering the host cell. A major virulence factor of *L. pneumophila* is a Type IVb secretion system termed Dot/Icm (33–35). Dot/Icm is a large macromolecular complex composed of 30 proteins that spans the bacterial inner membrane, periplasm, and outer membrane (35–40). The Dot/Icm machinery translocates more than 300 bacterial proteins, termed effectors into the host cell (41–45), to modulate numerous host cellular processes (46–49). The Dot/Icm complex localizes to the bacterial poles (50, 51), and its mis-localization impairs intracellular replication (50). Bacteria with defects in Dot/Icm function fail to remodel their phagosomes into replication compartments and instead traffic along the endocytic pathway to the lysosome (33, 52–54).

In a previous genetic screen, we identified the *L. pneumophila* gene *lpg1993*, predicted to encode a polysaccharide deacetylase, as important for *L. pneumophila* fitness in the amebal host *Acanthamoeba castellanii* (55). Here, we demonstrate that Lpg1993 is a NAG deacetylase that both confers resistance to lysozyme and restricts binding of the Dot/Icm subunit DotK to peptidoglycan, and as a consequence Dot/Icm to the bacterial pole. Thus, the loss of Lpg1993 thereby causes defects in the proper localization and function of the Dot/Icm secretion system. As a consequence, there is increased endocytic trafficking of *L. pneumophila* to the lysosome. Moreover, within the lysosome, the increased sensitivity of a Δ*lpg1993* mutant to the degradative enzyme lysozyme causes decreased bacterial survival. These results demonstrate a multifaceted role for a bacterial polysaccharide deacetylase in pathogenesis, linking the acetylation state of the *Legionella* peptidoglycan, Type IVb secretion system function, and the fate of the bacteria within host cells.

Results

Lpg1993 Encodes a Polysaccharide Deacetylase That Protects against Lysozyme. The Lpg1993 protein is predicted to be a periplasmic protein homologous to bacterial polysaccharide deacetylases of the CE-4/NodB family of proteins (*SI Appendix*, Figs. S1 and S2). To determine whether Lpg1993 functions as a polysaccharide deacetylase of peptidoglycan, structural analysis of peptidoglycan muropeptides isolated from wild-type (WT) bacteria and an Δ*lpg1993* mutant was performed. Bacteria were grown in bacteriological medium to post-exponential phase, which coincides with the onset of virulence (56, 57) and thus are used to challenge host cells when studying *Legionella* pathogenesis. Cell wall was then harvested from the bacteria and analyzed by ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) analysis. Peptidoglycan isolated from WT bacteria consisted of NAG subunits with and without *N*-linked acetylation (*SI Appendix*, Figs. S3–S5), whereas there was a significant reduction in the amount of deacetylated muropeptides from Δ*lpg1993* mutant bacteria (27.7 ± 2.7% vs. 15.3 ± 1.2%, respectively) (Fig. 1*A* and *SI Appendix*, Figs. S3–S5). Consistent with these observations, recombinant wild-type Lpg1993 protein ($Lpg1993_{WT}$) (*SI Appendix*, Fig. S6) exhibited dose-dependent deacetylation of purified peptidoglycan (Fig. 1*B*), whereas no activity was observed for a variant of Lpg1993 lacking a conserved catalytic aspartate residue ($Lpg1993_{D37A}$) (*SI Appendix*, Fig. S6 and Fig. 1*B*). To determine the impact of Lpg1993 on *L. pneumophila* resistance to digestion, peptidoglycan was isolated from WT and Δ*lpg1993* bacteria and subjected to lysozyme treatment. UPLC-coupled MS/MS analysis showed more extensive degradation of peptidoglycan isolated from the Δ*lpg1993* mutant than peptidoglycan from WT bacteria (59.1 ± 6.6% vs. 37.7 ± 7.3%, respectively) (Fig. 1*C*). Moreover, Δ*lpg1993* bacteria exhibited enhanced sensitivity to lysozyme, measured by bacterial survival when compared to WT bacteria, both in the absence (consistent with conditions in a lysosome during infection) (Fig. 1*D*) and presence of a permeabilizing agent (*SI Appendix*, Fig. S7*A*). The extent of the lysozyme resistance defect of Δ*lpg1993* bacteria (1.4 to 2.6-fold) was consistent with the decrease in NAG deacetylation (1.8-fold) (Fig. 1*A*), and could be rescued by WT Lpg1993 ($Lpg1993_{WT}$) but not catalytically inactive Lpg1993 variants ($Lpg1993_{D37A}$ and $Lpg1993_{D37N}$) (Fig. 1*B* and *SI Appendix*, Fig. S7*A*). Theoretically, the absence of *lpg1993* could indirectly alter outer membrane permeability, which could contribute to the increased sensitivity of Δ*lpg1993* bacteria to lysozyme-mediate killing. However, this does not appear to be the case as periplasmic

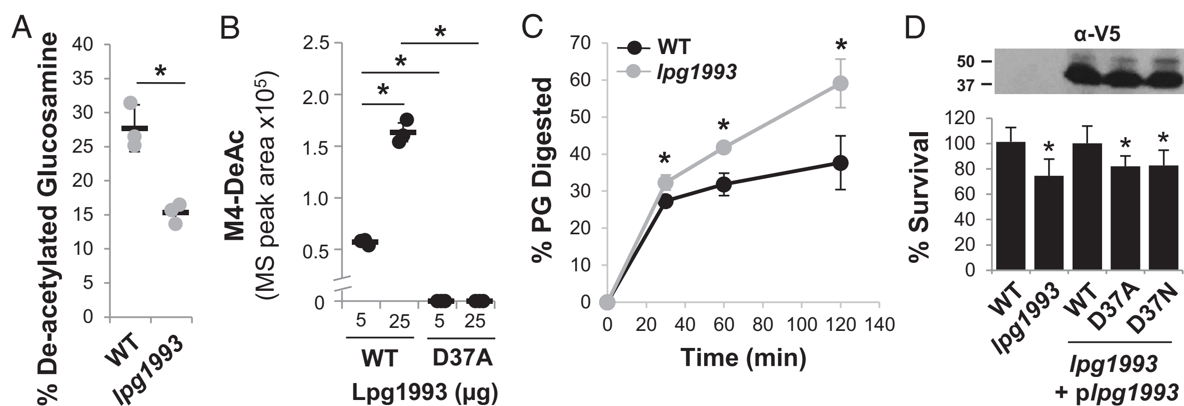

Fig. 1. Lpg1993 catalyzes the deacetylation of *N*-acetylglucosamine protecting *L. pneumophila* peptidoglycan from lysozyme digestion. (*A*) *lpg1993* encodes an *N*-linked *N*-acetylglucosamine deacetylase. Quantitative structural analysis of peptidoglycan muropeptides of wild-type (WT) and Δ*lpg1993* bacteria. (*B*) Lpg1993 catalyzes the deacetylation of peptidoglycan in vitro. Varying amounts of wild-type (WT) or catalytically inactive (D37A) Lpg1993 protein were incubated with purified peptidoglycan, and the amount of NAG deacetylation based on the abundance of deacetylated GlcN-MurNAc-tetrapeptide (M4-DeAc) was measured by UPLC-MS. (*C*) Peptidoglycan of Δ*lpg1993* bacteria is more sensitive to lysozyme digestion. Peptidoglycan isolated from WT and Δ*lpg1993* bacteria was treated with lysozyme, and the percentage of hydrolyzed peptidoglycan was measured by UPLC-MS. (*A*–*C*) Data are the mean ± SD of 3 biological replicates. (*D*) Lpg1993 protects *L. pneumophila* against lysosome-mediated killing. (*Top*) Western analysis of whole-cell lysates of WT and Δ*lpg1993* bacteria harboring empty vector or Δ*lpg1993* bacteria expressing V5-6×HIS dual epitope–tagged fusion proteins of wild-type Lpg1993 (WT) or variants lacking the conserved catalytic residue aspartate residue (D37A, D37N) grown to post-exponential phase. Data are representative of 3 biological replicates. (*Bottom*) Lysozyme sensitivity assay. Bacteria were treated with lysozyme, and percent survival was measured based on recovered colony-forming units (cfus) on solid medium comparing lysozyme treatment to no lysozyme control. Data are the mean ± SD of 6 biological replicates, each consisting of 3 technical replicates. (*A*–*D*). An asterisk indicates a Student's *t* test $P < 0.05$ relative to the WT strain, unless otherwise indicated.

proteins in Δ*lpg1993* bacteria were not more susceptible to digestion compared to those in WT bacteria when intact bacteria were exposed to a protease of similar size to lysozyme (18 kD vs. 15 kD, respectively) (*SI Appendix*, Fig. S7*B*). Collectively, these results demonstrate that Lpg1993 is a polysaccharide deacetylase that removes the acetyl group from NAG, and this activity protects the peptidoglycan layer against lysozyme digestion. Lpg1993 was thus named peptidoglycan deacetylase A (PgdA).

Loss of PgdA Restricts Bacterial Numbers at Early Stages of the Infection Cycle. A previous genetic screen identified *pgdA* as important for *L. pneumophila* fitness in the amoeba *A. castellanii* (55). The fitness defect could result from multiple factors, for example decreased survival due to defects in lysosomal avoidance or impaired bacterial replication. To determine the basis of the phenotype, survival of the Δ*pgdA* mutant in the first hour of infection was examined. *A. castellanii* were challenged with the WT or Δ*pgdA* mutant strain and the number of bacteria was determined at 20, 40, and 60 min post infection. For the WT strain, the number of bacteria accumulated over the course of 1 h (Fig. 2*A*). In contrast, the number of Δ*pgdA* mutant bacteria was significantly lower than that of the WT strain at all the 3 time points (Fig. 2*A*), reaching a 3.3-fold decrease by 60 min. The phenotype of the Δ*pgdA* mutant could be rescued by reintroducing a copy of *pgdA* on a self-replicating plasmid (Fig. 2*A*). Conversely, deleting *pgdA* did not inhibit *L. pneumophila* replication in *A. castellanii* (*SI Appendix*, Fig. S8*A*), demonstrating that the subset of bacteria that endure the first hour of infection are able to grow similar to the WT strain. These results demonstrated that *pgdA* plays an important role in survival at early stages of the infection cycle.

To determine whether PgdA is similarly important during infection of macrophages, bacterial numbers were examined at early time points in primary bone marrow–derived murine macrophages. In this host cell type, we observed high numbers of WT bacteria at 20 min that were maintained through 60 min (Fig. 2*B*). In contrast, significantly decreased numbers of Δ*pgdA* bacteria were observed at 20 min with a reduction in bacterial numbers through 40 and 60 min, reaching a 3-fold decrease by 60 min (Fig. 2*B*). The reduced number of Δ*pgdA* bacteria was not due to an increase in the cytotoxicity of the deacetylated peptidoglycan as no difference in host viability between WT and Δ*pgdA* mutant–infected macrophages was observed (*SI Appendix*, Fig. S9). Importantly, WT PgdA ($PgdA_{WT}$) but not catalytically inactive variants ($PgdA_{D37A}$ and $PgdA_{D37N}$) could restore Δ*pgdA* bacterial numbers at 60 min post infection to that of the WT strain (Fig. 2*C*), demonstrating that survival of Δ*pgdA* bacteria depends on the ability of PgdA to deacetylate the peptidoglycan. Similar to growth in *A. castellanii*, deleting *pgdA* had no effect on *L. pneumophila* replication in macrophages (*SI Appendix*, Fig. S8*B*). These results link peptidoglycan deacetylation to *L. pneumophila* survival through the first hour of the infection cycle and an important role for PgdA in both amoebae and macrophages.

Lpg0633 and Lpg1637 Impact *L. pneumophila* NAG Deacetylation but Differentially Effect Survival within Host Cells. NAG subunits are incorporated into the peptidoglycan in the acetylated form and the extent of their deacetylation is mediated by periplasmic polysaccharide deacetylases (58–60). The decreased but not abolished NAG deacetylation of Δ*pgdA* bacteria suggested the existence of another protein that contributes to this process. *L. pneumophila* is predicted to encode two additional polysaccharide deacetylases, Lpg0633 and Lpg1637, although Lpg1637 lacks many conserved active site residues characteristic of these enzymes (*SI Appendix*, Figs. S1 and S2). While loss of Lpg0633 alone did not significantly reduce NAG deacetylation (*SI Appendix*, Figs. S10*A*, S11, and S12), combined deletion of *pgdA* and *lpg0633* completely eliminated NAG deacetylation (*SI Appendix*, Fig. S10*A*), suggesting that Lpg0633 plays an accessory role in peptidoglycan modification. In the case of *lpg1637*, inactivation of the gene did not significantly affect NAG deacetylation, nor did it exacerbate the deacetylation defect of a Δ*pgdA* mutant (*SI Appendix*, Fig. S10*A*). However, surprisingly, deleting *lpg1637* in combination with *lpg0633* almost abolished NAG deacetylation (*SI Appendix*, Fig. S10*A*), indicating a convoluted interaction between their encoded proteins. Thus, PgdA, Lpg0633, and Lpg1637 each contribute to NAG deacetylation in some manner. Lpg0633 likely functions as an NAG deacetylase similar to PgdA. In contrast, Lpg1637 likely performs a regulatory role rather than an enzymatic one.

Despite its impact on NAG deacetylation, disrupting *lpg0633* did not result in decreased bacterial numbers within macrophages (*SI Appendix*, Fig. S13*A*), demonstrating that unlike *pgdA*, *lpg0633* is not important for *L. pneumophila* intracellular survival. In

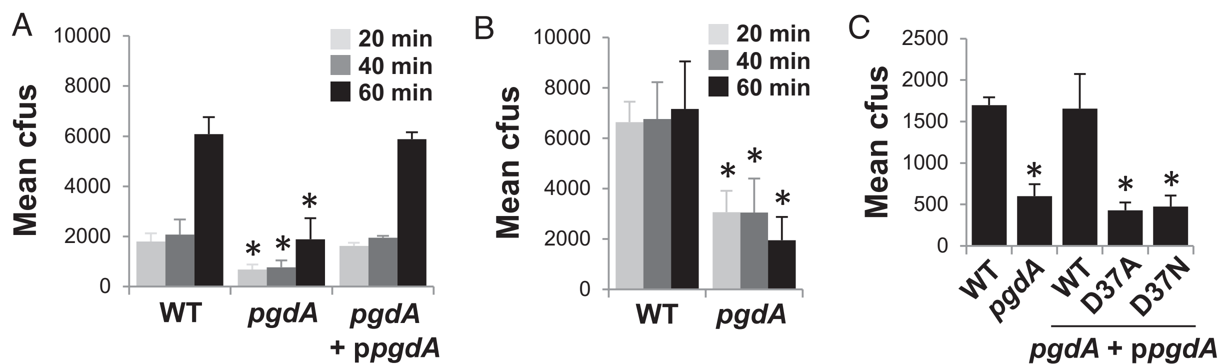

Fig. 2. Loss of PgdA results in reduced numbers of bacteria in host cells at early time points of the infection cycle. (*A*) Δ*pgdA* mutant bacteria show reduced numbers through the first hour of infection in the amoebal host *A. castellanii*. *A. castellanii* were challenged for 20, 40, or 60 min with the wild-type (WT) strain or a Δ*pgdA* mutant harboring empty vector or a Δ*pgdA*-complemented strain expressing *pgdA* from a self-replicating plasmid. Cells were rinsed and then bacterial numbers were quantified based on recovered cfus on solid medium from lysed host cells normalized to bacterial cfus from the inoculum. Data are the mean ± SD of 2 to 4 biological replicates, each consisting of 3 technical replicates. *$P < 0.02$. (*B*) Δ*pgdA* bacteria exhibit a survival defect in macrophages. Primary bone marrow–derived murine macrophages were challenged with the indicated strains, and bacterial numbers through the first hour of infection were quantified as in *A*. Data are the mean ± SD of 5 biological replicates, each consisting of 3 technical replicates. *$P < 0.01$. (*C*) The survival defect of Δ*pgdA* bacteria depends on PgdA polysaccharide deacetylase activity. Macrophages were challenged with the indicated strains for 60 min and then treated with gentamicin for 2 h to remove extracellular bacteria. Bacterial numbers were enumerated as in *A* comparing WT bacteria and a Δ*pgdA* mutant harboring empty vector to a Δ*pgdA* mutant expressing WT PgdA or catalytically inactive variants (D37A, D37N) from a self-replicating plasmid (p*pgdA*). Data are the mean ± SD of 3 biological replicates, each consisting of 3 technical replicates. *$P < 0.001$. (*A*–*C*) An asterisk indicates a two-tailed Student's *t* test *P* value as indicated relative to the WT strain.

contrast, the absence of Lpg1637 in the Δ*pgdA* mutant rescued its survival defect in macrophages (*SI Appendix*, Fig. S13*A*), consistent with a regulatory role for this protein. Notably, the decrease in NAG deacetylation of the Δ*pgdA* Δ*lpg0633* and Δ*lpg0633* Δ*lpg1637* double mutants did not coincide with an increase in lysozyme sensitivity when compared to a Δ*pgdA* single mutant (*SI Appendix*, Fig. S10*B*), alluding to the possibility that additional factors may contribute to lysozyme resistance when NAG deacetylation is severely impaired. Similar to PgdA, loss of Lpg0633 or Lpg1637 did not result in a replication defect in macrophages (*SI Appendix*, Fig. S13*B*). Consistent with this observation, impaired NAG deacetylation did not affect *L. pneumophila* growth in nutrient-rich bacteriological medium (*SI Appendix*, Fig. S13*C*). Thus, PgdA is important for survival within host cells, and its requirement is linked to the function of Lpg1637.

PgdA Is Required to Avoid Endocytic Trafficking. *L. pneumophila dot*/*icm* mutant–containing phagosomes are rapidly targeted to lysosomes, becoming decorated with the lysosomal marker lysosomal associated membrane protein 1 (LAMP-1) as early as 5 min post infection (30). One possible explanation for the decrease in Δ*pgdA* bacterial numbers at early stages of the infection is that a subset of bacteria is delivered to the lysosome and degraded. To test this, macrophages were challenged with WT, Δ*pgdA* mutant, or Dot/Icm translocation–deficient (*dot-*) bacteria and colocalization of LAMP-1 with LCVs was examined at 20, 40, and 60 min by fluorescence microscopy. At 20 and 40 min, the percentage of Δ*pgdA* mutant–containing vacuoles colocalizing with LAMP-1 was 2.5-fold higher than that of WT bacteria (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S14). $\mathrm{PgdA_{WT}}$ but not the catalytically inactive variants $\mathrm{PgdA_{D37A}}$ could rescue the trafficking defect of the Δ*pgdA* mutant (*SI Appendix*, Fig. S15), indicating that proper trafficking depends on PgdA deacetylase activity. Interestingly, by 60 min, the percentage of LAMP-1 decorated Δ*pgdA* mutant vacuoles was reduced to that of the WT bacteria (Fig. 3*B* and *SI Appendix*, Fig. S14), consistent with clearance of a subpopulation of Δ*pgdA* bacteria. In contrast, the majority of *dot-* bacteria colocalized with LAMP-1 at all time points tested (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S14). These data demonstrate that a subpopulation of Δ*pgdA* bacteria mistargets along the endocytic pathway, revealing a role for PgdA in the proper trafficking of the LCV during infection.

While *dot-* bacteria target to a LAMP-1-positive vacuole, they are not degraded (61). In contrast, inactivation of *pgdA* in a *dot-* mutant resulted in decreased numbers of bacteria at 20, 40, and 60 min post infection (Fig. 3*C*), demonstrating a role for *pgdA* in *L. pneumophila* survival within an LAMP-1-positive compartment.

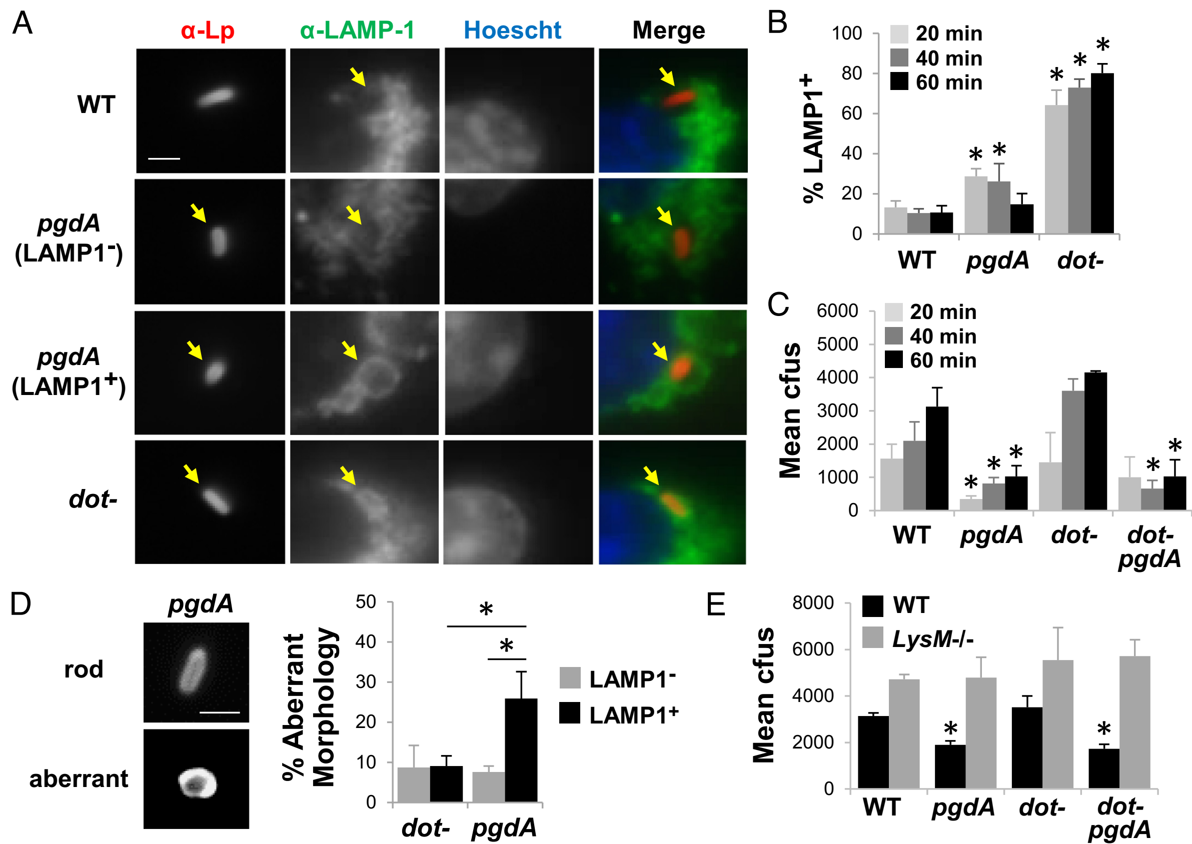

Fig. 3. The Δ*pgdA* mutant is defective for avoiding endocytic trafficking and survival in lysosomes. (*A*) Loss of PgdA results in endocytic trafficking of the LCV. Macrophages were infected with wild-type (WT), Δ*pdgA*, or Dot/Icm translocation–deficient (*dot-*) bacteria for 20, 40 (*SI Appendix*, Fig. S14), or 60 min (*SI Appendix*, Fig. S14), fixed, and visualized by fluorescence microscopy. (*B*) The number of LAMP-1-positive *Legionella*–containing vacuoles (LCVs) in *A* was scored, counting 100 vacuoles per replicate. Data are the mean ± SD of 4 biological replicates, each consisting of 3 technical replicates. *$P < 0.03$ relative to the WT strain. (*C*) Loss of *pgdA* in a *dot-* mutant background reduces bacterial survival in host cells. Macrophages were challenged with the indicated strains for 20, 40, or 60 min. Cells were rinsed, and bacterial numbers were quantified based on recovered cfus on solid medium from lysed host cells. Data are the mean ± SD of 5 biological replicates, each consisting of 3 technical replicates. *$P < 0.005$ relative to the WT strain (*pgdA*) and $P < 0.001$ relative to the *dot-* strain (*dot- pgdA*). (*D*) Δ*pgdA* bacteria that colocalize with LAMP-1 (*A* and *B*) show increased aberrant morphology. (*Left*) Fluorescence microscopy of *dot-* and Δ*pgdA* bacteria at 20 min post infection. (*Right*) The number of *dot-* and Δ*pgdA* bacteria with aberrant morphology was scored. *$P < 0.04$. (*E*) Inactivation of host lysozyme restores Δ*pgdA* bacterial numbers to WT levels. WT and *LysM*−/− macrophages were challenged with the indicated strains, and bacterial numbers at 60 min were examined as in *C*. Data are the mean ± SD of 3 biological replicates, each consisting of 3 technical replicates. *$P < 0.005$ for Δ*pgdA* bacteria relative to the WT strain and $P < 0.001$ for *dot-* Δ*pgdA* bacteria relative to the *dot-* strain. (*B*–*E*) An asterisk indicates a two-tailed Student's *t* test *P* value as indicated. (*A* and *D*) (Scale bar indicates 2 μm.)

Indeed, within LAMP-1-decorated vacuoles, a greater number of Δ*pgdA* bacteria exhibited aberrant morphology, including rounding and blebbing that is consistent with bacterial degradation, when compared to a *dot-* strain (Fig. 3*D*). Moreover, in macrophages isolated from *LysM*−/− mice that lack lysozyme (62), the number of Δ*pgdA* single-mutant and *dot-* Δ*pgdA* double-mutant bacteria was similar to that of WT and *dot-* bacteria, respectively (Fig. 3*E*), linking decreased survival of the Δ*pgdA* mutant to its increased sensitivity to lysozyme. Collectively, these data demonstrate that in addition to promoting lysosome avoidance, PgdA is important for protection against degradation within vacuoles that mistarget along the endocytic pathway to a lysosome.

Loss of PgdA Alters Dot/Icm Polar Localization and Function. Since the *L. pneumophila* Dot/Icm secretion system is essential for modulating endocytic trafficking events and preventing rapid phagosome–lysosome fusion (33, 52–54), we assayed whether loss of PgdA activity perturbs Dot/Icm function. To begin, survival of Δ*pgdA* bacteria upon exposure to moderate levels of sodium chloride was examined, as mutations that impair Dot/Icm function render the bacteria more resistance to salt (35). When compared to a *dot-* strain, the Δ*pgdA* mutant showed increased salt resistance relative to WT bacteria (*SI Appendix*, Fig. S16*A*). The phenotype was not due to altered abundance of the Dot/Icm machinery, as similar levels of the core complex proteins DotF and DotH were observed in WT and Δ*pgdA* bacteria (*SI Appendix*, Fig. S16*B*).

Next, we compared the ability of WT and Δ*pgdA* bacteria to translocate the effector LidA into host cells during infection. LidA is the only effector shown to be translocated as early as 5 min post infection using fluorescence microscopy and LidA-specific antibodies, to be maintained through the first 4 h of the infection cycle, and to be sequestered at the LCV (63), allowing effector translocation to be quantified at a single-cell level and within a time frame that coincides with Δ*pgdA* bacterial phenotypes. Macrophages were challenged with WT or Δ*pgdA* bacteria for 20 and 60 min and the relative amount of translocated LidA at individual LCVs was quantified based on fluorescence intensity. At 20 min, LidA could be detected at the majority of LCVs of each strain (Fig. 4 *A* and *B*). However, closer examination of the data revealed a subpopulation of Δ*pgdA* bacteria–containing vacuoles exhibiting diminished LidA translocation (Fig. 4*B*). These results were not due to lower levels of LidA in the Δ*pgdA* mutant compared to WT bacteria or alterations in the levels of the Dot/Icm machinery (*SI Appendix*, Fig. S17). In addition, there was a 3.5-fold increase in the number of Δ*pgdA* bacteria that failed to translocate detectable levels of LidA into host cells when compared to WT bacteria (Fig. 4*C*). Notably, these bacteria exhibited aberrant morphology, rounding, and blebbing, consistent with bacterial cell death (Fig. 4*D*). While we were able to detect LidA, the signal did not extend beyond the boundary of the bacteria and was likely due to the loss of cell wall integrity and/or increased sensitivity to solvent-induced permeabilization during staining. The number of LidA translocation–deficient Δ*pgdA* bacteria with aberrant morphology is consistent with the greater number of Δ*pgdA* mutant LCVs targeting to LAMP-1-positive compartments (Fig. 3), and the decrease in Δ*pgdA* mutant bacterial numbers at early time points (Fig. 2). At 60 min post infection, the amount of translocated LidA was increased for both strains (Fig. 4

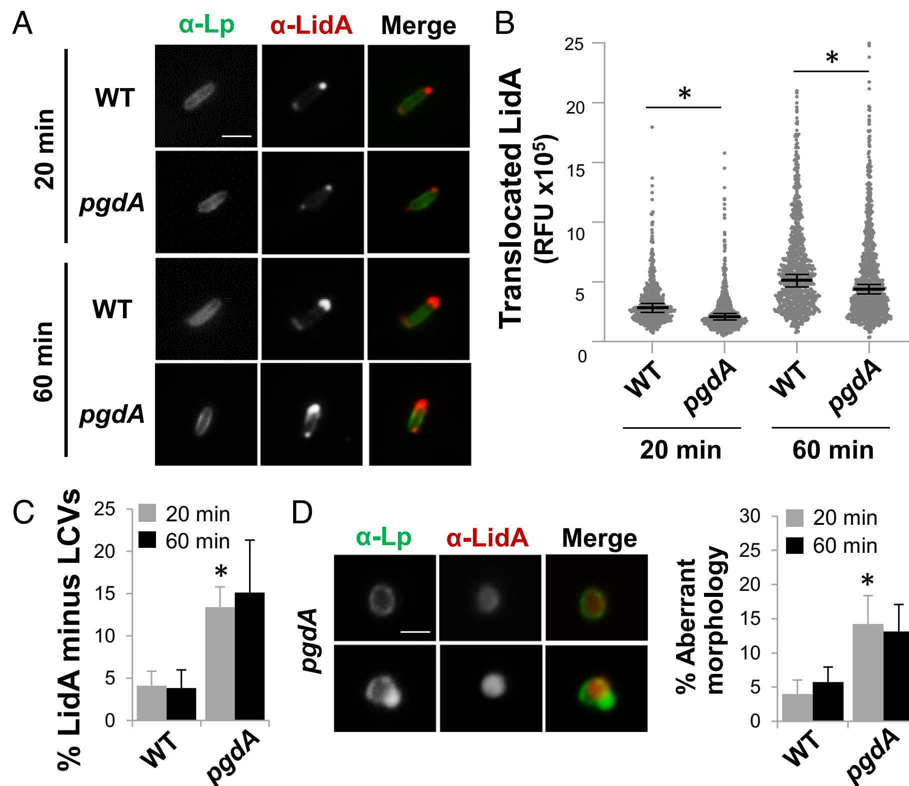

Fig. 4. The Δ*pgdA* mutant is defective for Dot/Icm function. (*A*) Loss of PgdA impairs effector translocation. Macrophages were infected with wild-type (WT) or Δ*pgdA* bacteria for 20 or 60 min, fixed, and visualized by fluorescence microscopy. (*B*) The relative amount of translocated LidA based on fluorescence signal intensity in *A* was quantified. Data are the individual measurements and median ± SD of 3 biological replicates scoring 400 to 700 LCVs each. An asterisk indicates a two-way ANOVA and a two-tailed, nonparametric *t* test with Welch correction $P < 0.0001$ relative to the WT strain. (*C*) The absence of PgdA renders a subset of bacteria incapable of effector translocation. For infected macrophages in *B*, the number of LCVs devoid of LidA translocation into host cells was scored. *$P < 0.02$. (*D*) Δ*pgdA* bacteria defective for LidA translocation exhibit aberrant morphology. (*Left*) Fluorescence microscopy of Δ*pgdA* bacteria. (*Right*) The number of WT and Δ*pgdA* bacteria with aberrant morphology was scored. *$P < 0.04$. (*C* and *D*) An asterisk indicates a two-tailed Student's *t* test *P* value relative to the WT strain. (*A* and *D*) (Scale bar indicates 2 μm.)

A and *B*), consistent with the survival of a subpopulation of Δ*pgdA* bacteria that grow intracellularly (Fig. 2 and *SI Appendix*, Fig. S8) but the amount of LidA detected for Δ*pgdA* bacteria was lower than that of WT bacteria, further highlighting the importance of PgdA for effector translocation. Collectively, these data demonstrate that loss of PgdA impairs Dot/Icm function, and the extent of the defect varies across the population which, in more severe cases, leads to endocytic trafficking of the LCV to the lysosome and subsequent bacterial death.

Δ*pgdA* Mutant Bacteria Exhibit Defects in Dot/Icm Polar Localization. To determine how loss of PgdA affects Dot/Icm activity, the cellular location of the Dot/Icm secretion system was examined, as it was previously shown to localize to the bacterial poles (37, 50) and that polar translocation of effectors is required for *L. pneumophila* virulence (50, 51). To do this, the distribution of the core complex subunit DotF was examined in WT bacteria and the Δ*pgdA* mutant by fluorescence microscopy. In WT bacteria, DotF distinctly partitioned to the bacterial poles, and at the mid-cell of dividing bacteria (Fig. 5*A*). In comparison, while many Δ*pgdA* bacteria showed a similar distribution pattern as WT bacteria, a significant portion of bacteria also exhibited DotF puncta along the length of the bacteria (Fig. 5 *A* and *B*). Similar results were also observed for another Dot/Icm subunit, DotH (*SI Appendix*, Fig. S18). Moreover, $PgdA_{WT}$ but not catalytically inactive $PgdA_{D37A}$ could restore aberrant DotF localization pattern of the Δ*pgdA* mutant to that of the WT strain (Fig. 5*B* and *SI Appendix*, Fig. S18), indicating that Dot/Icm polar localization depends on PdgA-mediated peptidoglycan deacetylation. Since the relative abundance of DotF and DotH was similar between WT and Δ*pgdA* bacteria (*SI Appendix*, Fig. S16*B*), these observations indicate that a subset of Dot/Icm translocons mislocalizes in the absence of PgdA. These results demonstrate a role for peptidoglycan deacetylation in the proper partitioning of the Dot/Icm machinery to the bacterial pole and a molecular basis for the effector translocation defect of Δ*pgdA* bacteria.

DotK Binds Peptidoglycan in an Acetylation-Dependent Manner That Promotes Dot/Icm Polar Localization. Since the assembly and function of Type II, Type VI, and Type IV pilin secretion systems (64–67) depend on peptidoglycan-binding proteins, one explanation for the impact of PgdA on Dot/Icm localization is that NAG deacetylation affects Dot/Icm anchoring to the cell wall. The lipoprotein DotK (40) is a putative peptidoglycan-binding protein, with structural homology to the OmpA-like domain that binds the *meso*-diaminopimelic acid subunit of the peptidoglycan (40, 51). To test whether DotK interacts with peptidoglycan and whether this depends on its acetylation state, DotK binding to peptidoglycan isolated from WT and Δ*pgdA* bacteria was compared. To do this, purified DotK (*SI Appendix*, Fig. S19*A*) was incubated with varying amounts of peptidoglycan, peptidoglycan was pelleted by ultracentrifugation, and the amount of DotK bound was determined by western analysis. In both cases, DotK was observed to bind the peptidoglycan in a dose-dependent manner (Fig. 5*C* and *SI Appendix*, Fig. S19*B*), demonstrating that DotK is a peptidoglycan-binding protein. Intriguingly, in

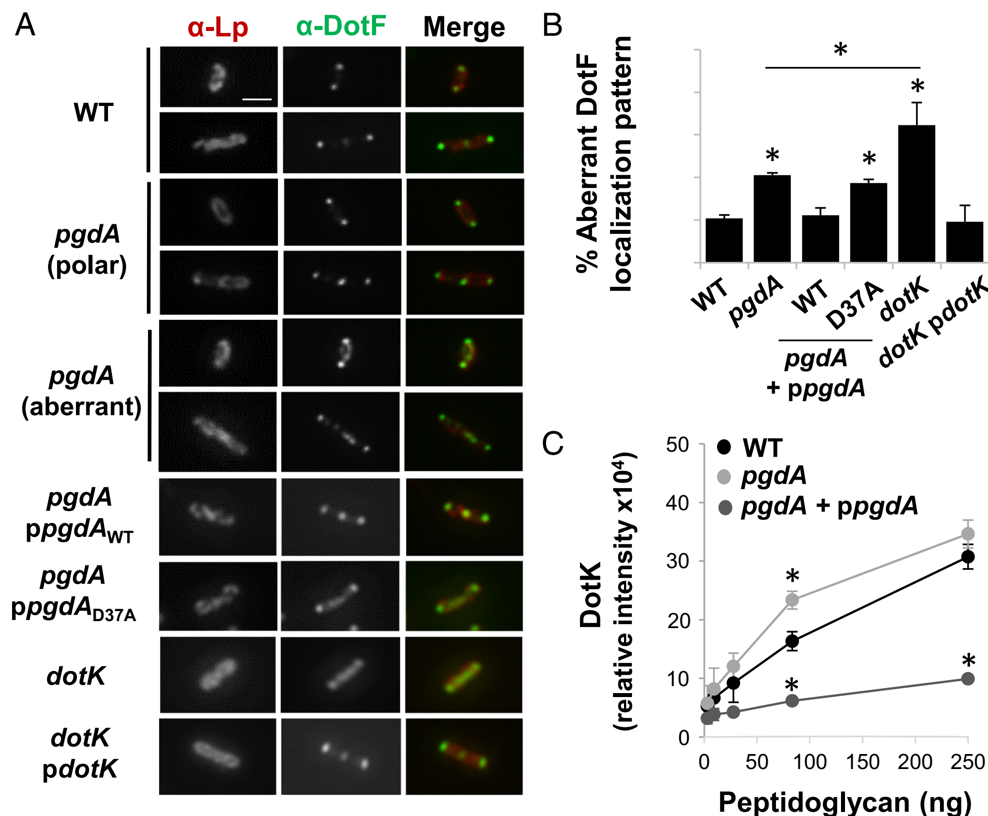

Fig. 5. PgdA promotes polar localization of the Dot/Icm secretion system by modulating DotK binding to peptidoglycan. (*A*) Δ*pgdA* and Δ*dotK* mutant bacteria show mislocalization of Dot/Icm. Bacteria were grown to late exponential phase, fixed, stained for DotF, and visualized by fluorescence microscopy. (Scale bar indicates 2 μm.) (*B*) The number of bacteria in *A* exhibiting aberrant (non-polar) distribution of the DotF was scored. Data are the mean ± SD of 3 biological replicates, scoring an average of 300 to 500 bacteria per replicate. *$P < 0.001$. (*C*) PgdA restricts DotK binding to peptidoglycan. Purified DotK was incubated with varying amounts of peptidoglycan isolated from the indicated strains, peptidoglycan was collected, and the amount of bound DotK based on western analysis (*SI Appendix*, Fig. S19*B*) was quantified. Data are the mean ± SD of 3 biological replicates, each consisting of independently isolated peptidoglycan. *$P < 0.05$. An asterisk indicates a two-tailed Student's *t* test *P* value as indicated relative to the wild-type (WT) strain.

comparison to WT peptidoglycan, we observed a 1.5-fold increase in DotK binding to Δ*pgdA* mutant peptidoglycan (Fig. 5*C* and *SI Appendix*, Fig. S19*B*). Furthermore, DotK binding to peptidoglycan from Δ*pgdA* bacteria overexpressing PgdA was severely impaired compared to peptidoglycan from WT bacteria (Fig. 5*C* and *SI Appendix*, Fig. S19*B*). Thus, not only does the interaction between DotK and peptidoglycan depend on the acetylation state of NAG, but its deacetylation by PgdA inhibits DotK binding.

Given the link between DotK and PgdA activity, the importance of DotK for Dot/Icm polar localization was examined. Similar to the Δ*pgdA* mutant, bacteria lacking *dotK* showed defects in DotF polar localization, a phenotype that could be rescued by reintroducing *dotK* on a self-replicating plasmid (Fig. 5 *A* and *B*). These results define a role for DotK in the proper localization of the Dot/Icm translocon at the bacterial poles. Notably, the number of Δ*dotK* bacteria exhibiting an aberrant DotF distribution pattern was higher than that of the Δ*pgdA* mutant. The discrepancy between the two strains may reflect the decreased but not abolished NAG deacetylation of Δ*pgdA* bacteria (Fig. 1*A* and *SI Appendix*, Fig. S10*A*). However, the number of Δ*pgdA* Δ*lpg0633* bacteria, which lack NAG deacetylation (*SI Appendix*, Fig. S10*A*) with mis-localized DotF, was not statistically significant compared to the Δ*pgdA* single mutant (*SI Appendix*, Fig. S20). Thus, there are likely other features that regulate DotK binding to peptidoglycan. Intriguingly, deletion of *lpg1637* restored DotF polar localization in Δ*pgdA* bacteria to WT levels (*SI Appendix*, Fig. S20), providing an explanation for the lack of a survival defect for Δ*pgdA* Δ*lpg1637* bacteria in macrophages (*SI Appendix*, Fig. S13*A*). Collectively, these data show that PgdA-mediated deacetylation of the peptidoglycan restricts DotK binding and thus, Dot/Icm localization to the bacterial poles.

Discussion

PgdA has multiple effects on *Legionella* pathogenesis through its ability to deacetylate peptidoglycan (*SI Appendix*, Fig. S21*A*). First, a reduction in NAG deacetylation causes defects in Dot/Icm localization (Fig. 5 and *SI Appendix*, Fig. S18) and function, impairing effector translocation into host cells (Fig. 4). As a consequence, bacteria lacking PgdA are more likely to traffic to a lysosomal compartment (Fig. 3 and *SI Appendix*, Figs. S14 and S15), preventing the formation of a replication vacuole and thus bacterial proliferation. Second, the inability to deacetylate the peptidoglycan decreases *L. pneumophila* resistance to lysozyme (Fig. 1), rendering Δ*pgdA* mutant bacteria more susceptible to degradation within lysosomes (Figs. 3 and 4*D*). Thus, peptidoglycan editing by PgdA impacts several key events in *L. pneumophila* pathogenesis: establishing a replication-permissive compartment, avoiding delivery to the lysosome, and lysozyme-mediated digestion within host cells. Consequently, bacteria that are unable to deacetylate their peptidoglycan are less likely to survive within host cells (Fig. 2), and thus, are less virulent. Collectively, these results demonstrate an unprecedented role for peptidoglycan modifications in *Legionella* pathogenesis and the importance of peptidoglycan deacetylation for the activity of a specialized secretion system.

PgdA promotes the proper localization and function of the Dot/Icm secretion system, as disruption of *pgdA* causes mislocalization of Dot components and impaired effector translocation into host cells (Figs. 4 and 5). Whether mislocalized Dot subunits represent partial or fully assembled translocons remains unclear. However, since all of the LidA detected in Δ*pgdA* bacteria was at the bacterial poles (Fig. 4), despite the increased percentage of bacteria with mistargeted Dot components (Fig. 5 and *SI Appendix*, Fig. S18), the most likely explanation for the lower level of effector translocation in Δ*pgdA* bacteria is that non-polar Dot/Icm components do not represent functional complexes. Consistent with this idea, the Δ*pgdA* mutant showed increased salt resistance, a hallmark of non-functional translocons, compared to WT bacteria (*SI Appendix*, Fig. S16). By deacetylating NAG, PgdA inhibits peptidoglycan binding by the Dot/Icm subunit DotK (Fig. 5*C*), a component required for proper localization of the Dot/Icm complex (Fig. 5 *A* and *B*). These results suggest that mislocalization of the Dot/Icm machinery in Δ*pgdA* bacteria occurs because DotK is no longer restricted to the poles. Thus, these studies have revealed that DotK, along with DotU and IcmF (68), is responsible for polar localization of this Type IVb secretion system. While DotU and DotF direct localized assembly of Dot/Icm at the mid-cell (68), which subsequently becomes the new poles, DotK likely functions to anchor the Dot/Icm machinery in place. Since newly synthesized peptidoglycan is incorporated at the mid-cell in the acetylated form, we propose that this trait promotes DotK binding to peptidoglycan at its site of insertion (*SI Appendix*, Fig. S21*B*). In parallel, we predict that PgdA-mediated deacetylation of NAG distal from the pole restricts DotK diffusion from the mid-cell, spatially confining Dot/Icm to the newly formed poles. Moving forward, the development of tools to examine the spatial patterns of peptidoglycan acetylation and deacetylation will allow further elucidation of the mechanisms governing Dot/Icm polar localization.

Although PgdA functions as the primary NAG deacetylase in *L. pneumophila*, Lpg0633 and Lpg1637 additionally contribute to this process. For example, inactivation of *lpg0633* on its own does not affect the acetylation status of the peptidoglycan, but Lpg0633 appears to function in combination with PgdA under certain conditions. The differential contributions of these proteins to NAG deacetylation could be due to differences in their individual activities, relative abundance, or subcellular distribution. For Lpg1637, the lack of conserved active site residues (*SI Appendix*, Fig. S1) would require a noncanonical mechanism of catalysis, and thus Lpg1637 is more likely to function in a regulatory role influencing the activities of PgdA and Lpg0633. While deleting different combinations of these proteins had varying effects on NAG deacetylation, the extent of NAG deacetylation did not fully correlate with the phenotypic defects in lysozyme sensitivity, Dot/Icm polar localization, and intracellular survival of the corresponding mutants (*SI Appendix*, Figs. S10, S13, and S20). One possible explanation is that different combinations of these proteins generate different patterns of acetylated and deacetylated NAG in three-dimensional space, which in turn dictate the interaction of lysozyme or DotK with peptidoglycan. Thus, the local organization of deacetylated subunits rather than the total amount of deacetylation would define the physiological impact of this modification. Moreover, lysozyme sensitivity, and thus intracellular survival, may be further complicated by additional mechanisms that protect against bacterial degradation (69), for example increased peptidoglycan cross-linking (70) or changes in cell envelope integrity through lytic transglycosylases (71) such as Lpg1994 that is encoded directly downstream of *pgdA* and also important for *L. pneumophila* fitness in amoebae (55). Collectively, our results establish roles for NAG deacetylases in *L. pneumophila* pathogenesis and begin to map the complex interplay between them.

The importance of PgdA and peptidoglycan deacetylation at early stages of the infection cycle may indicate a role for this cell wall modification in the evolution of *L. pneumophila* as an intracellular pathogen. Since humans are accidental and terminal hosts for *Legionella*, the adaptation of polysaccharide deacetylases to

avoid immune detection and antimicrobial defenses in humans is not the main driver in *Legionella* evolution. Previously, it has been proposed that *L. pneumophila* evolved in multiple steps to survive and replicate within protozoa in the environment (72, 73). A plausible first step in avoiding digestion would be modifying the cell envelope to increase resistance to killing, allowing enhanced survival in a lysosomal compartment. In a second step, acquisition of a Type IVb secretion system and its cognate effectors would provide the ability to disrupt endocytic trafficking of the phagosome while building a replicative-permission compartment, adding another layer of protection against degradation. Our data indicate that PgdA functions in both steps by increasing resistance to lysozyme and optimizing the function of the Dot/Icm secretion system. Thus, it is plausible that PgdA has contributed to the transition of *L. pneumophila* from prey to parasite in natural reservoirs and, as a consequence of its benefits in macrophages, the emergence of *L. pneumophila* as a human pathogen.

Materials and Methods

The materials and methods are described in detail in *SI Appendix*, *Material and Methods*, including Bacterial and Cell Culture Conditions, Construction of *L. pneumophila* Deletion Mutants and Expression Plasmids, In Vitro Growth Assays, Isolation and Structural Analysis of *L. pneumophila* Peptidoglycan, Purification of Lpg1993/PgdA, In Vitro Peptidoglycan Deacetylation, Lysozyme Digestion and Protease Protection Assays, Lysozyme Sensitivity Assays, Intracellular Survival and Growth Assays, Gentamycin Protection and Sensitivity Assays, Generation of α-*Legionella* Antibodies, Immunofluorescence Microscopy, Dot Protein Subcellular Localization, Quantification of LidA and Dot Protein Levels, and Purification of DotK and Peptidoglycan Binding Assays.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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