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Avoidance of Autophagy Mediated by PlcA or ActA Is Required for *Listeria monocytogenes* Growth in Macrophages

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***Listeria monocytogenes* is a facultative intracellular pathogen that escapes from phagosomes and grows in the cytosol of infected host cells. Most of the determinants that govern its intracellular life cycle are controlled by the transcription factor PrfA, including the pore-forming cytolysin listeriolysin O (LLO), two phospholipases C (PlcA and PlcB), and ActA. We constructed a strain that lacked PrfA but expressed LLO from a PrfA-independent promoter, thereby allowing the bacteria to gain access to the host cytosol. This strain did not grow efficiently in wild-type macrophages but grew normally in macrophages that lacked ATG5, a component of the autophagy LC3 conjugation system. This strain colocalized more with the autophagy marker LC3 (42% ± 7%) at 2 h postinfection, which constituted a 5-fold increase over the colocalization exhibited by the wild-type strain (8% ± 6%). While mutants lacking the PrfA-dependent virulence factor PlcA, PlcB, or ActA grew normally, a double mutant lacking both PlcA and ActA failed to grow in wild-type macrophages and colocalized more with LC3 (38% ± 5%). Coexpression of LLO and PlcA in a PrfA-negative strain was sufficient to restore intracellular growth and decrease the colocalization of the bacteria with LC3. In a cell-free assay, purified PlcA protein blocked LC3 lipidation, a key step in early autophagosome biogenesis, presumably by preventing the formation of phosphatidylinositol 3-phosphate (PI3P). The results of this study showed that avoidance of autophagy by *L. monocytogenes* primarily involves PlcA and ActA and that either one of these factors must be present for *L. monocytogenes* growth in macrophages.**

Listeria monocytogenes is a Gram-positive facultative intracellular bacterial pathogen that has been used for decades as a model organism for studying basic aspects of host-pathogen interactions (1–3). Subsequent to internalization by macrophages, the bacteria escape from phagosomes and access the host cytosol, a process that requires the pore-forming cytolysin listeriolysin O (LLO) (4). Two other virulence factors, a phosphatidylinositol-specific phospholipase C (PlcA) and a broad-range phospholipase C (PlcB), also participate in the escape from phagosomes (5–7). *L. monocytogenes* then grows rapidly in the host cytosol and expresses high levels of the surface protein ActA. ActA recruits host proteins (e.g., the Arp2/3 complex and Ena-VASP proteins) that mediate actin polymerization and allow bacteria to move inside host cells and to spread from cell to cell (8). Most of the virulence factors that play a role in the intracellular life cycle of *L. monocytogenes* (e.g., ActA, LLO, PlcA, and PlcB) are under the control of the Crp family member transcription factor PrfA (9, 10). Although the PrfA regulon is absolutely required for *L. monocytogenes* pathogenesis, it is not clear which PrfA-dependent factors contribute to growth of *L. monocytogenes* in the macrophage cytosol.

Autophagy is a catabolic process that targets intracellular material to the lysosomal pathway for degradation and recycling (11). Autophagy also plays a role in both innate and adaptive host immunity and is a cell-autonomous innate defense mechanism that directly controls the replication of intracellular microbes (12). Macroautophagy sequesters invading microbes in double-membrane vesicles called autophagosomes and targets these microbes for lysosomal degradation. An essential step in macroautophagy is cleavage and coupling of LC3 proteins to phosphatidylethanolamine (PE) on early autophagosome structures. LC3-PE (LC3-II) then interacts with adaptor proteins that recognize microbes earmarked for autophagic degradation. Importantly, the class III phosphatidylinositide 3-kinase, VPS34, catalyzes the synthesis of phosphatidylinositol 3-phosphate (PI3P) by the phosphorylation of phosphatidylinositol (PI) and plays a central role in the regulation of autophagosome formation and autophagic flux (13). Some components of the autophagy machinery also contribute to antibacterial defenses by mechanisms that do not rely on autophagosome formation, such as LC3-associated phagocytosis (LAP) (14). LAP is a process at the convergence of phagocytosis and autophagy during which LC3 is directly conjugated to single-membrane phagosomes in order to promote acidification and fusion with lysosomes (15). Not surprisingly, many pathogens have adopted strategies to interfere with or exploit the autophagy machinery to promote pathogenesis (16–18).

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TABLE 1 *L. monocytogenes* strains used in this study

Strain	Description	Reference
10403S	Wild type	62
DP-L2261	Δ*hly*	32
DH-L991	Δ*hly* cLLO (pHpPL3-*hly*)	35
DP-L4317	Δ*prfA*	63
DP-L6170	Δ*hly* Δ*prfA*	This study
DP-L6172	Δ*hly* Δ*prfA* pHpPL3	This study
DP-L6173	Δ*hly* Δ*prfA* cLLO (pHpPL3-*hly*)	This study
DP-L6174	Δ*hly* Δ*prfA* pHpPL3-*hly* (no terminator)	This study
DP-L6175	Δ*hly* Δ*prfA* cLLO cPlcA (pHpPL3-*hly*-*plcA*)	This study
DP-L1552	Δ*plcA*	7
DP-L1935	Δ*plcB*	6
DP-L3078	Δ*actA*	31
DP-L1936	Δ*plcA* Δ*plcB*	6
DP-L6176	Δ*plcA* Δ*plcB* pPL2-*plcA*	This study
DP-L6177	Δ*plcA* Δ*plcB* pPL2-P_{actA}-*plcB* (pERS1018)	This study
DP-L6171	Δ*actA* Δ*plcA*	This study
DP-L6178	Δ*actA* Δ*plcA* pPL2-*actA*	This study
DP-L6179	Δ*actA* Δ*plcA* pPL2-*plcA*	This study
DP-L4066	Δ*actA* Δ*plcB*	64
DP-L2160	Δ*actA* Δ*plcA* Δ*plcB*	This study

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]
hly-FWD	ATATATCGGCCGATAAAGCAAGCATATAATATTGCGTT
*hly*noTT-REV	ATATATCTGCAGTTATTCGATTGGATTATCTACTTTATTA
plcA-FWD	ATATATGATATCATATACTAATCAAAGGAGGGGGC
plcA-REV	ATATATGTCGACAGAGTTAGTATATGGTTCCGAGG
*actA*comp-FWD	ATATATCGGCCGGGGAAGCAGTTGGGGTTAACT
*actA*comp-REV	ATATATCTCGAGCTCACTTTTTTCTTTCGTTCTGTGTT
*plcA*comp-FWD	ATATATGATATCGCTATCCTTTTGACGTCATTAACA

[a] Underlining indicates restriction enzyme sites.

L. monocytogenes replicates similarly in wild-type and autophagy-defective bone marrow-derived macrophages (BMDM) (19), suggesting that the bacteria can circumvent the host cell autophagy machinery (20). One proposed mechanism is that *L. monocytogenes* avoids autophagic recognition by recruiting host proteins to the bacterial surface using either ActA or InlK (21, 22). However, InlK is not expressed during *in vitro* cell infection (21), and the effect of ActA on LC3 recruitment requires that bacterial protein synthesis be inhibited (14, 23, 24), suggesting that additional factors are involved. *L. monocytogenes* phospholipases C (PLCs) also contribute to autophagy evasion, but the mechanism has remained elusive (19, 23, 25). A recent study suggested that PLCs prevent autophagy targeting of *L. monocytogenes* by reducing autophagic flux, depleting host PI3P, and inhibiting the maturation of preautophagosomal structures (26). Importantly, PlcA seemed to be more important than PlcB in mediating the accumulation of cytoplasmic granules with characteristics of preautophagosomal structures during *L. monocytogenes* infection (26). The relative contribution of ActA, PlcA, and PlcB, either alone or in combination, in evasion of the autophagy pathway by *L. monocytogenes* is still ambiguous. Furthermore, it is still unclear to what extent autophagy avoidance contributes to growth of *L. monocytogenes* in the host cell. This study clearly demonstrates that autophagy avoidance is required for *L. monocytogenes* replication in macrophages and is mediated by either PlcA or ActA.

MATERIALS AND METHODS

Bacterial strains, growth medium, and cell culture. *L. monocytogenes* strains used in this study are listed in Table 1. Strains were grown in brain heart infusion (BHI) medium at 30°C overnight prior to all experiments. Bone marrow-derived macrophages (BMDM) were prepared and cultured using standard protocols (27). *Atg5*$^{flox/flox}$ (28), *Atg5*$^{flox/flox}$-Lyz-*Cre* (29), and green fluorescent protein (GFP)-conjugated LC3 (30) mice were described previously. HEK293T cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Deletions of *hly* and *actA* in Δ*prfA* and Δ*plcA* backgrounds, respectively, were achieved as previously reported (31, 32). Plasmids pPL2 (33), pPL2-P_{actA}-*plcB* (pERS1018) (34), pHpPL3 (35), and pHpPL3-*hly* (cLLO) (35) have already been described. To generate pHpPL3-*hly* (no terminator), the 5′ untranslated region (5′ UTR) and the coding sequence of *hly* were amplified without transcriptional terminator by PCR (primers *hly*-FWD and *hly*noTT-REV), digested with EagI and PstI, and inserted into pHpPL3 downstream of the hyper-P_{spac} promoter (P_{hyper}). The 5′ UTR and the coding sequence of *plcA* were then amplified by PCR (primers *plcA*-FWD and *plcA*-REV), digested with EcoRV and SalI, and inserted in pHpPL3-*hly* (no terminator) in order to generate pHpPL3-*hly*-*plcA* (cLLO cPlcA). For genetic complementation experiments, *actA* and *plcA* were amplified by PCR (primers *actA*comp-FWD and *actA*comp-REV and primers plcAcomp-FWD and *plcA*-REV, respectively) and inserted into pPL2 with their native promoters. The *actA* amplicon was digested with EagI and XhoI, and the *plcA* amplicon was digested with EcoRV and SalI. Primers used in this study are listed in Table 2. Inserts were sequenced, transformed into *Escherichia coli* SM10, and conjugated into *L. monocytogenes* strains.

Intracellular growth curves. Intracellular growth curves were performed as previously described (36). Briefly, BMDM were infected at a multiplicity of infection (MOI) of 0.25 (1 bacterium per 4 macrophages), which results in the infection of approximately 8% of the cells. Thirty minutes after infection, cells were washed and fresh medium was added. At 1 h postinfection, 50 μg/ml of gentamicin was added to the medium in order to kill extracellular bacteria. Replication was quantified by enumerating intracellular CFU. When specified, 5 mM 3-methyladenine (3-MA) (Sigma, St. Louis, MO) was added to infected cells at 1 h postinfection.

Immunofluorescence, microscopy, and image analysis. GFP-LC3 BMDM were infected at an MOI of 0.4 (2 bacteria per 5 macrophages), resulting in the infection of approximately 13% of the cells, as described above. When specified, 5 mM 3-MA was added to infected cells at the time of infection. At various time points, coverslips were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and incubated for at least 30 min in permeabilization/blocking buffer (PB buffer; PBS containing 2% bovine serum albumin [BSA] and either 0.1% saponin or 0.1% Triton X-100). Coverslips were then incubated for 1 h in PB buffer containing mouse anti-GFP antibody (no. 11814460001; 1:200 dilution; Roche, Indianapolis, IN) and/or rabbit anti-*Listeria* antibody (no. 223021; 1:1,000 dilution; BD Biosciences, San Jose, CA). Coverslips were then washed 6 times and incubated for 45 min in PB buffer containing Alexa Fluor 488 or 647 goat anti-mouse IgG (1:2,000 dilution; Invitrogen, Grand Island, NY), rhodamine Red-X goat anti-rabbit IgG (1:2,000 dilution; Invitrogen) and Alexa Fluor 647 rat anti-mouse LAMP1 (no. 121609; 1:250 dilution; BioLegend, San Diego, CA), when required. Coverslips were washed 6 times and mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cells were imaged with an Olympus IX71 epifluorescence microscope using the 100× objective. Several frames per time point were randomly selected, and images were collected and color combined using MetaMorph software (Universal imaging). Images from at least 3 inde-

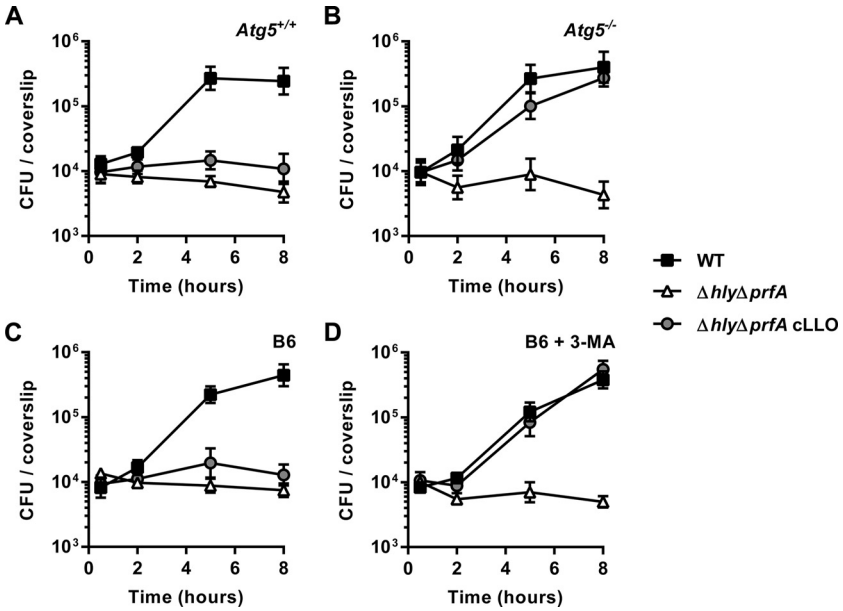

FIG 1 Intracellular growth of a Δ*prfA* strain expressing LLO. Kinetics of intracellular growth for wild-type, Δ*hly* Δ*prfA* (with the empty integrated vector pHpPL3), and Δ*hly* Δ*prfA* cLLO in *Atg5*$^{+/+}$ BMDM (A), *Atg5*$^{-/-}$ BMDM (B), B6 BMDM (C), and B6 BMDM exposed to 3-MA (D) are shown. Results are expressed as means and standard deviations obtained from at least 3 independent experiments.

pendent experiments were analyzed using ImageJ (National Institutes of Health), and a minimum of 100 bacteria, or GFP-LC3^{+} bacteria, were scored for colocalization with GFP-LC3 or LAMP1, respectively, for each tested condition. Images are representative of observed results.

Protein purification. Genes *plcA* and *plcB* were cloned into vector pTYB21 (New England BioLabs, Ipswich, MA). Recombinant proteins were expressed in *E. coli* strain BL21-AI (Life Technologies, Grand Island, NY). Bacteria were grown to an optical density of about 0.6, and protein expression was induced with both L-arabinose and IPTG, at final concentrations of 0.2% of L-arabinose and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were shaken at 225 rpm and 20°C for 6 h before harvest. Target proteins were expressed with an N-terminal intein tag, which harbored a chitin-binding domain (CBD) for affinity purification. Protein purification was undertaken with protocols suggested by the manufacturer (37). Ultimately, the desired protein was eluted from chitin resin with the native N terminus following thiol-induced intein self-splicing on the column. The excessive thiol in the protein elute was removed by dialysis with storage buffer (20 mM HEPES, 150 mM NaCl [pH 8.5]). Purified proteins were aliquoted and stored at 4°C or frozen with liquid nitrogen in 20% glycerol and stored at −80°C. All of the purification procedures were undertaken at 4°C. Site-directed point mutation of proteins [PlcA(W49A) and PlcB(D55N)] was conducted with the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). The biochemical properties of PlcA(W49A) have previously been described (38). The activity of the PlcB(D55N) mutant was 43-fold lower than that of wild-tye (WT) PlcB when 5 mM dihexanoylphosphatidylcholine was used as a substrate in 20 mM HEPES buffer, 150 mM NaCl, 0.1 mg/ml BSA (pH 6) and with a method based on the detection of inorganic phosphate that has been previously described (39).

Phospholipase treatment of membrane. A 25,000 × *g* pellet membrane, enriched in lipidation activity, was collected by differential centrifugation and suspended in B88 buffer as previously described (40). For treatment with PlcA and PlcA(W49A), the membrane fraction was diluted to a final concentration of 0.2 mg/ml of phosphatidylcholine (PC) content and incubated with the indicated concentrations of enzymes. For treatment with PlcB and PlcB(D55N), the membrane fraction was diluted and incubated with enzymes, as described above, but 50 μM zinc acetate was included to enhance PlcB activity (41). The mixtures were then incubated at 30°C for 1 h and pelleted at 25,000 × *g*. Finally, the membrane was washed once with B88 buffer, pelleted again, and used for lipidation reactions.

Cell-free LC3 lipidation and immunoblotting. The lipidation and immunoblotting procedure was carried out as previously described (40), with subtle modifications. In brief, cytosol (2-mg/ml final concentration) collected from starved HEK293T cells, an ATP regeneration system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM ATP), GTP (0.15 mM), T7-LC3 (amino acids 1 to 120), and the phospholipase-treated membrane fractions (0.2-mg/ml PC content, final concentration) were incubated in a final volume of 30 μl. Reactions were performed at 30°C for 1 h, and LC3 lipidation was detected by immunoblotting as previously described (40, 42, 43). Antibodies included mouse anti-PDI (Enzo Life Sciences, Farmingdale, NY), mouse anti-GST (Santa Cruz, Dallas, TX), mouse anti-T7 (EMD, Billerica, MA), rabbit anti-ERGIC53 (Sigma), and rabbit anti-VPS34 (Cell Signaling, Boston, MA).

Quantification of phospholipids. For PC and PE measurements, the 25,000 × *g* membrane fraction was collected, and phospholipase digestions were performed as described above. The digested membranes were collected and incubated with cytosol, ATP regeneration system, GTP, and T7-LC3, as described above. The membranes were then collected by centrifugation at 25,000 × *g*, and suspended in B88 buffer. Membrane PC and PE levels were measured as previously described (40, 43). For PI3P measurement, the 25,000 × *g* membrane fraction was collected, digested with phospholipases, and incubated with cytosol, ATP regeneration system, GTP and T7-LC3, as described above, in the presence of 2 μM GST-FYVE. GST-FYVE binds specifically to PI3P (40). The membrane fraction was collected by pelleting, washed once with B88 buffer, and collected again for immunoblot analysis of the bound GST-FYVE level.

Statistical analysis. Statistical analyses were carried out with the GraphPad Prism software (v.6.02). CFU were transformed to base 10 logarithm values before being used for statistical analyses. Statistical tests used for the analysis of each experiment are specified in the figure legends.

Ethical statement. This study was performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the

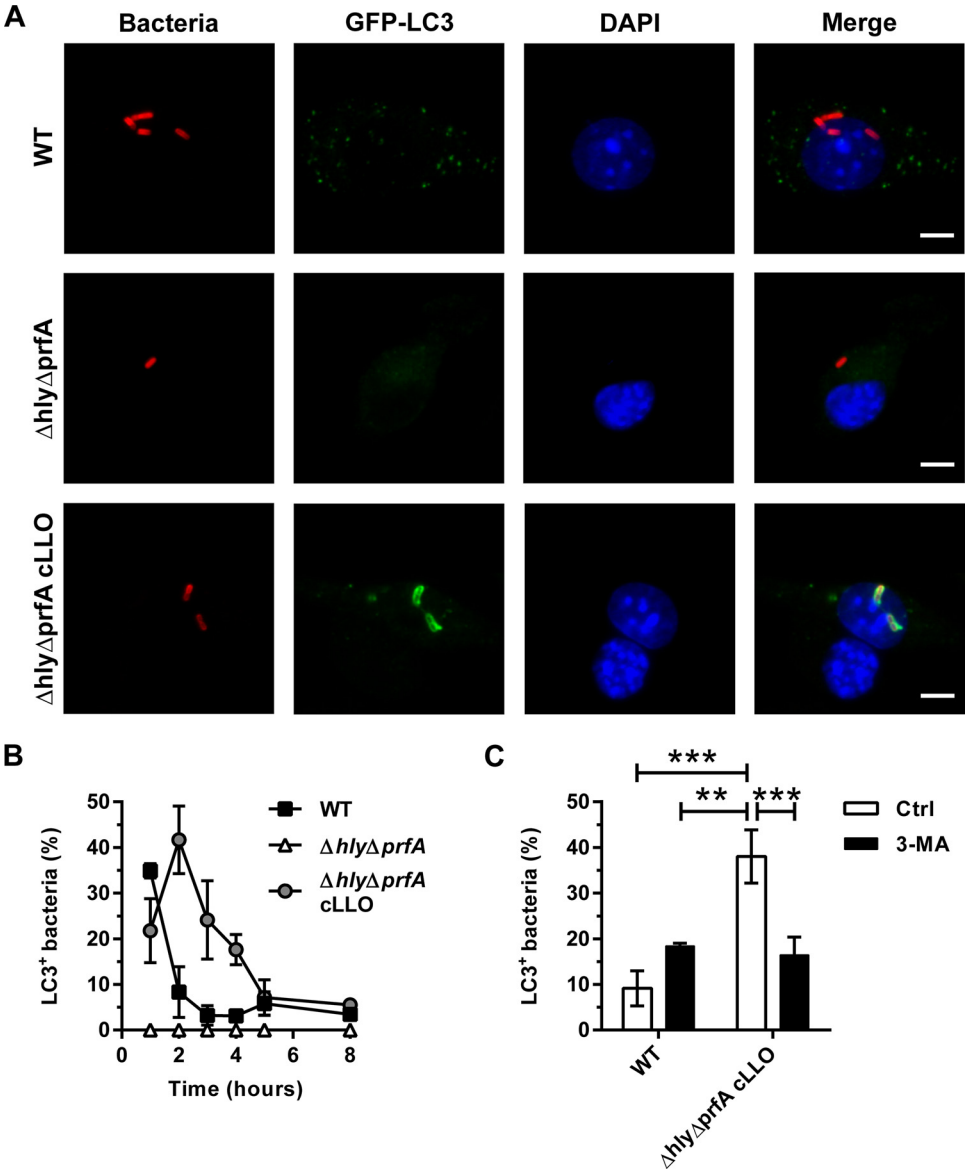

FIG 2 Colocalization of LC3 with a Δ*prfA* strain expressing LLO. (A) Representative micrographs of GFP-LC3 BMDM infected for 2 h with 10403S, Δ*hly* Δ*prfA*, and Δ*hly* Δ*prfA* cLLO. Infected cells were stained for *L. monocytogenes* (red), GFP-LC3 (green), and DNA (blue). (B) Colocalization kinetics of GFP-LC3 with WT, Δ*hly* Δ*prfA* (pHpPL3), and Δ*hly* Δ*prfA* cLLO. Proportions of GFP-LC3$^+$ bacteria are expressed as a percentage of total intracellular *L. monocytogenes*. The Δ*hly* Δ*prfA* cLLO strain showed increased colocalization with LC3 in comparison to the WT strain from 2 to 4 h postinfection ($P < 0.0001$ for each time points; two-way ANOVA with Dunnett's posttest). (C) Effect of 3-MA on the colocalization of GFP-LC3 with WT and Δ*hly* Δ*prfA* cLLO at 2 h postinfection. Relevant statistically significant differences are indicated (**, $P < 0.01$; ***, $P < 0.001$ [ANOVA with Tukey's posttest]). Results are expressed as means and standard deviations obtained from at least 3 independent experiments. Bars = 5 μm.

National Institutes of Health (44). Protocols were approved by the Animal Care and Use Committee of the University of California, Berkeley.

RESULTS

PrfA is required for *L. monocytogenes* growth and autophagy evasion in BMDMs. We hypothesized that *L. monocytogenes* actively evades autophagy during infection by using PrfA-dependent factors. To investigate the impact of autophagy on the ability of *L. monocytogenes* to grow in C57BL/6 (B6) BMDM in the absence of PrfA-dependent virulence factor expression, we adopted a strategy previously described by Birmingham et al. (23) based on the use of a constitutively expressed allele of the gene encoding LLO (*hly*) (P_{hyper}-*hly*; cLLO). Integration of the P_{hyper}-*hly* allele in the genome of a Δ*hly* strain resulted in a strain that replicated at the same rate as the wild-type (WT) strain in BMDM (see Fig. S1 in the supplemental material). In contrast, introduction of this allele into a double *hly* and *prfA* deletion mutant resulted in a strain that was hemolytic (data not shown) but did not grow in BMDM (Fig. 1). Microscopic analysis revealed that most of the macrophages infected with the Δ*hly* Δ*prfA* cLLO strain showed only one or very few bacteria, although a small subset (~8%) contained actively replicating bacteria. Strikingly, we observed

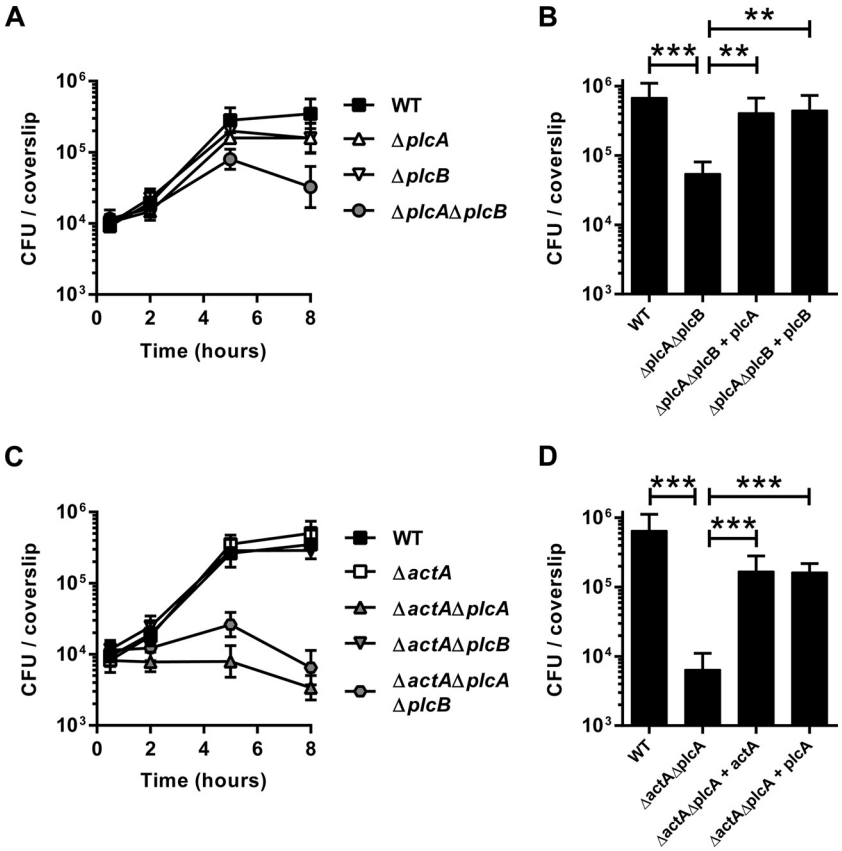

FIG 3 Intracellular growth of Δ*actA*, Δ*plcA*, and Δ*plcB* strains. (A) Kinetics of intracellular growth for WT, Δ*plcA*, Δ*plcB*, and Δ*plcA* Δ*plcB* strains in BMDM. (B) CFU recovered from BMDM infected with WT, Δ*plcA* Δ*plcB*, Δ*plcA* Δ*plcB* pPL2-*plcA*, and Δ*plcA* Δ*plcB* pPL2-P_{actA}-*plcB* organisms for 8 h. Statistically significant differences between strains are indicated (**, $P < 0.01$; ***, $P < 0.001$ [one-way ANOVA with Tukey's posttest]). (C) Kinetics of intracellular growth for WT, Δ*actA*, Δ*actA* Δ*plcA*, Δ*actA* Δ*plcB* and Δ*actA* Δ*plcA* Δ*plcB* strains in BMDM. (D) CFU recovered from BMDM infected with WT, Δ*actA* Δ*plcA*, Δ*actA* Δ*plcA* pPL2-*actA*, and Δ*actA* Δ*plcA* pPL2-*plcA* strains for 8 h. Statistically significant differences between strains are indicated (***, $P < 0.001$ [one-way ANOVA with Tukey's posttest]). Results are expressed as means and standard deviations obtained from at least 3 independent experiments.

that the Δ*hly* Δ*prfA* cLLO strain replicated at a rate similar to that of the WT strain in BMDM from *Atg5*$^{flox/flox}$-Lyz-*Cre* mice (referred to here as *Atg5*$^{-/-}$ macrophages) (Fig. 1A and B), suggesting that the intracellular growth of this strain was constrained by the host autophagy machinery. In addition, the Δ*hly* Δ*prfA* cLLO strain grew in B6 BMDM exposed to 5 mM 3-MA, a molecule that blocks LC3 lipidation by inhibiting type III phosphatidylinositol 3-kinases (Fig. 1C and D).

To directly evaluate targeting by the autophagy machinery, BMDM derived from GFP-LC3 transgenic mice were infected with *L. monocytogenes* strains, and the association of bacteria with LC3 was examined at defined times (1 to 5 and 8 h postinfection) (Fig. 2A and B). As previously described (14, 19), the WT strain transiently colocalized with LC3 early in infection (i.e., peak at 1 h postinfection), while a Δ*hly* mutant (represented here by the Δ*hly* Δ*prfA* mutant) failed to colocalize with LC3 (19). The Δ*hly* Δ*prfA* cLLO strain showed increased colocalization with LC3 in comparison to the WT strain from 2 to 4 h postinfection (Fig. 2B), and colocalization was reduced by 3-MA (Fig. 2C). By 5 h, colocalization with LC3 decreased significantly. GFP-LC3-II proteins are ultimately digested by the lysosomal degradative pathway (30), which may explain the decreased colocalization of GFP-LC3 with the Δ*hly* Δ*prfA* cLLO strain as a function of time. Indeed, the proportion of LC3^{+} Δ*hly* Δ*prfA* cLLO bacteria that were also positive for the lysosomal marker LAMP1 was 77% ± 6% at 2 h postinfection (see Fig. S2 in the supplemental material). Overall, these results strongly suggested that evasion of the autophagy pathway was essential for bacterial growth in BMDM and that *L. monocytogenes* used one or several PrfA-dependent factors to avoid targeting by the autophagy machinery.

ActA and PlcA interfere with the autophagy pathway. The PrfA-regulated virulence factors that have been associated with evasion from the autophagy pathway are ActA, PlcA, and PlcB (20, 22, 26). However, single deletions of each had minimal effects on the growth of *L. monocytogenes* in BMDM (Fig. 3). A mutant lacking both PlcA and PlcB grew intracellularly but showed a defect at 5 and 8 h postinfection (Fig. 3A and B). Strikingly, a strain lacking *plcA* and *actA* failed to replicate in BMDM (Fig. 3C and D), but inhibition of host actin polymerization did not affect the intracellular growth of the Δ*plcA* strain (see Fig. S3 in the supplemental material). In contrast, a Δ*actA* Δ*plcB* strain grew like the wild type, and the intracellular replication/survival ability of the Δ*actA* Δ*plcA* Δ*plcB* mutant was similar to that of the Δ*actA* Δ*plcA* strain. The Δ*actA* Δ*plcA*, Δ*plcA* Δ*plcB*, and Δ*plcA* Δ*plcB* Δ*actA* strains replicated efficiently in *Atg5*$^{-/-}$ macrophages (Fig. 4A, B, and C), confirming that the intracellular growth defect of these strains is

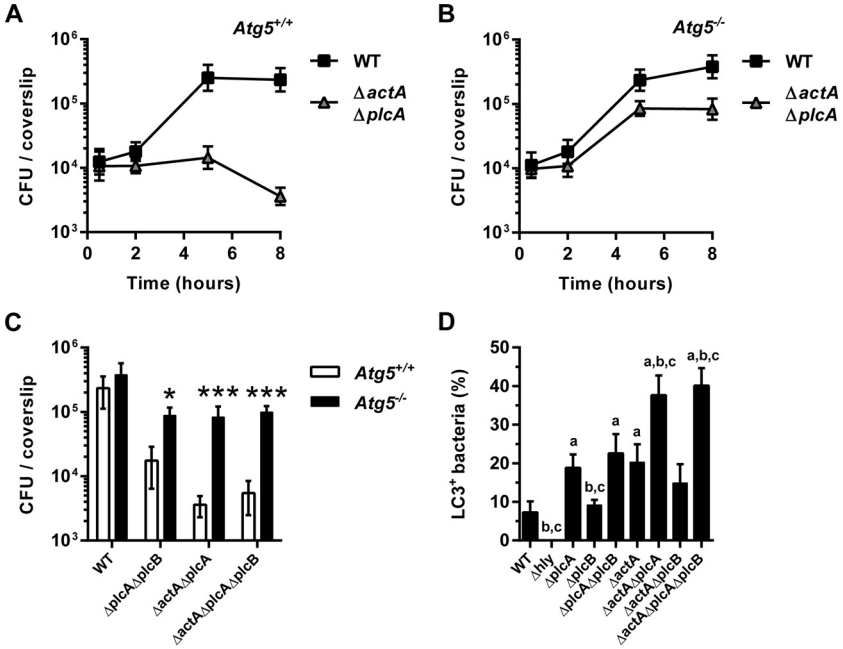

FIG 4 Intracellular growth of Δ*actA*, Δ*plcA*, and Δ*plcB* strains in *Atg5*$^{-/-}$ BMDM and colocalization with LC3. Kinetics of intracellular growth for WT and Δ*actA* Δ*plcA* strains in *Atg5*$^{+/+}$ (A) and *Atg5*$^{-/-}$ (B) BMDM are shown. (C) CFU recovered from *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ BMDM infected with WT, Δ*plcA* Δ*plcB*, Δ*actA* Δ*plcA*, and Δ*actA* Δ*plcA* Δ*plcB* strains for 8 h. Statistically significant differences between *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ BMDM are indicated for each strain (*, $P < 0.05$; ***, $P < 0.001$; unpaired *t* test). (D) Colocalization of GFP-LC3 with WT, Δ*hly*, Δ*plcA*, Δ*plcB*, Δ*plcA* Δ*plcB*, Δ*actA*, Δ*actA* Δ*plcA*, Δ*actA* Δ*plcB*, and Δ*actA* Δ*plcA* Δ*plcB* strains at 2 h postinfection. Proportions of GFP-LC3^{+} bacteria are expressed as a percentage of total intracellular *L. monocytogenes*. Statistically significant differences in comparison to WT, Δ*actA* and Δ*plcA* strains are indicated by the letters a, b, and c, respectively ($P < 0.05$ [one-way ANOVA with Tukey's posttest]). Results are expressed as means and standard deviations obtained from at least 3 independent experiments.

linked to autophagy. In order to determine the direct role of each virulence factor in autophagy evasion, the association between bacteria and GFP-LC3 was evaluated at 2 h postinfection (Fig. 4D). While deletion of both *actA* and *plcA* significantly increased colocalization with LC3, no significant increase was observed for the *plcB* mutant. Furthermore, deletion of both *plcA* and *actA* had an additive effect on the association of bacteria with GFP-LC3. No additive effect was observed by combining mutations in *plcB* with mutations in *actA* and *plcA*. Overall, these results demonstrated that ActA, PlcA, and, to a much lesser extent, PlcB contributed to the ability of *L. monocytogenes* to interfere with autophagy and to grow in BMDM.

We hypothesized that expression of PlcA in the Δ*hly* Δ*prfA* cLLO strain would promote bacterial replication in BMDM. To test this hypothesis, *hly* and *plcA* genes were inserted in tandem, downstream of the P_{hyper} promoter. The ability of the Δ*hly* Δ*prfA* cLLO cPlcA to grow in BMDM was similar to that of the WT strain (Fig. 5A). Furthermore, the expression of *plcA* in the Δ*hly* Δ*prfA* cLLO strain significantly decreased the association of bacteria with GFP-LC3, although not to the level of the WT strain (Fig. 5B). Overall, these results confirmed that PlcA is involved in autophagy escape and demonstrated that LLO and PlcA are sufficient to promote the intracellular growth of an *L. monocytogenes* Δ*prfA* strain in BMDM.

Effect of PlcA and PlcB on *in vitro* LC3 lipidation, membrane integrity, and PI3P levels. We next evaluated the ability of PlcA and PlcB to directly interfere with autophagy induction using a previously described *in vitro* assay (40, 45, 46) that monitors the cleavage and lipidation of the LC3 protein, a key step in early autophagosome formation. PlcA, PlcB, and mutant controls [PlcA(W49A), which has impaired interfacial binding to membranes (38), and PlcB(D55N) (see Materials and Methods)] were expressed, purified, and added to the LC3 lipidation assay. PlcA,

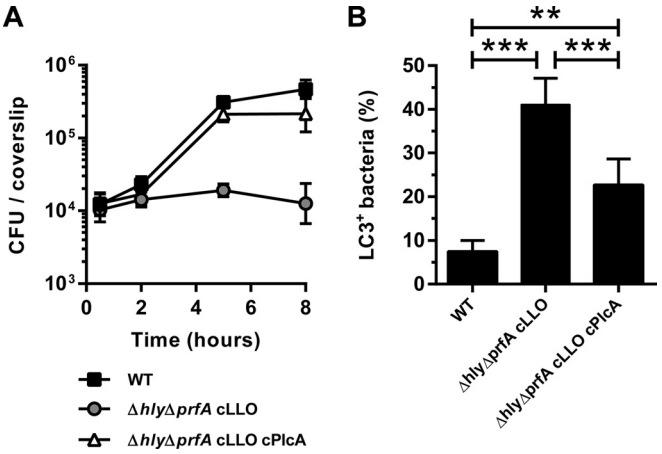

FIG 5 Intracellular growth and colocalization with LC3 of a Δ*prfA* strain expressing LLO and PlcA. (A) Kinetic of intracellular growth for WT, Δ*hly* Δ*prfA* cLLO, and Δ*hly* Δ*prfA* cLLO cPlcA strains in BMDM. (B) Quantification of GFP-LC3^{+} bacteria for WT, Δ*hly* Δ*prfA* cLLO, and Δ*hly* Δ*prfA* cLLO cPlcA strains expressed as a percentage of total intracellular *L. monocytogenes* at 2 h postinfection. Significant differences between strains are indicated (**, $P < 0.01$; ***, $P < 0.001$ [one-way ANOVA with Tukey's posttest]). Results are expressed as means and standard deviations obtained from at least 3 independent experiments.

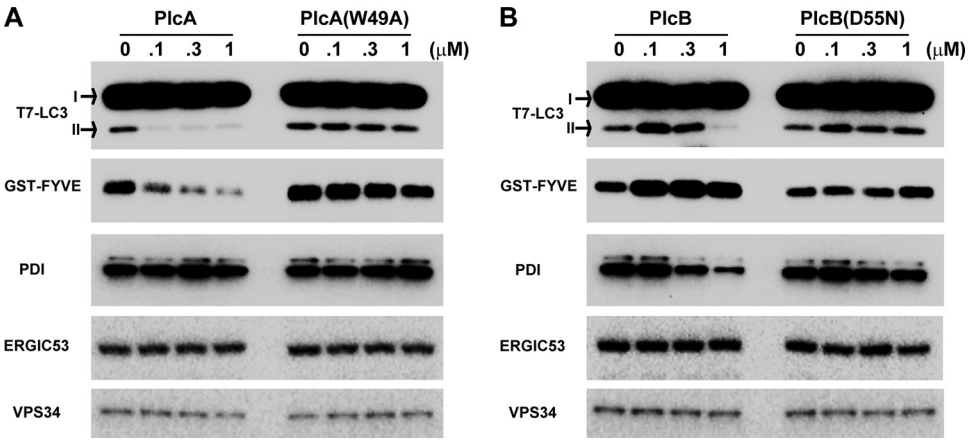

FIG 6 Effect of PlcA and PlcB on *in vitro* LC3 lipidation, membrane integrity, and PI3P levels. The membrane fraction was digested with the indicated concentrations of PlcA and PlcA(W49A) (A) or PlcB and PlcB(D55N) (B). The postdigestion membranes were then collected and subjected to *in vitro* LC3 lipidation assay and PI3P measurement followed by immunoblotting with the indicated antibodies. Membrane integrity was evaluated by measuring the levels of the intraluminal protein disulfide isomerase (PDI) in the membrane fraction. ERGIC-53 is the membrane loading control. Membrane levels of VPS34 were also evaluated.

but not PlcA(W49A), strongly inhibited LC3 lipidation *in vitro* (Fig. 6A; also, see Fig. S4 in the supplemental material [for quantification]). The inhibition of LC3 lipidation was associated with a decrease in membrane PI3P as detected by a GST-FYVE probe (Fig. 6A). Importantly, PlcA-treated membranes remained intact, as revealed by levels of the intraluminal protein disulfide isomerase (PDI) in the membrane fraction (Fig. 6A). In contrast, PlcB inhibited LC3 lipidation, but only at higher concentrations, and inhibition was associated with membrane damage, as revealed by a decrease in PDI in the membrane fraction (Fig. 6B). In accordance with the known broad-range activity of PlcB (41, 47), PC and PE levels were decreased in the membrane fraction treated with PlcB [but not in membrane factions treated with PlcA, PlcA(W49A), and PlcB(D55N)] (see Fig. S5 in the supplemental material). No impact on the levels of the membrane loading control ERGIC-53 or VPS34 was detected (Fig. 6). These results suggested that PlcA specifically interfered with autophagy by decreasing PI3P levels, while PlcB interfered with LC3 lipidation at higher concentrations by affecting membrane integrity.

DISCUSSION

The results of this study support previous observations that *L. monocytogenes* utilizes ActA and PLCs to avoid autophagy during infection of host cells. Here we show that *L. monocytogenes* lacking ActA or PlcA grew similarly to wild-type bacteria but that a mutant lacking both ActA and PlcA was targeted by the autophagy LC3 conjugation system and failed to grow in BMDM macrophages. Additionally, purified PlcA prevented the formation of PI3P and blocked LC3 lipidation in a cell-free assay. Overall, this study demonstrated that interference with autophagy is required for *L. monocytogenes* intracellular growth and depends upon either ActA or PlcA.

Previous studies have examined the effects of PlcA on host phosphoinositide metabolism during infection (48, 49). Tattoli et al. (26) showed that *L. monocytogenes* PLCs are associated with reduction of host PI3P, a signaling molecule that plays a critical role in autophagy (13) and is enriched in subcellular structures where antibacterial autophagy occurs (50). Considering that PI3P is required for LC3 lipidation (40, 51), we speculated that PLCs, especially PlcA, decreased LC3 lipidation. Accordingly, our results are in agreement that PlcA inhibits autophagy induction by decreasing PI3P levels, most likely by cleaving PI (5), the substrate of class III PI3Ks (13, 52). However, it is noteworthy that seven different host polyphosphoinositides are derived from PI that impact functions ranging from membrane trafficking to actin cytoskeleton dynamics (53). As a result, pathogens target host cell phosphoinositide metabolism for many purposes (54, 55), and it is conceivable that PlcA has multiple effects on host cells by modulating different phosphoinositide pools. Therefore, it is possible that PlcA acts in a vacuole to counteract autophagy (25) and/or acts globally to impact both autophagy (26) and/or other functions. For instance, PlcA activity might affect actin-based motility, since PI(3,5)P_2 and PI(3,4,5)P_3 bind to ActA (56, 57).

L. monocytogenes has two PLCs; PlcA is specific for PI, while PlcB cleaves a broad range of phospholipid substrates but not PI (5). The role of each PLC in autophagy escape has been difficult to dissociate (23, 25, 26). The results of this study suggested that PlcB plays a minor role in autophagy evasion. Purified PlcB inhibited LC3 lipidation *in vitro*, but only at concentrations that caused nonspecific membrane damage. However, the possibility that PlcB affects autophagy by cleaving PE remains attractive. Indeed, PE is the phospholipid anchoring LC3 proteins on early autophagosomal structures, and it is possible that PlcB decreases LC3 lipidation by removing PE head groups. Interestingly, the *Legionella pneumophila* effector RavZ interferes with autophagy by directly uncoupling LC3 proteins on autophagosomal membranes (58). PlcB might also act on later steps of the autophagy pathway, perhaps mediating bacterial escape from autophagosomes and/or autolysosomes.

It is now established in the literature that both *L. monocytogenes* and *Shigella flexneri* avoid autophagy in the host cell cytosol by masking their surfaces (16, 22, 59). During *L. monocytogenes* infection, the recruitment of the host Arp2/3 complex and Ena/VASP proteins by ActA prevents autophagy recognition, but actin-based motility is not required for autophagy avoidance (22). *S.*

flexneri escapes autophagy by secreting IcsB, a protein that competitively inhibits the binding of ATG5 to VirG/IcsA, a bacterial protein required for actin-based motility (59). However, recent data suggest that IcsB acts by inhibiting LAP and/or LC3 recruitment to vacuolar membrane remnants early during infection (60). The LAP pathway also targets *L. monocytogenes* (14), and the induction of autophagy by *L. monocytogenes* requires the pore-forming cytolysin LLO (14, 19, 26). Therefore, it is possible that *L. monocytogenes*, like *S. flexneri*, is targeted by autophagy exclusively in a damaged phagosome, not free in the cytosol.

Although it is clear that *L. monocytogenes* requires either ActA or PlcA to grow in host cells, the contribution of each is not yet fully appreciated. The simplest model is that each determinant acts at a different time and place: PlcA acts in a phagocytic vacuole, and ActA acts in the cytosol. However, if this model was correct, one would predict that single mutants would also exhibit bacterial growth defects and that the contribution of PlcA and ActA would be additive, not synergistic. Since both PlcA and ActA may also contribute to vacuolar escape (5, 61), perhaps the double mutant escapes more slowly, thereby allowing time for the LAP pathway to contain the infection. Alternatively, the autophagy machinery may be recruited by membrane remnants or directly at the bacterial surface. In these scenarios, ActA might block autophagy recognition while PlcA interferes with autophagy flux locally and/or globally. Future studies using real-time imaging and electron microscopy are required to better define the relationship between escape from the phagosome, membrane remnants, and the recruitment of the autophagy machinery to *L. monocytogenes* during infection.

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