Legionella pneumophila Is Directly Sensitive to 2-Deoxyglucose-Phosphate via Its UhpC Transporter but Is Indifferent to Shifts in Host Cell Glycolytic Metabolism

Jordan V. Price,[a] Kallie Jiang,[a] Abigail Galantowicz,[a] Alana Freifeld,[b] Russell E. Vance[b,c]

[a]Department of Biology, Oberlin College, Oberlin, Ohio, USA
[b]Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, and Cancer Research Laboratory, University of California, Berkeley, California, USA
[c]Howard Hughes Medical Institute, University of California, Berkeley, California, USA

ABSTRACT   Toll-like receptor (TLR) stimulation induces a pronounced shift to increased glycolytic metabolism in mammalian macrophages. We observed that bone marrow-derived macrophages (BMMs) increase glycolysis in response to infection with *Legionella pneumophila*, but the role of host macrophage glycolysis in terms of intracellular *L. pneumophila* replication is not currently understood. Treatment with 2-deoxyglucose (2DG) blocks *L. pneumophila* replication in mammalian macrophages but has no effect on bacteria grown in broth. In addition, we found that 2DG had no effect on bacteria grown in amoebae. We used a serial enrichment strategy to reveal that the effect of 2DG on *L. pneumophila* in macrophages requires the *L. pneumophila* hexose-phosphate transporter UhpC. Experiments with UhpC-deficient *L. pneumophila* revealed that mutant bacteria are also resistant to growth inhibition following treatment with phosphorylated 2DG in broth, suggesting that the inhibitory effect of 2DG on *L. pneumophila* in mammalian cells requires 2DG phosphorylation. UhpC-deficient *L. pneumophila* replicates without a growth defect in BMMs and protozoan host cells and also replicates without a growth defect in BMMs treated with 2DG. Our data indicate that neither TLR signaling-dependent increased macrophage glycolysis nor inhibition of macrophage glycolysis has a substantial effect on intracellular *L. pneumophila* replication. These results are consistent with the view that *L. pneumophila* can employ diverse metabolic strategies to exploit its host cells.

IMPORTANCE   We explored the relationship between macrophage glycolysis and replication of an intracellular bacterial pathogen, *Legionella pneumophila*. Previous studies demonstrated that a glycolysis inhibitor, 2-deoxyglucose (2DG), blocks replication of *L. pneumophila* during infection of macrophages, leading to speculation that *L. pneumophila* may exploit macrophage glycolysis. We isolated *L. pneumophila* mutants resistant to the inhibitory effect of 2DG in macrophages, identifying a *L. pneumophila* hexose-phosphate transporter, UhpC, that is required for bacterial sensitivity to 2DG during infection. Our results reveal how a bacterial transporter mediates the direct antimicrobial effect of a toxic metabolite. Moreover, our results indicate that neither induction nor impairment of host glycolysis inhibits intracellular replication of *L. pneumophila*, which is consistent with a view of *L. pneumophila* as a metabolic generalist.

KEYWORDS   2-deoxyglucose, *Legionella pneumophila*, macrophages, metabolism

Mammalian macrophages and dendritic cells exhibit dramatic metabolic remodeling in response to pathogen-associated molecular patterns detected by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (1–3). PRR-stimulated macrophages display a characteristic "Warburg"-type metabolism with elevated levels

of glycolysis that can be measured through increased uptake of glucose and increased secretion of lactate (4). Increased glycolysis is thought to allow macrophages to rapidly process carbon from glucose and glutamine to generate biomolecules such as cytokines, chemokines, and other inflammatory mediators during the acute immune response to infection (1, 5, 6). In addition to providing for the energetic and biosynthetic requirements of macrophages during an immune response to pathogens, it is increasingly clear that the metabolic changes that occur in macrophages can also directly influence the ability of intracellular pathogens to survive and replicate within macrophages as host cells (7–9).

The intracellular bacterial pathogen *Legionella pneumophila* naturally replicates in protozoan host amoebae. *L. pneumophila* is an accidental pathogen of mammals and can cause a severe pneumonia in humans, called Legionnaires' disease, via infection of lung macrophages (10–12). Upon uptake by phagocytosis, *L. pneumophila* employs a type IV secretion system (Dot/Icm) to translocate effector proteins into the host cell cytosol, a subset of which hijack host trafficking machinery and allow the bacteria to establish a replicative vacuole (13, 14). While flagellin produced by wild-type (WT) *L. pneumophila* can result in restriction of bacterial replication via activation of the NAIP/NLRC4 inflammasome in infected mouse cells, *L. pneumophila* that lack flagellin (Δ*flaA*) replicate to high levels in mouse bone marrow-derived macrophages (BMMs) (15–21).

Here, we report that BMMs infected with *L. pneumophila* display a characteristic increase in glycolysis, similar to observations noted in a recent report (22). We speculated that increased host cell glycolysis may benefit *L. pneumophila* because the glycolysis inhibitor 2-deoxyglucose (2DG) has been reported to block *L. pneumophila* replication in mammalian cells, though the mechanism(s) underlying this effect remains obscure (23–25). It has been suggested that 2DG acts on host cells during *L. pneumophila* infection by modulating autophagy, inhibiting uptake of bacteria by interfering with phagocytosis, or altering the intracellular metabolic environment (22–25). Consistent with the notion that 2DG acts via an effect on host cells, the inhibitory effect of 2DG on *L. pneumophila* is specific to infection of mammalian cells, since 2DG has no effect on bacterial replication in broth (23), and we observed that 2DG does not inhibit bacterial growth in protozoan host cells. However, the idea that inhibition of macrophage glycolysis affects intracellular replication of *L. pneumophila* is complicated by the observation that another glycolysis inhibitor, sodium fluoride, did not similarly affect bacterial growth in macrophages (23). Indeed, we tested multiple independent glycolysis-restricting strategies in macrophages and did not observe inhibitory effects on intracellular *L. pneumophila* replication. Since 2DG is frequently used to investigate metabolic aspects of immunity, including recent investigations of the metabolic relationships between intracellular pathogens and host cells (8, 9), including *L. pneumophila* (22), a better understanding the effects of 2DG in these systems is needed.

To distinguish whether the inhibitory effect of 2DG acts through modulating host macrophage metabolism or instead acts more directly on *L. pneumophila* during infection of macrophages, we used a serial enrichment approach to identify *L. pneumophila* mutants with the ability to replicate in 2DG-treated macrophages. This approach revealed that the *L. pneumophila* hexose-phosphate transporter UhpC is required for bacterial sensitivity to 2DG during infection of macrophages. We went on to demonstrate that *L. pneumophila* UhpC is required for sensitivity to phosphorylated 2DG (2DG-6-phosphate [2DGP]). These results suggest that phosphorylation of 2DG and the subsequent bacterial uptake of 2DGP from host macrophages via UhpC is the mechanism by which 2DG treatment restricts *L. pneumophila* replication in macrophages. The isolation of a 2DG-resistant *uhpC* mutant allowed us to assess for the first time whether intracellular replication of *L. pneumophila* is affected by 2DG-mediated inhibition of host glycolysis. We found that *L. pneumophila* can replicate without a growth defect in 2DG-treated macrophages with severely impaired glycolysis. Overall, our results indicate that host cell glycolysis has little effect on *L. pneumophila* intracel-
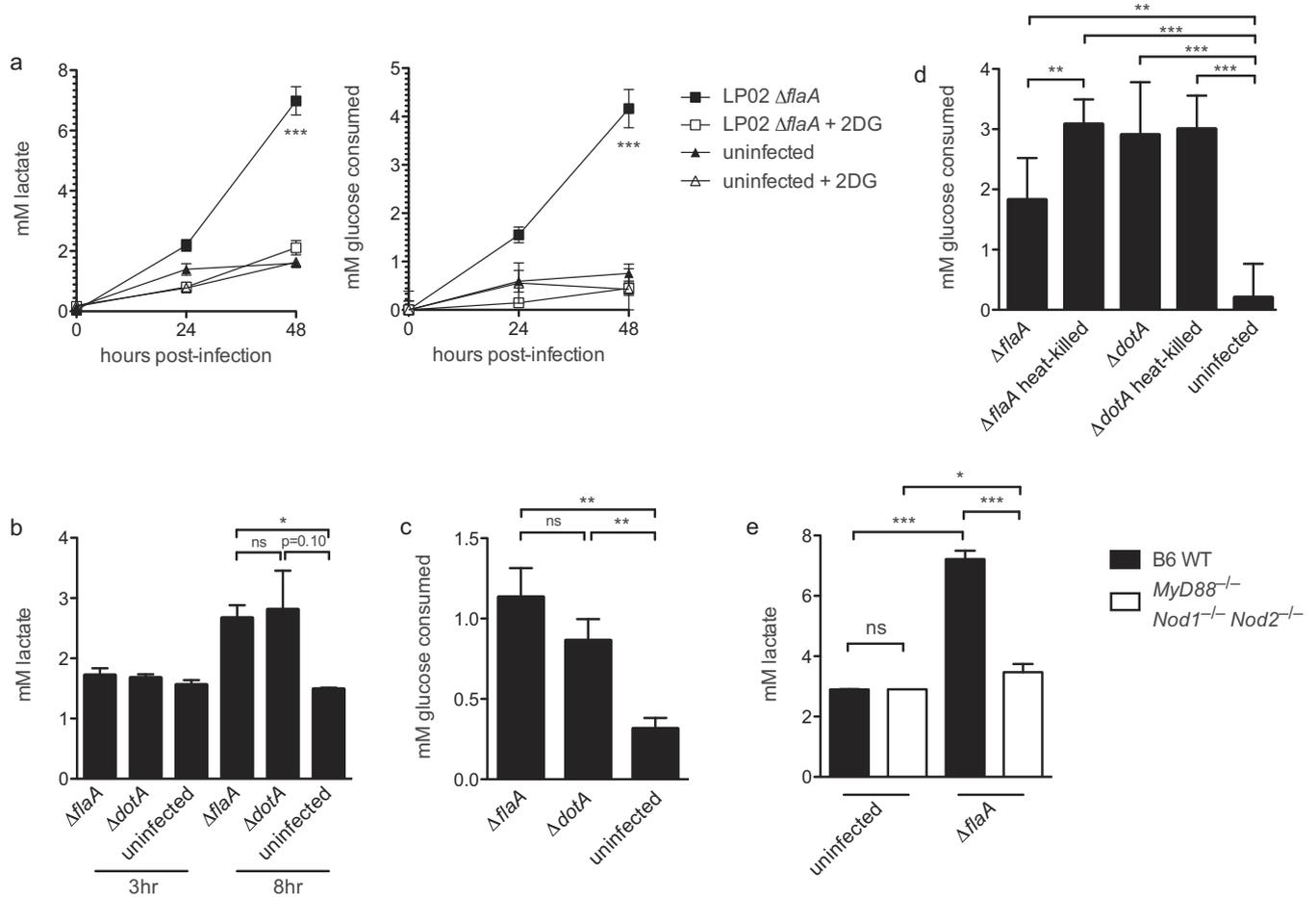
FIG 1 Increased glycolysis in BMMs during *L. pneumophila* infection. (a) Lactate levels and glucose consumption over 48 h in culture supernatant from uninfected BMMs (derived from wild-type C57BL/6 mice unless otherwise indicated) and BMMs infected with LP02 Δ*flaA* with or without 1.0 mM 2DG added 1 h postinfection (MOI = 0.05). (b) Lactate levels in culture supernatant from uninfected BMMs and BMMs infected with LP01 Δ*flaA* or LP01 Δ*dotA L. pneumophila* (MOI = 2.0). (c) Glucose consumption by uninfected BMMs or BMMs infected with LP01 Δ*flaA* or LP01 Δ*dotA L. pneumophila* (MOI = 2.0) at 8 h postinfection. Levels were calculated by measuring the change in glucose concentration at 8 h relative to 0-h controls. (d) Glucose consumed at 24 h postinfection by uninfected BMMs or BMMs infected with live and heat-killed LP02 Δ*flaA* and LP02 Δ*dotA L. pneumophila* (MOI = 1.0). The levels were calculated by measuring the change in glucose concentration at 24 h relative to 0-h controls. (e) Lactate levels in culture supernatant from uninfected wild-type BMMs and *MyD88*^{−/−} *Nod1*^{−/−} *Nod2*^{−/−} BMMs and infected BMMs of both genotypes at 48 h postinfection with LP02 Δ*flaA L. pneumophila* (MOI = 0.05). Data are representative of at least three independent experiments. In panel a, symbols and error bars represent means $\pm$ the standard deviations from three technical replicates. Curves in panel a depicting BMMs infected with LP02 Δ*flaA* are significantly distinct from other curves in each graph ($P < 0.001$, two-way analysis of variance [ANOVA]). For histograms, bars and error bars represent means $\pm$ the standard deviations from at least three technical replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant (two-tailed Student *t* test).

lular replication and are consistent with the view that *L. pneumophila* is a generalist that encodes redundant systems to capture metabolites from its environment.

RESULTS

L. pneumophila-infected macrophages upregulate glycolysis. To determine whether macrophages increase glycolysis during infection with *L. pneumophila*, we measured the lactate levels in the supernatant of BMMs infected with LP02 Δ*flaA L. pneumophila*. In comparison to culture media containing uninfected BMMs, we observed increased lactate in culture media of infected BMMs over time (Fig. 1a). Infected BMMs also consumed an increased amount of glucose over the course of infection, suggesting that infected BMMs are increasing glycolysis in response to infection (Fig. 1a). Increased secretion of lactate and consumption of glucose by *L. pneumophila*-infected BMMs was largely blocked by the addition of the glycolysis inhibitor 2DG, reinforcing the idea that *L. pneumophila* infection triggers increased glycolysis in host macrophages (Fig. 1a). To determine whether an intact bacterial type IV secretion

system is required for increased BMM glycolysis during infection with *L. pneumophila*, we measured the lactate levels in the supernatant of BMMs infected with LP01 Δ*flaA L. pneumophila* or Dot/Icm-deficient LP01 Δ*dotA L. pneumophila* at 3 and 8 h postinfection. At 8 h postinfection, we observed increased lactate secretion by BMMs infected with both strains (Fig. 1b). Increased secretion of lactate at 8 h corresponded with increased uptake of glucose by BMMs infected with both LP01 Δ*flaA* and LP01 Δ*dotA L. pneumophila* strains (Fig. 1c). These results suggest that bacterial replication is not required for increased glycolysis in BMMs infected with *L. pneumophila*. Furthermore, triggering of increased glycolysis did not require live bacteria, since exposure to live and heat-killed LP02 Δ*flaA* and Δ*dotA L. pneumophila* had similar effects on macrophage glucose consumption (Fig. 1d). Decreased glucose consumption by BMMs infected with live versus heat-killed LP02 Δ*flaA L. pneumophila* likely reflects increased host cell death observed under conditions with actively replicating bacteria. Lactate secretion was not induced to the same extent in infected BMMs derived from mice lacking the PRRs MyD88 and nucleotide-binding oligomerization domain-containing proteins 1 and 2 ($MyD88^{-/-}$ $Nod1^{-/-}$ $Nod2^{-/-}$ triply deficient mice) compared to WT BMMs (Fig. 1e). $MyD88^{-/-}$ $Nod1^{-/-}$ $Nod2^{-/-}$ triply deficient mice fail to activate TLR or NOD signaling in response to *L. pneumophila* (26). In sum, these results indicate that increased glycolysis in BMMs in response to infection by *L. pneumophila* is independent of bacterial replication or bacterial metabolism and relies on intact host macrophage TLR/NOD signaling.

2DG blocks *L. pneumophila* replication in WT and $Atg5^{-/-}$ macrophages but not in broth or protozoan host cells. To test the effect of blocking glycolysis on *L. pneumophila* replication in BMMs, we treated BMMs infected with of LP02 Δ*flaA* expressing a luminescence cassette containing *lux* genes from *P. luminescens* (27) (LP02 Δ*flaA* lux) with 2DG at 1 h postinfection. In accord with previous observations (23–25), we found that *L. pneumophila* replication was potently inhibited by the addition of 2DG to the infection culture media (Fig. 2a). Inhibition of *L. pneumophila* replication in 2DG-treated BMMs was not due to increased host death in response to 2DG since BMM cell viability was not adversely affected by 0.2 mM 2DG up to 48 h (data not shown). Matsuda et al. proposed that induction of host autophagy by 2DG was responsible for the restriction of *L. pneumophila* replication in BMMs (25). To test this, we infected *Atg5*-deficient BMMs with *L. pneumophila* and treated infected cultures with 2DG. ATG5 is considered essential for autophagy (28). While we observed lower levels of *L. pneumophila* replication in $Atg5^{-/-}$ BMMs, 2DG-dependent restriction was unaffected (Fig. 2b). This result indicated that 2DG is unlikely to restrict *L. pneumophila* replication in BMMs via increased host autophagy. In addition, 2DG did not have an adverse effect on *L. pneumophila* growth in broth, in agreement with a previous report (Fig. 2c) (23), and also did not restrict *L. pneumophila* growth in a laboratory model of its natural protozoan host cells *Acanthamoeba castellanii* (Fig. 2d). These data suggest that 2DG inhibition of *L. pneumophila* replication requires a mammalian host cell and does not rely on induction of host autophagy.

Treatment with other inhibitors of glycolysis, substitution of glucose for galactose, and removal of glucose from cell culture media do not affect intracellular replication of *L. pneumophila* in macrophages. Previous work found that 2DG but not another glycolysis inhibitor, sodium fluoride (NaF), had an inhibitory effect on *L. pneumophila* replication in macrophages (23). To further investigate whether host macrophage glycolysis inhibition negatively affects *L. pneumophila* replication during infection, we perturbed glycolysis using NaF, a lactate dehydrogenase inhibitor sodium oxamate (NaO), or by altering the availability of glucose during infection of wild-type BMMs with LP02 Δ*flaA* lux *L. pneumophila*. In agreement with Ogawa et al., we did not observe an effect of NaF on intracellular replication of *L. pneumophila* (Fig. 2e) (23). Similarly, NaO did not have an inhibitory effect on *L. pneumophila* replication (Fig. 2f). Replacement of glucose in the infection media with galactose, a sugar known to inhibit the transition to increased glycolysis in macrophages during bacterial infection (29), or removal of glucose from the infection media had no inhibitory effect on *L. pneumophila*
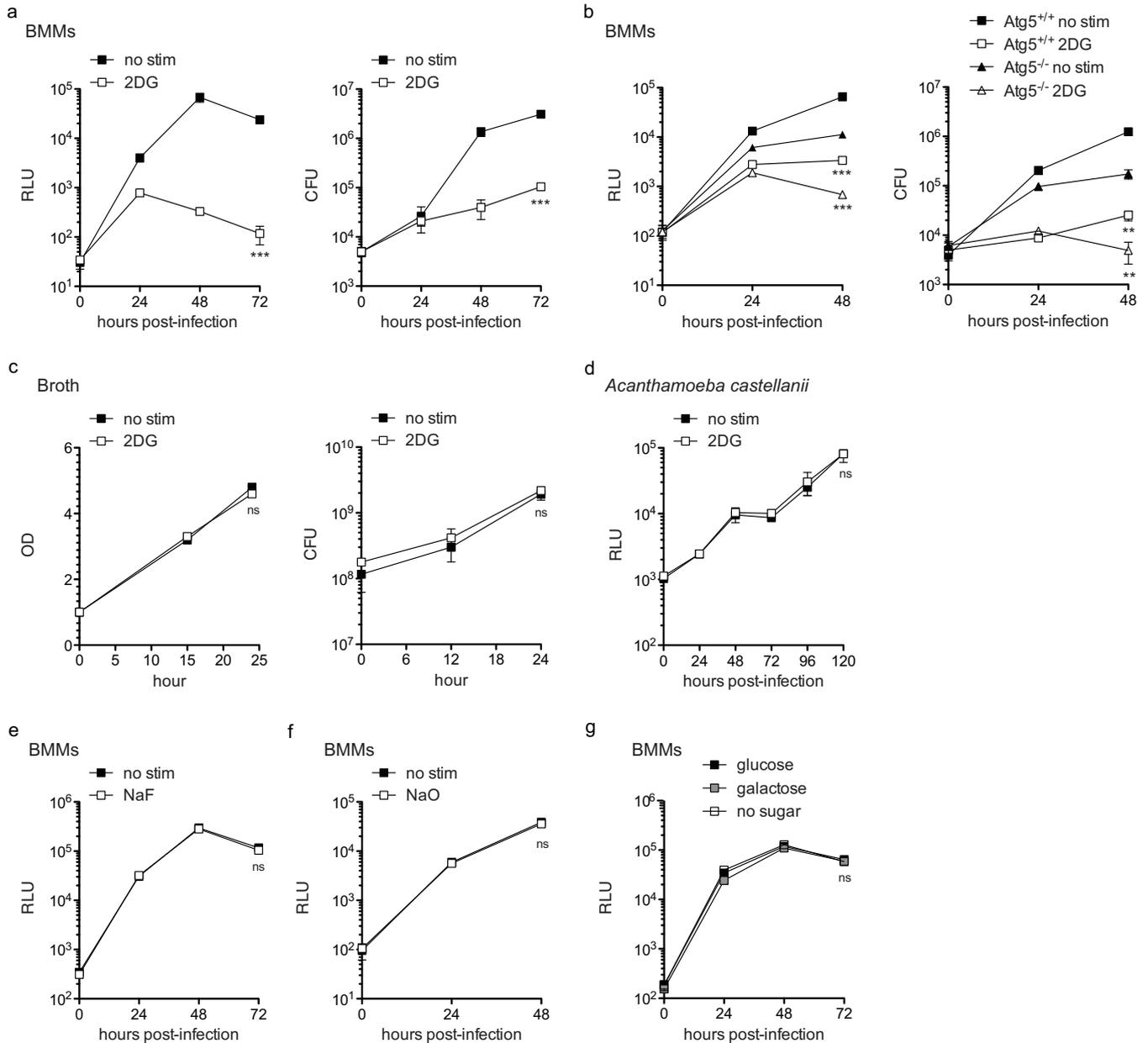
FIG 2 2DG restricts *L. pneumophila* replication in BMMs independent of Atg5, and perturbation of macrophage glycolysis via other inhibitors or glucose substitution does not affect intracellular replication of *L. pneumophila* in macrophages. (a) LP02 Δ*flaA* lux *L. pneumophila* replication measured by light emission (relative light units [RLU]) detected via luminometer (left) or CFU (right) in unstimulated BMMs (no stim) or in the presence of 0.2 mM 2DG (MOI = 0.01). (b) LP02 Δ*flaA* lux *L. pneumophila* replication (RLU and CFU) in Atg5$^{+/+}$ BMMs (derived from *LysMCre*$^{+/+}$ C57BL/6 mice) or Atg5$^{-/-}$ BMMs (derived from *LysMCre*$^{+/+}$ Atg5$^{fl/fl}$ C57BL/6 mice) with or without 0.2 mM 2DG (MOI = 0.01). (c) LP02 Δ*flaA* *L. pneumophila* replication in broth with or without 0.2 mM 2DG from a starting OD of 1.0 as measured by OD (left) and CFU (right). (d) LP02 Δ*flaA* (pJB908) (*thyA*$^{+}$) lux *L. pneumophila* replication in *A. castellanii* with or without 0.2 mM 2DG as measured by light emission (RLU). **, $P < 0.01$; ***, $P < 0.001$; ns, not significant (comparing growth in the presence of 2DG to that with no stimulation by two-way ANOVA). Symbols and error bars represent means ± the standard deviations from at least three technical replicates. (e) LP02 Δ*flaA* lux *L. pneumophila* replication (RLU) in WT BMMs with or without 1.0 mM sodium fluoride (NaF; MOI = 0.05). (f) LP02 Δ*flaA* lux *L. pneumophila* replication (RLU) in WT BMMs with or without 1.25 mM sodium oxamate (NaO; MOI = 0.05). (g) LP02 Δ*flaA* lux *L. pneumophila* replication (RLU) in WT BMMs in culture conditions containing 11.11 mM glucose or 11.11 mM galactose in the absence of glucose or in the absence of any sugar (no sugar). In each graph, no curves differ significantly (ns, not significant) as assessed by two-way ANOVA. Data are representative of at least two independent experiments.

replication in BMMs (Fig. 2g). These results indicate that *L. pneumophila* is able to replicate in host macrophages exhibiting a wide range of glycolytic states and that host glycolysis *per se* does not affect bacterial replication. Further, these data strongly suggest that the inhibitory effect of 2DG on *L. pneumophila* growing in macrophages is not due to the effect of 2DG on macrophage glycolysis.
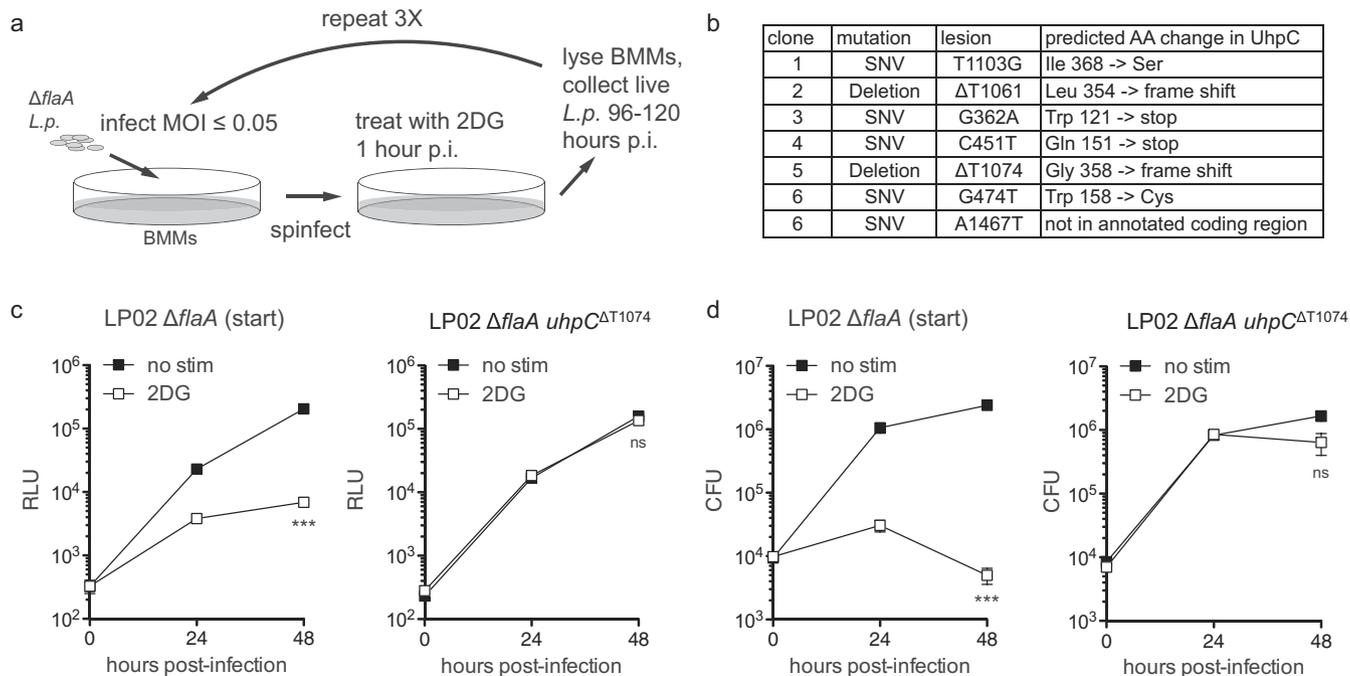
FIG 3 Enrichment of *uhpC*-mutant *L. pneumophila* following serial infection of BMMs treated with 2DG. (a) Strategy for serial passaging of LP02 Δ*flaA* lux *L. pneumophila* in 2DG-treated BMMs. *L.p.*, *Legionella pneumophila*; p.i., postinfection. (b) Table of mutations in *uhpC* and corresponding predicted amino acid (AA) changes in UhpC protein observed in independent clones of LP02 Δ*flaA* lux *L. pneumophila* with the ability to replicate in 2DG-treated BMMs. SNV, single nucleotide variant. (c) LP02 Δ*flaA* lux (starting strain before the serial enrichment experiment) and LP02 Δ*flaA* *uhpC*$^{\Delta T1074}$ lux replication in BMMs with or without 0.5 mM 2DG as measured by light emission (RLU). (d) LP02 Δ*flaA* lux and LP02 Δ*flaA* *uhpC*$^{\Delta T1074}$ lux replication in BMMs with or without 0.5 mM 2DG as measured by CFU. ***, $P < 0.001$; ns, not significant (comparing growth in the presence of 2DG to that with no stimulation by two-way ANOVA). Data are representative of at least two independent experiments. Symbols and error bars represent means ± the standard deviations from at least three technical replicates.

Mutations in hexose-phosphate transporter gene *uhpC* allow *L. pneumophila* to replicate in 2DG-treated macrophages. 2DG-mediated inhibition of *L. pneumophila* replication in macrophages may act through changes in host macrophage metabolism or could result from a more direct effect on *L. pneumophila* during infection of macrophages. To determine whether *L. pneumophila* has the ability to overcome the replication block imposed by 2DG during infection of macrophages, we serially passaged LP02 Δ*flaA* lux *L. pneumophila* in BMMs treated with 2DG (Fig. 3a). After three rounds of enrichment in 2DG-treated BMMs, we were able to purify several independent clones of LP02 Δ*flaA* lux *L. pneumophila* with the ability to replicate without a growth defect in 2DG-treated BMMs. Whole-genome sequencing of six independent clones of *L. pneumophila* with the ability to replicate in 2DG-treated BMMs revealed that each clone harbored mutations in a single shared gene, the hexose-phosphate transporter *uhpC* (NCBI Gene ID 19834118) (Fig. 3b). Several of the mutations observed in *uhpC* were predicted to introduce premature stop codons or frameshifts for the translated UhpC protein, suggesting that mutations resulting in nonfunctional UhpC protect *L. pneumophila* from 2DG-mediated restriction in BMMs (Fig. 3b). Analysis of an isolated clone from the serial enrichment experiment, a thymidine deletion at position 1074 of *uhpC* (LP02 Δ*flaA* *uhpC*$^{\Delta T1074}$ lux), demonstrated that compared to its parental strain, LP02 Δ*flaA* *uhpC*$^{\Delta T1074}$ lux *L. pneumophila* replicates in 2DG-treated BMMs without a growth defect (Fig. 3c and d). These results suggest that *L. pneumophila* sensitivity to 2DG during infection of BMMs depends on interaction of 2DG or a 2DG-derived molecule directly with *L. pneumophila* via the bacterial protein UhpC.

Δ*uhpC* *L. pneumophila* is resistant to 2DG-mediated growth inhibition in BMMs and does not display a growth defect in broth or in protozoan host cells. To confirm that loss of functional UhpC is responsible for the rescue of *L. pneumophila* replication in 2DG-treated BMMs, we generated in-frame deletions of *uhpC* in LP02 Δ*flaA* and LP01 Δ*flaA* strains of *L. pneumophila*. Compared to LP02 Δ*flaA*, LP02 Δ*flaA* Δ*uhpC* was
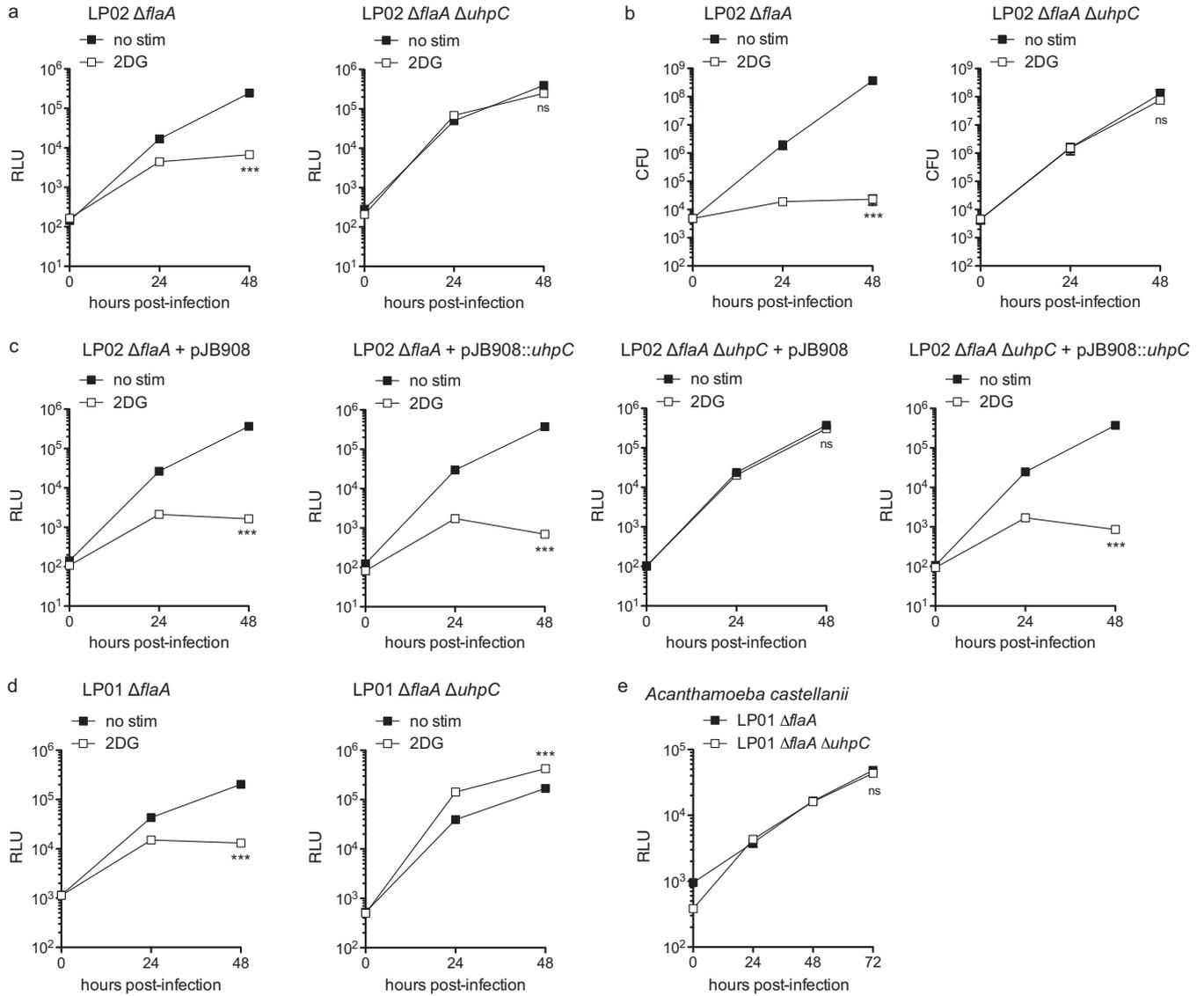
FIG 4 In-frame deletion of *uhpC* rescues 2DG-mediated inhibition of *L. pneumophila* replication in BMMs. (a) LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* replication in BMMs with or without 1.0 mM 2DG as measured by light emission (RLU). (b) LP02 Δ*flaA* and LP02 Δ*flaA* Δ*uhpC L. pneumophila* replication in BMMs with or without 1.0 mM 2DG as measured by CFU. (c) Replication of LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* complemented with pJB908 or pJB908 expressing wild-type (LP02) *uhpC* in BMMs with or without 0.5 mM 2DG as measured by light emission (RLU). (d) LP01 Δ*flaA* lux and LP01 Δ*flaA* Δ*uhpC* lux *L. pneumophila* replication in BMMs with or without 1.0 mM 2DG as measured by light emission (RLU). (e) LP01 Δ*flaA* lux and LP01 Δ*flaA* Δ*uhpC* lux *L. pneumophila* replication in *Acanthamoeba castellanii* as measured by light emission (RLU). ***, $P < 0.001$; ns, not significant (comparing growth curves by two-way ANOVA). Data are representative of at least two independent experiments. Symbols and bars represent means ± the standard deviations from at least three technical replicates.

insensitive to the inhibitory effect of 2DG during replication in BMMs (Fig. 4a and b). To verify that *L. pneumophila uhpC* is responsible for 2DG sensitivity, we transformed LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux with the expression vector pJB908, which rescues the thymidine auxotrophy of LP02 *L. pneumophila* (30), or pJB908 expressing *uhpC* cloned from WT LP02 *L. pneumophila*. We observed that pJB908::*uhpC* conferred 2DG sensitivity to LP02 Δ*flaA* Δ*uhpC* during infection of BMMs (Fig. 4c). LP01 Δ*flaA* Δ*uhpC* did not display a defect in replication during infection of BMMs or *A. castellanii* (Fig. 4d and e). All Δ*uhpC* strains of *L. pneumophila* grow without defect in broth (data not shown). These results indicate that functional UhpC is responsible for 2DG-mediated inhibition of growth in BMMs. Notably, *L. pneumophila* does not appear to require functional UhpC to replicate in any laboratory conditions tested, including BMMs treated with high levels of 2DG.

2DG-phosphate inhibits wild-type but not Δ*uhpC L. pneumophila* replication in broth. Given that the predicted function of *L. pneumophila* UhpC is to transport hexose-phosphates and that 2DG is itself a hexose sugar analog, we hypothesized that the inhibitory effect of 2DG in BMMs requires phosphorylation to produce 2DG 6-phosphate (2DGP). In support of this hypothesis, we saw that LP02 Δ*flaA* lux displayed a profound growth defect in broth in the presence of 2DGP but not 2DG (Fig. 5a). The inhibitory effect of 2DGP on *L. pneumophila* growth in broth was largely *uhpC*-dependent, as growth of LP02 Δ*flaA* Δ*uhpC* lux was not potently inhibited by the presence of 2DGP in broth (Fig. 5a). We observed that the addition of glucose had no effect on *L. pneumophila* replication in replete broth or in broth containing a low level of cysteine (Fig. 5a and b). Given that UhpC is a predicted hexose-phosphate transporter, we tested whether *L. pneumophila* growth in replete broth or cysteine-limited broth could be enhanced by phosphorylated glucose (glucose-6-phosphate [G6P]) and whether this effect depended on UhpC. However, we observed that the addition of G6P did not increase the ability of either LP02 Δ*flaA* or LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* to replicate in either replete or cysteine-limited conditions and, in fact, slightly decreased the bacterial replication of both strains (Fig. 5a and b). We verified that 2DGP inhibited the growth of LP02 Δ*flaA* lux but not LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* growth in broth by measuring CFU at 48 h (Fig. 5c). Complementation of LP02 Δ*flaA* Δ*uhpC* lux with pJB908::*uhpC* restored sensitivity 2DGP in broth (Fig. 6). These results demonstrate that *L. pneumophila* UhpC is required for sensitivity to 2DGP and strongly suggest that the inhibitory effect of 2DG on *L. pneumophila* replication in macrophages is mediated by phosphorylated 2DG.

DISCUSSION

There is an increasing appreciation for how changes in macrophage metabolism are critical for the immune response against pathogens, particularly intracellular bacterial pathogens (7, 31). Increased glycolysis is part of a characteristic metabolic shift in macrophages in response to bacterial pattern recognition signaling. This metabolic change is thought to allow macrophages to produce the high levels of inflammatory mediators necessary for initiation of an immune response. We observed that BMMs upregulate glycolysis in response to infection with *L. pneumophila*, a response we characterized to be largely dependent on intact macrophage pattern recognition signaling. Elevated glycolysis is associated with proinflammatory activities in a variety of immune cells, and increased glycolysis has been associated with restriction of intracellular bacterial replication in macrophages (29, 32). Because *L. pneumophila* is able to replicate in glycolytic macrophages, we initially hypothesized that *L. pneumophila* was taking advantage of macrophage glycolysis during intracellular replication. This hypothesis was supported by our result showing that the glycolysis inhibitor 2DG blocked *L. pneumophila* replication in BMMs, an effect that has been reported in other studies (22–25). However, other experiments in which we inhibited host macrophage glycolysis had no effect on intracellular *L. pneumophila* replication and demonstrated that the inhibitory effect of 2DG on intracellular *L. pneumophila* was unlikely via induction of a shift in host cell metabolism. Ultimately, we observed that *L. pneumophila* hexose-phosphate transporter UhpC was required for bacterial sensitivity to 2DG during infection of BMMs, defining a potential mechanism for the sensitivity of *L. pneumophila* to 2DG during intracellular growth.

We propose that in BMMs mammalian hexokinase phosphorylates 2DG to produce 2DGP, which is taken up via *L. pneumophila* UhpC. Once inside the bacterium, 2DGP interferes with *L. pneumophila* growth through an as-yet-undetermined mechanism. The lack of effect of 2DG on *L. pneumophila* replication in broth may be explained by the inability of certain bacteria to phosphorylate 2DG (33). Similarly, 2DG phosphorylation may not occur in *A. castellanii*. This revised model of how 2DG restricts *L. pneumophila* replication in BMMs is supported by our result that 2DGP potently inhibits the growth of Δ*flaA* but not Δ*flaA* Δ*uhpC L. pneumophila* in broth.

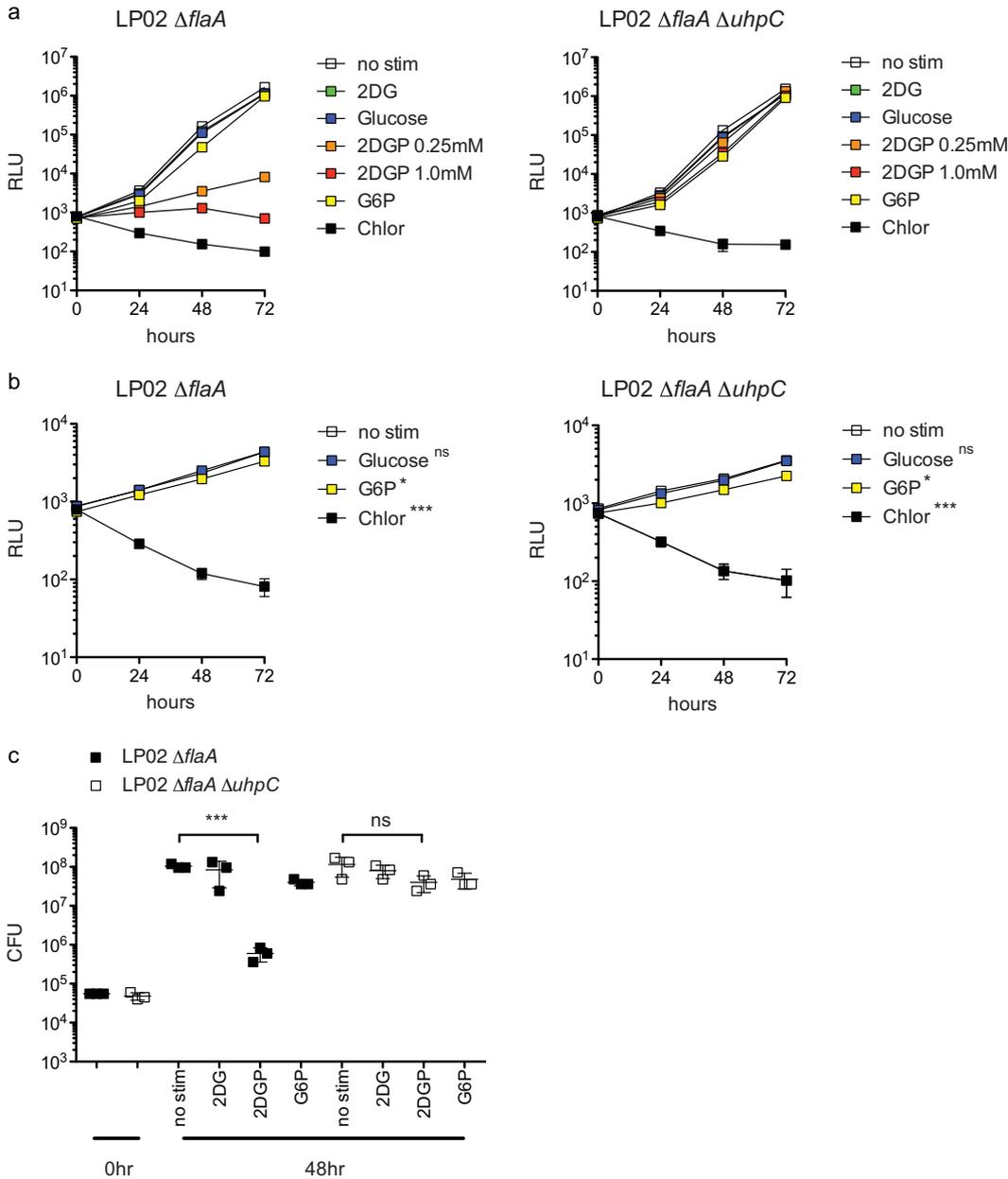We did not observe defects in Δ*uhpC L. pneumophila* replication in BMMs, broth, or

FIG 5 Restriction of *L. pneumophila* replication by 2DGP in broth is *uhpC* dependent. (a) LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* growth in replete broth in the presence of no stimulation (no stim), 1.0 mM 2DG, 1.0 mM glucose, 0.25 mM 2DGP, 1.0 mM 2DGP, 1.0 mM G6P, and 10.0 μg/ml chloramphenicol (Chlor) as measured by light emission (RLU). Growth under all stimulation conditions differs from growth in no stimulation conditions ($P < 0.05$ by two-way ANOVA with Bonferroni posttest to control for multiple comparisons). (b) LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* growth in broth containing 1/16 normal amount of cysteine (0.21 mM) in the presence of no stimulation (no stim), 1.0 mM glucose, 1.0 mM G6P, and 10.0 μg/ml chloramphenicol (Chlor) as measured by light emission (RLU). *, $P < 0.05$; ***, $P < 0.001$; ns, not significant (two-way ANOVA with Bonferroni posttest to control for multiple comparisons). (c) CFU of LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* at 0 h and after 48 h in broth in the presence of no stimulation (no stim), 0.25 mM 2DG, 0.25 mM 2DGP, or 0.25 mM G6P. ***, $P < 0.001$; ns, not significant (two-tailed Student *t* test). Data represent at least two independent experiments. Error bars reflect the standard deviations from three technical replicates.

in *Acanthamoeba castellanii*, indicating that this gene is not required for *L. pneumophila* growth in normal laboratory conditions. Of particular note, Δ*uhpC L. pneumophila* replicates without a growth defect in BMMs treated with high levels of 2DG. This result indicates that in contrast to our initial hypothesis, increased macrophage glycolysis is not required to support intracellular *L. pneumophila* growth. Increased macrophage glycolysis may be part of a coordinated multicellular immune response in response to
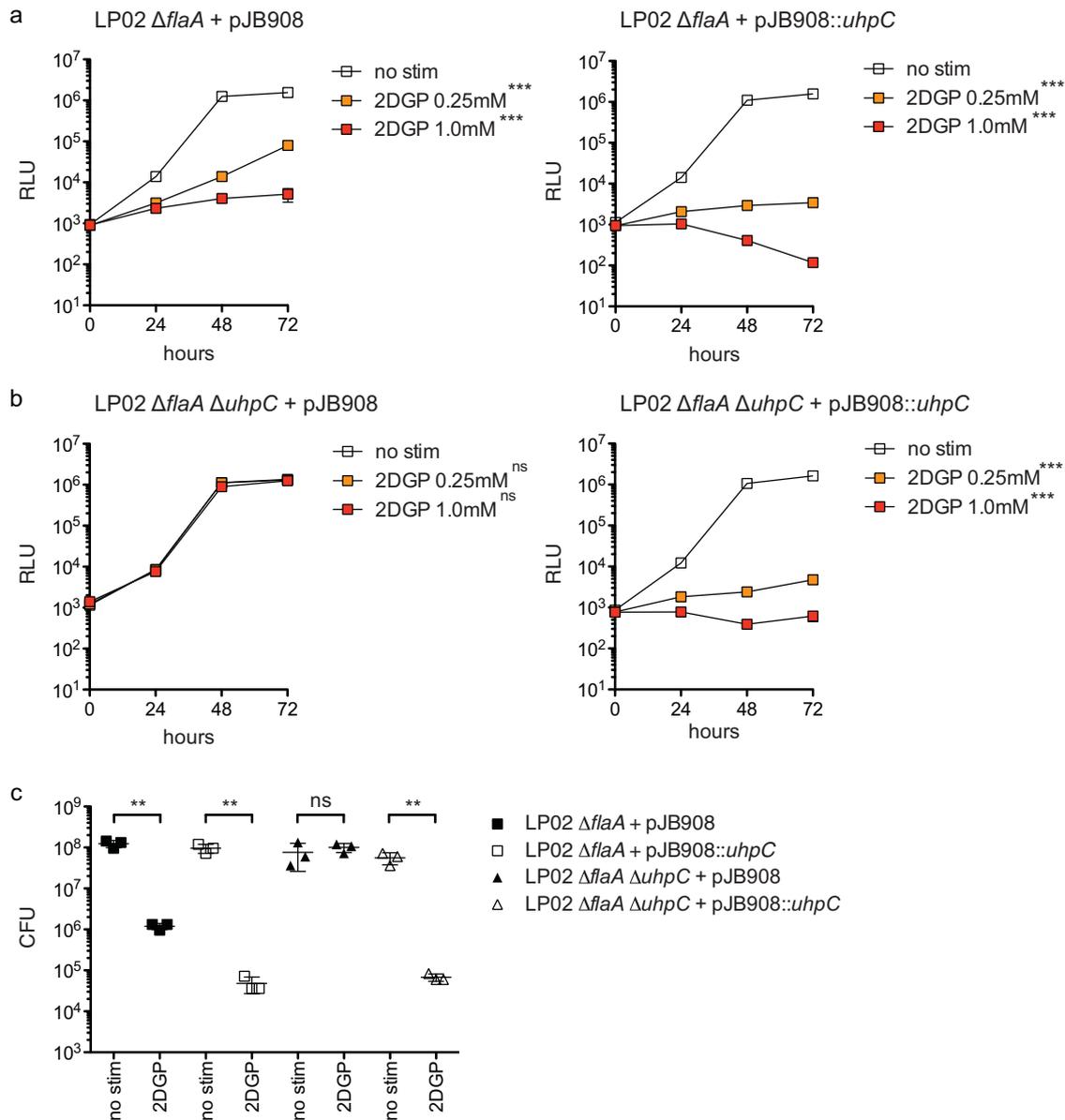
FIG 6 Complementation of Δ*uhpC L. pneumophila* with wild-type *uhpC* confers sensitivity to 2DG-phosphate in broth. (a) Replication of LP02 Δ*flaA* lux complemented with pJB908 or pJB908 expressing wild-type (LP02) *uhpC* in broth in the presence of no stimulation (no stim), 0.25 mM 2DGP, and 1.0 mM 2DGP as measured by light emission (RLU). (b) Replication of LP02 Δ*flaA* Δ*uhpC* lux complemented with pJB908 or pJB908 expressing wild-type (LP02) *uhpC* in broth in the presence of no stimulation (no stim), 0.25 mM 2DGP, and 1.0 mM 2DGP as measured by light emission (RLU). ***, $P < 0.001$, ns, not significant (two-way ANOVA with Bonferroni posttest to control for multiple comparisons in panels a and b). (c) CFU of LP02 Δ*flaA* lux and LP02 Δ*flaA* Δ*uhpC* lux *L. pneumophila* complemented with pJB908 or pJB908 expressing wild-type (LP02) *uhpC* after 48 h in broth in the presence of no stimulation (no stim) and 0.25 mM 2DG. **, $P < 0.01$; ns, not significant (two-tailed Student *t* test). Data represent at least two independent experiments. Error bars reflect the standard deviations from three technical replicates.

L. pneumophila infection *in vivo*. Accordingly, the identification of 2DG-resistant *L. pneumophila* may provide a useful tool for the study of metabolic responses to infection in more complex experimental models.

During intracellular and extracellular growth, *L. pneumophila* is thought to depend on amino acids as a carbon source (34, 35). While the *L. pneumophila* genome is predicted to contain intact glucose metabolism pathways and the bacteria have been shown to incorporate carbon from glucose into its central carbon metabolism, the addition of glucose does not boost *L. pneumophila* growth in broth and cannot replace amino acids as a carbon source (35–37). The importance of *L. pneumophila* glucose

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)[a]
uhpC deletion construct in pSR47S	
uhpC forward	TAAGCA**GGATCC**CGTATGCCGATAGCTTGAAACG (BamHI)
uhpC internal reverse	CTAAGCAAATTCGGATTTGCCTGAAGGTTTTAGAAAACTCAATAATGCCAT
uhpC internal forward	ATGGCATTATTGAGTTTTCTAAAACCTTCAGGCAAATCCGAATTTGCTTAG
uhpC reverse	TGCTTA**GCGGCCGC**ATCCAGGTGCCTTGGTATGTCC (NotI)
uhpC complementation construct in pJB908	
uhpC comp forward	TAAGCA**GGATCC**TTTTCATGATCATGGACAATC (BamHI)
uhpC comp reverse	ATGTA**GTCGAC**GAAGCCAATCACTAAGCAAAT (SalI)

[a]Restriction enzymes are indicated in parentheses; restriction sites are indicated in boldface.

metabolism during intracellular replication remains obscure, since a range of defects have been reported in *L. pneumophila* harboring mutations in glucose metabolism pathways (37, 38). *L. pneumophila uhpC* is a putative hexose-phosphate transporter gene based on sequence similarity to characterized transporters in other species of bacteria, including *Chlamydia*, *Salmonella*, and *Escherichia coli* (39, 40). In *Listeria monocytogenes*, the hexose-phosphate transporter gene *hpt* is a virulence (PrfA)-regulated gene, and *hpt* mutant bacteria display impaired intracellular growth and a virulence defect during mouse infection (41). The prevalence of hexose-phosphate transporter genes across species of pathogenic bacteria indicates that hexose-phosphates may be an advantageous nutrient source during intracellular replication. Presumably, *L. pneumophila* UhpC facilitates the uptake of phosphorylated hexose sugars, similar to UhpC-like proteins in other bacteria. However, we did not observe increased *L. pneumophila* growth in broth supplemented with glucose-6-phosphate. Inhibition of *L. pneumophila* replication by 2DGP suggests that poisoning *L. pneumophila* glucose metabolic pathways does have a deleterious effect on bacterial growth. Alternatively, 2DGP may have off-target toxic effects on *L. pneumophila*. Future metabolite profiling of wild-type and UhpC-deficient *L. pneumophila* may reveal a role for UhpC in terms of facilitating uptake of carbon and/or phosphate-containing molecules, which may allow *L. pneumophila* to access a variety of metabolites that could be critical for persistence or replication in specific environments or host species encountered in nature.

MATERIALS AND METHODS

Ethics statement. We conducted this study in accordance with guidelines contained in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (42) and under a protocol approved by the Animal Care and Use Committee at the University of California, Berkeley (protocol AUP-2014-09-6665).

Cell culture. We used BMMs from WT C57BL/6J mice for all experiments unless otherwise indicated. We derived macrophages from the bone marrow of C57BL/6J mice (Jackson Laboratory) or $Myd88^{-/-}$ $Nod1^{-/-}$ $Nod2^{-/-}$ mice on the C57BL/6J background (26) in RPMI medium supplemented with 10.0% fetal bovine serum, 2.0 mM L-glutamine, and 100 μM streptomycin (all from Life Technologies), as well as 5.0% supernatant from 3T3 cells expressing macrophage colony-stimulating factor (generated in-house). BMMs from LysMCre$^{+/+}$ and LysMCre$^{+/+}$Atg5$^{fl/fl}$ mice in a C57BL/6J background were provided by the lab of Daniel Portnoy at the University of California, Berkeley.

Bacterial strains and culture. LP01 is a streptomycin-resistant derivative of a clinical isolate of Philadelphia-1 strain *L. pneumophila* (43). LP02 is derived from LP01 *L. pneumophila* and is a thymidine auxotroph (43). Δ*flaA*, Δ*dotA*, and lux strains of *L. pneumophila* have been described previously (16, 27, 44). We cultured *L. pneumophila* in AYE (ACES-buffered yeast extract broth) or on ACES-buffered charcoal-yeast extract (BCYE) agar plates at 37°C. We made in-frame deletion of *uhpC* (Δ*uhpC*) using the sucrose-sensitive suicide plasmid pSR47S as described previously (45). We complemented Δ*uhpC* strains with wild-type *uhpC* cloned from LP02 genomic DNA and expressed from the plasmid pJB908, which encodes thymidine synthetase for selection. We list sequences of primers used to generate deletion and complementation constructs in Table 1. To measure growth of *L. pneumophila* in AYE broth, we diluted bacteria to an optical density at 600 nm (OD$_{600}$) of 1.0 with or without 2-deoxyglucose (2DG; Abcam) at the indicated concentrations and measured bacterial growth by measuring the OD$_{600}$ or diluting bacteria to enumerate CFU on BYCE agar plates. To measure lux strain *L. pneumophila* growth in broth, we diluted bacteria to an estimated 5.0×10^4 *L. pneumophila* cells/well in opaque white TC-treated 96-well microtiter plates (Corning) in AYE broth in the presence of glucose (Sigma), glucose-6-phosphate (G6P; Santa Cruz Biotechnology), 2DG, 2DG-6-phosphate (2DGP; Santa Cruz Biotechnology), or chloramphenicol (Sigma) as indicated. We measured bacterial growth by detection of luminescence at λ470 using a Spectramax L luminometer (Bio-Rad) or by dilution on BYCE agar plates for enumeration of CFU. For

experiments using heat-killed bacteria, we incubated *L. pneumophila* in phosphate-buffered saline (PBS) at 100°C for 15 min to kill bacteria before performing infections. We verified death of bacteria by incubation on BYCE agar plates.

Infection of BMMs. For measurement of intracellular *L. pneumophila* growth by luminescence, we plated 1.0×10^5 BMMs/well in opaque white TC-treated 96-well microtiter plates and infected the samples with *L. pneumophila* at a multiplicity of infection (MOI) of 0.01 or 0.05 as indicated. For measurement *L. pneumophila* growth by CFU, we plated 5.0×10^5 BMMs/well in TC-treated 24-well plates (Corning) and infected with *L. pneumophila* at an MOI of 0.01. At 1 h postinfection by centrifugation at $287 \times g$, we replaced the media of infected BMMs with media containing 2DG, sodium fluoride (NaF, Sigma), or sodium oxamate (NaO; Sigma) at the indicated concentrations. We maintained 2DG or other drugs in the infection media for the duration of the experiment. At the indicated times after infection, we measured bacterial growth by detection of luminescence or by dilution of infected cultures on BYCE agar plates for the enumeration of CFU. To measure lactate and glucose in the supernatant of BMMs infected with *L. pneumophila*, we plated 5.0×10^5 BMMs/well in 1.0 ml/well in TC-treated 24-well plates (Fig. 1a, d, and e) or 2.0×10^6 BMMs/well in 2.0 ml/well in TC-treated 6-well plates (Fig. 1b and c) and infected them with *L. pneumophila* as described above. At 1 h postinfection, we replaced the media of infected BMMs with media with or without 2DG at the indicated concentrations. We measured lactate and glucose in the supernatant of BMMs cultures using assay kits (Sigma) according to the manufacturer's instructions. For altered-sugar experiments, we performed infections using glucose-free RPMI (Life Technologies). We added 11.11 mM galactose (Sigma) as indicated.

Serial enrichment of *L. pneumophila* mutants. We plated 1.0×10^6 BMMs/well in 6-well TC-treated plates (Corning) for a total of 12 wells. We infected each well with LP02 Δ*flaA* lux *L. pneumophila* at an MOI of 0.05 as described above. At 1 h postinfection, we replaced the media of infected BMMs in 11 wells with medium containing 0.2 mM 2DG and one well with medium containing no 2DG. At 96 to 120 h postinfection, we removed the culture supernatant and lysed the remaining BMMs in sterile H_2O. We combined *L. pneumophila* from BMM lysis with *L. pneumophila* collected from the culture supernatant for reinfection of fresh BMMs. We diluted *L. pneumophila* for reinfection based on the luminescence signal measured from recovered bacteria with the aim of reinfecting at an MOI between 0.01 and 0.05. We repeated the infection procedure as described above at least three times in two independent experiments. At each reinfection step, a fraction of *L. pneumophila* recovered from each well were tested in a luminescence growth assay using 1.0×10^5 BMMs/well in opaque white TC-treated 96-well microtiter plates with or without 2DG. After the third round of reinfection, when we observed more robust growth of *L. pneumophila* in 2DG-treated BMMs from several independent source wells versus the control well, we isolated individual clones of *L. pneumophila* from each of the 12 wells by dilution on BYCE agar plates. After testing of these clonal populations of *L. pneumophila* for the ability to replicate in 2DG-treated BMMs, we extracted genomic DNA using a blood and tissue DNA extraction kit (Qiagen) and submitted the DNA for library preparation and paired-end sequencing on a MiSeq instrument at the QB3 functional genomics facility at UC Berkeley (http://qb3.berkeley.edu/fgl/). We analyzed the sequencing data for variations compared to the Philadelphia-1 *L. pneumophila* reference genome (GenBank accession no. CP013742.1) using CLC Genomics Workbench software (Qiagen).

Infection of *Acanthamoeba castellanii*. We maintained *A. castellanii* strain Neff (ATCC 30010) in PYG broth (2.0% proteose-peptone [BD Biosciences], 0.1% yeast extract [BD Biosciences], 0.1 M glucose, 4.0 mM $MgSO_4$ [Fisher Scientific], 0.4 M $CaCl_2$ (Fisher Scientific) 0.1% sodium citrate (Sigma), 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ [Sigma], 2.5 mM NaH_2PO_4 [Fisher Scientific], 2.5 mM K_2HPO_4 [Fisher Scientific], and 100 μM streptomycin [Life Technologies]) at room temperature. For infection, we plated 1.0×10^5 *A. castellanii* cells/well in opaque white TC-treated 96-well microtiter plates in PBS (Life Technologies). We diluted *L. pneumophila* to the desired MOI in PBS, followed by centrifugation at $287 \times g$. At 1 h postinfection, we replaced the media of infected *A. castellanii* with PBS containing 2DG at the indicated concentrations and maintained infected cultures at 37°C. At the indicated times after infection, we measured the growth of *L. pneumophila* by the detection of luminescence.

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