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Aggravating genetic interactions allow a solution to redundancy in a bacterial pathogen

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Abstract

Interactions between hosts and pathogens are complex, so understanding the events that govern these interactions requires the analysis of molecular mechanisms operating in both organisms. Many pathogens use multiple strategies to target a single event in the disease process, confounding the identification of the important determinants of virulence. We developed a genetic screening strategy called insertional Mutagenesis And Depletion (iMAD) that combines bacterial mutagenesis and RNA interference, to systematically dissect the interplay between a pathogen and its host. We used this technique to resolve the network of proteins secreted by the bacterium *Legionella pneumophila* to promote intracellular growth, a critical determinant of pathogenicity of this organism. This strategy is broadly applicable, allowing the dissection of any interface between two organisms involving numerous interactions.

The outcome of most parasitic relationships is decided by a series of molecular interactions involving hundreds of proteins. Through specialized secretion systems, intracellular pathogens deploy an arsenal of proteins that modulate numerous host cell processes to establish growth (1). For many pathogens, redundancy complicates the analysis of these secreted virulence proteins (2), and no systematic strategy exists that allows specific roles to be ascribed to proteins that, based on mutant analysis, appear to be dispensable for growth within the host.

Legionella pneumophila is a parasite of a broad range of free-living amoebae (3). Human disease occurs after inhalation of contaminated water aerosols followed by replication of the bacteria in alveolar macrophages (4) which results in pneumonia. Within the host cell, *L. pneumophila* establishes replication within a membrane-bound compartment by preventing the delivery of this vacuole to the lysosome (5), while recruiting host material from the endoplasmic reticulum (ER) (6). This latter event is accomplished by manipulating host proteins involved in the early secretory pathway (7), including the small GTPases Rab1, Arf1 and Sar1 as well as Sec22 and Bet5, proteins involved in tethering and fusion of ER-derived vesicles to target membranes (7–9).

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Intracellular replication of *L. pneumophila* depends on the Dot/Icm Type IVb secretion system (10, 11). To date, over 270 *L. pneumophila* proteins have been shown to be substrates of Dot/Icm (12), several of which directly modulate the activity of GTPases that control host vesicle trafficking. Although the biochemical activity of more than a dozen Dot/Icm translocated substrates (TS) and their host targets are known, their roles in disease are unclear, because deletion of their encoding genes rarely causes a detectable defect in intracellular growth (13). The most likely explanation for this is redundancy, which extends beyond multiple paralogs that can perform the same function (13, 14) to the use of seemingly unrelated proteins to manipulate complementary host cell pathways (9). Although this has impeded understanding how these proteins contribute to pathogenesis, there has been no attempt to systematically resolve this problem.

Studies using RNA interference (RNAi) in cultured *Drosophila* cells demonstrate that wild-type *L. pneumophila* grow efficiently in cells depleted of one of the membrane trafficking proteins Arf1, Sec22 or Bet5 (9); however, simultaneous depletion of pairs of these proteins impairs intracellular replication of the bacterium (9). These data are consistent with several different pathways promoting intracellular replication, with bacterial growth inhibition requiring disruption of at least two pathways. We reasoned that mutation of a bacterial gene encoding a Dot/Icm TS that targets a host pathway distinct from one targeted by RNAi should effectively abolish the contribution of both pathways (Fig. S1).

Using transposon site hybridization (TraSH) (15), a library of *L. pneumophila* transposon mutants (16) was screened for mutations that render the bacterium defective for growth in *Drosophila* cells depleted of various component proteins of the early secretory system (Fig. S1). A total of 678 genes were identified as being important for intracellular growth in *Drosophila* cells in the presence of dsRNA treatment (Table S1). Many of these encode proteins that are not predicted to function in the host cytoplasm. For example, several genes encode proteins involved in nutrient acquisition and metabolism (Table S1), consistent with disruptions in vacuole remodeling limiting the availability of metabolites required for growth. To address the inability to connect phenotypes with mutations in Dot/Icm TS genes, one of the central problems in the field, we decided to focus on the 55 Dot/Icm TS-encoding genes identified (Fig. 1A), 44 of which had not previously been shown to have a role in promoting intracellular growth of *L. pneumophila*.

Data from the iMAD screen could be recapitulated for selected genes when *Drosophila* cells treated with the appropriate dsRNA were challenged with *L. pneumophila* deletion mutants (Fig. 1B). For example, a *L. pneumophila* Δ*wipB* (*lpg0642*) mutant was defective for growth in cells depleted of Sec22 but not in untreated *Drosophila* cells or in cells depleted of Bet5, as predicted from our screen. Similarly, growth of Δ*ceg32/sidI* (*lpg2504*) was more defective in cells depleted of Arf1 than in untreated cells or cells depleted of Sar1 (Fig. 1B). Thus, we have mapped a series of aggravating genetic interactions (17) that identify specific host factors required for intracellular replication of bacterial mutants. These results defined host conditions under which individual bacterial proteins were important for growth and provided the basis for identifying Dot/Icm TS that function in complementary host pathways.

We predicted that if a set of Dot/Icm TS targets a particular host pathway, mutations in individual members of that set should result in similar phenotypes. Our iMAD screen examined growth of *L. pneumophila* mutants in *Drosophila* cells under five different conditions of dsRNA treatment. Using these data, we performed cluster analysis to group bacterial mutations based on common behavior patterns. This identified 14 distinct functional groups of Dot/Icm TS with more than one member (Fig. 1A; Groups I–XIV). For example, mutations in the *L. pneumophila* gene *lidA* impaired intracellular growth if either

Bet5 or Sec22 was depleted, and mutations in *legA3* (*lpg2300*) behaved similarly, placing *legA3* and *lidA* in the same functional group (Fig. 1A; Group XI). Conversely, mutation of *wipB* caused defective intracellular growth in cells depleted of either Arf1 or Sec22, placing it in a separate functional group (Fig. 1A; Group IX). If two functional groups consist of bacterial proteins that modulate redundant host pathways, we predicted that the combined deletion of one bacterial gene from each functional group should result in defective intracellular replication of *L. pneumophila* in untreated *Drosophila* cells. LidA and WipB clustered to separate functional groups predicting that they act on potentially redundant host cell pathways, so growth of a Δ*wipB*Δ*lidA* double mutant was assessed in untreated *Drosophila* cells. Consistent with the interpretation that these two proteins target redundant pathways, the double mutant showed reduced intracellular replication in untreated *Drosophila* cells compared to either wild-type *L. pneumophila* or strains bearing mutations in only one of these genes (Fig. 1C). Mutations in a newly identified Dot/Icm TS-encoding gene *lpg2395* (Fig. S2), clustered to the same functional group as *wipB* mutations (Fig. 1A: Group IX), and as predicted by this strategy, deletion of *lpg2395* impaired growth of *L. pneumophila* when combined with Δ*lidA* (Fig. 1C). In contrast, a strain containing lesions in two genes assigned to the same functional group, Δ*wipB*Δ*lpg2395*, grew as proficiently as the wild-type strain in untreated *Drosophila* cells, consistent with the model that growth defects are only observed when targeting of multiple host pathways by Dot/Icm TS is disrupted (Fig. 1D). This demonstrated that cluster analysis can predict pairs of seemingly unrelated Dot/Icm TS that perform compensatory functions.

Defective intracellular growth was not limited to strains that combine mutations from functional groups IX and XI. Mutations in *mavP* (*lpg2884*) clustered in a third functional group, Group XII (Fig. 1A), and the combined deletion of *mavP* with *lidA* similarly impaired bacterial growth in untreated *Drosophila* cells (Fig. 1C). We also observed specificity between functional groups. For example, reduced growth of *L. pneumophila* was observed when *legC8* (*lgt2*/*lpg2862*) (Group XIV) was deleted in combination with *mavP* (Fig. 1E). However, neither Δ*legC8*Δ*wipB* nor Δ*legC8*Δ*lpg2395* double mutants were attenuated despite loss of gene pairs from separate functional groups (Fig. 1E). Instead, we observed a positive interaction between mutations in *wipB* and *legC8* in which intracellular growth was enhanced by combining the two mutations (Fig. 1E). Thus, cluster analysis of mutations allows functional relationships between bacterial proteins to be revealed.

By analyzing genetic interactions between members of several functional groups, we were able to identify a network of functional relationships between *L. pneumophila* proteins, in which pairs of mutations in Dot/Icm TS genes were predicted to have aggravating effects on intracellular growth (Fig. 1F, lines represent predicted aggravating genetic interactions). Many of these predicted interactions were verified by constructing defined double deletions (Fig. 1F, blue font). The cluster analysis also assigned genes encoding proteins of unknown function, such as *lpg2182* and *lpg1098* (Table S1), to specific functional groups (Fig. 1F) allowing these proteins to be assigned to particular steps in intracellular growth, defined by the members of their respective functional groups.

To show that the functional relationships between Dot/Icm TS identified using iMAD were similarly important in a natural host of *L. pneumophila,* the growth of double mutants in *Acanthamoebae castellanii* was examined. Each of the double mutants shown to be defective for replication in *Drosophila* cells exhibited impaired intracellular replication in the amoebal host (Fig. 2A and Fig. S3). Growth of all of the double mutants could be rescued by reintroducing either of the deleted genes individually, and no growth defects were observed for any of the double mutants when grown in bacteriological medium (Fig. S3). A more detailed examination through a single round of infection in *A. castellanii* revealed that all three double mutants generated vacuoles that contained fewer bacteria than the wild-type

strain (Fig. 2B and Fig. S3). This phenotype correlated with inefficient recruitment of ER-derived material, as determined in *Dictyostelium discoideum* by measuring accumulation of the GFP-HDEL protein about the *Legionella*-containing vacuole (LCV). GFP-HDEL is a fusion protein of the green fluorescent protein (GFP) and the ER localization signal HDEL, which is used as a proxy for the presence of ER-resident proteins in amoebae (5, 18, 19). In *D. discoideum* harboring GFP-HDEL, fewer Δ*wipB*Δ*lidA*-containing vacuoles stained positively for this marker compared to amoebae challenged with either wild-type or bacteria containing a single mutation (Fig. 2C) supporting the model that the activities of WipB and LidA can compensate for each other's absence. As expected, the Δ*wipB*Δ*lidA* mutant grew less robustly than either the wild-type or single deletion strains in this host (Fig. 2D). These results are consistent with at least two routes for generating a replication compartment that are targeted by *L. pneumophila* proteins in all the host cells examined. The partial defect in GFP-HDEL recruitment and intracellular growth of the Δ*wipB*Δ*lidA* mutant likely reflects the existence of additional pathways that can partially compensate for loss of WipB and LidA.

Mutations placed in the same group by hierarchical cluster analysis identify proteins that participate in the same process in the cell or comprise a single protein complex (20). We applied the same reasoning to the cluster analysis data obtained from our iMAD screen in *Drosophila* cells, which indicated that mutations in *wipB* and *lpg2395* belonged to the same functional group as *sdhA* mutations (Fig. 1A), consistent with all three proteins contributing to a common step in LCV formation. SdhA plays a crucial role in maintaining LCV integrity in bone marrow-derived murine macrophages (21). Vacuoles harboring Δ*sdhA* mutant bacteria are more permeable than those harboring wild-type bacteria (21), and as a consequence co-localize with host proteins associated with partially degraded phagosomes (21, 22). This vacuolar disruption leads to host cell death, bacterial degradation and poor bacterial growth in macrophages challenged with Δ*sdhA* mutants (21, 23). Therefore, we tested whether mutations that cluster with *sdhA* lesions could identify other *L. pneumophila* proteins that promote vacuole integrity. Consistent with other cell types, the Δ*wipB*Δ*lidA* double mutant exhibited reduced replication in bone marrow-derived murine macrophages relative to either the wild-type strain or strains having single mutations in each of these genes (Fig. 3A). Since a *L. pneumophila* mutant lacking 31% of Dot/Icm TS grows robustly in macrophages (16) this finding confirmed the power of the cluster analysis to identify functionally related Dot/Icm TS. The growth defect was associated with a loss of membrane integrity of the LCV based on colocalization with Galectin-3, a marker for vacuole lysis (22). Macrophages challenged with the Δ*wipB*Δ*lidA* mutant showed an increase relative to wild-type bacteria in the number of vacuoles staining positively for Galectin-3 (Fig. 3B). Localization of this marker was comparable to challenge with the Δ*sdhA* mutant, while the Δ*wipB* and Δ*lidA* single deletion mutants resembled the wild-type strain in this assay (Fig. 3B).

Analysis of morphological markers was consistent with the Δ*wipB*Δ*lidA* strain causing a breakdown in vacuole integrity (21). We observed an increase in the number of infected host cells undergoing cell death relative to cells harboring wild-type bacteria (Fig. 3C), indicated by aberrant nuclear morphology characteristic of apoptosis, while the number of bacteria appearing degraded, based on aberrant bacterial morphology (Fig. 3D), was similarly increased. The amount of bacterial degradation and host cell death was less extensive than that observed for the Δ*sdhA* mutant, consistent with the relative severity of the growth defects of these two strains. For the Δ*sdhA* mutant, the vast majority of vacuoles contained a single bacterium, while vacuoles harboring the Δ*wipB*Δ*lidA* mutant often had more than one bacterium, many of which appeared degraded (Fig. 3D). This is consistent with failure of the double mutant to maintain vacuole integrity, even though there was sufficient vacuolar remodeling to support initial replication events (Fig. 3D and 3E). Enhanced recruitment of

Galectin-3 and host cell death relative to the wild-type strain was similarly observed in macrophages challenged with the Δ*mavP*Δ*lidA* mutant, accompanying defective growth of this mutant in macrophages (Fig. S4). Assigning genes to specific functional groups based on the behavior of their corresponding mutants, therefore, identified microbial proteins that were required for similar steps during replication vacuole biogenesis.

Despite its severe growth defect in macrophages, an *L. pneumophila* Δ*sdhA* mutant is only partially attenuated for growth in *D. discoideum*, presumably because the host cell death pathways that inhibit growth of this mutant in macrophages are not present in amoebae (23). The cluster analysis predicted that this partial defect should be potentiated by the addition of a second mutation known to aggravate mutations that belong to the same functional group as *sdhA* (Group IX) (Fig. 1A). As mutations in Group IX (*wipB* and *lpg2395*) were aggravated by mutations in Group XI (*lidA* and *legA3*) (Fig. 2B and 2D), we predicted that a Group XI mutation, such as deletion of *lidA*, should exacerbate the growth defect of the Δ*sdhA* mutant in *D. discoideum*. Indeed, the Δ*sdhA*Δ*lidA* double mutant exhibited reduced growth relative to the Δ*sdhA* mutant alone (Fig. 4A). Moreover, a mutant lacking *sdhA* and *legA3*, another member of Group XI, was also attenuated for growth in this host (Fig. 4A). Thus, the presence of Group XI proteins could compensate for loss of SdhA in this amoebal host.

Phenotypic analysis of *L. pneumophila* double mutants showed that Group XI in its entirety was required to bypass the loss of single members of Group IX, however the extent to which individual members were able to compensate varied. For example, in *A. castellanii*, the Δ*sdhA*Δ*lidA* mutant was more attenuated for growth than the Δ*sdhA*Δ*legA3* mutant (Fig. 4B), arguing for a more significant role for LidA than LegA3 in promoting growth in the absence of SdhA in this species. This phenomenon was dependent on the host examined, as we did not observe this hierarchial relationship in *D. discoideum* (Fig. 4A). Similarly, in bone marrow-derived macrophages, loss of *mavP* had no effect on the growth of a Δ*legA3* mutant (Fig. 4C) whereas the Δ*mavP*Δ*legA3* mutant showed reduced growth in *A. castellanii* (Fig. 4D). Thus, optimal growth in a particular host depends on a specific set of Dot/Icm TS. Similar to the partial defect in GFP-HDEL recruitment observed for the Δ*wipB*Δ*lidA* mutant (Fig. 2C), the residual growth of specific double mutants in *A. castellanii* or macrophages (Fig. 4C and 4D) is consistent with existence of additional compensatory pathways promoted by other functional groups.

Maintaining an extensive repertoire of Dot/Icm TS enables *L. pneumophila* to grow in a broad assortment of amoebal hosts (16). As it is common for many TS to be present in only a subset of isolates (24), much of the selective pressure for preserving Dot/Icm TS appears to be centered on maintaining sets of functions, rather than individual proteins. For example, activation of Rab1 appears to be a central feature of *L. pneumophila* pathogenesis. A key player in this event in the Philadelphia 1 strain is SidM, which acts at two levels to activate and recruit Rab1 to the LCV (25, 26), but is only encoded by a subset of clinical strains (24). Presumably, the presence of TS such as AnkX, which activates Rab1 via a phosphocholine transfer mechanism (27), allows conservation of function. Therefore, activation of Rab1 is conserved without conserving specific proteins, or even the enzymatic activities they perform to activate this modulator of vesicle trafficking.

The accumulation of a large repertoire of Dot/Icm TS to cope with host variation allows different combinations of proteins to promote growth, arming the bacterium with multiple mechanisms for accomplishing a single task as long as the appropriate host targets are present. This flexibility is limited however, as it was possible to impair intracellular growth by disrupting more than one pathway (Fig. 1C). The host cell also restricts the degree of flexibility the bacterium can exert, as the specific combinations of Dot/Icm TS that were required for optimal growth varied between hosts (Fig. 4).

We have developed a strategy to elucidate the network of functional interactions central to *Legionella*'s manipulation of host cells. This approach defines sets of proteins that participate in common pathways, groups of proteins that modulate redundant virulence strategies and predicts cell biological defects based on hierarchical clustering. For pathogens that employ redundant virulence mechanisms, similar to *L. pneumophila*, simple strategies such as the construction of strains with random sets of genetic lesions, have failed to identify critical bacterial determinants of pathogenicity and have given no information on the interrelationships between members of a large group of proteins known to interact with the host cell. Our integrated approach can be applied to other bacterial pathogens, as well as other systems that have convergent biochemical pathways, and shows that it is now possible to define roles for individual proteins and their relative contributions to pathogenesis in different hosts. The utility of this strategy can be extended to any interface between two organisms involving a large number of interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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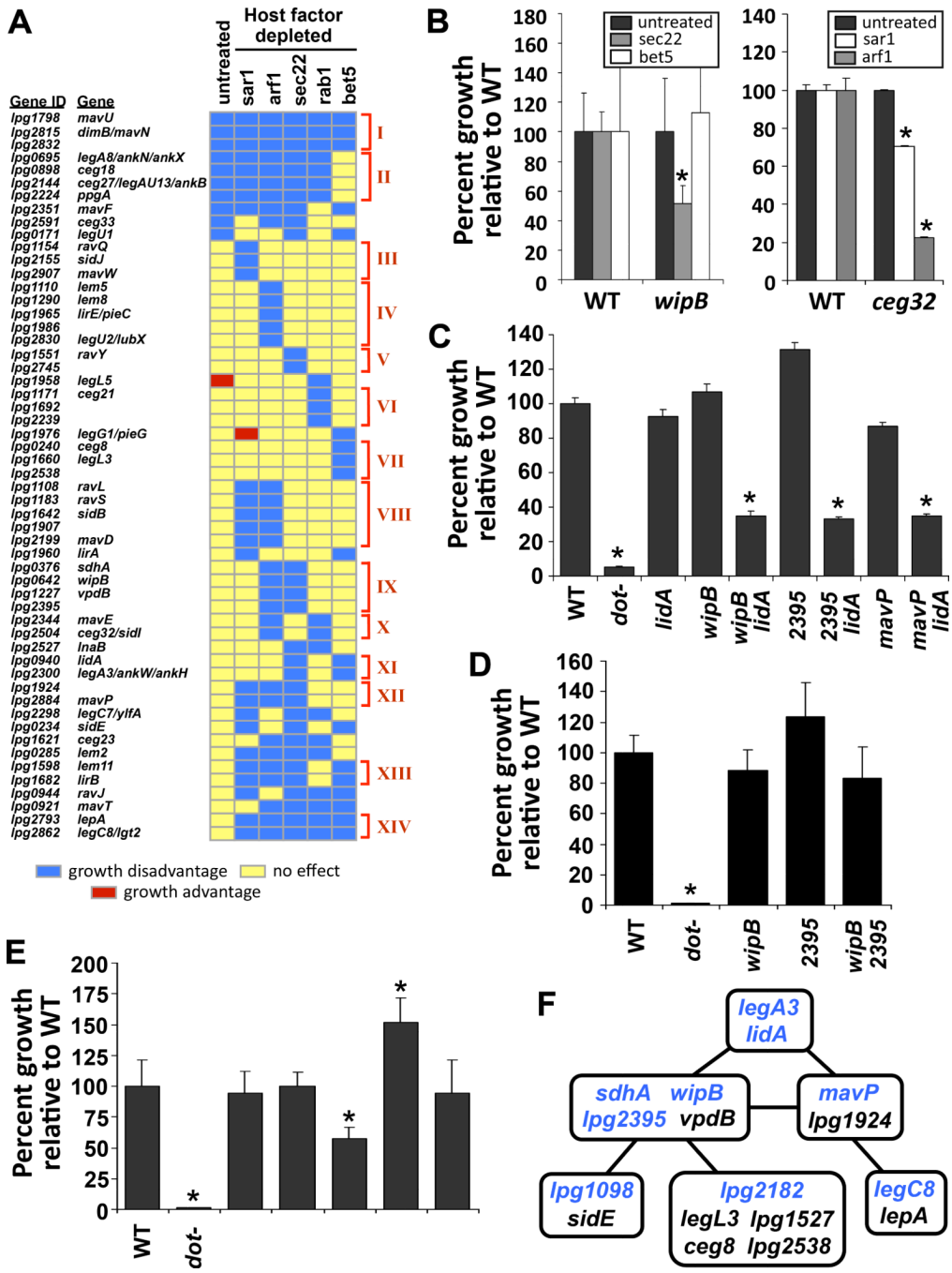

Figure 1. iMAD identifies redundant relationships between Dot/Icm translocated substrates

(**A**) Defective intracellular growth of *L. pneumophila* resulting from mutations in Dot/Icm TS genes combined with depletion of host cell proteins associated with the early secretory pathway. Cultured *Drosophila* cells treated with dsRNA were challenged with a library of *L. pneumophila* transposon mutants and the relative intracellular growth under each depletion condition was determined by TraSH. A growth disadvantage is defined as a 3.7 ± 0.2-fold growth defect, equivalent to an average TraSH ratio > 1.75 ± 0.1 standard deviations from the mean, which varies depending on the dsRNA treatment. Individual genes are clustered into distinct functional groups (red brackets, I-XIV) based on common behavioral patterns

across all host conditions examined. (**B**) Host-condition specific growth defects for *L. pneumophila* null mutants. Growth of *wipB* (*lpg0642*) (Group IX) (left panel) and *ceg32/sidI* (*lpg2504*) (Group X) (right panel) null mutants in cultured *Drosophila* cells depleted of the indicated proteins by RNAi was compared to the wild-type (WT) strain. (**C**) The combined deletion of bacterial genes from separate functional groups impaired intracellular replication of *L. pneumophila* in untreated *Drosophila* cells. (**D**) Deletion of genes belonging to the same functional group did not adversely affect bacterial growth in untreated *Drosophila* cells. (**E**) Genetic interactions define distinct relationships between different functional groups. (**F**) Summary of redundant relationships between individual functional groups defined by genetic interaction mapping. A solid black line indicates aggravating genetic interactions on intracellular growth of the bacterium in untreated *Drosophila* cells when two *L. pneumophila* genes, one from each of the corresponding functional groups (indicated by blue lettering), are deleted in combination. (B–E) Bacterial growth was determined by colony forming units (cfus) on solid media from lysed host cells 48 hours post infection relative to the number of cfus recovered 1 hour post infection. Data are means from at least 2 independent experiments ± standard deviation of 3 replicates. *$p < 0.05$ relative to the wild-type (WT) strain.

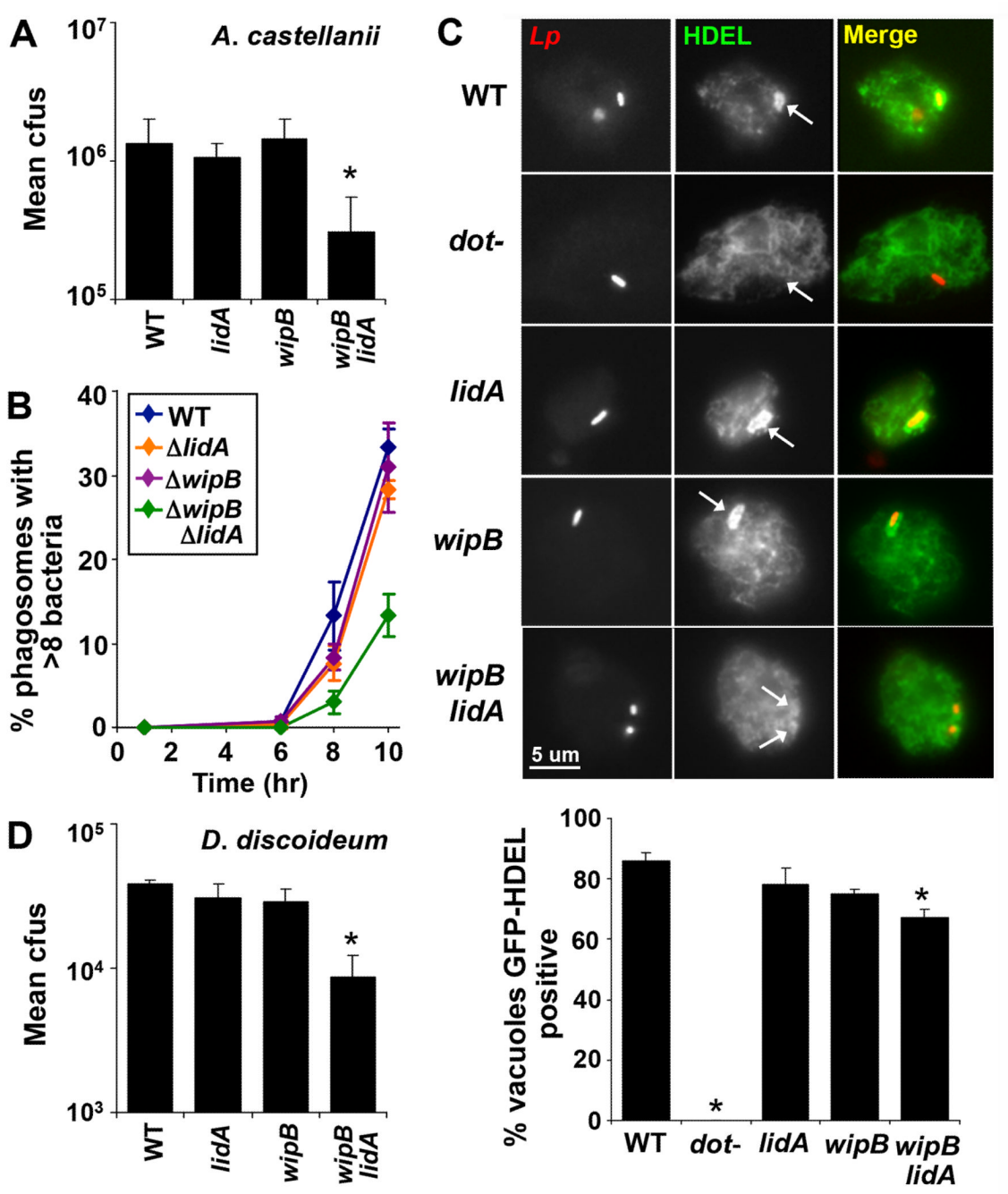

Figure 2. Impaired growth of *L. pneumophila* mutants in natural hosts

(**A**) The Δ*wipB*Δ*lidA* mutant shows a growth defect in *A. castellanii* relative to the wild-type (WT) and corresponding single deletion strains. (**B**) The Δ*wipB*Δ*lidA* mutant is impaired in the accumulation of vacuoles containing large numbers of bacteria. *A. castellanii* were infected with *L. pneumophila* strains expressing GFP, fixed 1, 6, 8 or 10 hours post infection and the number of bacteria per phagosome was scored using fluorescence microscopy. (**C**) The Δ*wipB*Δ*lidA* mutant is defective for recruitment of the ER-targeted fusion protein GFP-HDEL in *D. discoideum*. *D. discoideum* expressing GFP-HDEL were infected with *L. pneumophila* strains expressing the red fluorescent protein tdTomato for 4 hours, fixed and

visualized by fluorescence microscopy (upper panel). The number of GFP-HDEL positive *Legionella* vacuoles was scored, counting 100 vacuoles per replicate (lower panel). (**D**) Defective intracellular growth of the Δ*wipB*Δ*lidA* mutant in *D. discoideum*. (A, D) *A. castellanii* and *D. discoideum* were infected with *L. pneumophila* and bacterial growth was monitored as described in Fig. 1 over 3 days, equivalent to 3 consecutive rounds of replication and plotted as in Fig. 1. Data are representative of at least 2 independent experiments ± standard deviation of 3 replicates. *p <0.05 relative to the wild-type strain.

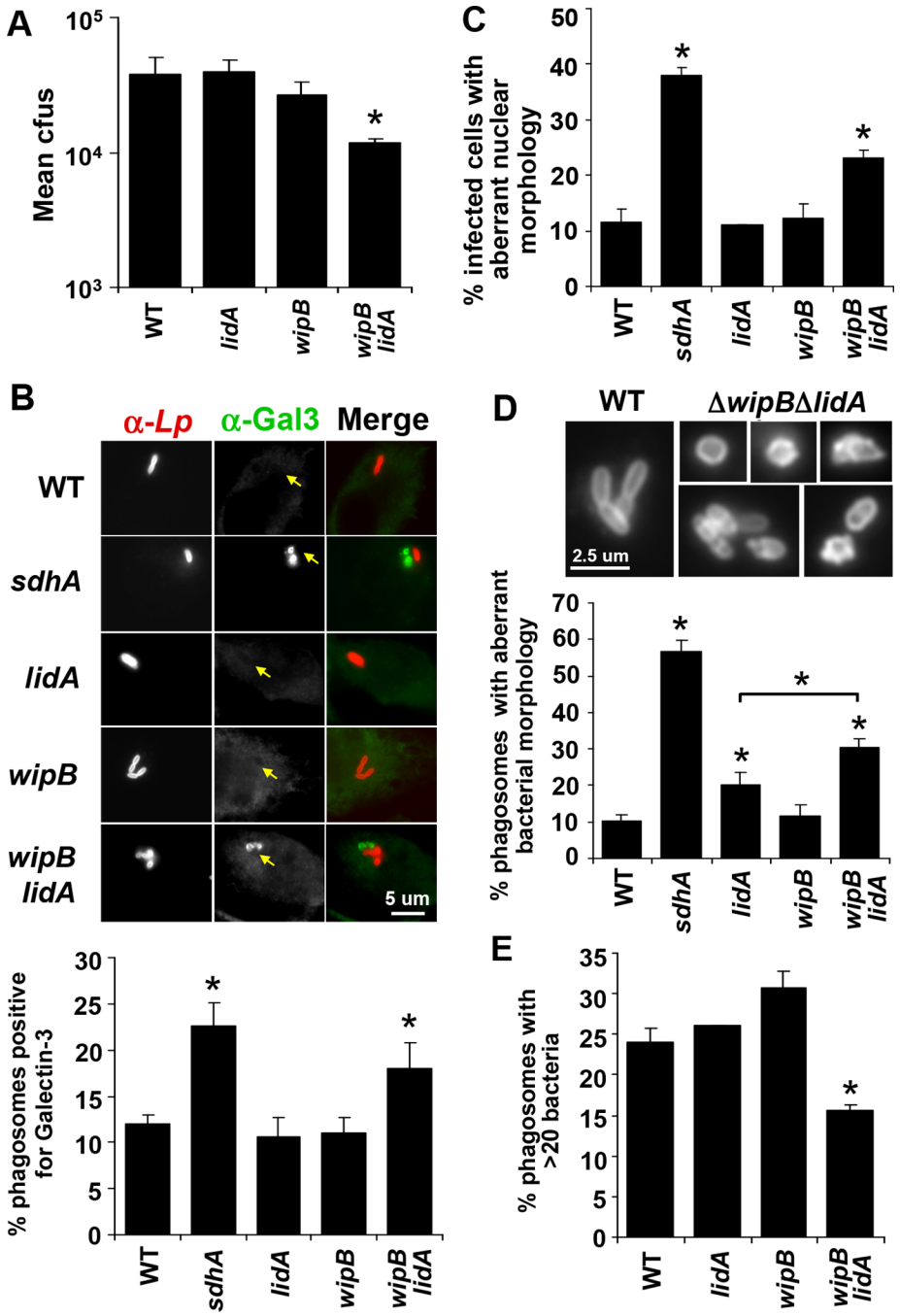

Figure 3. Hierarchial cluster analysis predicts double mutants defective in maintaining vacuole integrity

(**A**) Growth of the Δ*wipB*Δ*lidA* mutant was reduced in bone marrow-derived murine macrophages compared to the wild-type (WT) and single deletion mutant strains. Bacterial growth was determined as described in Fig. 2. (**B**) Δ*wipB*Δ*lidA* mutant-containing vacuoles showed enhanced recruitment of Galectin-3. Macrophages were infected with *L. pneumophila* for 6 hours, fixed and stained for *Legionella* and Galectin-3 then visualized by fluorescence microscopy (upper panel). The number of *Legionella* vacuoles staining positive for Galectin-3 were scored (lower panel). (**C**) Cells infected with the Δ*wipB*Δ*lidA* mutant exhibit increased host cell death based on aberrant nuclear morphology characteristic of

apoptosis. Macrophages were infected with *L. pneumophila* for 8 hours, fixed and stained for *Legionella* then treated with Hoechst stain. (**D**) Δ*wipB*Δ*lidA* mutant bacteria exhibited aberrant morphology after challenge of macrophages. Bacteria visualized by fluorescence microscopy as in (B) showed both swelling and blebbing in vacuoles containing either single or multiple bacteria relative to the smooth rod shaped morphology of wild-type bacteria (upper panel). The number of vacuoles in which at least one bacterium exhibited aberrant morphology was scored (bottom panel). (**E**) The Δ*wipB*Δ*lidA* mutant showed a defect in the number of vacuoles containing large numbers of bacteria in a macrophage host. Macrophages were infected with *L. pneumophila* for 10 hours, fixed and stained for *Legionella* and the number of bacteria per vacuole was scored. (A–E) Data are representative of at least 2 independent experiments ± standard deviation of 3 replicates, scoring 100 vacuoles (B, D, E) or infected cells (C) per replicate. *p <0.05 relative to the wild-type strain unless indicated otherwise.

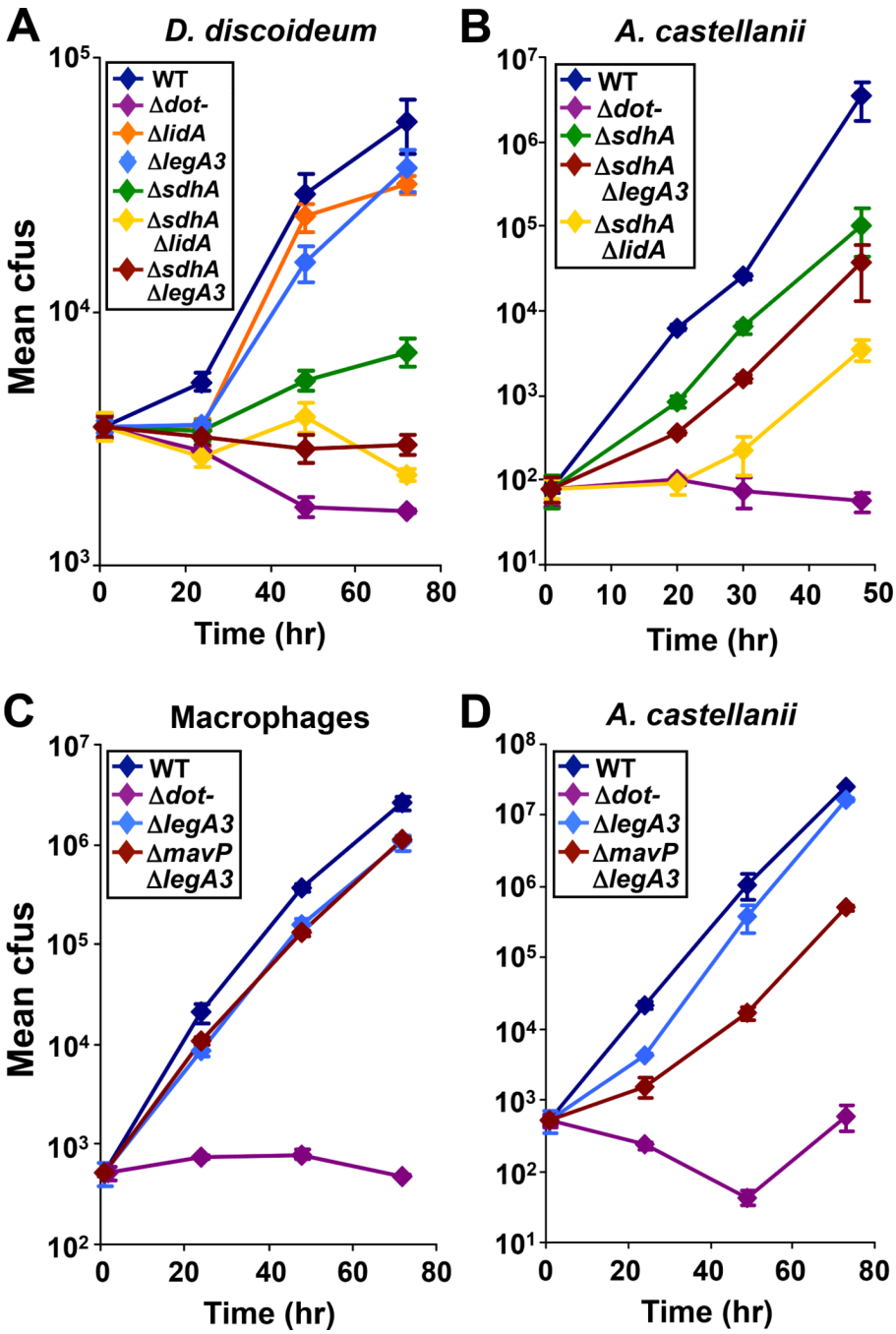

Figure 4. Host-specific aggravating genetic interactions between mutations in genes encoding Dot/Icm translocated substrates

(**A**) Deletion of *lidA* or *legA3* (Group XI) attenuated the growth defect of the Δ*sdhA* mutant (Group IX) in *D. discoideum*. (**B**) The Δ*sdhA*Δ*lidA* mutant was more defective for growth than the Δ*sdhA*Δ*legA3* mutant in *A. castellanii*. (**C**) A Δ*mavP*Δ*legA3* mutant grew as well as a Δ*legA3* single mutant in bone marrow-derived murine macrophages. (**D**) The Δ*mavP*Δ*legA3* mutant showed reduced growth relative to the Δ*legA3* mutant in *A. castellanii*. (A–D) Bacterial growth was monitored as described in Fig. 2. WT: wild-type. Data are representative of at least 2 independent experiments ± standard deviation of 3 replicates.