Supporting Information

Mitchell et al. 10.1073/pnas.1716055115

SI Materials and Methods

Bacterial Strains, Growth Medium, Cell Culture, and Intracellular Growth Curves. *L. monocytogenes* strains used in this study (Table S1) were derived from 10403S (36). The Δ*hly* strain (DP-L2161) was previously described (37). *L. monocytogenes* was grown overnight in brain heart infusion (BHI) medium at 30 °C before each experiment, except if otherwise indicated. BMMs were prepared and cultured using standard protocols (20). Green fluorescent protein (GFP)-LC3 (5), GFP-LC3 *Beclin 1*$^{flox/flox}$ (*Becn1*$^{+/+}$) (5), GFP-LC3 *Beclin 1*$^{flox/flox}$-Lyz-*Cre* (*Becn1*$^{-/-}$) (5), GFP-LC3 *Fip200*$^{flox/flox}$ (*Fip200*$^{+/+}$) (5), GFP-LC3 *Fip200*$^{flox/flox}$-Lyz-*Cre* (*Fip200*$^{-/-}$) (5), *p62*$^{-/-}$ (38), *Tnfr1*$^{-/-}$ (28), *Tbk1*$^{-/-}$ *Tnfr1*$^{-/-}$ (28), and *Ulk1*$^{-/-}$ (5) mice were previously described. *Ndp52*$^{-/-}$ mice were obtained through the Knockout Mouse Project (KOMP) Repository [Allele name: Calcoco2$^{tm1(KOMP)Wtsi}$].

For intracellular growth curves, BMMs were infected as previously described (20) at a multiplicity of infection (MOI) of 0.25 (one bacterium per four macrophages), which results in the infection of ∼8% of the cells. At the indicated time points, intracellular bacteria were quantified by enumerating CFU. The proportion of tetracycline resistant bacteria was determined by replica plating on BHI containing 2 μg/mL tetracycline.

Correlative Light-Electron Microscopy. GFP-LC3 BMMs were seeded on gridded coverslips (MatTek) and infected at an MOI of 1 (one bacterium per cell), as previously described (20). At 1 or 2 h after infection, cells were washed with PBS and fixed for 15 min in 4% PFA. Fluorescence and DIC images were acquired in PBS using a Nikon Eclipse TE2000-E microscope, and 10, 40, and 60× objectives (CRL Molecular Imaging Center, UC Berkeley). The localization of several cells containing LC3^{+} bacteria was noted, and samples were further fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were postfixed with 1% osmium tetroxide and 1.6% potassium ferrocyanide, dehydrated in graded series of ethanols, and embedded in epon araldite resin. Samples were then trimmed, sectioned, and stained with 2% uranyl acetate and lead citrate. Images were acquired on a FEI Tecnai 12 transmission electron microscope.

Immunofluorescence, Microscopy, and Image Analysis. BMMs were infected at a MOI of 0.4 (two bacteria per five macrophages) or 1.6 (eight bacteria per five macrophages), as previously described (20). At the time points indicated in the figure legends, coverslips were washed with PBS, fixed in PFA, and immunostained using saponin permeabilization, as previously described (20). Antibodies that recognize *L. monocytogenes* (1:1000 dilution; BD Biosciences), ActA [rabbit polyclonal antibody against the N terminus of ActA (39); 1:200 dilution], GFP (no. 11814460001; 1:200 dilution; Roche), LLO (anti-LLO B3-19, a kind gift of Pascale Cossart, Institut Pasteur, Paris; 1:250 dilutions), p62 (no. 20R-PP001; 1:200 dilutions; Fitzgerald) and polyubiquitylated proteins (FK1, no. PW8805; 1:200 dilution; Enzo Life Sciences) were used and detected with secondary antibodies conjugated with fluorophores (1:2000 dilution; Invitrogen).

For the detection of endogenous LC3, infected cells were washed in PBS and fixed in −20 °C cold MeOH for 5 min. Blocking and antibody incubations were performed in PBS containing 2% BSA at room temperature. Primary antibodies that bind *L. monocytogenes* (BD Biosciences) and LC3B (no. 0260-100/LC3-2G6; 1:200 dilutions; nanoTools) were used and detected with secondary antibodies conjugated with fluorophores (1:2,000 dilution; Invitrogen).

Coverslips were mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen) and imaged with an Olympus IX71 epifluorescence microscope or a BZ-X710 KEYENCE fluorescent microscope using the 100× objective. Several frames per condition were randomly selected, and images were collected and color combined using MetaMorph software (Molecular Devices) or the BZ-X analyzer software. For Fig. 4*C*, images were Z-stacked of 10 Z-positions covering 5 μm of thickness and were posttreated using the haze reduction function of the BZ-X analyzer software. Images were analyzed using ImageJ (National Institutes of Health), and a minimum of 100 bacteria were considered for each tested condition.

Plasmids and Strains Construction. *E. coli* strains used in this study are listed in Table S2. For vector construction, plasmids were introduced into *E. coli* TOP10 (Invitrogen) or XL1 (Stratagene). Plasmids were then transferred in *E. coli* SM10 (40), and then into *L. monocytogenes* via conjugation, as previously described (41). Recommendations from the manufacturers were followed for plasmid DNA isolation and purification of DNA fragments (QIAGEN). Unless otherwise stated, all molecular reagents were purchased from New England Biolabs. PCRs were performed using Kapa polymerase (Kapa Biosystems) and primers listed in Table S3. Positive clones were verified by Sanger sequencing.

To generate Δ*actA*Δ*plcA* (DP-L6580), the DNA insert from pKSV7Δ*plcA* (42) was subcloned in pKSV7-*oriT* (43) and transferred in DP-L3078 (44). For the generation of Δ*plcB* (DP-L6574) and Δ*plcA*Δ*plcB* (DP-L6578), pKSV7-*oriT* Δ*plcB*^{WT} was transferred in 10403S and DP-L1552 (42). The plasmid pKSV7-*oriT* Δ*plcB*^{WT} was constructed by joining together two DNA fragments of 650 bp flanking the region to be deleted using splicing by overlap extension (SOE) PCR (and primers GM1070-1073), as previously described (43), and by cloning the resulting fragment into pKSV7-*oriT* using EcoRI and PstI restriction enzymes. For the generation of Δ*actA*Δ*plcB* (DP-L6582) and Δ*actA*Δ*plcA*Δ*plcB* (DP-L6584), pKSV7-*oriT* Δ*plcB*$^{\Delta actA}$ was transferred in DP-L3078 and DP-L6580. The plasmid pKSV7-*oriT* Δ*plcB*$^{\Delta actA}$ was constructed by joining together two DNA fragments of 650 bp, amplified from DP-L3078, using SOE PCR (and primers GM1071-1074), and by cloning the resulting fragment into pKSV7-*oriT* using EcoRI and PstI restriction enzymes. To generate Δ*actA* PlcA^{H86A} (DP-L6581), the DNA insert from pKSV7Δ*actA* (44) was subcloned in pKSV7-*oriT* using EcoRI and PstI restriction enzymes, and the resulting plasmid was conjugated into DP-L3430 (45). For PlcA^{H86A} PlcB^{H69G} (DP-L6579), Δ*actA* PlcB^{H69G} (DP-L6583) and Δ*actA* PlcA^{H86A} PlcB^{H69G} (DP-L6586), pKSV7-*oriT* PlcB^{H69G} was transferred in DP-L3430, DP-3078, and DP-L6581. The plasmid pKSV7-*oriT* PlcB^{H69G} was constructed by amplifying and cloning *plcB* from DP-L3177 (46) into pKSV7-*oriT* using primers GM1047 and GM1048, and EcoRI and PstI restriction enzymes. Allelic replacements were achieved as previously described (42, 43).

Strains that delete *actA* upon access to the host cytosol were engineered using the Cre-*lox* system, as previously described (25). The *cre* gene was previously cloned in pPL2e, downstream of the *actA* promoter (P_{actA}), which is robustly and specifically up-regulated in the host cytosol (25). The *actA* gene was cloned in pPL1 (41) and flanked by lox66/lox71 *loxP* sites in an orientation that resulted in excision of the floxed (*fl*) cassette upon Cre expression. To monitor loss of *actA* during infection, a tetracycline resistance gene (*tetL*) was also inserted within

the floxed cassette, thus generating (*actA-tetL*)^{fl}. The expression of both ActA and Cre was induced early during infection, but the (*actA-tetL*)^{fl} cassette was deleted from the *L. monocytogenes* genome later during infection, upon access to the host cytosol. The plasmid pPL1-(*actA-tetL*)^{fl} (also referred to as pPL1-*actA*^{fl}) was constructed in a multistep process using both restriction enzymes and Gibson assembly cloning, and consisted of three units organized in the following order and flanked by lox66/lox71 sites: (*i*) the *actA* gene [including the coding sequence as well as both proximal and distal (*mpl*) promoters] amplified by PCR from the genome of DP-L2296 (Δ*mpl*) (47) using primers BN030 and BN031, (*ii*) a series of transcriptional terminators from pPL2t-term (43), and (*iii*) a tetracycline resistance gene (*tetL*) from pPL2t (43). The (*actA-tetL*)^{fl} cassette was inserted between the KpnI and EagI restriction sites in the multiple cloning site of pPL1.

E. coli SM10 carrying pHPL3-mcherry (48), pPL2e-P_{actA}-*cre* (25), and pPL1-(*actA-tetI*)^{fl} were conjugated with *L. monocytogenes* strains, as previously described (41). For the conjugation of pPL1-(*actA-tetI*)^{fl}, phage cured strains were generated and/or used, as previously described (41).

Time-Lapse Microscopy. GFP-LC3 BMMs were infected for 3 min with exponentially grown mCherry bacteria resuspended in PBS, unless otherwise indicated. Exponentially grown bacteria were obtained by diluting an overnight culture 1/12 in BHI, and further incubating the resulting culture for 1 h 45 min at 37 °C with shaking. When required, 400 μg/mL of 10,000 molecular weight (MW) dextran conjugated to Alexa Fluor 647 (Invitrogen) was added during infection. Infected cells were washed three times with PBS and imaging was performed in BMM media without phenol red containing ProLong Antifade Reagents for Live Cells (Invitrogen) using the fluorescent microscope BZ-X710 equipped with an incubation chamber (37 °C and 5% CO_2). Fluorescent signals were obtained from five Z-positions covering 5 μm of thickness using an autofocus system. Images were Z-stacked and posttreated using the haze reduction function of the BZ-X analyzer software.

Statistical Analysis. The GraphPad prism software (v.7.00) was used to carry out statistical analysis. CFU were transformed to base 10 logarithm values before being used for statistical analyses. Statistical tests and number of independent experiments are specified in figure legends.

Table S1. *L. monocytogenes* strains used in this study

Strain	Description	Source
10403S	Wild type	(36)
DP-L6573	10403S + pHPL3-mCherry	(48)
DP-L2161	Δ*hly*	(37)
DP-L2296	Δ*mpl*	(47)
DP-L1552	Δ*plcA*	(42)
DP-L3430	PlcA^{H86A}	(45)
DP-L6574	Δ*plcB*	This study
DP-L3177	PlcB^{H69G}	(46)
DP-L3078	Δ*actA* (ActA$^{Δ7–633}$)	(44)
DP-L4029	DP-L3078, phage cured	(41)
DP-L6575	DP-L4029 + pPL1-(*actA-tetL*)^{fl}	This study
DP-L6576	DP-L4029 + pPL2-P_{actA}-*cre*	This study
DP-L6577	DP-L6575 + pPL2-P_{actA}-*cre*	This study
DP-L6578	Δ*plcA*Δ*plcB*	This study
DP-L6579	PlcA^{H86A}PlcB^{H69G}	This study
DP-L6580	Δ*actA*Δ*plcA*	This study
DP-L6581	Δ*actA* PlcA^{H86A}	This study
DP-L6582	Δ*actA*Δ*plcB*	This study
DP-L6583	Δ*actA* PlcB^{H69G}	This study
DP-L6584	Δ*actA*Δ*plcA*Δ*plcB*	This study
DP-L6592	DP-L6584 + pHPL3-mCherry	This study
DP-L6586	Δ*actA* PlcA^{H86A}PlcB^{H69G}	This study
DP-L6587	DP-L6586 + pHPL3-mCherry	This study
DP-L6588	DP-L6586, phage cured	This study
DP-L6589	DP-L6588 + pPL1-(*actA-tetL*)^{fl}	This study
DP-L6590	DP-L6588 + pPL2-P_{actA}-*cre*	This study
DP-L6591	DP-L6589 + pPL2-P_{actA}-*cre*	This study

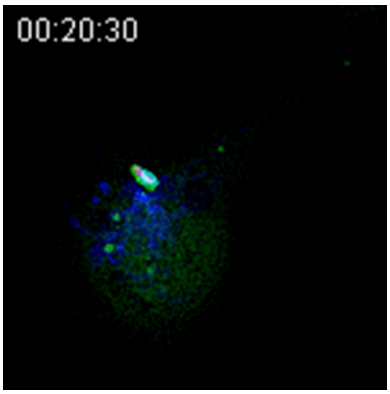
Table S2. *E. coli* strains used in this study

Strain	Description	Source
TOP10	For vector construction	Invitrogen
XL1	For vector construction	Stratagene
SM10	Conjugation strain	(40)
DP-E6566	SM10 + pKSV7-*oriT* Δ*plcA*	This study
DP-E6567	SM10 + pKSV7-*oriT* Δ$plcB^{WT}$	This study
DP-E6568	SM10 + pKSV7-*oriT* Δ$plcB^{\Delta actA}$	This study
DP-E6569	SM10 + pKSV7-*oriT* Δ*actA*	This study
DP-E6570	SM10 + pKSV7-*oriT* $PlcB^{H69G}$	This study
DP-E6571	SM10 + pPL1-(*actA-tetL*)^{fl}	This study
DP-E6572	SM10 + pHPL3-mCherry	(48)
DP-E6233	SM10 + pPL2e-P_{actA}-*cre*	(25)

Table S3. Oligonucleotides used in this study

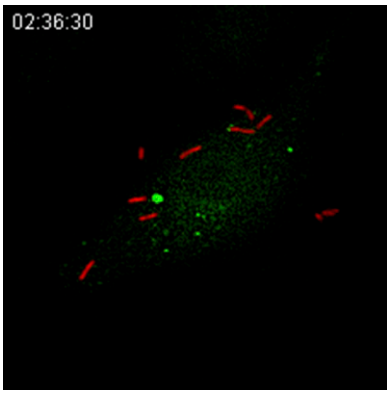
Primer	Sequence (5′-3′)*	Description
BN030	CTTCTACATTCGAGGTAAAACCGCTTAACACACACG	For pPL1-(*actA-tetl*)^{fl} (*actA* fragment)
BN031	TTTTATGCCGCATAGCTCACTTTTTTCTTTCGTTCTGTG	For pPL1-(*actA-tetl*)^{fl} (*actA* fragment)
GM1047	ATATATGAATTCAAACACAGAACGAAAGAAAAAAGTGAG	$PlcB^{H69G}$ Knock-in
GM1048	ATATATCTGCAGATCCACCCGCTAACGAGTG	$PlcB^{H69G}$ Knock-in
GM1070	ATATATGAATTCAGCCCTGTTCCTTCATTAACTC	Δ$plcB^{WT}$ primer A
GM1071	CAAGCACATACCTAGAACCACA	Δ*plcB* primer B
GM1072	TGTGGTTCTAGGTATGTGCTTGTCACAGCAAACTTTGGCAGG	Δ*plcB* primer C
GM1073	ATATATCTGCAGCCACACGCGACTAACATAACT	Δ*plcB* primer D
GM1074	ATATATGAATTCGGATGTCTGTGTGCGTGG	Δ$plcB^{\Delta actA}$ primer A

*Underlined sequences indicate restriction sites.



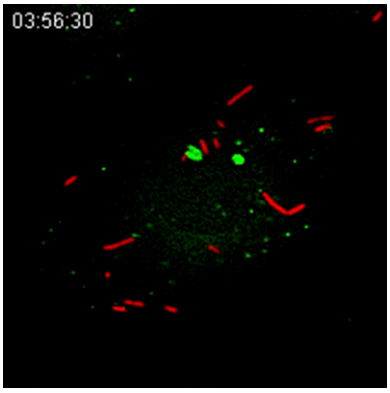
Movie S1. GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red) in the presence of fluorescent dextran (blue). Images are posttreated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s). Micrographs presented in Fig. 1*H* were selected from this movie.

Movie S1



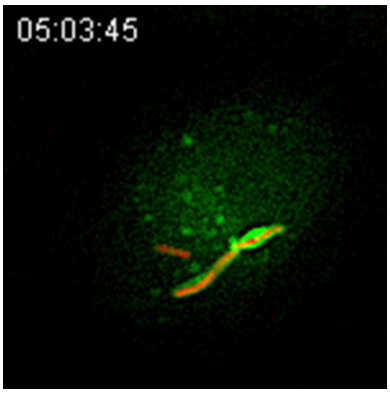
Movie S2. GFP-LC3 (green) BMMs infected with exponentially grown mCherry WT *L. monocytogenes* (red). Images are posttreated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s). Micrographs presented in Fig. 3*A* were selected from this movie.

Movie S2



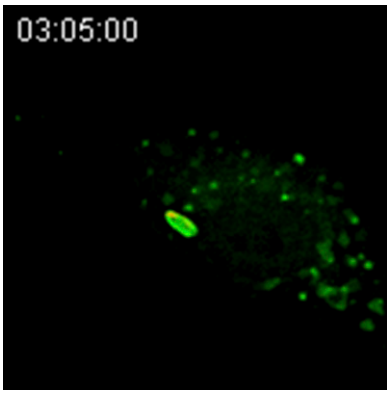
Movie S3. GFP-LC3 (green) BMMs infected with exponentially grown mCherry WT *L. monocytogenes* (red). Images are posttreated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s).

Movie S3



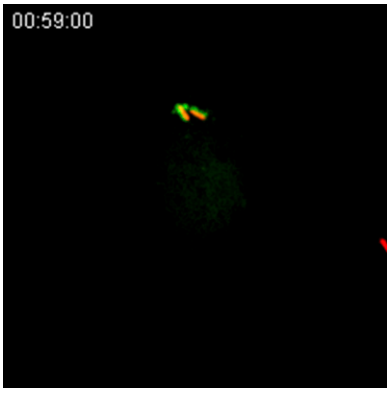
Movie S4. GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red). Images are posttreated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s). Micrographs presented in Fig. 3*B* were selected from this movie.

Movie S4



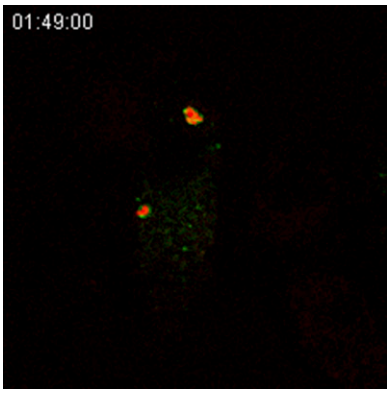
Movie S5. GFP-LC3 (green) BMMs infected with an overnight culture of the triple mutant expressing mCherry (red). Images are posttreated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s).

Movie S5



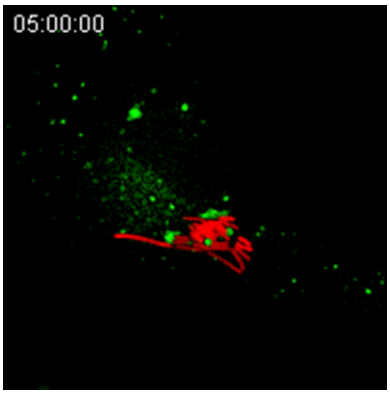
Movie S6. FIP200$^{+/+}$ GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red). Images are post-treated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s).

Movie S6



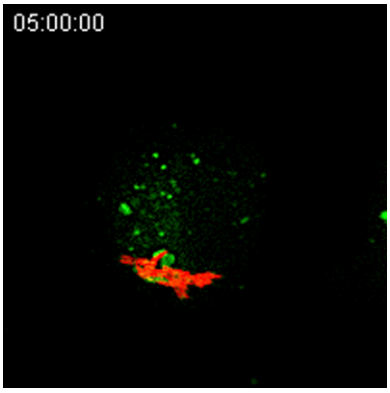
Movie S7. FIP200$^{+/+}$ GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red). Images are post-treated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s).

Movie S7



Movie S8. FIP200$^{-/-}$ GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red). Images are post-treated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s). Micrographs presented in Fig. 3*C* were selected from this movie.

Movie S8



Movie S9. FIP200$^{-/-}$ GFP-LC3 (green) BMMs infected with an exponentially grown culture of the triple mutant expressing mCherry (red). Images are post-treated Z-stacks covering 5 μm of thickness. Time is indicated in the upper left corner (h:min:s).

Movie S9