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The major role of *Listeria monocytogenes* folic acid metabolism during infection is the generation of N-formylmethionine

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ABSTRACT Folic acid and its derivatives (folates) play central roles in one-carbon metabolism, necessary for the synthesis of purines, pyrimidines, and some amino acids. Antifolate drugs are widely used as broad-spectrum antibiotics for bacterial infections, including listeriosis, a disease caused by the facultative intracellular pathogen *Listeria monocytogenes*. However, folate-derived metabolites required during bacterial infection are poorly understood. Here, we report that *L. monocytogenes* encodes two enzymes, methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (FolD) and formyltetrahydrofolate synthetase (Fhs), that catalyze the formation of N10-formyltetrahydrofolate, a critical intermediate in folate metabolism. N10-formyltetrahydrofolate is an essential carbon donor for biosynthesis of purines and N-formylmethionine, the amino acid used during initiation of bacteria translation. While *L. monocytogenes* mutants lacking Fhs had no observable defects and mutants lacking FolD had moderate virulence defects, mutants lacking both were highly attenuated in mice, especially in the liver. We compared the growth and virulence of mutants that were unable to synthesize folates with mutants unable to generate folate downstream products, including purines, thymidine, and N-formylmethionine. Mutants unable to synthesize N-formylmethionine behaved almost identically to FolD/Fhs double mutants during growth in broth or macrophages, but most importantly, were similarly attenuated to mutants unable to make folates, both of which had approximately 4-log₁₀ less colony-forming units in the livers compared with wild-type *L. monocytogenes*. The purine auxotrophic mutants were only 1.5-log₁₀ less virulent, and the thymidine mutants were fully virulent in the mouse infection model. These results strongly suggest that the main role of *L. monocytogenes* folates during infection is the generation of N-formylmethionine.

IMPORTANCE Folic acid is an essential vitamin for bacteria, plants, and animals. The lack of folic acid leads to various consequences such as a shortage of amino acids and nucleotides that are fundamental building blocks for life. Though antifolate drugs are widely used for antimicrobial treatments, the underlying mechanism of bacterial folate deficiency during infection is unclear. This study compares the requirements of different folic acid end-products during the infection of *Listeria monocytogenes*, a facultative intracellular pathogen of animals and humans. The results reveal the critical importance of N-formylmethionine, the amino acid used by bacteria to initiate protein synthesis. This work extends the current understanding of folic acid metabolism in pathogens and potentially provides new insights into antifolate drug development in the future.

KEYWORDS folic acid, *Listeria monocytogenes*, N-formylmethionine, purine, tetrahydrofolate

Folic acid is an essential B vitamin in all three domains of life that is synthesized by most bacteria, some archaea, plants, but not mammals (1–4). The bioactive

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derivatives of folic acid, tetrahydrofolates (THFs), play central roles in one-carbon (1C) metabolism by donating carbon groups necessary for the synthesis of purines, pyrimidines, amino acids, and N-formylmethionine (fMet), the first amino acid used during protein translation in bacteria and mitochondria (5, 6). Synthesis of THFs and their downstream metabolites are proven antibiotic targets (7–9). Sulfa drugs target the folate precursor para-aminobenzoic acid (PABA) biosynthesis, while trimethoprim targets dihydrofolate reductase and is generally used together as broad-spectrum antibiotics for many bacterial infections, including listeriosis, a disease caused by a facultative intracellular pathogen *Listeria monocytogenes* (10, 11). Despite the importance of current and new antifolate drugs, and the extensive studies of folic acid metabolism, especially in humans, the functions of folic acid metabolism during infection by intracellular pathogens remain poorly understood.

L. monocytogenes is a rapidly growing, Gram-positive facultative intracellular pathogen that can cause serious, sometimes fatal, disease following ingestion of contaminated food in a wide range of animals, including immunocompromised and pregnant humans (12–14). *L. monocytogenes* also serves as a highly tractable model organism for studying intracellular pathogens and cell-mediated immunity (12, 15). In a survey to identify *L. monocytogenes* transposon mutants that formed small plaques in monolayers of tissue culture cells, we identified two genes that encode enzymes in folic acid metabolism (16). Mutants in *pabBC*, which encodes two enzymes that catalyze the biosynthesis of the folic acid precursor, PABA, were severely attenuated for *L. monocytogenes* virulence in tissue culture and mouse infection models (17), although the precise reasons for its virulence were not established. Additionally, we identified *folD*, which encodes bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase, that catalyzes the reversible conversion of two major 1C-carrying folates, N⁵,N¹⁰-methylene-THF and N¹⁰-formyl-THF. The 1C group of N⁵,N¹⁰-methylene-THF is used to generate serine, glycine, and deoxythymidine monophosphate (dTMP) while that of N¹⁰-formyl-THF is used to synthesize purines and fMet (Fig. 1). *FolD* is essential in many bacteria, although some facultative anaerobes, including *L. monocytogenes*, can also synthesize N¹⁰-formyl-THF using formyltetrahydrofolate synthetase (formate tetrahydrofolate ligase, Fhs) (Fig. 1) (18). In bacteria and mitochondria, methionyl-tRNA formyltransferase (FMT) transfers the formyl-group from N¹⁰-formyl-THF to a methionine bound to the initiator tRNA which in turn promotes the initiation of translation (19, 20).

In this study, we sought to explore the functional mechanisms underlying the requirement of folate metabolism during *L. monocytogenes* infection. We characterized the roles of *FolD* and *Fhs* during bacterial growth in media, tissue culture, and animal infection models. *FolD* played a dominant role in making N¹⁰-formyl-THF and was critical to *L. monocytogenes* aerobic growth and pathogenesis, although mutants lacking both *FolD* and *Fhs* were much more highly attenuated. By comparing the phenotypes of mutants in different steps in folic acid metabolism, we concluded that fMet was the most critical folate end-product required during infection *in vivo*.

RESULTS

Characterization of *folD* and *fhs* mutants in media and in tissue culture models of infection

In an effort to understand the role of *L. monocytogenes* folic acid metabolism during infection, we characterized a previously identified transposon insertion mutant in *folD* (*folD::Tn*), which encodes a central enzyme of folic acid metabolism (Fig. 1), that catalyzes the formation of N¹⁰-formyl-THF, a bioactive folate that donates carbon groups essential for both purines and N-formylmethionine synthesis. We noted that *L. monocytogenes* encodes another enzyme, formate tetrahydrofolate ligase (*Fhs*), which also catalyzes the formation of N¹⁰-formyl-THF. We constructed in-frame deletions in *L. monocytogenes folD* ($\Delta folD$), in *fhs* (Δfhs), and double mutants lacking both ($\Delta fhs folD::Tn$ and $\Delta folD fhs::Tn$). Consistent with our previous findings characterizing the *folD::Tn* mutant (16), the $\Delta folD$ deletion

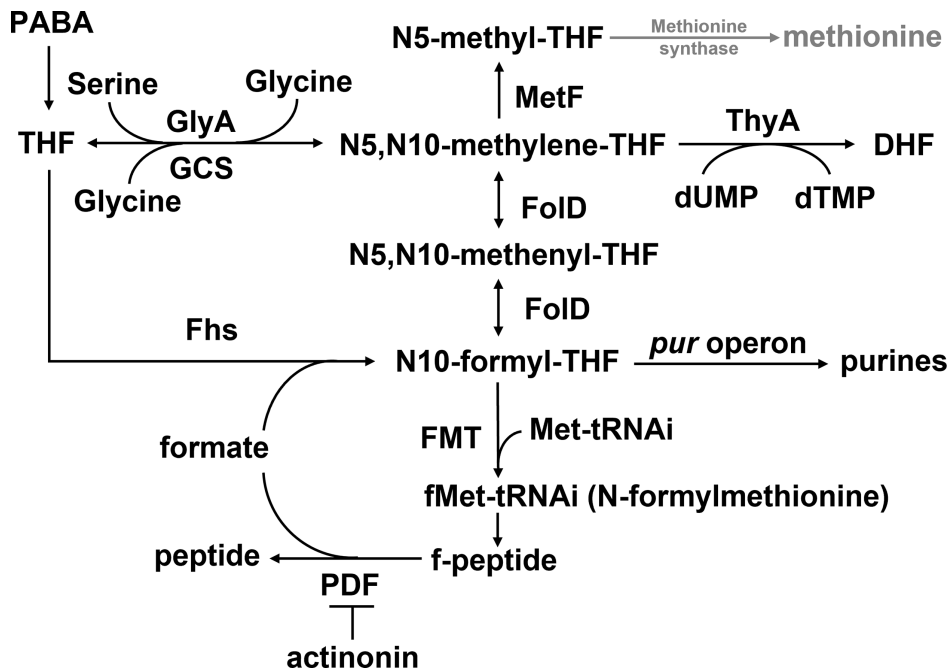


FIG 1 Simplified view of folate metabolism in *L. monocytogenes*. The pathway involves tetrahydrofolates carrying one-carbon units, and N-formylmethionine, purine, and thymidine as key products. Gray, enzymes and products that are absent in *L. monocytogenes* 10403S. dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FoID, methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase; GCS, glycine cleavage system; GlyA, serine hydroxymethyltransferase; MetF, 5,10-methylene-THF reductase; THF, tetrahydrofolate; ThyA, thymidylate synthase.

mutant formed very small plaques in monolayers of murine L2 fibroblasts (Fig. 2A). The Δfhs strain formed plaques indistinguishable from wild-type (WT), and the double mutants $\Delta fhs folD::Tn$ formed plaques similar to those formed by $\Delta folD$ (Fig. 2A), indicating that FoID plays the major role in generating N10-formyl-THF during host cell infection *in vitro*. Neither single mutant exhibited a growth defect in rich media, while the double mutants grew significantly slower compared to WT, with a 1.8-fold increased doubling time (Fig. 2B; Table S1). The growth defect observed in strains lacking *folD* and *fhs* was fully rescued

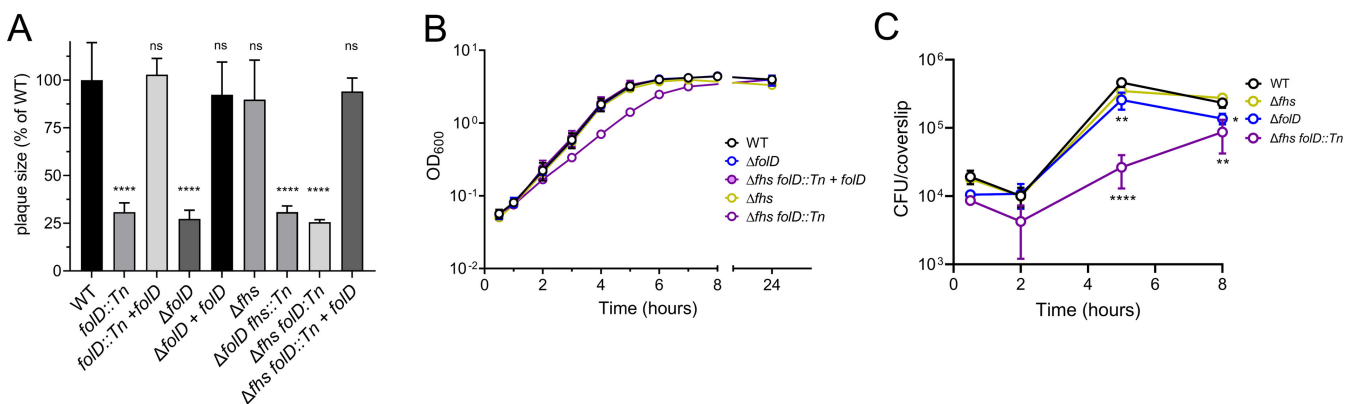


FIG 2 N10-formyl-THF is required for optimal growth in broth and host cells. (A) Plaque formation of indicated strains in L2 cells measured 3-d post-infection as a percentage of wild type. (B) Broth growth curve in brain heart infusion medium cultured at 37°C with agitation. Growth of *L. monocytogenes* strains was measured spectrophotometrically. (C) Intracellular growth in bone marrow-derived macrophages. BMMs were infected at a multiplicity of infection (MOI) of 0.25 for 30 min. Extracellular growth of bacteria was prevented by adding 50 µg/mL gentamicin at 1-h post-infection. Growth was enumerated by plating the colony-forming units (CFUs) at indicated time points. (A–C) Data are mean ± SD. Three independent experiments were combined. One-way analysis of variance (ANOVA), multiple comparisons with WT; ns, not significant; **P* < 0.05; ***P* < 0.01; *****P* < 0.0001.

by complementing with the *folD* gene expressed from a *pHyper* promoter (21) (Fig. 2A and B). We also examined the requirements of *folD* and *fhs* during infection of mouse bone marrow-derived macrophages (BMMs). Although the $\Delta folD$ strain had no defect when cultured in broth, it showed a small but significant defect at 5- and 8-h post-infection in BMMs (Fig. 2C). Notably, the intracellular growth rate of the $\Delta fhs folD::Tn$ double mutant was highly impaired, although it was able to grow until it reached a similar terminal OD_{600} as WT in broth (Fig. 2B and C; Table S1). Taken together, these data demonstrated that N10-formyl-THF is critical for *L. monocytogenes* growth and host cell infection. Although FoLD is the dominant enzyme required for the synthesis of N10-formyl-THF, Fhs plays a measurable role in the absence of FoLD.

Characterization of *folD* and *fhs* mutants in mice

To reveal the requirements of N10-formyl-THF during *in vivo* infection, CD-1 mice were infected intravenously, and bacterial burdens were determined in the livers and spleens 48-h post-infection. The $\Delta folD$ mutant exhibited an organ-specific defect that presented as a 2- \log_{10} virulence attenuation in the livers compared with WT and the complemented strains but showed no difference in colony-forming units (CFUs) in the spleens (Fig. 3). A main difference between these two organs is that in the spleen, *L. monocytogenes* grows in phagocytic cells, while much of the growth in the liver is in hepatocytes (22). To address if the liver-specific defect was due to poor growth in hepatocytes, the *folD::Tn* transposon was transduced into a strain lacking ActA and InlB, which prevents the bacteria from spreading into hepatocytes or entering via InlB-induced internalization (23). In the $\Delta actA \Delta inlB$ background, lack of *folD* still resulted in attenuation in the livers (Fig. S1A). In addition, *folD* played a negligible role in plaque formation in a murine hepatocyte cell line TIB-73 (Fig. S1B). These data suggested that the organ-specific defect of mutants lacking *folD* was unlikely attributed to the infection of hepatocytes.

L. monocytogenes virulence factor expression is controlled by the transcription factor PrfA (24, 25), and the biosynthesis of PABA, the folic acid precursor, is activated by PrfA (17). We wondered if the virulence defect of a *folD* mutant was due to lack of PrfA activation or if PrfA activation could compensate the defect of a *folD* mutant. To evaluate

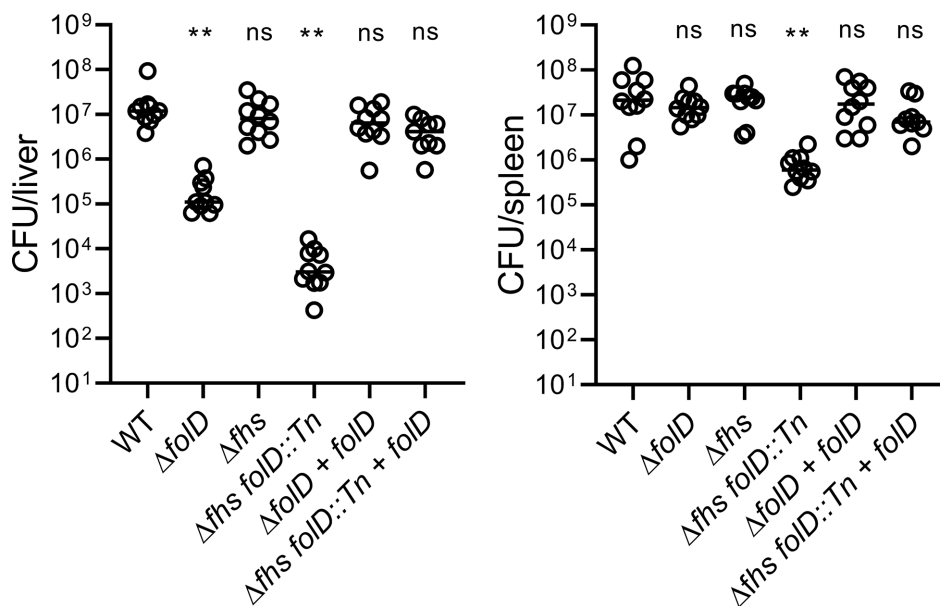


FIG 3 N10-formyl-THF is critical for virulence in mice. Eight-wk-old CD-1 mice (Charles River) were infected intravenously with 1×10^5 CFUs of indicated strains. Bacterial burdens in livers and spleens were measured 48-h post-infection by plating homogenized organs. Each circle represents an individual mouse. Lines present medians. Two biological repeats were combined with a total of 8–10 mice per strain. One-way ANOVA, multiple comparisons with WT; ns, not significant; ** $P < 0.01$.

the role of PrfA activation, we introduced the $\Delta folD$ deletion mutation into a *PrfA** strain where PrfA has a mutation that locks it in its active conformation (25). The *PrfA** $\Delta folD$ double mutant remained attenuated in the livers (Fig. S2), indicating that the defect of $\Delta folD$ was probably related to poor growth rather than loss of virulence gene activation.

Mutants lacking both *folD* and *fhs* ($\Delta fhs folD::Tn$) were severely attenuated *in vivo*, with approximately 4-log_{10} fewer CFUs in infected livers compared with WT and a 1.5-log_{10} defect in the spleens (Fig. 3). Complementation of $\Delta fhs folD::Tn$ with *folD* fully restored virulence (Fig. 3), again highlighting the dominant role of *folD* during infection. As observed in broth and BMMs, loss of *fhs* had no observable phenotype in the presence of *folD* but had a synergistic virulence defect when combined with a *folD* mutation during *in vivo* infection. We speculated that the role of Fhs during aerobic growth and during infection was limited due to the absence of its substrate formate, which is produced mostly during fermentation (26). Indeed, the growth defect of a $\Delta folD$ mutant growing aerobically in a chemically defined medium was completely restored if supplemented with formate, while restoration by formate was not observed in the absence of *fhs* as shown by the $\Delta fhs folD::Tn$ strain (Fig. S3).

Genetic screen to identify mutations that rescue the virulence defect of a $\Delta folD$ mutant and the role of purine biosynthesis during infection

We next sought to understand the mechanisms underlying the virulence defect of *L. monocytogenes* deficient in making N10-formyl-THF. THFs are carbon donors for various molecules, thus perturbations in folate metabolism could result in pleiotropic phenotypes (5, 6, 27). We showed that N10-formyl-THF made by FolD and Fhs was critical for *L. monocytogenes* growth and pathogenesis (Fig. 2 and 3), but it was unclear which factor(s) led to the virulence defect of the N10-formyl-THF deficient strains. To address this question, we generated a transposon library in a *folD* mutant background and screened for mutants that formed larger plaques in tissue culture. A total of 20 insertion mutants representing eight genes were identified that displayed increased plaque size from over 35,000 screened. Candidate suppressors were confirmed by transducing single insertions into WT and $\Delta folD$ background (Table S2).

Insertions disrupting *purR* led to the largest plaque restoration of $\Delta folD$ (Table S2). The *purR* gene encodes a transcriptional repressor that controls gene expression in response to the availability of purines (28–30). The major role of PurR is to repress the transcription of genes in the *pur* operon that encodes enzymes for the *de novo* synthesis of purines, although it represses other genes as well (28, 30–32). N10-formyl-THF is used as a cofactor for two *pur*-encoded enzymes that each donates a carbon unit during the construction of the purine ring. We hypothesized that purine shortage might lead to growth and virulence defects during N10-formyl-THF limitation, and de-repression of purine synthetic genes by the *purR* mutation replenished purine levels and consequently restored the growth and virulence of $\Delta folD$. As shown in Fig. S3, the growth of $\Delta folD$ was greatly impaired in chemically defined media without N10-formyl-THF end-products. Adding exogenous purines into the media fully restored the growth of $\Delta folD$ and partially rescued the $\Delta fhs folD::Tn$ strain as well (Fig. 4A). A *purR* in-frame deletion was generated in WT and $\Delta folD$ backgrounds, and consistent with the transposon mutants, the $\Delta folD \Delta purR$ mutants formed WT plaques (Fig. 4B). However, the *purR* mutation did not rescue plaque formation in a strain lacking *folD* and *fhs*, or the virulence of $\Delta folD$ in mice (Fig. 4B and C).

In order to directly evaluate the role of *de novo* purine biosynthesis, we generated a purine auxotrophic strain introducing an in-frame deletion of *purE* and *purK* ($\Delta purEK$), the first two genes in the *pur* operon. As expected, the $\Delta purEK$ strain failed to grow in media lacking purines (Fig. S4), but it grew like WT in both the plaque assay and in BMMs (Fig. 4B and Fig. 5D). However, the $\Delta purEK$ mutant did have an approximately 1.5-log_{10} virulence defect in mouse livers, although it was much less attenuated than a *folD/fhs* double mutant (Fig. 3 and Fig. 4C). Therefore, it is reasonable to conclude that N10-formyl-THF does contribute to purine biosynthesis during *in vivo* infection, and

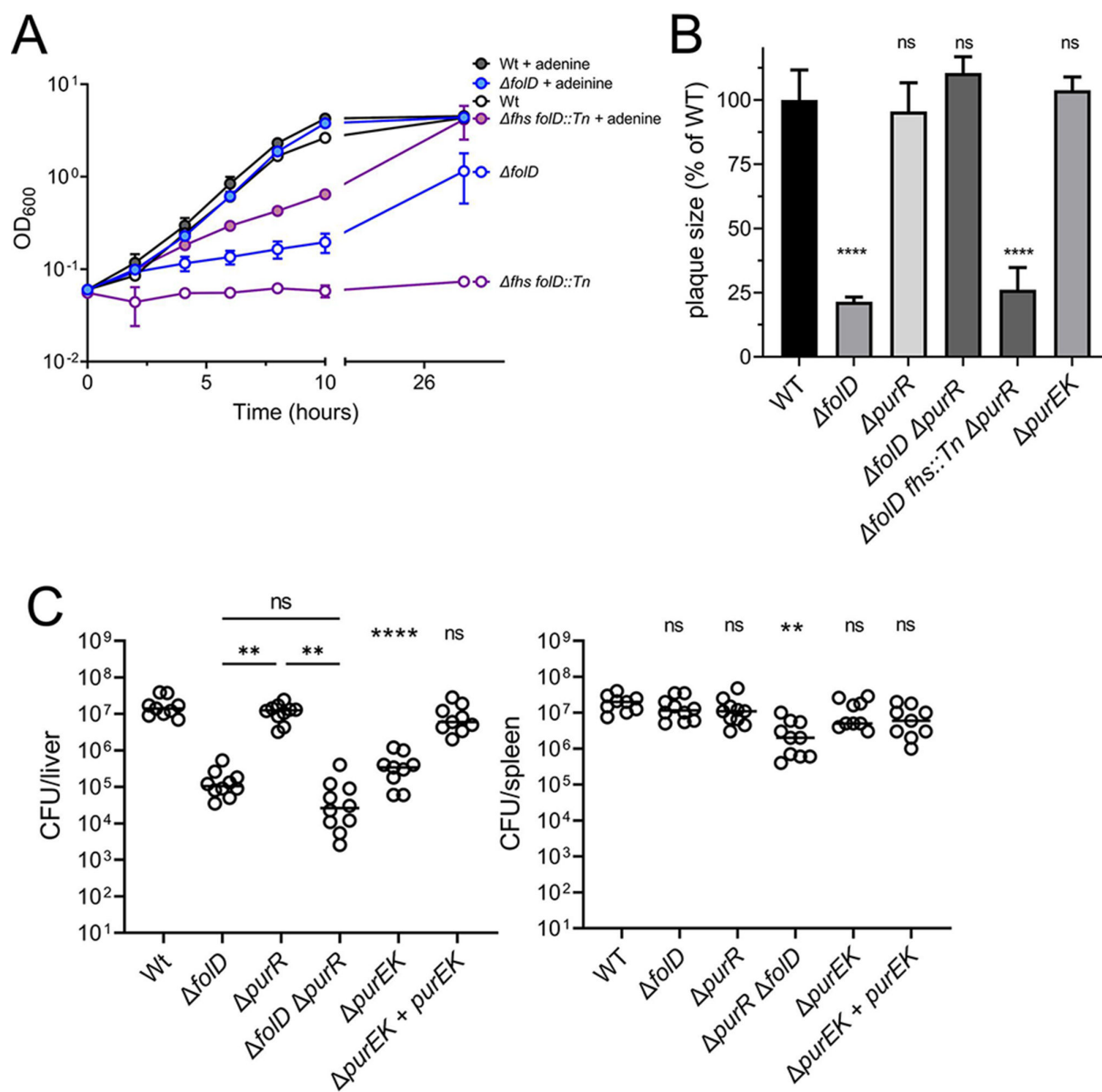


FIG 4 De-repression of PurR restores plaque forming of $\Delta foiD$ but not virulence in mice. (A) Broth growth curve in synthetic medium supplemented with adenine. Strains were cultured in the *Listeria* synthetic medium with or without 1 mM adenine at 37°C. Growth was measured spectrophotometrically. (B) Plaque formation of indicated strains in L2 cells. Plaques were measured on the third day after infection and presented as a percentage of wild type. Three independent experiments were combined for (A) and (B). Data are mean \pm SD. One-way ANOVA, multiple comparisons with WT; ns, not significant; **** $P < 0.0001$. (C) Virulence of indicated *L. monocytogenes* strains presented by CFUs in infected mouse livers and spleens. Two biological repeats were combined with a total of 10 mice per strain. Lines present medians. One-way ANOVA, multiple comparisons with WT control indicated on top of each strain, comparisons among the three mutant strains indicated by lines; ns, not significant; ** $P < 0.01$; **** $P < 0.0001$.

purine insufficiency is partially responsible for the severe attenuation of strains lacking FoiD and Fhs.

Comparison of mutants lacking different steps of the folic acid cycle suggests a critical role of N-formylmethionine during infection

In addition to purine biosynthesis, the other one-carbon recipient of N10-formyl-THF is methionine bound to the initiation tRNA (Met-tRNAⁱ), catalyzed by methionyl-tRNA formyltransferase (FMT) (Fig. 1). Although *fmt* is essential in some bacteria, it has

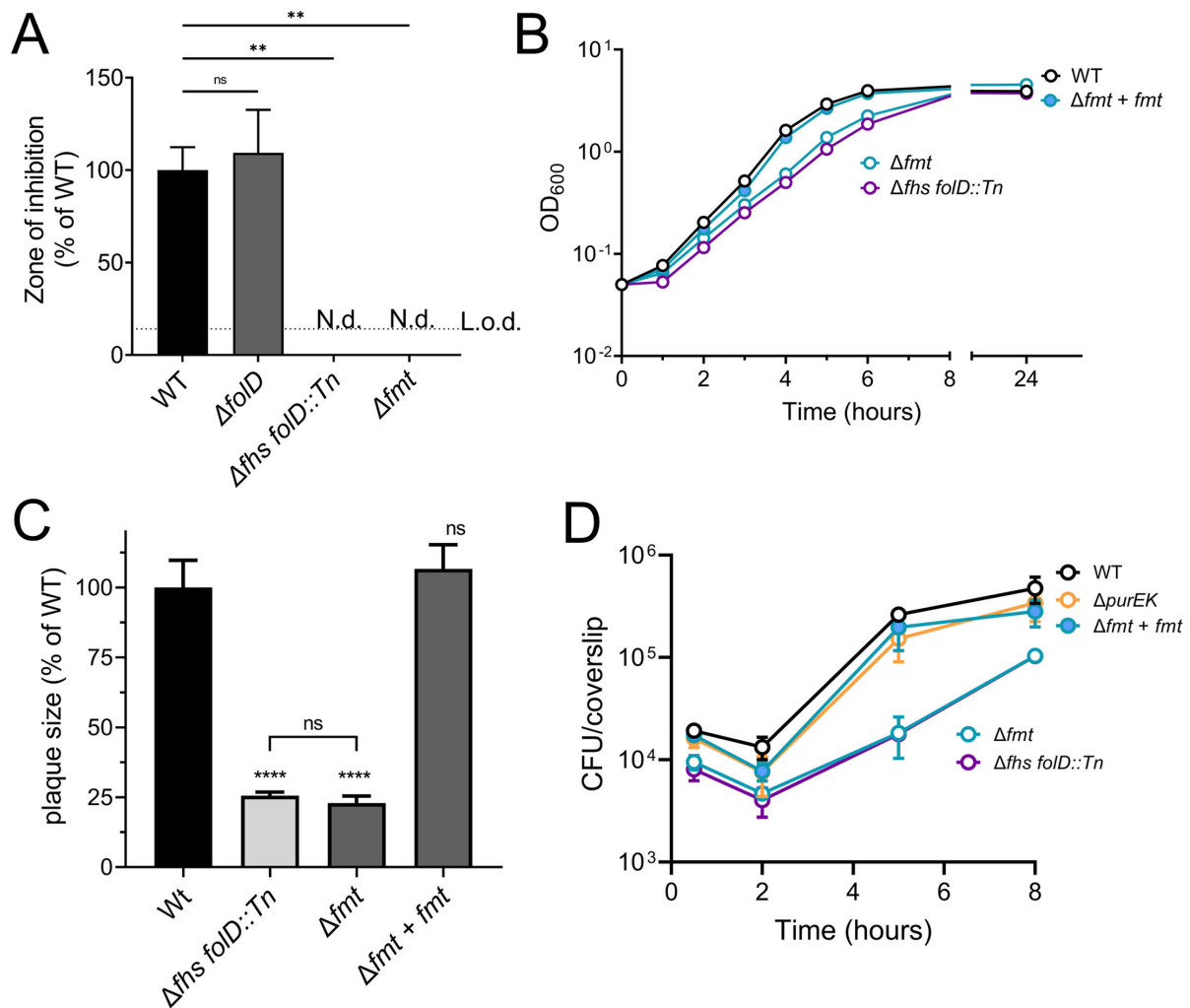


FIG 5 Lack of fMet results in defects in broth growth and host cell infection. (A) Antibiotic sensitivity measured by disk diffusion containing 500 μ g actinonin on brain heart infusion (BHI)-agar. L.o.d., limit of detection; N.d., not detectable. (B) Broth growth curve of indicated strains grown in BHI media at 37°C with agitation. (C) Plaque formation in murine fibroblast L2 cells measured 3-d post-infection of the indicated strains as a percentage of WT. (D) Intracellular growth in BMMs. Host cells were infected with indicated strains for 30 min at an MOI of 0.25. Antibiotic gentamicin was added to prevent extracellular bacteria at 1-h post-infection. (A–D) Three independent experiments were combined. Data are mean \pm SD. One-way ANOVA, multiple comparisons with WT; ns, not significant; $^{***}P < 0.01$; $^{****}P < 0.0001$.

been mutated in many bacteria, including *L. monocytogenes* (33–39). Accordingly, we constructed a strain lacking formylated Met-tRNAⁱ (fMet-tRNAⁱ) by in-frame deletion of *fmt* (Δfmt). Removal of the formyl-group from nascent peptides by peptide deformylase (PDF) is essential but is dispensable in strains lacking N-formylmethionine (i.e., fMet-tRNAⁱ). Mutants lacking N-formylmethionine are resistant to actinonin, an antibiotic targeting PDF (33). We tested actinonin sensitivity of $\Delta fhs fold::Tn$ and Δfmt in a disk-diffusion assay and found that both strains were resistant to actinonin (Fig. 5A), strongly suggesting that N-formylmethionine was not being synthesized. We then compared the $\Delta fhs fold::Tn$ and Δfmt mutants in various assays, and strikingly, the Δfmt strain behaved almost identical to $\Delta fhs fold::Tn$ in broth growth, plaque formation, growth in BMMs, and virulence in mice (Fig. 5B through D; Fig. 6). The defects observed in the mutants lacking *fmt* were fully rescued by complementation with the *fmt* gene (Fig. 5B through D; Fig. 6).

We next sought to evaluate the relative importance of N-formylmethionine compared with other folate end-products. In addition to purines and N-formylmethionine, the carbon units carried by different folate molecules can be used in the synthesis of serine,

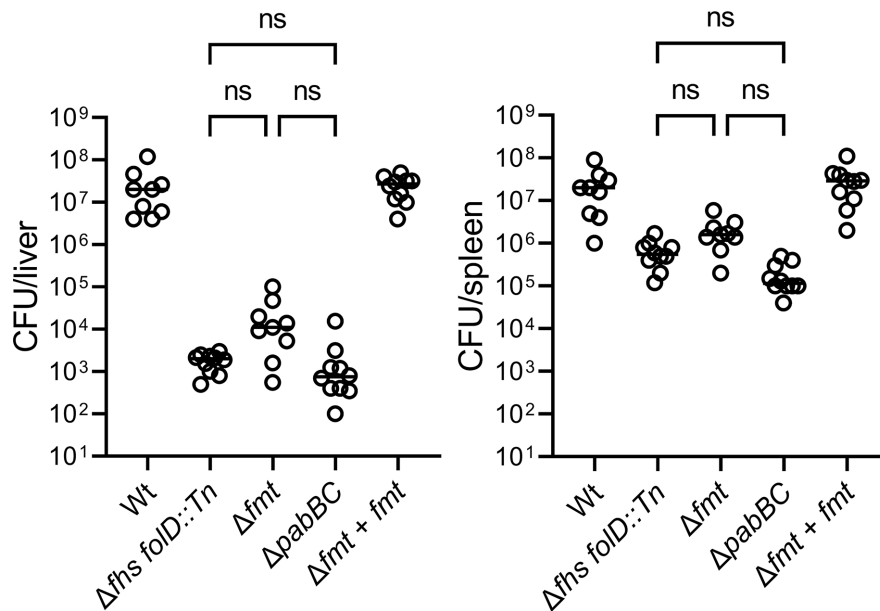


FIG 6 Strains that fail to make fMet are similarly attenuated *in vivo*. CD-1 mice ($n = 10$) were infected with 1×10^5 CFUs of indicated *L. monocytogenes* strains. Bacteria CFUs in mouse livers and spleens were enumerated 2-d post-infection. One-way ANOVA, multiple comparisons among the three mutant strains were indicated; ns, not significant.

glycine, and dTMP, depending on the needs of bacteria (Fig. 1). Previous ^{13}C -isotopologue profiling demonstrated that *L. monocytogenes* imports glycine and serine from the host cell cytoplasm (40). Methionine is also a folate product in many species, but *L. monocytogenes* 10403S lacks methionine synthase (Fig. 1) (41, 42), so it was not addressed here. We examined the requirement of thymidine during infection by using a mutant lacking *thyA* (43), which encodes the enzyme transferring the carbon unit from N⁵,N¹⁰-methylene-THF to produce dTMP (Fig. 1). The ΔthyA mutants do not grow in BMMs (Fig. S5A) but were fully virulent in the mouse infection model (Fig. S5B), suggesting that generation of dTMP from folates is not necessary during *L. monocytogenes in vivo* infection.

Finally, we compared the virulence defect of an *L. monocytogenes* strain unable to synthesize PABA (ΔpabBC) and, therefore, lacks folate synthesis. The folate biosynthesis defective strain, ΔpabBC , is severely attenuated *in vivo*, and purine supplementation partially replenished its growth in defined media (17). In the mouse infection model, the ΔpabBC and $\Delta\text{fhs fold::Tn}$ mutants were similarly attenuated, although the Δfmt mutant had a slightly higher, though not statistically significant, bacterial burden (Fig. 6).

DISCUSSION

Folic acid metabolism represents a critical aspect of central metabolism, being required for the synthesis of purines, pyrimidines, some amino acids, and critically, formylated methionine (N-formylmethionine). In addition, folic acid metabolism is an established target for antimicrobial therapy. However, despite the wide application of antifolate antibiotics, the precise role of folic acid metabolism during bacterial pathogenesis has remained obscure. The goal of this study was to identify which specific folic acid-derived metabolites are required during infection of the facultative intracellular pathogen *L. monocytogenes*.

This study began with the identification of an *L. monocytogenes* mutant that formed very small plaques in tissue culture caused by disruption of *fold*, which encodes bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (Fig. 1) (16). *Fold* is a bifunctional enzyme that plays a central role in the formation of most folic acid metabolites and is essential in many bacteria, although some bacteria, including *L. monocytogenes*, possess another enzyme,

formyltetrahydrofolate synthetase (Fhs) that also catalyzes the formation of N10-formyltetrahydrofolate. The results of this study showed that *folD* mutants had significant defects during growth in tissue culture cells and a 2-log_{10} defect in the livers of mice. Fhs mutants had no apparent defects during growth in tissue culture cells or during infection of mice, but mutants lacking both *FolD* and Fhs were severely attenuated during infection of mice. Since Fhs uses formate as its substrate and *L. monocytogenes* only produces formate during anaerobic growth (44), bacteria may be experiencing low oxygen conditions *in vivo*, leading to the Fhs-dependent production of N10-formyltetrahydrofolate. *L. monocytogenes* may also benefit from having an alternative way of making N10-formyl-THF by Fhs during its natural route of infection, which requires growth in the low oxygen environment of the intestinal tract.

During a genetic screen looking for $\Delta folD$ suppressors, we found that loss of *purR*, the purine synthesis repressor, restored WT plaque formation in L2 cells, suggesting that *folD* mutants are defective in purine biosynthesis. There are numerous examples of bacterial purine auxotrophs having growth and virulence defects *in vivo* (45). For example, *Staphylococcus aureus* purine auxotrophs cannot replicate in human blood and serum and have defects in animal models (46, 47). Purine auxotrophs of *Bacillus anthracis* are dramatically attenuated in a murine infection model (48). Purine auxotrophic mutants of *Salmonella typhimurium* have a 4-log_{10} higher LD₅₀ compared with WT (49, 50). In contrast to *Salmonella*, *L. monocytogenes* purine auxotrophic mutants are only moderately attenuated in terms of LD₅₀ (51). The $\Delta purEK$ mutants generated in this study exhibited only an approximately 1.5-log_{10} defect in the livers of infected mice (Fig. 4C). Notably, this moderate defect suggests that *L. monocytogenes* can acquire purines from the host environment, although it remains to be investigated whether the defect of *L. monocytogenes* purine mutants is due to insufficient host purines during intracellular and/or extracellular growth. However, although $\Delta purEK$ mutants were attenuated, they were more than 100-fold more virulent than mutants lacking both *FolD* and Fhs in mice, indicating that purine deficiency is not the major defect when *L. monocytogenes* lacks N10-formyl-THF during infection.

In addition to purine biosynthesis, 1C metabolism mediated by folates integrates carbon units from serine, glycine, and sometimes formate to generate thymidine and fMet. In this study, we characterized *L. monocytogenes* mutants unable to synthesize thymidine (*thyA*), fMet (*fmt*), or folate precursor PABA (*pabBC*). Synthesis of dTMP by *ThyA* was essential for *L. monocytogenes* growth in BMMs (Fig. S5A) but dispensable during mouse infection (Fig. S5B). Strikingly, the *fmt* mutants led to the highest degree of attenuation *in vivo*, statistically identical to the $\Delta pabBC$ strain (Fig. 6), indicating that lack of fMet was the major defect of folate deficiency during infection. Previous studies demonstrated that antifolate antibiotics cause thymineless death of bacteria in nutrient-rich condition and inhibit bacteria growth by glycine and purine depletion in nutrient-limited conditions (52, 53). The observations of this study suggest that antifolate drugs also deplete fMet production during bacteria pathogenesis. However, our results do not eliminate the possible contribution of folates to serine and glycine generation during *L. monocytogenes* infection, since serine and glycine not only serve as carbon sources of folates but are also folate end-products if needed (6). Previous ¹³C-isotopologue profiling studies demonstrated that significant fractions (50%–100%) of bacterial amino acids, including serine and glycine, were from the host cell cytosol during intracellular growth (41, 54). We propose that the *in vivo* host environment provides some or all of a number of folate metabolites, including purines, pyrimidines, and amino acids but not fMet.

N-formylmethionine is a modified amino acid used by bacteria and mitochondrial proteins. The formylated methionine carried by initiator tRNA interacts with initiation factor 2 with a higher affinity than an unformylated methionine and thereby fMet facilitates high efficacy and fidelity of peptide translation in bacteria and mitochondria (19, 20, 35). Although fMet is important for *L. monocytogenes* infection, protein translation in *L. monocytogenes* is not strictly dependent on fMet. Mutants lacking *fmt* grew slower in brain heart infusion (BHI) media and were less attenuated in mouse

spleens than livers. Similar to our findings in *L. monocytogenes*, fMet is not essential in many other bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Mycobacterium bovis*, *Staphylococcus aureus*, *Salmonella enterica*, and *Bacillus subtilis*, but loss of *fmt* often leads to slow growth and hypersensitivity to stress (34–39). The differences we observed *in vivo* indicate that the efficiency and fidelity in protein translation are more strictly important for *L. monocytogenes* in the liver than the spleen, although the nature of the differences is not clear. While *fmt* is not essential in many bacteria, it is predicted to be essential in *Streptococcus pneumoniae* and *M. tuberculosis* (55–57). The stringency of fMet dependence in different bacteria under various conditions shall be taken into consideration in the development and evaluation of new antifolate antibiotics.

Although fMet is not essential in many bacteria, removal of the formyl-group from a nascent peptide by PDF is essential across bacterial species, making PDF an attractive target of antibiotics such as actinonin (58). The critical role of folates in fMet generation is also revealed in other bacteria, as mutants resistant to actinonin not only mapped to the *fmt* gene but also to *folD* and *glyA* (59, 60) (see Fig. 1). Although PDF removes fMet from most proteins during translation, some formyl-groups escape removal and are released as short peptides that are key targets of the innate immune system recognized by chemotactic receptors on neutrophils and monocytes, both critical immune effector cells required for constraining and resolving bacterial infections (61–66). Mice deficient in formylated peptide receptors are more susceptible to *L. monocytogenes* infection, probably due to the lack of a rapid wave of neutrophil infiltration that occurs early upon infection (67, 68). Therefore, another potential consequence of antifolate antibiotics is that by blocking production of fMet they also block the release of formylated peptides which would prevent detection of bacteria by phagocytic cells.

MATERIALS AND METHODS

Bacterial growth conditions

All *L. monocytogenes* strains used in this study were derived from WT strain 104035 (Table S3). Strains were propagated in filter-sterilized BHI medium (BD) at 37°C with shaking. When needed, bacterial culture media supplements were used at the following concentrations: streptomycin at 200 µg/mL, chloramphenicol at 7.5 µg/mL, erythromycin at 1 µg/mL, carbenicillin at 100 µg/mL, actinonin (MedChemExpress) at 100 µg/mL, and tetracycline at 2 µg/mL. The *Listeria* synthetic medium (LSM) was made using a previously described recipe with 20 amino acids added (69). When needed adenine was removed from the LSM recipe, or 10 mM sodium formate and 1 mM adenine were added to the medium. All reagents were purchased from Sigma-Aldrich unless specified.

Broth growth curves were performed with *L. monocytogenes* strains from overnight cultures grown at 37°C with agitation (220 rpm). BHI and LSM growth curves were started at an optical density (OD₆₀₀) of 0.05. Growth was spectrophotometrically measured until saturated.

Plasmid and strain construction

All strains used in this study are listed in Table S3. Plasmids were introduced into *L. monocytogenes* by conjugation, using a donor *E. coli* SM10 and a compatible *L. monocytogenes* strain. In-frame deletion of genes was performed using allelic exchange as previously described (70). The double mutants lacking both *folD* and *fhs* were generated by U153 phage transduction (71). Transductants were selected on erythromycin. The Δ *fmt* strain was selected in the presence of 100 µg/mL actinonin. The complemented strains were generated by integrating a pPL2 vector encoding genes under control of the p*Hyper* promoter (21) into mutant strain genomes and selecting for tetracycline-resistant transconjugants (72).

Plaque assay

Plaque assays were performed as previously described (73). In brief, L2 fibroblasts and TIB73 hepatocytes were propagated in high-glucose Gibco Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific) plus 10% fetal bovine serum (FBS) (Avantor-Seradigm), 1 mM sodium pyruvate (Corning), and 2 mM L-glutamine (Corning) were plated 1.2×10^6 cells/well in a six-well tissue-culture treated plate and incubated overnight. Prior to infection, TIB73 was washed twice with phosphate-buffered saline (PBS) and incubated in cell culture medium containing 0.1% FBS. *L. monocytogenes* strains were grown at 30°C overnight without agitation. Bacteria were diluted, 1:500 (for TIB73), 1:10,000 (for L2), in prewarmed media and overlaid on host cells. After 1 h, the cells were washed with PBS twice and overlaid with media plus 0.7% agarose and gentamicin at 10 µg/mL. Cells were stained with Neutral Red for at least 6 h prior to imaging 3-d post-infection. Plaque areas were measured using ImageJ software (74), collecting more than four plaques per strain per experiment.

BMMs and intracellular growth curves

BMMs were prepared by collecting bone marrow from 8-wk-old female C57BL/6J mice (The Jackson Laboratory) and differentiated as previously described (75). All BMMs used in the experiments were cultured in DMEM with 20% FBS, 10% macrophage colony-stimulating factor, 1% L-glutamine, 1% sodium pyruvate, and 14 mM 2-mercaptoethanol (Thermo Fisher Scientific).

The intracellular growth curves were performed as previously described (75). Prior to infection, 3×10^6 BMMs were seeded in 60-mm non-tissue culture-treated dishes (MIDSCI) containing 14 12-mm glass coverslips (Thermo Fischer Scientific) overnight. *L. monocytogenes* strains were grown at 30°C overnight without agitation. On the day of infection, bacteria were diluted in sterile PBS and infected BMMs at an MOI of 0.25. At 30-min post-infection, BMMs were washed twice with PBS. At 1-h post-infection, 50 µg/mL gentamicin was added to the cell culture media to prevent bacteria from growing extracellularly. Bacteria growth was enumerated by plating the average CFUs on three coverslips at each desired time point.

Mouse infections

Eight-wk-old CD-1 mice (Charles River) were infected intravenously with 1×10^5 CFUs in 200 µL of PBS. Animals were sacrificed at 48-h post-infection, and spleens and livers were harvested in 5 or 10 mL 0.1% IGEPAL CA-630 in water, respectively, and plated for enumeration of bacterial burdens. The virulence of $\Delta inlB \Delta actA$ and $\Delta inlB \Delta actA \Delta foID::Tn$ was determined by challenging 1×10^7 CFUs per mouse.

$\Delta foID$ suppressor screen

The $\Delta foID$ strain was mutagenized with *himar1* transposons as previously described (76, 77). The $\Delta foID$ libraries were cultured in BHI for 4 h before infecting L2 cells. Plaques visibly larger than those formed by $\Delta foID$ were picked and recovered in BHI then plaque again. The insertions of suppressor strains were transduced into the unmutagenized $\Delta foID$ parent strain, and the plaque assay was performed again to verify a single transposon insertion could repeat the increased plaque size. Transposon locations were determined using arbitrarily primed PCR as described previously (16).

Disk diffusions

Antibiotic susceptibility was determined as previously described (78). In brief, 10^7 bacteria were immobilized in 3 mL of top-agar (0.8% agar and 0.8% NaCl in water) and evenly distributed on 20-mL BHI-agar plate. Sterile filter paper disks were placed in the center of the

agar plate, soaked with 10 μ L actinonin stock solution (50 mg/mL in DMSO), and incubated overnight at 37°C. Clear zone areas were measured using ImageJ software (74).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.2 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com.

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AUTHOR CONTRIBUTIONS

Ying Feng, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing | Daniel A. Portnoy, Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

DIRECT CONTRIBUTION

This article is a direct contribution from Daniel A. Portnoy, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by Anat Herskovits, Tel Aviv University, and Sebastian Winter, University of California Davis.

ETHICS APPROVAL

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council of the National Academy of Sciences. All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (AUP-2016-05-8811).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Fig. S1 (mBio.01074-23-s0001.pdf). Lack of *foiD* does not lead to defects in hepatocytes.

Fig. S2 (mBio.01074-23-s0002.pdf). Virulence in CD-1 mice of *L. monocytogenes* mutants lacking *foiD* in a PrfA* background.

Fig. S3 (mBio.01074-23-s0003.pdf). Broth growth curve in *Listeria* synthetic media (LSM) supplemented with or without 10 mM sodium formate.

Fig. S4 (mBio.01074-23-s0004.pdf). Bacterial growth on *Listeria* synthetic media (LSM) agarose plates with or without 1 mM adenine added.

Fig. S5 (mBio.01074-23-s0005.pdf). Virulence of *L. monocytogenes* mutants lacking *thyA*.

Table S1 (mBio.01074-23-s0006.pdf). Doubling time of *L. monocytogenes* strains.

Table S2 (mBio.01074-23-s0007.pdf). Transposon mutations that suppress $\Delta fold$ plaque defect.

Table S3 (mBio.01074-23-s0008.docx). List of strains.

REFERENCES

- Bermingham A, Derrick JP. 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *Bioessays* 24:637–648. <https://doi.org/10.1002/bies.10114>
- Hanson AD, Gregory JF. 2002. Synthesis and turnover of folates in plants. *Curr Opin Plant Biol* 5:244–249. [https://doi.org/10.1016/s1369-5266\(02\)00249-2](https://doi.org/10.1016/s1369-5266(02)00249-2)
- White RH. 1988. Analysis and characterization of the folates in the nonmethanogenic Archaeobacteria. *J Bacteriol* 170:4608–4612. <https://doi.org/10.1128/jb.170.10.4608-4612.1988>
- Levin I, Giladi M, Altman-Price N, Ortenberg R, Mevarech M. 2004. An alternative pathway for reduced folate biosynthesis in bacteria and halophilic archaea. *Mol Microbiol* 54:1307–1318. <https://doi.org/10.1111/j.1365-2958.2004.04339.x>
- Shetty S, Varshney U. 2021. Regulation of translation by one-carbon metabolism in bacteria and eukaryotic organelles. *J Biol Chem* 296:100088. <https://doi.org/10.1074/jbc.REV120.011985>
- Ducker GS, Rabinowitz JD. 2017. One-carbon metabolism in health and disease. *Cell Metab* 25:27–42. <https://doi.org/10.1016/j.cmet.2016.08.009>
- Minato Y, Thiede JM, Kordus SL, McKlveen EJ, Turman BJ, Baughn AD. 2015. *Mycobacterium tuberculosis* folate metabolism and the mechanistic basis for para-aminosalicylic acid susceptibility and resistance. *Antimicrob Agents Chemother* 59:5097–5106. <https://doi.org/10.1128/AAC.00647-15>
- Hajian B, Scocchera E, Shoen C, Krucinska J, Viswanathan K, G-Dayananandan N, Erlandsen H, Estrada A, Mikušová K, Korduláková J, Cynamon M, Wright D. 2019. Drugging the folate pathway in *Mycobacterium tuberculosis*: the role of multi-targeting agents. *Cell Chem Biol* 26:781–791. <https://doi.org/10.1016/j.chembiol.2019.02.013>
- Fernández-Villa D, Aguilar MR, Rojo L. 2019. Folic acid antagonists: antimicrobial and immunomodulating mechanisms and applications. *Int J Mol Sci* 20:4996. <https://doi.org/10.3390/ijms20204996>
- Spitzer PG, Hammer SM, Karchmer AW. 1986. Treatment of *Listeria monocytogenes* infection with trimethoprim-sulfamethoxazole: case report and review of the literature. *Rev Infect Dis* 8:427–430. <https://doi.org/10.1093/clinids/8.3.427>
- Winslow DL, Pankey GA. 1982. *In vitro* activities of trimethoprim and sulfamethoxazole against *Listeria monocytogenes*. *Antimicrob Agents Chemother* 22:51–54. <https://doi.org/10.1128/AAC.22.1.51>
- Radoshevich L, Cossart P. 2018. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol* 16:32–46. <https://doi.org/10.1038/nrmicro.2017.126>
- Buchanan RL, Gorris LGM, Hayman MM, Jackson TC, Whiting RC. 2017. A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control* 75:1–13. <https://doi.org/10.1016/j.foodcont.2016.12.016>
- Allerberger F, Wagner M. 2010. Listeriosis: a resurgent foodborne infection. *Clin Microbiol Infect* 16:16–23. <https://doi.org/10.1111/j.1469-0691.2009.03109.x>
- Pamer EG. 2004. Immune responses to *Listeria monocytogenes*. *Nat Rev Immunol* 4:812–823. <https://doi.org/10.1038/nri1461>
- Anaya-Sanchez A, Feng Y, Berude JC, Portnoy DA. 2021. Detoxification of methylglyoxal by the glyoxalase system is required for glutathione availability and virulence activation in *Listeria monocytogenes*. *PLoS Pathog* 17:e1009819. <https://doi.org/10.1371/journal.ppat.1009819>
- Zhang Y, Anaya-Sanchez A, Portnoy DA. 2022. Para-aminobenzoic acid biosynthesis is required for *Listeria monocytogenes* growth and pathogenesis. *Infect Immun* 90:e0020722. <https://doi.org/10.1128/iai.00207-22>
- Rabinowitz JC, Pricer WE. 1962. Formyltetrahydrofolate synthetase. *J Biol Chem* 237:2898–2902. [https://doi.org/10.1016/S0021-9258\(18\)60248-0](https://doi.org/10.1016/S0021-9258(18)60248-0)
- Lahry K, Gopal A, Sah S, Shah RA, Varshney U. 2020. Metabolic flux of N¹⁰-formyltetrahydrofolate plays a critical role in the fidelity of translation initiation in *Escherichia coli*. *J Mol Biol* 432:5473–5488. <https://doi.org/10.1016/j.jmb.2020.08.003>
- Piatkov KI, Vu TTM, Hwang C-S, Varshavsky A. 2015. Formyl-methionine as a degradation signal at the N-termini of bacterial proteins. *Microb Cell* 2:376–393. <https://doi.org/10.15698/mic2015.10.231>
- Shen A, Higgins DE. 2005. The 5' untranslated region-mediated enhancement of intracellular Listeriolysin O production is required for *Listeria monocytogenes* pathogenicity. *Mol Microbiol* 57:1460–1473. <https://doi.org/10.1111/j.1365-2958.2005.04780.x>
- Conlan JW, North RJ. 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. *Infect Immun* 60:5164–5171. <https://doi.org/10.1128/iai.60.12.5164-5171.1992>
- Brockstedt DG, Giedlin MA, Leong ML, Bahjat KS, Gao Y, Luckett W, Liu W, Cook DN, Portnoy DA, Dubensky TW. 2004. *Listeria*-based cancer vaccines that segregate immunogenicity from toxicity. *Proc Natl Acad Sci U S A* 101:13832–13837. <https://doi.org/10.1073/pnas.0406035101>
- Reniere ML, Whiteley AT, Hamilton KL, John SM, Lauer P, Brennan RG, Portnoy DA. 2015. Glutathione activates virulence gene expression of an intracellular pathogen. *Nature* 517:170–173. <https://doi.org/10.1038/nature14029>
- Miner MD, Port GC, Freitag NE. 2008. Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA. *Microbiology (Reading)* 154:3579–3589. <https://doi.org/10.1099/mic.0.2008/021063-0>
- Romick TL, Fleming HP, McFeeters RF. 1996. Aerobic and anaerobic metabolism of *Listeria monocytogenes* in defined glucose medium. *Appl Environ Microbiol* 62:304–307. <https://doi.org/10.1128/aem.62.1.304-307.1996>
- Sah S, Aluri S, Rex K, Varshney U. 2015. One-carbon metabolic pathway rewiring in *Escherichia coli* reveals an evolutionary advantage of 10-formyltetrahydrofolate synthetase (Fhs) in survival under hypoxia. *J Bacteriol* 197:717–726. <https://doi.org/10.1128/JB.02365-14>
- Rappu P, Shin BS, Zalkin H, Mäntsälä P. 1999. A role for a highly conserved protein of unknown function in regulation of *Bacillus subtilis* *purA* by the purine repressor. *J Bacteriol* 181:3810–3815. <https://doi.org/10.1128/JB.181.12.3810-3815.1999>
- Weng M, Nagy PL, Zalkin H. 1995. Identification of the *Bacillus subtilis* *pur* operon repressor. *Proc Natl Acad Sci U S A* 92:7455–7459. <https://doi.org/10.1073/pnas.92.16.7455>
- Saxild HH, Brunstedt K, Nielsen KI, Jarmer H, Nygaard P. 2001. Definition of the *Bacillus subtilis* PurR operator using genetic and bioinformatic tools and expansion of the PurR regulon with *glyA*, *guaC*, *pbuG*, *Xpt-pbuX*, *yqhZ-fold*, and *pbuO*. *J Bacteriol* 183:6175–6183. <https://doi.org/10.1128/JB.183.21.6175-6183.2001>
- Cho B-K, Federowicz SA, Embree M, Park Y-S, Kim D, Palsson BØ. 2011. The PurR regulon in *Escherichia coli* K-12 Mg1655. *Nucleic Acids Res* 39:6456–6464. <https://doi.org/10.1093/nar/gkr307>

32. Sause WE, Balasubramanian D, Irnov I, Copin R, Sullivan MJ, Sommerfield A, Chan R, Dhabaria A, Askenazi M, Ueberheide B, Shopsis B, van Bakel H, Torres VJ. 2019. The purine biosynthesis regulator PurR moonlights as a virulence regulator in *Staphylococcus aureus*. Proc Natl Acad Sci U S A 116:13563–13572. <https://doi.org/10.1073/pnas.1904280116>
33. D'Orazio SEF, Shaw CA, Starnbach MN. 2006. H2-M3-restricted CD8+ T cells are not required for MHC class Ib-restricted immunity against *Listeria monocytogenes*. J Exp Med 203:383–391. <https://doi.org/10.1084/jem.20052256>
34. Meinel T, Guillon JM, Mechulam Y, Blanquet S. 1993. The *Escherichia coli* *fmt* gene, encoding methionyl-tRNA (fMet) formyltransferase, escapes metabolic control. J Bacteriol 175:993–1000. <https://doi.org/10.1128/jb.175.4.993-1000.1993>
35. Steiner-Mosonyi M, Creuzenet C, Keates RAB, Strub BR, Mangroo D. 2004. The *Pseudomonas aeruginosa* initiation factor IF-2 is responsible for formylation-independent protein initiation in *P. aeruginosa*. J Biol Chem 279:52262–52269. <https://doi.org/10.1074/jbc.M408086200>
36. Vanunu M, Lang Z, Barkan D. 2017. The gene *fmt*, encoding tRNA^{fMet}-formyl transferase, is essential for normal growth of *M. Bovis*, but not for viability. Sci Rep 7:15161. <https://doi.org/10.1038/s41598-017-15618-9>
37. Zorzet A, Andersen JM, Nilsson AI, Møller NF, Andersson DI. 2012. Compensatory mutations in *agrC* partly restore fitness *in vitro* to peptide deformylase inhibitor-resistant *Staphylococcus aureus*. J Antimicrob Chemother 67:1835–1842. <https://doi.org/10.1093/jac/dks168>
38. Nilsson AI, Zorzet A, Kanth A, Dahlström S, Berg OG, Andersson DI. 2006. Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. Proc Natl Acad Sci U S A 103:6976–6981. <https://doi.org/10.1073/pnas.0602171103>
39. Cai Y, Chandrangsu P, Gaballa A, Helmann JD. 2017. Lack of formylated methionyl-tRNA has pleiotropic effects on *Bacillus subtilis*. Microbiology (Reading) 163:185–196. <https://doi.org/10.1099/mic.0.000413>
40. Grubmüller S, Schauer K, Goebel W, Fuchs TM, Eisenreich W. 2014. Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism. Front Cell Infect Microbiol 4:156. <https://doi.org/10.3389/fcimb.2014.00156>
41. Sauer J-D, Herskovits AA, O'Riordan MXD. 2019. Metabolism of the Gram-positive bacterial pathogen *Listeria monocytogenes*. Microbiol Spectr 7:4. <https://doi.org/10.1128/microbiolspec.GPP3-0066-2019>
42. Tsai HN, Hodgson DA. 2003. Development of a synthetic minimal medium for *Listeria monocytogenes*. Appl Environ Microbiol 69:6943–6945. <https://doi.org/10.1128/AEM.69.11.6943-6945.2003>
43. Tang Q, Precit MR, Thomason MK, Blanc SF, Ahmed-Qadri F, McFarland AP, Wolter DJ, Hoffman LR, Woodward JJ. 2022. Thymidine starvation promotes c-di-AMP-dependent inflammation during pathogenic bacterial infection. Cell Host Microbe 30:961–974. <https://doi.org/10.1016/j.chom.2022.03.028>
44. Rivera-Lugo R, Deng D, Anaya-Sanchez A, Tejedor-Sanz S, Tang E, Reyes Ruiz VM, Smith HB, Titov DV, Sauer JD, Skaar EP, Ajo-Franklin CM, Portnoy DA, Light SH. 2022. *Listeria monocytogenes* requires cellular respiration for NAD⁺ regeneration and pathogenesis. Elife 11:11. <https://doi.org/10.7554/eLife.75424>
45. Goncheva MI, Chin D, Heinrichs DE. 2022. Nucleotide biosynthesis: the base of bacterial pathogenesis. Trends Microbiol 30:793–804. <https://doi.org/10.1016/j.tim.2021.12.007>
46. Connolly J, Boldock E, Prince LR, Renshaw SA, Whyte MK, Foster SJ. 2017. Identification of *Staphylococcus aureus* factors required for pathogenicity and growth in human blood. Infect Immun 85:e00337-17. <https://doi.org/10.1128/IAI.00337-17>
47. Goncheva MI, Flannagan RS, Heinrichs DE. 2020. *De novo* purine biosynthesis is required for intracellular growth of *Staphylococcus aureus* and for the hypervirulence phenotype of a *purR* mutant. Infect Immun 88:e00104-20. <https://doi.org/10.1128/IAI.00104-20>
48. Samant S, Lee H, Ghassemi M, Chen J, Cook JL, Mankin AS, Neyfakh AA. 2008. Nucleotide biosynthesis is critical for growth of bacteria in human blood. PLoS Pathog 4:e37. <https://doi.org/10.1371/journal.ppat.0040037>
49. Fields PI, Swanson RV, Haidaris CG, Heffron F. 1986. Mutants of *Salmonella typhimurium* that can not survive within the macrophage are avirulent. Proc Natl Acad Sci U S A 83:5189–5193. <https://doi.org/10.1073/pnas.83.14.5189>
50. Leung KY, Finlay BB. 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. Proc Natl Acad Sci U S A 88:11470–11474. <https://doi.org/10.1073/pnas.88.24.11470>
51. Marquis H, Bouwer HGA, Hinrichs DJ, Portnoy DA. 1993. Intracytoplasmic growth and virulence of *Listeria monocytogenes* auxotrophic mutants. Infect Immun 61:3756–3760. <https://doi.org/10.1128/iai.61.9.3756-3760.1993>
52. Kwon YK, Higgins MB, Rabinowitz JD. 2010. Antifolate-induced depletion of intracellular glycine and purines inhibits thymineless death in *E. coli*. ACS Chem Biol 5:787–795. <https://doi.org/10.1021/cb100096f>
53. Estrada A, Wright DL, Anderson AC. 2016. Antibacterial Antifolates: from development through resistance to the next generation. Cold Spring Harb Perspect Med 6:a028324. <https://doi.org/10.1101/cshperspect.a028324>
54. Eylert E, Schär J, Mertins S, Stoll R, Bacher A, Goebel W, Eisenreich W. 2008. Carbon metabolism of *Listeria monocytogenes* growing inside macrophages. Mol Microbiol 69:1008–1017. <https://doi.org/10.1111/j.1365-2958.2008.06337.x>
55. Margolis P, Hackbarth C, Lopez S, Maniar M, Wang W, Yuan Z, White R, Trias J. 2001. Resistance of *Streptococcus pneumoniae* to deformylase inhibitors is due to mutations in *defB*. Antimicrob Agents Chemother 45:2432–2435. <https://doi.org/10.1128/AAC.45.9.2432-2435.2001>
56. Griffin JE, Gawronski JD, Dejesus MA, Proeger TR, Akerley BJ, Sassetti CM. 2011. High-resolution phenotypic profiling defines genes essential for Mycobacterial growth and cholesterol catabolism. PLoS Pathog 7:e1002251. <https://doi.org/10.1371/journal.ppat.1002251>
57. Lamichhane G, Zignol M, Blades NJ, Geiman DE, Dougherty A, Grosset J, Broman KW, Bishai WR. 2003. A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 100:7213–7218. <https://doi.org/10.1073/pnas.1231432100>
58. Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, Margolis PS, Wu C, Ni ZJ, Trias J, White RJ, Yuan Z. 2000. Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 39:1256–1262. <https://doi.org/10.1021/bi992245y>
59. Duroc Y, Giglione C, Meinel T. 2009. Mutations in three distinct loci cause resistance to peptide deformylase inhibitors in *Bacillus subtilis*. Antimicrob Agents Chemother 53:1673–1678. <https://doi.org/10.1128/AAC.01340-08>
60. Yang N, Sun C. 2016. The inhibition and resistance mechanisms of actinonin, isolated from marine *Streptomyces* sp. Nhf165, against *Vibrio anguillarum*. Front Microbiol 7:1467. <https://doi.org/10.3389/fmicb.2016.01467>
61. Southgate EL, He RL, Gao J-L, Murphy PM, Nanamori M, Ye RD. 2008. Identification of formyl peptides from *Listeria monocytogenes* and *Staphylococcus aureus* as potent chemoattractants for mouse neutrophils. J Immunol 181:1429–1437. <https://doi.org/10.4049/jimmunol.181.2.1429>
62. Shi C, Hohl TM, Leiner I, Equinda MJ, Fan X, Pamer EG. 2011. Ly6G+ neutrophils are dispensable for defense against systemic *Listeria monocytogenes* infection. J Immunol 187:5293–5298. <https://doi.org/10.4049/jimmunol.1101721>
63. Okunnu BM, Berg RE. 2019. Neutrophils are more effective than monocytes at phagosomal containment and killing of *Listeria monocytogenes*. Immunohorizons 3:573–584. <https://doi.org/10.4049/immunohorizons.1900065>
64. Witter AR, Okunnu BM, Berg RE. 2016. The essential role of neutrophils during infection with the intracellular bacterial pathogen *Listeria monocytogenes*. J Immunol 197:1557–1565. <https://doi.org/10.4049/jimmunol.1600599>
65. Carr KD, Sieve AN, Indramohan M, Break TJ, Lee S, Berg RE. 2011. Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during *Listeria monocytogenes* infection. Eur J Immunol 41:2666–2676. <https://doi.org/10.1002/eji.201041363>
66. Chen K, Bao Z, Gong W, Tang P, Yoshimura T, Wang JM. 2017. Regulation of inflammation by members of the formyl-peptide receptor family. J Autoimmun 85:64–77. <https://doi.org/10.1016/j.jaut.2017.06.012>
67. Liu M, Chen K, Yoshimura T, Liu Y, Gong W, Wang A, Gao J-L, Murphy PM, Wang JM. 2012. Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*. Sci Rep 2:786. <https://doi.org/10.1038/srep00786>

68. Gao JL, Lee EJ, Murphy PM. 1999. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med* 189:657–662. <https://doi.org/10.1084/jem.189.4.657>
69. Portman JL, Dubensky SB, Peterson BN, Whiteley AT, Portnoy DA. 2017. Activation of the *Listeria Monocytogenes* virulence program by a reducing environment. *mBio* 8:e01595-17. <https://doi.org/10.1128/mBio.01595-17>
70. Camilli A, Tilney LG, Portnoy DA. 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol Microbiol* 8:143–157. <https://doi.org/10.1111/j.1365-2958.1993.tb01211.x>
71. Rae CS, Geissler A, Adamson PC, Portnoy DA. 2011. Mutations of the *Listeria monocytogenes* peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways. *Infect Immun* 79:3596–3606. <https://doi.org/10.1128/IAI.00077-11>
72. Lauer P, Chow MYN, Loessner MJ, Portnoy DA, Calendar R. 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. *J Bacteriol* 184:4177–4186. <https://doi.org/10.1128/JB.184.15.4177-4186.2002>
73. Sun AN, Camilli A, Portnoy DA. 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect Immun* 58:3770–3778. <https://doi.org/10.1128/iai.58.11.3770-3778.1990>
74. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>
75. Nguyen BN, Chávez-Arroyo A, Cheng MI, Krasilnikov M, Louie A, Portnoy DA. 2020. TLR2 and endosomal TLR-mediated secretion of IL-10 and immune suppression in response to phagosome-confined *Listeria monocytogenes*. *PLoS Pathog* 16:e1008622. <https://doi.org/10.1371/journal.ppat.1008622>
76. Zemansky J, Kline BC, Woodward JJ, Leber JH, Marquis H, Portnoy DA. 2009. Development of a mariner-based transposon and identification of *Listeria monocytogenes* determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype. *J Bacteriol* 191:3950–3964. <https://doi.org/10.1128/JB.00016-09>
77. Whiteley AT, Pollock AJ, Portnoy DA. 2015. The PAMP c-di-AMP is essential for *Listeria monocytogenes* growth in rich but not minimal media due to a toxic increase in (p)ppGpp. [corrected]. *Cell Host Microbe* 17:788–798. <https://doi.org/10.1016/j.chom.2015.05.006>
78. Whiteley AT, Garelis NE, Peterson BN, Choi PH, Tong L, Woodward JJ, Portnoy DA. 2017. c-di-AMP modulates *Listeria monocytogenes* central metabolism to regulate growth, antibiotic resistance and osmoregulation. *Mol Microbiol* 104:212–233. <https://doi.org/10.1111/mmi.13622>