RibU is an essential determinant of *Listeria* pathogenesis that mediates acquisition of FMN and FAD during intracellular growth

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Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are essential riboflavin-derived cofactors involved in a myriad of redox reactions across all forms of life. Nevertheless, the basis of flavin acquisition strategies by riboflavin auxotrophic pathogens remains poorly defined. In this study, we examined how the facultative intracellular pathogen *Listeria monocytogenes*, a riboflavin auxotroph, acquires flavins during infection. A *L. monocytogenes* mutant lacking the putative riboflavin transporter (RibU) was completely avirulent in mice but had no detectable growth defect in nutrient-rich media. However, unlike wild type, the RibU mutant was unable to grow in defined media supplemented with FMN or FAD or to replicate in macrophages starved for riboflavin. Consistent with RibU functioning to scavenge FMN and FAD inside host cells, a mutant unable to convert riboflavin to FMN or FAD retained virulence and grew in cultured macrophages and in spleens and livers of infected mice. However, this FMN- and FAD-requiring strain was unable to grow in the gallbladder or intestines, where *L. monocytogenes* normally grows extracellularly, suggesting that these sites do not contain sufficient flavin cofactors to promote replication. Thus, by deleting genes required to synthesize FMN and FAD, we converted *L. monocytogenes* from a facultative to an obligate intracellular pathogen. Collectively, these data indicate that *L. monocytogenes* requires riboflavin to grow extracellularly in vivo but scavenge FMN and FAD to grow in host cells.

Riboflavin (vitamin B₂) is a water-soluble vitamin essential to all organisms and the precursor of the biologically active flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fig. 1A), which are necessary for a diverse array of oxidation-reduction reactions (1–3). While plants, fungi, and most bacteria and archaea synthesize riboflavin, some bacteria and all mammals lack the genes to make this vitamin de novo (4–6). Many organisms encode transporters that allow them to obtain riboflavin from the environment (5–8). Several bacterial and all eukaryotic intracellular pathogens require an exogenous source of riboflavin (9–11), and interestingly, some also lack the enzymes that catalyze the conversion of riboflavin to FMN and FAD (11, 12). How these riboflavin auxotrophic intracellular pathogens fulfill their flavin requirement in host cells is poorly understood. Most of these intracellular pathogens encode annotated riboflavin transporters. Interestingly, however, riboflavin is scarce in host cells while FMN and FAD are abundant (13–15). To better understand flavin acquisition and requirements for pathogenesis, here we focused on the riboflavin auxotrophic bacterium *Listeria monocytogenes*.

*L. monocytogenes* is a gram-positive bacterium that lives both as an environmental saprophyte and as a facultative intracellular pathogen of mammals including humans (16). Once *L. monocytogenes* enters a cell, it escapes from a phagosome and gains access to the cytosol, where it acquires nutrients and rapidly divides (17). Although *L. monocytogenes* has few growth requirements (18), it lacks the riboflavin biosynthetic genes. To obtain this vitamin, *L. monocytogenes* encodes an energy-coupling factor (ECF) transporter, RibU, that is annotated as a riboflavin transporter (19, 20). RibU is the substrate binding subunit of an ECF transporter, which is also composed of two ATPases and a transmembrane subunit that together form a complex for riboflavin import (21) (*SI Appendix*, Fig. S1). RibU from *L. monocytogenes* binds riboflavin (21) and rescues the growth of a riboflavin auxotrophic *Bacillus subtilis* strain (20). Additionally, *L. monocytogenes* encodes the enzymes RibC and RibF, which are involved in the biosynthesis of FMN and FAD (20, 22). RibC is a bifunctional enzyme that catalyzes the phosphorylation of riboflavin to FMN and the adenylylation of FMN to form FAD. RibF also converts FMN to FAD by adenylylation. RibU, RibC, and RibF are

**Significance**

Riboflavin (vitamin B₂) is converted into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential cofactors for many redox reactions across all domains of life. *Listeria monocytogenes* is a facultative intracellular pathogen that cannot synthesize riboflavin and must therefore obtain flavins from the host. In this study, we show that a previously identified riboflavin transporter (RibU) is essential for virulence and intracellular growth, but rather than transporting riboflavin, RibU transports FMN and FAD directly from the host cell cytosol. Mutants unable to convert riboflavin to FMN and FAD retained their capacity to grow intracellularly and were virulent, but they were unable to grow extracellularly and were thus converted from facultative to obligate intracellular pathogens.
Fig. 1. RibU is essential for virulence but dispensable for growth in nutrient-rich media. (A) Structures of riboflavin (black), FMN (red), and FAD (purple). Riboflavin is phosphorylated by riboflavin kinases to produce FMN. FAD synthases adenylylate FMN to generate FAD. (B) Broth growth curves of *L. monocytogenes* strains grown in nutrient-rich media. OD600 was used to determine cell density. The means and SDs of three independent experiments are shown. Note: both the WT and ΔribU mutant growth curves are superimposable. (C and D) Bacterial burdens in CD-1 mice infected intravenously with *1 × 10⁵* CFUs of indicated *L. monocytogenes* strains. At 48 h postinfection, the spleens (C) and livers (D) were harvested, homogenized, and plated to determine the CFUs per organ. The data show the median CFUs for each strain. The dashed line represents the limit of detection. Statistical significance of logarithmically transformed CFU values was determined using one-way ANOVA and Dunnett’s posttest using WT as the control. ****P < 0.0001; ns, not significant, P > 0.05.

the only proteins known to control flavin metabolism in *L. monocytogenes* (SI Appendix; Fig. S1).

Recently, there has been renewed interest in flavin metabolism in the context of pathogenesis stemming from the discovery that intermediates of riboflavin biosynthesis activate innate-like mucosal-associated invariant T (MAIT) cells (23). In addition, we recently discovered that flavins are implicated in distinct extracellular redox activities in thousands of bacterial species (24). These bacterial redox systems allow bacteria to transfer electrons from the cytosol to various electron acceptors. We previously described bacterial redox activities in *L. monocytogenes* (25). Among the *L. monocytogenes* transposon mutants that lacked EET were mutations in genes encoding the flavin acquisition, since de novo riboflavin synthesis was not essential. To confirm that RibU was not essential for growth, and possibly assess flavin acquisition and requirements during pathogenesis, we generated a *L. monocytogenes* strain with an in-frame deletion in ribU (ΔribU). Like the transposon mutant, the ΔribU strain had no detectable growth defect in nutrient-rich media compared to wild-type (WT) *L. monocytogenes* (Fig. 1B). In contrast, the ΔribU strain had a 5-log virulence defect in the spleens of mice compared to WT *L. monocytogenes* (Fig. 1C), and no colony-forming units (CFUs) could be recovered from the livers of infected mice (Fig. 1D). Complementation of the ΔribU mutant with a ribU gene with its endogenous promoter (ΔribU + ribU) fully restored virulence in vivo (Fig. 1 C and D).

To determine if the virulence defect of the ΔribU strain was caused by riboflavin starvation in vivo, we engineered the ΔribU mutant to synthesize riboflavin by inserting the riboflavin operon ribDEAHT into the closely related gram-positive bacterium *B. subtilis* (27) into the *L. monocytogenes* chromosome. This strain grew in colorless chemically defined synthetic media without riboflavin supplementation and turned the media yellow, the natural color of flavins (SI Appendix; Fig. S2). Expression of the ribDEAHT operon rescued the ΔribU strain’s virulence to WT *L. monocytogenes* levels in the spleens and livers of mice (Fig. 1 C and D). Based on these observations, we concluded that RibU is essential for *L. monocytogenes* pathogenesis and that its function relates to flavin acquisition, since de novo riboflavin production in the RibU-minus strain completely bypassed RibU’s essentiality in vivo.

*L. monocytogenes* Uses RibU to Grow in Macrophages. To study why RibU was essential for growth of *L. monocytogenes* in mice, we performed infections in vitro using bone marrow-
derived macrophages (BMMs). At 2 h postinfection, the ΔribU mutant had a small but significant growth advantage over WT L. monocytogenes (Fig. 2A), which was associated with an increase in phagosomal escape (SI Appendix, Fig. S3A). However, during exponential growth (2 to 5 h postinfection), the ΔribU mutant had an apparent defect in replication rate and showed a loss in CFUs during the late stages of infection (5 to 8 h postinfection) (Fig. 2A). Complementation of the ΔribU strain with the ribo gene or the ribDEAHT operon completely restored the growth defects in BMMs (SI Appendix, Fig. S3B).

To test if the ΔribU strain has an inherent intracellular virulence defect not related to riboflavin, BMMs were incubated with excess riboflavin (10 μM) prior to infection to increase the concentration of intracellular riboflavin. In this condition of riboflavin excess, the ΔribU mutant replicated to WT levels (Fig. 2B). To assess if the growth of the ΔribU strain observed during the exponential growth phase in BMMs (Fig. 2A) is due to residual flavins from the medium, we infected riboflavin-deficient BMMs with riboflavin-starved bacteria. In this experiment, the ΔribU mutant and WT L. monocytogenes strains were incubated in chemically defined synthetic media lacking flavins for 16 to 18 h prior to infection. The BMM cell culture media was replaced with media lacking riboflavin, and the macrophages were incubated for 3 h prior to infection with the riboflavin-starved bacteria. We observed that riboflavin-starved WT L. monocytogenes were able to grow in riboflavin-deficient BMMs. However, the riboflavin-starved ΔribU mutant was unable to replicate in riboflavin-depleted BMMs (Fig. 2C). The riboflavin-starved ΔribU mutant was able to grow in riboflavin-deficient BMMs supplemented with 1 μM riboflavin just prior to infection (SI Appendix, Fig. S3C).

To further characterize the growth dynamics of the ΔribU mutant, we focused on the late stage of infection and the loss of CFUs observed between 5 and 8 h postinfection (Fig. 2A). We hypothesized that the loss of CFUs was due to the inability of the ΔribU mutant to obtain riboflavin from the cytosol of host cells and that riboflavin starvation led to bacterial and/or host cell death. To test if infection with the ΔribU strain led to host cell death, we performed a lactate dehydrogenase (LDH) release assay. The results demonstrated that ΔribU caused a significant increase in host cell death (Fig. 2D). In dying host cells, gentamicin from the media can enter the cytoplasm and kill intracellular bacteria. Based on previous studies (28), we hypothesized that the ΔribU strain was lysing in the macrophage cytosol and releasing DNA that activated the DNA-dependent AIM2 inflammasome, resulting in pyroptotic cell death. To test if ΔribU was triggering AIM2-dependent pyroptosis, we performed LDH release assays using AIM2 knockout (KO) BMMs and observed that the ΔribU mutant did not lead to LDH release (Fig. 2E). As a control, L. monocytogenes

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**Fig. 2.** L. monocytogenes requires RibU to grow in riboflavin-starved macrophages. (A–C and F) Intracellular growth curves of L. monocytogenes strains in murine BMMs. BMMs were infected at a multiplicity of infection (MOI) of 0.1, and CFUs were enumerated at the indicated times. (A) Intracellular growth curves of indicated L. monocytogenes strains in WT BMMs. The data show the means and SEMs of four independent experiments. (B) Intracellular growth curves of indicated L. monocytogenes strains in WT BMMs incubated with cell culture media containing excess (10 μM) riboflavin during infection. The means and SEMs of three independent experiments are shown. (C) Intracellular growth curves of indicated flavin-starved L. monocytogenes strains in riboflavin-deficient WT BMMs. The data represent the means and SEMs of three independent experiments. (D and E) Cell death of WT (D) or AIM2 KO (E) BMMs infected with specified L. monocytogenes strains. LDH released to the cell culture media was used as an indicator of cell death. LDH release values were normalized to 1% Triton-X-treated cells which represent 100% lysis. BMMs were infected at an MOI of 4. The data show the means and SEMs of three technical replicates from at least two (D) and four (E) independent experiments. Statistical significance was determined using one-way ANOVA and Dunnett’s posttest using WT as the control. ***p < 0.0001; **p < 0.01; ns, not significant, p > 0.05. (F) Intracellular growth curves of indicated L. monocytogenes strains in AIM2 KO BMMs. The means and SEMs of five independent experiments are shown. WT + l.p.fla, WT L. monocytogenes expressing Legionella pneumophila flagellin A under the control of the actA promoter (29). Holin-Lysin, WT L. monocytogenes expressing the bacteriophage proteins Holin and Lysin under the control of the actA promoter, leading to lysis of the bacteria in the cytosol of the host cell (28).
securing flagellin (WT + L.p flaA), which activates the NLR C4 inflammasome (29), still mediated cell death in infected AIM2 KO BMMs (Fig. 2B). Since the ΔribU mutant did not lead to cell death in AIM2 KO BMMs, we hypothesized that the loss of CFUs in the ΔribU mutant during the later stages of infection in WT BMMs should be rescued in AIM2 KO BMMs. Indeed, there was no loss of CFUs of the ΔribU mutant at 8 h postinfection in AIM2 KO BMMs (Fig. 2F). These data suggest that the RibU-minus strain lysed to some extent in vivo and activated AIM2-dependent pyroptosis, which negatively impacted the virulence of the strain.

L. monocytogenes Uses RibU to Scavenge FMN and FAD from the Cytosol of Host Cells. Our observation that riboflavin-starved WT L. monocytogenes grew in riboflavin-deprived macrophages (Fig. 2C) led us to question how L. monocytogenes fulfills its flavin requirements under these conditions. Interestingly, we did not observe any difference in the ability of WT L. monocytogenes to replicate intracellularly in the absence of riboflavin. The doubling time of riboflavin-starved WT L. monocytogenes in riboflavin-deficient BMMs was very similar to the doubling time of WT L. monocytogenes growing in BMMs with riboflavin: 48.6 and 51.5 min, respectively (SI Appendix, Fig. S3D). Based on these results, and the fact that mammalian cells rapidly convert riboflavin to FMN and FAD upon import (13, 30), we hypothesized that WT L. monocytogenes imports FMN and/or FAD intracellularly to grow, using RibU for their transport. To test if L. monocytogenes can import FMN and FAD to support growth, we used chemically defined synthetic media supplemented with riboflavin, FMN, or FAD as the sole flavin source and found that WT L. monocytogenes grew in media containing each of the three flavins (Fig. 3A). By contrast, the ΔribU strain did not replicate in chemically defined media with FMN or FAD (Fig. 3B) and had only a slight defect in growth in media containing riboflavin. These results suggested that RibU is responsible for growth on FMN and FAD and that riboflavin can enter cells using RibU and/or another, yet to be identified, riboflavin transporter.

To test the hypothesis that L. monocytogenes utilizes RibU to scavenge FMN and FAD from the cytosol of host cells, we sought to generate a L. monocytogenes FMN and FAD auxotroph by constructing strains lacking ribC, ribF, or both (ΔribCΔribF), enzymes responsible for converting riboflavin to FMN and FAD. Since FMN and FAD are essential cofactors, construction of this strain was performed in nutrient-rich media containing excess FMN and FAD to circumvent synthetic lethality. As predicted, the ΔribCΔribF mutant was unable to replicate in chemically defined media with riboflavin as the sole flavin source (Fig. 3C).

We reasoned that if L. monocytogenes imports FMN and FAD from the host cytosol, the ΔribCΔribF mutant should not be impaired for intracellular growth. Indeed, these strains replicated intracellularly in BMMs to WT L. monocytogenes levels (Fig. 3D). To test if the ΔribC, ΔribF, and the ΔribCΔribF mutant L. monocytogenes strains grew in vivo, we performed mouse virulence assays. The ΔribC, ΔribF, and ΔribCΔribF mutants maintained their virulence and grew to high levels in both the spleens and livers of mice, although the ΔribC and ΔribCΔribF strains had statistically significant 2-log defects in the liver (Fig. 3 E and F). Complementation of the ΔribCΔribF strain with the ribC gene with its endogenous promoter was able to restore most of the growth in the spleens and livers of infected mice (Fig. 3 E and F). Thus, these results support a model in which L. monocytogenes uses RibU to import FMN and FAD from the cytosol of host cells.

The ΔribCΔribF Mutant Cannot Grow in Blood, Gallbladders, or the Gastrointestinal Tract. The observation that the ΔribCΔribF strain replicated similarly to WT L. monocytogenes in the spleens but not in the livers of mice (Fig. 3 E and F) prompted us to examine if this strain was able to colonize and grow in other sites of infection in mice and use growth as an indicator of flavin availability. WT L. monocytogenes can grow extracellularly in the gallbladder, blood, and the gastrointestinal (GI) tract of mice (31–34). During infection, L. monocytogenes colonizes the lumen of the gallbladder, which is connected to the liver through biliary ducts, and rapidly replicates extracellularly in the bile, establishing this organ as a bacterial reservoir (31, 32). The ΔribCΔribF mutant was unable to colonize the gallbladder, while the ΔribC and ΔribF strains grew to WT L. monocytogenes levels (Fig. 4A). Complementation of the ΔribCΔribF mutant with a ribC gene with its endogenous promoter completely rescued the growth of the ΔribCΔribF strain in the gallbladder.

To assess if the ΔribCΔribF mutant strain can grow extracellularly in blood, we performed a growth curve in defibrinated sheep’s blood and found that the ΔribCΔribF mutant did not replicate and had a 2- to 3-log CFU loss by 24 h postinoculation (Fig. 4B). Finally, to test if the ΔribCΔribF mutant can grow extracellularly in the lumen of the GI tract, mice were pretreated for 2 d prior to the infection with streptomycin and avins and then infected with WT L. monocytogenes at 24 h postinfection (Fig. 4C). No CFUs were recovered from the ΔribCΔribF mutant–infected mice following day 1 postinfection (Fig. 4C). Collectively, these observations suggest that the ΔribCΔribF strain cannot grow extracellularly in the gallbladder, blood, or the GI tract and that this mutant is restricted to intracellular growth in vivo.

Discussion

The riboflavin derivatives FMN and FAD are redox active cofactors essential to all organisms (1–3), including pathogens which either synthesize riboflavin or import it from their host (35–37). Little is known about how pathogens that cannot synthesize riboflavin acquire this vitamin during infection. Here we show that the riboflavin auxotrophic, facultative intracellular bacterium L. monocytogenes uses the riboflavin transporter RibU to acquire FMN and FAD, but not riboflavin, from the cytoplasm of host cells. The finding that L. monocytogenes imports FMN and FAD to grow in host cells using RibU is supported by our data showing that a mutant lacking RibU is avirulent in vivo (Fig. 1 C and D) but grew in nutrient-rich media and chemically defined media supplemented with riboflavin and not with FMN and FAD (Fig. 3B). Furthermore, a mutant lacking the enzymes that convert riboflavin to FMN and FAD (ribCribF double mutant) grew like WT L. monocytogenes in BMMs (Fig. 3D) and in vivo in the spleen (Fig. 3E). Collectively, these observations led us to conclude that (1) RibU is a flavin transporter that, in addition to riboflavin, can import FMN and FAD; (2) L. monocytogenes might encode another, unknown riboflavin transporter that is unable to import FMN and FAD; and (3) L. monocytogenes primarily acquires FMN and FAD in vivo to support its intracellular growth.

The reliance of L. monocytogenes on host cell FMN and FAD is not surprising given the fact that the concentration of these flavins inside cells far surpasses that of their precursor riboflavin.
We report that *L. monocytogenes* can obtain FMN and FAD from the host cytosol using RibU. We speculate that these pathogens must acquire FMN and FAD from the host to satisfy their flavin requirements. Similarly, we hypothesize that riboflavin auxotrophic vacuolar parasites, like members of the Apicomplexa phylum and *Leishmania* species, might rely on FMN and FAD synthetases (11, 12).
FAD import to grow intracellularly as well. These vacuolar parasites likely employ different mechanisms to scavenge these flavins from the cytosol, as has been shown for other micronutrients (38–40).

The evolutionary path to becoming an obligate intracellular pathogen often results in genome reduction (41–43) involving loss of a pathogen’s ability to synthesize its own metabolites and rely on import of nutrients from their hosts. The active cofactors FMN and FAD are highly prevalent in host cells (13–15), which might explain why some intracellular pathogens do not synthesize riboflavin or encode enzymes that convert it to FMN and FAD. In this study, we demonstrate that a L. monocytogenes mutant lacking the ribC and ribF enzymes required supplementation of FMN and FAD to grow and was unable to replicate in the blood (Fig. 4B), gallbladder (Fig. 4A) and GI tract (Fig. 4C) of mice, which represent environments where WT L. monocytogenes grows extracellularly (31–34). In contrast, the ribC/ribF double mutant grew intracellularly in macrophages and the spleen and liver of mice. Thus, we show the apparent conversion of a facultative intracellular pathogen into an obligate intracellular pathogen by eliminating two genes involved in the flavin biosynthesis pathway. This study provides an example of how pathogens, as an evolutionary adaptation, might lose their biosynthetic capabilities, by discarding genes involved in the production of metabolites prevalent in their hosts, and instead rely on acquisition of nutrients directly from the host, ultimately becoming obligate intracellular pathogens. Our findings may also have practical applications by providing an additional safety measure to L. monocytogenes strains used as therapeutic cancer vaccines by preventing extracellular growth and limiting dissemination (44).

Considering that L. monocytogenes thrives as a ubiquitous environmental saprophyte and has few nutritional requirements, it is curious that these bacteria lack the capacity to synthesize riboflavin. We hypothesize that the lack of riboflavin biosynthesis may provide L. monocytogenes an evolutionary advantage during infection of mammalian hosts, which almost universally have a prevalent population of MAIT cells (45) that are activated by an environmental pathogen and can potentially limit dissemination (44).

Tissue Culture and Growth Media. BMMs were prepared by collecting bone marrow from 8-wk-old female WT (Jackson Laboratory) and AIM2 KO (a gift from Dr.41) at 30°C overnight in 14-mL round polypropylene tubes (Thermo Fisher Scientific) at a slanted position. The bacteria were washed and diluted in sterile 1× phosphate-buffered saline (PBS), and BMMs were infected at an MOI of 0.25. At 3 min postinfection, the cells were washed twice with 1× PBS. At 1 h postinfection, 50 μg/mL gentamicin sulfate (Sigma-Aldrich) was added to the cell culture media to kill/prevent bacteria from growing extracellularly. The growth curves then proceeded as previously described (55).

Intracellular Growth Curves in Media Lacking Riboflavin. To deplete the intracellular flavins in L. monocytogenes, bacterial cultures were started 2 d prior to BMM infection in chemically defined media containing 1 μM riboflavin and grown at 37°C with shaking. The bacteria were washed twice 16 to 18 h prior to infection with 1× PBS and then diluted into chemically defined media lacking flavins and grown at 37°C with shaking.

Macrophages were washed twice, 3 h prior to BMM infection, with 1× PBS, and the cell culture media was replaced with high-glucose DMEM lacking riboflavin (Millipore Sigma), a gift from Dr.41, with 20% dia- lyzed FBS using SnakeSkin dialysis tubing. 3.5K molecular weight cutoff (MWCO) (Thermo Fisher Scientific), and other components as described in Tissue Culture and Growth Media. The riboflavin-starved L. monocytogenes were washed and diluted in sterile 1× PBS, and the BMMs were infected at an MOI of 0.25. These growth curves were performed without the addition of riboflavin unless otherwise stated in the legend. The growth curve experiments then proceeded as previously described (55).

Cell Death (LDH Release) Assay. From 16 to 18 h before infection, 5 × 10^5 BMMs per well were seeded in 24-well plates with 100 ng/mL Pam3CSK4 (Inviv oGen) in DMEM. Before infecting the BMMs, the cell culture media was replaced with DMEM with 5% FBS. L. monocytogenes strains were grown overnight, slanted, at 30°C. For the infection, bacteria were diluted in 1× PBS, and BMMs were infected at an MOI of 4. At 30 min postinfection, the cells were washed twice with 1× PBS and DMEM with 5% FBS and 50 μg/mL gentamicin was added to wells. The experiment was conducted as previously described (28).

Mouse Intravenous Infections. Each 8-wk-old female CD-1 mouse (Charles River Laboratories) was infected via the tail vain with 200 μL of PBS containing 1 × 10^5 logarithmically growing bacteria. The mice were euthanized 48 h postinfection, and the spleen, liver, and gallbladder were collected, homogenized, and plated to determine the number of CFU per organ.

Blood Growth Curve. The growth of L. monocytogenes strains in blood was determined using defibrinated sheep’s blood (HemoStat Laboratories). The bacteria were grown logarithmically for 2.5 h, washed, and resuspended in 3 mL of defibrinated sheep’s blood at a concentration of 1 × 10^9 per milliliter. Blood cultures were incubated at 37°C with shaking. The growth of L. monocytogenes in blood was monitored for 3 d by diluting the blood in 1× PBS and plating to determine the number of CFU in total blood.
Mouse Oral Infections. Mice were given 5 mg/mL streptomycin sulfate salt (Sigma-Aldrich) in drinking water 48 h prior to infection, as previously described (32). Mice were transferred to clean cages 18 to 24 h prior to infection, and the food source (mouse colony chow) was removed to start the overnight fast. The day of the infection, a 3-mm piece of bread was inoculated with $1 \times 10^7$ logarithmically growing bacteria in 1X PBS and covered with 3 ml of butter. Each 8-wk-old female CD-1 mouse (Charles River Laboratories) was then fed a single piece of infected bread. The streptomycin sulfate water was replaced with standard drinking water and the chow was restored. Fecal samples were collected everyday postinfection for 5 d, weighed, vortexed at 4 °C for 10 min, and plated to determine the number of CFU per gram of stool.

Phagosomal Escape Assay. BMMs were seeded in 24-well plates containing 12-mm glass coverslips (Thermo Fisher Scientific) and cultured overnight. BMMs were treated with 250 ng/mL cytochalasin D (Sigma-Aldrich) 30 min prior to infection. At 1 h 15 min postinfection, the BMMs were slanted position. At 1 h 15 min postinfection, the BMMs were washed twice with DMEM. The secondary antibodies used were rhodamine red-X goat anti-rabbit immunoglobulin G (IgG) (catalog no. R6394, Invitrogen-Thermo Fisher Scientific) at 1:2,000 dilution and Alexa Fluor-647 goat anti-rabbit Alexa Fluor-594 (catalog no. A21450, Invitrogen-Thermo Fisher Scientific) at a 1:2,000 dilution. At least 100 bacteria per condition were quantified for analysis.

Animal Use Ethics Statement. The mice were maintained by University of California, Berkeley Office of Laboratory Animal Care, personnel according to institutional guidelines. Animal studies were performed in accordance with the guidance and recommendations of the University of California, Berkeley Office of Laboratory Animal Care, and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used in this study were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (AUP-2016-05-8811).

Statistical Analysis. All statistical analyses were performed using GraphPad Prism version 9.2 for MacOS, GraphPad Software.

Data Availability. All study data are included in the article and/or SI Appendix.

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