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 cytolyisin 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.

L. monocytogenes is a facultative intracellular 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 cytolyisin 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). Macrophagocyte 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 phosphatidylinositol 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).

Received 29 January 2015. Returned for modification 17 February 2015. Accepted 9 March 2015.


Editor: C. R. Roy
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00110-15.

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

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<th>Strain</th>
<th>Description</th>
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**TABLE 2 Primers used in this study**

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<tr>
<td>plcAcomp-REV</td>
<td>ATATATGGGAAACAGATCTTTCGTTGTTCTG</td>
</tr>
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</table>

*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). Atg5floxtfloxt (28), Atg5floxtfloxt-Lyz-Cre (29), and green fluorescent protein (GFP)-conjugated Lc3 (30) mice were described previously. HEK293T cells were grown at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Deletions of hly and actA in ΔprfA and ΔplcA backgrounds, respectively, were achieved as previously reported (31, 32). Plasmids pPL2 (33), pPL2-ΔαctAΔpCcb (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 hlynoTT-REV), digested with Eagl and Pstl, and inserted into pHpPL3 downstream of the hyper-P_poc promoter (P_hyp). The 5′ UTR and the coding sequence of plcA were then amplified by PCR (primers plcA-FWD and plcA-REV), digested with EcoRV and Sall, and inserted into pHPPL3-hly (no terminator) in order to generate pHPPL3-hly-ΔpCcA (cLLO CplcA). For genetic complementation experiments, actA and plcA were amplified by PCR (primers actAcomp-FWD and actAcomp-REV and primers plcAcomp-FWD and plcAcomp-REV, respectively) and inserted into pPL2 with their native promoters. The actA amplicon was digested with Eagl and Xhol, and the plcA amplicon was digested with EcoRV and Sall. 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 15% 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 (DAP) (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-
Bacteria were grown to an optical density of about 0.6, and proteins were expressed in pTYB21 (New England BioLabs, Ipswich, MA). Recombinant 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 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 digests 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. The membranes were then collected by centrifugation at 25,000 × g, and suspended in B88 buffer. Membrane PI3P and PE levels were measured as previously described (40, 43).

**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 1** Intracellular growth of a ∆prfA strain expressing LLO. Kinetics of intracellular growth for wild-type, ∆hly ∆prfA (with the empty integrated vector pHpPL3), and ∆hly ∆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.
RESULTS

PrfA is required for \textit{L. monocytogenes} growth and autophagy evasion in BMDMs. We hypothesized that \textit{L. monocytogenes} actively evades autophagy during infection by using PrfA-dependent factors. To investigate the impact of autophagy on the ability of \textit{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 (\textit{hly}) (\textit{P\textsubscript{hyper-hly}}; cLLO). Integration of the \textit{P\textsubscript{hyper-hly}} allele in the genome of a \textit{Δhly} 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 \textit{hly} and \textit{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 \textit{Δhly ΔprfA} cLLO strain showed only one or very few bacteria, although a small subset (∼8%) contained actively replicating bacteria. Strikingly, we observed...
that the Δhly ΔprfA cLLO strain replicated at a rate similar to that of the WT strain in BMDM from Atg5<sup><ins>fl</ins></sup><ins>-<ins>Lys-Cre</ins></sup> mice (referred to here as Atg5<sup><ins>-/-</ins></sup> macrophages) (Fig. 1A and B), suggesting that the intracellular growth of this strain was constrained by the host autophagy machinery. In addition, the Δhly ΔprfA 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). As previously described (14, 19), the WT strain transiently colocalized with LC3 early in infection (i.e., peak at 1 h postinfection), while a Δhly mutant (represented here by the Δhly ΔprfA mutant) failed to colocalize with LC3 (19). The Δhly ΔprfA 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 transiently colocalized with LC3 postinfection, while a Δhly ΔprfA mutant (represented here by the Δhly ΔprfA cLLO strain) replicated efficiently in Atg5<sup><ins>-/-</ins></sup> macrophages (Fig. 4A, B, and C), confirming that the intracellular growth defect of these strains is

**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 PlcA or 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 ΔplcA strain (see Fig. S3 in the supplemental material). In contrast, a ΔactA ΔplcB strain grew like the wild type, and the intracellular replication/survival ability of the ΔactA ΔplcA ΔplcB mutant was similar to that of the ΔactA ΔplcA strain. The ΔactA ΔplcA, ΔplcA ΔplcB, and ΔplcA ΔplcB ΔactA strains replicated efficiently in Atg5<sup><ins>-/-</ins></sup> macrophages (Fig. 4A, B, and C), confirming that the intracellular growth defect of these strains is...
Results are expressed as means and standard deviations obtained from at least 3 independent experiments.

GFP-LC3, although not to the level of the WT strain (Fig. 5B). cLLO strain significantly decreased the association of bacteria with actA4D. While deletion of both the colocalization with LC3, no significant increase was observed for differences in comparison to WT,cleavage (Fig. 5A). Furthermore, the expression of virulence factor in autophagy evasion, the association between bacteria and GFP-LC3 was evaluated at 2 h postinfection (Fig. 5D). As previously described intracellular growth of an escape and demonstrated that LLO and PlcA are sufficient to pro-
mute the intracellular growth of an

linked to autophagy. In order to determine the direct role of each virulence factor in autophagy evasion, the association between bacteria and GFP-LC3 in Atg5+/+ was evaluated at 2 h postinfection (Fig. 4D). While deletion of both plcA and actA significantly increased colocalization with LC3, no significant increase was observed for 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 Δhly ΔprfA cLLO strain would promote bacterial replication in BMDM. To test this hypothesis, hly and plcA genes were inserted in tandem, downstream of the Phyper promoter. The ability of Δhly ΔprfA cLLO cPlcA to grow in BMDM was similar to that of the WT strain (Fig. 5A). Furthermore, the expression of plcA in the Δhly ΔprfA 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 ΔprfA 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 4 Intracellular growth of ΔactA, ΔplcA, and ΔplcB strains in Atg5+/− BMDM and colocalization with LC3. Kinetics of intracellular growth for WT and ΔactA ΔplcA strains in Atg5+/+ (A) and Atg5−/− (B) BMDM are shown. (C) CFU recovered from Atg5+/+ and Atg5−/− BMDM infected with WT, ΔplcA ΔplcB, ΔactA ΔplcA, and ΔactA ΔplcA ΔplcB 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, Δhly, ΔplcA, ΔplcB, ΔplcA ΔplcB, ΔactA, ΔactA ΔplcA, ΔactA ΔplcB, and ΔactA ΔplcA ΔplcB 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, ΔactA and ΔplcA 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.

FIG 5 Intracellular growth and colocalization with LC3 of a ΔprfA strain expressing LLO and PlcA. (A) Kinetic of intracellular growth for WT, Δhly ΔprfA cLLO, and Δhly ΔprfA cLLO cPlcA strains in BMDM. (B) Quantification of GFP-LC3+ bacteria for WT, Δhly ΔprfA cLLO, and Δhly ΔprfA 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.
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 fractions 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). Tat-toli 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 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

**REFERENCES**


**ACKNOWLEDGMENTS**

Femurs from Atg5fl/fl, Atg7fl/fl, LAMP2-/-, and GFP-LC3 mice were generously provided by Jeffery S. Cox, Anita Sil, and Michele Swanson. We thank Hélène Marquis for providing plasmid pE8RS108 and Gregory A. Smith for the generation of the ΔactA ΔplcA ΔplcB strain.

This work was supported by National Institutes of Health grants 1PO1 AI63302 (D.A.P.) and 1RO1 AI27655 (D.A.P.). G.M. was supported by fellowships from Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), Fonds de recherche santé Québec (FRSQ), and the Natural Sciences and Engineering Research Council of Canada (NSERC). L.G. was supported by a fellowship from the Jane Coffin Childs Fund (JCCF). R.S. is an Investigator of the HHMI and a Senior Fellow of the UC Berkeley Miller Institute.

Daniel A. Portnoy has a consulting relationship with and a financial interest in Aduro Biotech. Both he and the company stand to benefit from the commercialization of the results of this research.

**Iai.asm.org May 2015 Volume 83 Number 5**


