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Intracellular *Listeria monocytogenes* Comprises a Minimal but Vital Fraction of the Intestinal Burden Following Foodborne Infection

Grant S. Jones  
*University of Kentucky*, grantjones@uky.edu

Kate M. Bussell  
*University of Kentucky*, kate.bussell@uky.edu

Tanya Myers-Morales  
*University of Kentucky*, tmyer0@uky.edu

Abigail M. Fieldhouse  
*University of Kentucky*

Elsa N. Bou Ghanem  
*University of Kentucky*

See next page for additional authors

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Intracellular *Listeria monocytogenes* Comprises a Minimal but Vital Fraction of the Intestinal Burden following Foodborne Infection

Grant S. Jones, Kate M. Bussell, Tanya Myers-Morales, Abigail M. Fieldhouse, Elsa N. Bou Ghanem, Sarah E. F. D’Orazio

Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky College of Medicine, Lexington, Kentucky, USA

*Listeria monocytogenes* is a highly adaptive bacterial pathogen that can grow in diverse environments, including the cytosol of mammalian cells (1, 2). Much research effort has focused on defining the factors that allow cell-to-cell spread of *L. monocytogenes* without encountering the extracellular environment, since this is thought to be the primary virulence strategy of *L. monocytogenes in vivo*. However, as a facultative intracellular pathogen, *L. monocytogenes* can readily survive and multiply in extracellular spaces, and there may be multiple environments that harbor extracellular bacteria during infection. For example, we previously showed that extracellular *L. monocytogenes* was present in the lamina propria of both the ileum and the colon after foodborne infection (3). The liver, spleen, and placenta were each shown to contain gentamicin-sensitive *L. monocytogenes* during systemic infection of mice or guinea pigs (4, 5). There is also evidence that *L. monocytogenes* replicates extracellularly in the lumen of the murine gallbladder, and it was suggested that the presence of these organisms may prolong intestinal infection if infected bile is released into the small intestine (6). These previous studies indicate that extracellular *L. monocytogenes* can be present in a variety of tissues during mammalian infection; however, the relative proportion of extracellular *L. monocytogenes* and a role in virulence have not been clearly established.

Studies using signature-tagged bacteria have been fundamental in modeling the systemic spread of enteric pathogens by highlighting two routes of spread from the gut (7–9). One route presumably involves direct invasion of the bloodstream and dissemination via the portal vein since it results in colonization primarily in the liver. The mechanisms used by bacteria to promote this invasion are unclear, and it is possible that the use of excessively large inocula or physically traumatic transmission methods can facilitate rapid spread by this route. Bacteria can also reach the spleen and liver after colonizing the draining mesenteric lymph nodes (MLN) (7–9). Melton-Witt et al. showed that this indirect route of spread led to continual seeding of the spleen, presumably due to the flow of efferent lymphatic fluid into the bloodstream via the thoracic duct (9). They found that MLN contained the highest percentage of bacterial clones of all other organs tested and proposed that the MLN represented a bottleneck for a secondary wave of *L. monocytogenes* dissemination to the spleen and liver (9). To spread via this indirect route, *L. monocytogenes* could be transported inside a migratory phagocyte, or it is possible that extracellular *L. monocytogenes* could traffic within afferent lymphatic vessels to the MLN. Lymph nodes also represent an important bottleneck for systemic spread during bacterial infections that occur via other routes of transmission. For example, Gonzalez et al. recently showed that extracellular *Yersinia pestis* disseminated from the dermis of the skin to draining lymph nodes and that only a subset of the clones that reached the lymph nodes could spread to the spleen (10).

Based on retrospective analysis of foodborne listeriosis outbreaks, the infectious dose for humans is thought to be approximately $1 \times 10^6$ CFU (11). Mice appear to be more resistant to oral infection than humans, and this has led investigators to use much higher inocula ($10^9$ to $10^{11}$ CFU) to establish an intestinal infection with *L. monocytogenes*. The relative resistance of mice has been attributed mainly to the species specificity of the interaction between E-cadherin expressed on intestinal epithelial cells and the
TABLE 1 Plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Antibiotic resistance</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKSV7</td>
<td>Temp-sensitive shuttle vector</td>
<td>Cb, Cm</td>
<td>18</td>
</tr>
<tr>
<td>pPL2</td>
<td>Site-specific integration vector</td>
<td>Cm</td>
<td>43</td>
</tr>
<tr>
<td>pIMC3ery, pIMC3tet, pIMC3kan</td>
<td>Site-specific integration vectors with IPTG-induced expression of Ery, Tet, Kan</td>
<td>Cm, Ery, Tet, Kan</td>
<td>44</td>
</tr>
<tr>
<td>pAD1-cGFP</td>
<td>Phpyer-driven expression of GFP (constitutive) in pPL2 derivative</td>
<td>Cm</td>
<td>45</td>
</tr>
<tr>
<td>pTM2</td>
<td>InLA™ subcloned into PalI/BamHI-digested pKSV7</td>
<td>Ch, Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>pGL-cGFP</td>
<td>Phelp from pIMCl subcloned into SacI/EagI-digested pAD1-cGFP</td>
<td>Cm, Cm, Tet</td>
<td>This study</td>
</tr>
<tr>
<td>pAF1a</td>
<td>0.98-kb fragment upstream of lpaI (−988 to −1) in pKSV7</td>
<td>Cm, Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>pAF1-1</td>
<td>1.042-kb fragment from bp 954 of lpaI to bp +1001 downstream adjacent to 0.98-kb fragment upstream of lpaI in pAF1a</td>
<td>Cm, Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>pTML1</td>
<td>lpaI plus 988 bp of upstream DNA from L. monocytogenes InLA™ in Sall/PstI-digested pIMC3ery</td>
<td>Cm, Ery</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Bacterial strains**

**E. coli**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Antibiotic resistance</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>DH5x</td>
<td>F− endA1 hisD17(rKm− mcr−) supE44 thi-11 λ− recA1 gyrA96 relA1 φ80lacZΔM15</td>
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<td>M. N. Starnbach</td>
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<tr>
<td>SURE</td>
<td>mcrA mcrCB mcrF mrr hsdR endA recB recJ</td>
<td>Kan, Tet, Cm</td>
<td>Agilent Technologies</td>
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</tbody>
</table>

**L. monocytogenes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Antibiotic resistance</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGDe</td>
<td>Wild-type L. monocytogenes</td>
<td>None</td>
<td>C. G. Gahan</td>
</tr>
<tr>
<td>ΔinLA</td>
<td>inLA deletion mutant derived from strain EGDe</td>
<td>None</td>
<td>C. G. Gahan</td>
</tr>
<tr>
<td>InLA™</td>
<td>Mouse-adapted L. monocytogenes; InLA S192N; Y369S</td>
<td>None</td>
<td>W.-D. Schubert</td>
</tr>
<tr>
<td>SD1902</td>
<td>InLA™; pIMC3ery</td>
<td>Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>SD2000</td>
<td>ΔinLA; pTM2 (InLA™)</td>
<td>None</td>
<td>This study</td>
</tr>
<tr>
<td>SD2001</td>
<td>SD2000; pIMC3kan</td>
<td>Cm, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>SD2002</td>
<td>SD2000; pIMC3ery</td>
<td>Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>SD2300</td>
<td>SD2000 ΔlpaI</td>
<td>None</td>
<td>This study</td>
</tr>
<tr>
<td>SD2301</td>
<td>SD2300; pIMC3kan</td>
<td>Cm, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>SD2302</td>
<td>SD2300; pTML1 (+ lpaI)</td>
<td>Cm, Ery</td>
<td>This study</td>
</tr>
<tr>
<td>SD2710</td>
<td>SD2000; pGI-cGFP</td>
<td>Cm</td>
<td>This study</td>
</tr>
<tr>
<td>SD2800</td>
<td>ΔinLA; pIMC3tet</td>
<td>Cm, Tet</td>
<td>This study</td>
</tr>
<tr>
<td>SD2900</td>
<td>EGDe; pIMC3ery</td>
<td>Cm, Ery</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a* Cb, carbenicillin; Cm, chloramphenicol; Ery, erythromycin; Kan, kanamycin; Tet, tetracycline.

bacterial surface protein internalin A (InLA) (12, 13). Two approaches have been developed to circumvent this species barrier. The Lecuit group generated “humanized” mice with a single amino acid substitution (E16P) in murine E-cadherin (14). Wollert et al. constructed a mouse-adapted L. monocytogenes strain expressing a modified InLA protein (InLA™) that binds murine E-cadherin with a similar affinity as native InLA binds human E-cadherin (15). Using this mouse-adapted strain, intestinal infection can be established with doses as low as 10⁶ to 10⁷ CFU in mice, but it is not entirely clear how that may affect dissemination to the MLN during foodborne infection (16).

In this study, we used a foodborne model of listeriosis to test how both the mouse-adapted and wild-type L. monocytogenes strains spread to the MLN. Surprisingly, we found that there was very little intracellular L. monocytogenes within the MLN during the first few days after infection. This led us to investigate whether intracellular growth was essential for the dissemination of L. monocytogenes to the MLN or other peripheral tissues. The results presented here using lipoate protein ligase A1 (lpaI1)-deficient bacteria, which are unable to replicate in cells, demonstrate that the minimal fraction of intracellular L. monocytogenes present during the intestinal phase of the infection is crucial for efficient spread to the MLN, spleen, and liver after foodborne infection.

**MATERIALS AND METHODS**

**Bacteria.** L. monocytogenes EGDe and an isogenic inLA deletion (ΔinLA) mutant were provided by Cormac Gahan (University College Cork, Ireland). All L. monocytogenes isolates used in this study were derivatives of this strain. All strains and plasmids used are listed in Table 1. L. monocytogenes was routinely grown in brain heart infusion (BHI) broth or agar (Difco). Intestinal homogenates were plated on BHI agar supplemented with 15 g/liter LiCl and 10 g/liter glycine (BHI/LG) to inhibit the growth of intestinal microbiota. Suspect colonies were confirmed to be L. monocytogenes by plating on CHROMagar Listeria plates. For selection of L. monocytogenes, antibiotics were used at the following concentrations: chloramphenicol, 5 µg/ml (pKSV7) or 7.5 µg/ml (pPL2 or pIMC3); erythromycin, 5 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 10 µg/ml. For selection of Escherichia coli, the following antibiotic concentrations were used: carbenicillin, 100 µg/ml; chloramphenicol, 10 µg/ml (pGI-cGFP) or 100 µg/ml (pTML1); erythromycin, 250 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 10 µg/ml. IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration, 1 mM) was added to induce the expression of antibiotic resistance genes carried on pIMC3 derivatives. Recombinant plasmids were transformed into E. coli DH5α or E. coli SURE. All DNA purifications were done using Qiagen kits. For each strain, aliquots were prepared and stored at −80°C and thawed prior to use in either in vivo or in vitro infections (17).
Construction of recombinant L. monocytogenes strains. PCR primers were purchased from Integrated DNA Technologies (Coralville, IA) or Sigma-Aldrich (St. Louis, MO). The temperature-sensitive shuttle vector pKS7V was used to generate integrations and deletions on the L. monocytogenes chromosome as described previously (18). Electrocompetent L. monocytogenes strains were generated as described by Monk et al. (19) using either filter-sterilized BHI or vegetable peptone broth (VGP; Oxoid) supplemented with 500 mM sucrose to improve bacterial growth rate and electroporation efficiency. After electroporation, bacteria were immediately recovered in 1 ml of room temperature BHI or VGP supplemented with 500 mM sucrose (filter sterilized) and incubated statically for 1.5 h at 37°C (or 30°C for pKS7V) prior to plating.

(i) Isogenic InlAΔ and ΔinlA strains. A 4.4-kb DNA fragment comprising InlAΔ with flanking regions (~1 kb upstream and downstream) was amplified from mouse-adapted L. monocytogenes strain EGDe (15) using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and primers that had PstI and BamHI sites added. The PCR product was ligated into PstI- and BamHI-digested pKS7V and transformed into E. coli SURE, resulting in pTM2. InlAΔ was integrated into the chromosome of L. monocytogenes ΔinlA to create L. monocytogenes SD2000, and the integration was confirmed by determining the DNA sequence of the region spanning 500 bp upstream and 924 bp downstream of InlAΔ.

(ii) GFP-expressing L. monocytogenes. Phyer was excised from pAD-cGFP by digestion with SacI and EagI. The Phyer promoter was amplified from the plasmid backbone (bp 4379 to 4581; GenBank accession number AM940001.1) using primers containing SacI and NotI sites to create compatible ends and ligated to SacI- and EagI-digested pAD-cGFP. The resulting plasmid, pGf-cGFP, was electroporated into SD2000 to create L. monocytogenes SD2710. Green fluorescent protein (GFP) expression was verified using flow cytometry.

(iii) Ilp1A-deficient L. monocytogenes. A DNA fragment spanning from −988 bp to −1 bp upstream of ilp1A was amplified from L. monocytogenes InlAΔ and ligated into HindIII- and XbaI-digested pKS7V, resulting in pFAl1. Next, a 1.042-kb DNA fragment spanning from 954 bp of the ilp1A coding sequence to 1.001 bp downstream of ilp1A was amplified from L. monocytogenes InlAΔ using primers that had EcoRI and XbaI sites added. The PCR product was ligated into EcoRI- and XbaI-digested pFA1, resulting in pFA1-1. L. monocytogenes SD2000 was electroporated with pFA1-1 to create SD2300. After the recovery of Cmu− mutants, the chromosomal deletion of Ilp1A was confirmed by determining the DNA sequence of the region spanning 988 bp upstream and 192 bp downstream of ilp1A. To complement the Ilp1A mutation, Ilp1AΔ plus 988 bp of upstream DNA was amplified from L. monocytogenes InlAΔ and ligated into PstI- and Sall-digested pMCGery, resulting in pFML1.

Mice. Female BALB/CByJ (BALB/C) and C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age. Mice were housed in a specific-pathogen free facility with a 10-h dark cycle. Mice were infected using a natural feeding model as previously described (17, 20). Briefly, mice were transferred to cages with raised wire flooring to prevent coprophagy and fasted for 16 to 24 h. Aliquots of late-exponential-phase L. monocytogenes SD2000 as a negative gating control. For GFP+ L. monocytogenes experiments, flow cytometry data were acquired using an iCyt Synergy sorter and analyzed using FlowJo software (Tree Star). Debris and cell aggregates were excluded by using forward scatter (FSC) versus side scatter (SSC) and FSC-A versus FSC-H parameters, respectively, resulting in “singlets.” The percentage of GFP+ cells in each population was determined by using mice infected with L. monocytogenes SD2001 as a negative gating control.

Determination of intracellular and extracellular CFU. For phagocyte enrichment experiments, the number of extracellular bacteria (“supermatant”) was determined after MLN cells were centrifuged for 10 min at 300 × g. The supernatant was collected, and serial dilutions were prepared in sterile water and plated on BHI. The number of intracellular bacteria associated with enriched phagocyte populations was determined after gentamicin-treated cells were lysed in sterile water.

Following enzymatic processing of MLN, intracellular L. monocytogenes was identified by centrifuging a sample of MLN cells (10% of total volume) for 6 min at 300 × g. The cell pellet was suspended in 1 ml of RP-10 medium supplemented with gentamicin. Cells were incubated statically for 20 min at 37°C in 7% CO2, and then washed once with RP-10 medium. The cells were centrifuged for 8 min (20,000 × g) and suspended in sterile water before being plated on BHI. To quantify L. monocytogenes, a sample of untreated MLN cells (10% of total volume) was centrifuged for 8 min at 20,000 × g. Bacteria and cells were suspended in sterile water, and serial dilutions were plated on BHI. The number of extracellular L. monocytogenes was calculated by subtracting the number of intracellular CFU from the total number of CFU recovered from the MLN of each mouse.
For determination of minimal bactericidal concentration, exponential-phase *L. monocytogenes* SD2000 was resuspended in PBS at $4 \times 10^5$ ml and seeded in triplicate (25 μl/well) in a 96-well plate. RP-10 medium supplemented with various concentrations of gentamicin was added, and the plate was incubated for 20 min at 37°C in 7% CO₂. Serial dilutions were prepared in sterile water and plated on BHI. For *in vivo* gentamicin experiments, mice were given a single intraperitoneal (i.p.) injection of 2 mg gentamicin in PBS and control mice received an injection of 500 μl PBS. To determine if residual gentamicin present in tissue homogenates could kill *L. monocytogenes* during *in vitro* processing, uninfected mice were treated with gentamicin or PBS for 12 h and then tissue homogenates were prepared. *L. monocytogenes* SD2000 (1.5 $\times 10^8$ CFU) was added to each homogenate and then incubated for 1 h on ice (to mimic normal harvest conditions) before plating on BHI.

**Lipoate starvation.** For lipoate starvation, *L. monocytogenes* was grown in IMM, which was prepared fresh from concentrated stocks and used within 2 weeks (21). Fresh isolated colonies grown on BHI agar were used to inoculate 3 ml of BHI broth in 16- by 150-mm glass tubes and incubated at 37°C in a rotating rack for 8 h. Growth was normalized by optical density at 600 nm (OD₆₀₀), and bacteria were washed with PBS before inoculating 20 ml IMM without lipoic acid and incubated for ~16 h at 37°C shaking. For growth curves, lipoate-starved bacteria were back-diluted to an OD₆₀₀ of 0.05 and growth was monitored over time. Aliquots of lipoate-starved *L. monocytogenes* were prepared as previously described for animal infections (17) with the exceptions that *L. monocytogenes* was suspended in IMM without lipoic acid before storage at ~80°C and was recovered in IMM before use in infections.

**Intracellular growth assays.** J774 cells were maintained in RP-10 medium supplemented with penicillin and streptomycin. The day before infection, cells were washed once with warm RPMI and suspended in IMM without antibiotics. Cells ($5 \times 10^6$/ml) were seeded on 24-well plates. Lipoate-starved *L. monocytogenes* was added at various multiplicities of infection (MOI), and the plates were centrifuged for 5 min at 300 x g to synchronize infection. After 30 min, cells were washed three times with pre-warmed PBS. Cells were suspended in RP-10 medium plus 10 μg/ml gentamicin and incubated at 37°C in 7% CO₂. At each time point indicated, coverslips were removed, placed in 5 ml sterile water, and vortexed for 30 s, and serial dilutions were plated on BHI agar.

**Statistics.** Statistical analysis was performed using Prism software for Macintosh (version 6; Graph Pad), and the specific tests used are indicated in each figure legend. *P* values of <0.05 were considered significant and are indicated as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

**RESULTS**

Wild-type *L. monocytogenes* EGDe spreads beyond the intestine similarly to a murinized strain after foodborne infection in mice. The mouse-adapted strain of *L. monocytogenes* is ideal for studying oral transmission of listeriosis in mice because it can be used at doses that are 10- to 100-fold lower than the $10^9$ CFU typically required to establish intestinal infection with wild-type *L. monocytogenes* EGDe (3, 15). However, Tsai et al. recently showed that the InlA<sup>m</sup> protein expressed by the mouse-adapted strain altered the tropism for *L. monocytogenes* in the intestinal epithelium by promoting binding to N-cadherin as well as E-cadherin (16). It is unclear how this may affect subsequent dissemination to peripheral tissues and the remainder of the infection in mice. To find out if wild-type *L. monocytogenes* EGDe had a similar course of infection to that previously published using the murinized strain (3, 15), we fed both susceptible BALB/cByJ (BALB) and resistant C57BL/6 (B6) mice $3 \times 10^9$ CFU and determined the bacterial loads in various tissues over the course of 8 days. As expected, a small percentage of the initial inoculum was recovered from the ileum and colon 24 h postinfection (Fig. 1A). Three days postinfection, the colon harbored more *L. monocytogenes* than the ileum in both mouse strains. These findings were similar to what was previously observed in the gut after mice were fed a 10-fold-lower dose ($10^6$ CFU) of the mouse-adapted *L. monocytogenes* strain (3). However, *L. monocytogenes* EGDe did not continue to multiply exponentially in the intestines of susceptible BALB mice (Fig. 1A).
Once L. monocytogenes EGDe disseminated beyond the intestines, the growth curves in the spleen and liver (Fig. 1A) closely mimicked previously published time course experiments that were performed using L. monocytogenes InlA<sup>m</sup> (3). Exponential growth of L. monocytogenes was observed in the gallbladders of susceptible BALB, but not resistant B6 mice (Fig. 1A). The peak bacterial burden occurred 5 days postinfection in all three of these tissues. Small numbers of L. monocytogenes EGDe were detected in the brain starting at 5 days postinfection in both BALB and B6 mice (Fig. 1A). Together, these results suggested that oral infection with wild-type L. monocytogenes resulted in a pattern of dissemination from the gut similar to that of the mouse-adapted strain shown in a previous study, but wild-type L. monocytogenes required a 10-fold-higher inoculum to achieve bacterial loads comparable to that of the InlA<sup>m</sup> strain.

Although the course of infection in mice fed 10<sup>9</sup> CFU L. monocytogenes EGDe was similar to previous results using L. monocytogenes InlA<sup>m</sup>, there was one noticeable difference. During foodborne infection with 10<sup>9</sup> CFU of the mouse-adapted strain, there was a reproducible delay of at least 36 to 48 h until L. monocytogenes reached the spleen or liver (3). In contrast, when mice were fed 3 × 10<sup>8</sup> L. monocytogenes EGDe CFU, spread to the liver occurred within 18 h in about half of the mice (Fig. 1B). To determine if this was related to the bacterial strain difference or the higher inoculum size, BALB and B6 mice were fed 3 × 10<sup>8</sup> CFU of the mouse-adapted strain, and total CFU in the spleen and liver was determined 1 day later. As shown in Fig. 1B, L. monocytogenes InlA<sup>m</sup> spread to the liver in 3 of 8 mice tested. Thus, an inoculum of 10<sup>9</sup> CFU promoted rapid spread to the liver, regardless of which bacterial strain was used, suggesting that inocula greater than or equal to 10<sup>9</sup> CFU may overwhelm innate immune defenses and promote direct spread from the gut to the liver via the portal vein. Therefore, a key advantage of using the L. monocytogenes InlA<sup>m</sup> strain is that it can be fed to mice at low enough doses to study the bottleneck that occurs in the gut as bacteria spread via the MLN to the spleen.

The majority of L. monocytogenes in the MLN was extracellular. We previously showed that when BALB mice were coinfected with wild-type EGDe and the mouse-adapted strain, the wild-type L. monocytogenes had a bimodal distribution in the MLN, with some mice having low or undetectable numbers while others had high bacterial loads comparable to mice infected with L. monocytogenes InlA<sup>m</sup> (3). The bimodal distribution suggested that there was a bottleneck in the intestinal lamina propria and that expression of InlA<sup>m</sup> enhanced dissemination of L. monocytogenes to the MLN. One mechanism to explain this could be that InlA<sup>m</sup> promoted invasion of a migratory cell type that could transport intracellular L. monocytogenes from the intestinal lamina propria to the MLN.

To test this, we assessed the amount of L. monocytogenes associated with either CD11c<sup>+</sup> cells, which represent primarily migratory subsets of dendritic cells, or F4/80<sup>+</sup> cells, which represent mainly tissue-resident macrophages. BALB mice were coinfected with an equal ratio of L. monocytogenes InlA<sup>m</sup> and an InlA deletion (ΔinlA) mutant strain that had been tagged with two different antibiotic resistance genes. Three days postinfection, CD11c<sup>+</sup> or F4/80<sup>+</sup> cells were enriched from the MLN by positive selection, and the total number of either InlA<sup>m</sup> or ΔinlA mutant CFU associated with these cells was determined. As shown in Fig. 2A, CD11c-enriched MLN cells harbored approximately 10-fold more InlA<sup>m</sup> than ΔinlA mutant CFU. In contrast, similar numbers of InlA<sup>m</sup> and ΔinlA mutant CFU were recovered from F4/80-enriched MLN cells (Fig. 2B). These results suggested that expression of InlA<sup>m</sup> may enhance invasion of dendritic cells but not macrophages. However, the combined number of L. monocytogenes CFU associated with either CD11c<sup>+</sup> or F4/80<sup>+</sup> cells was surprisingly small, ranging from ~400 to 2,300 CFU per mouse. Typically, the total number of L. monocytogenes cells found in the MLN 3 days postinfection for either the wild type (Fig. 2C) or the mouse-adapted strain (3) is approximately 10<sup>5</sup> CFU. To find out if the remaining CFU were associated with other cell types or were simply present in the extracellular environment, supernatant fractions from the processing of the CD11c<sup>+</sup> and F4/80<sup>+</sup> enriched cells were collected and plated. As shown in Fig. 2A and B, the majority of the total CFU was found in the supernatant. Since the number of CFU recovered from the intracellular and supernatant fractions was approximately equal to the total MLN burdens shown in Fig. 2C, this suggests that adherent CFU were likely to be only a minor proportion of L. monocytogenes in the MLN.

It was possible that the large proportion of extracellular L. monocytogenes in the MLN was an artifact of the ex vivo processing

![FIG 2](http://iai.asm.org/) Intracellular L. monocytogenes was a minor proportion of total CFU in the MLN. BALB mice were coinfected with equal proportions of L. monocytogenes SD1902 (InlA<sup>m</sup>) and L. monocytogenes SD2800 (ΔinlA) for a total inoculum of 5 × 10<sup>9</sup> CFU, and MLN were harvested 3 days postinfection. The number of extracellular CFU (supernatant) and the total number of intracellular (gentamicin<sup>+</sup>) CFU associated with either CD11c<sup>+</sup> (A) or F4/80<sup>+</sup> (B) MLN cells are shown. CFU for individual mice are shown; horizontal lines indicate mean values. Statistical significance was determined by two-tailed Mann-Whitney analysis. (C) BALB mice were infected with 2 × 10<sup>5</sup> to 3 × 10<sup>5</sup> CFU of L. monocytogenes SD2900 (Ery<sup>r</sup> EGDe), and the total number of CFU in the MLN was determined. Mean values (± SD) for pooled data from 9 mice (1 and 8 days postinfection [dpi]), 4 mice (2 dpi), or 10 mice (3 and 5 dpi) are shown. Dashed lines indicate limits of detection.
The majority of *L. monocytogenes* in the MLN was predominantly gentamicin sensitive. (A) BALB mice were fed 3 × 10^6 to 8 × 10^8 CFU *L. monocytogenes* InLA<sup>m</sup>. Mean percentages (±SD) of intracellular (gent<sub>10<sup>6</sup></sub>) and extracellular CFU in collagenase-treated MLN of mice harvested 2 (n = 6) and 3 (n = 11) days postinfection are shown. (B) Mean number (±SD) of *L. monocytogenes* SD2000 CFU surviving after 20 min of incubation at 37°C with CO2 at the indicated concentration of gentamicin. (C) BALB mice were fed 2 × 10^8 to 7 × 10^8 CFU *L. monocytogenes* InLA<sup>m</sup>, and MLN were dissociated using only enzymatic digestion (n = 7 to 11 per group). Mean percentages (±SD) of intracellular (gent<sub>10<sup>6</sup></sub>/H11006) and extracellular CFU in the MLN are shown. (D) BALB mice were fed 3 × 10<sup>6</sup> or 3 × 10<sup>8</sup> *L. monocytogenes* EGDe (n = 4 per group), and MLN were dissociated using enzymatic digestion 3 days postinfection. Mean percentages (±SD) of intracellular (gent<sub>10<sup>6</sup></sub>/H9262) and extracellular CFU in the MLN are shown. (E) BALB mice were fed 2 × 10<sup>8</sup> to 5 × 10<sup>8</sup> CFU of *L. monocytogenes* SD2710 (GFP<sup>+</sup>) or *L. monocytogenes* SD2001. MLN were collected 2 days postinfection and analyzed by flow cytometry. Representative dot plots showing the percentage of GFP<sup>+</sup> cells in each myeloid-derived MLN population (n = 5 mice) are shown. Statistical significance was determined by two-tailed Mann-Whitney analysis. (H) Symbols indicate the total number of GFP<sup>+</sup> cells identified in the MLN of each mouse; the shaded bar represents the mean value for the group.

Gentamicin treatment is commonly used to selectively kill extracellular bacteria; however, gentamicin can penetrate mammalian cells when used at high enough concentrations. To determine the minimal bactericidal concentration for our experimental conditions, we exposed 1 × 10<sup>8</sup> CFU of *L. monocytogenes* InLA<sup>m</sup> to increasing concentrations of gentamicin. The lowest concentration that killed 100% of the inoculum was 10 μg/ml (Fig. 3B). We repeated the analysis of intracellular and extracellular *L. monocytogenes* in the MLN 2 and 3 days postinfection using 10 μg/ml gentamicin and recovered approximately 10-fold-higher percentages of intracellular *L. monocytogenes* (Fig. 3C versus 3A). Thus, 10% of the bacterial burden in the MLN was intracellular, and approximately 90% was extracellular (gentamicin resistant). To ensure that these results applied to both the wild-type and murinized strains, we fed groups of mice two different doses of *L. monocytogenes* EGDe. Slightly higher proportions of intracellular *L. monocytogenes* EGDe than *L. monocytogenes* InLA<sup>m</sup> were found, but the majority of the bacteria in the MLN were still extracellular (Fig. 3D).

To confirm these findings, flow cytometry was used to quantify cell-associated *L. monocytogenes* in the MLN. Mice were fed 10<sup>8</sup> CFU of either an *L. monocytogenes* InLA<sup>m</sup> derivative that constitutively expressed GFP (*L. monocytogenes* SD2710) or a vector control strain (*L. monocytogenes* SD2001). As expected, only a small fraction of MLN cells were infected. It was difficult to detect the small number of GFP<sup>+</sup> cells when bulk populations were analyzed, due to some autofluorescence of MLN cells using a 525/50-nm filter (Fig. 3E). Therefore, myeloid-derived cell subsets were analyzed by gating on either CD11c<sup>hi</sup> cells (dendritic cells) or CD11b<sup>−</sup>CD11c<sup>−</sup> cells, which included monocytes, macrophages, and neutrophils (Fig. 3F). As shown in representative dot
plots (Fig. 3E) and in collected data from groups of mice (Fig. 3G), clear shifts in GFP expression were visible when analyzing these myeloid-derived subsets. However, even when these two subsets were combined, an average of only $4.3 \times 10^4$ GFP+ MLN cells were detected in each mouse (Fig. 3H), even though the total number of *L. monocytogenes* recovered from the MLN in these experiments ranged from $1.5 \times 10^5$ to $7 \times 10^5$ CFU. This suggested either that each infected cell contained at least 4 and up to 16 bacteria or that some portion of the total CFU burden was extracellular.

**In vivo treatment with gentamicin significantly reduced *L. monocytogenes* burdens following foodborne challenge.** Since all *in vitro* approaches involve some degree of processing and handling, we next used an *in vivo* approach to assess the degree to which extracellular *L. monocytogenes* was present in the gut and the draining lymph nodes. To do this, mice were fed *L. monocytogenes* InlAm, and 3 days later, half of the animals received an intraperitoneal injection of 2 mg of gentamicin and the other half were injected with PBS. The number of viable bacteria present in each tissue was determined 12 h later. As shown in Fig. 4A, significantly lower numbers of *L. monocytogenes* were recovered from mice treated with gentamicin than from control mice. The average number of *L. monocytogenes* CFU recovered from the colons of gentamicin-treated mice represented only 14% of the bacterial load in the colons of PBS-treated mice. Likewise, a significantly reduced number of CFU were observed in the MLN, spleens, and livers of mice treated with gentamicin (Fig. 4A). In each tissue, gentamicin-sensitive *L. monocytogenes* represented approximately 60 to 80% of the bacterial load recovered. It was possible that the lower number of CFU recovered from gentamicin-treated mice occurred because of residual gentamicin present in the tissue homogenates, which may have killed intracellular *L. monocytogenes* that was released during cell lysis and plating. To test this, uninfected mice were treated with gentamicin or PBS. Tissue homogenates were prepared 12 h later and inoculated *in vitro* with *L. monocytogenes*. As shown in Fig. 4B, there was no inhibition of *L. monocytogenes* growth in the gentamicin-treated homogenates compared to the PBS-treated homogenates. Therefore, the results of both the *in vitro* and *in vivo* approaches strongly suggested that intracellular *L. monocytogenes* represented only a minimal fraction of the total *L. monocytogenes* burden during the first few days following oral challenge.

**Intracellular replication was not required for *L. monocytogenes* to establish intestinal infection.** The relatively small proportion of intracellular *L. monocytogenes* in the intestines and MLN led us to question if intracellular replication was necessary during the intestinal phase of foodborne infection. Therefore, we constructed a mutant strain of mouse-adapted *L. monocytogenes* that had a defect only in intracellular replication, with normal growth in extracellular environments. O’Riordan et al. previously showed that lipoate protein ligase A1 (LplA1)-deficient *L. monocytogenes* strains were unable to scavenge lipoate from host cell-derived lipoyl peptides and, thus, had a significant defect in intracellular growth in J774 macrophages (22). Therefore, we generated an InlAm*-expressing Δ*lplA1* mutant to study when intracellular replication was important during foodborne infection.

*L. monocytogenes* stores large quantities of lipoic acid, and the intracellular growth phenotype of the Δ*lplA1* mutant can be observed only when these reserves have been depleted (23). To do this, *L. monocytogenes* was grown overnight in improved minimal media (IMM) in the absence of lipoic acid. *L. monocytogenes Δ*lplA1* grew as well as either the parental strain InlAm* (L. monocytogenes SD2000) or the complemented strain (+*lplA1*) when lipoate-starved bacteria were transferred to rich media (BHI) (Fig. 5A). During further growth in minimal medium with limiting quantities of nutrients, all strains had an extended lag phase of 12 to 15 h (Fig. 5B and C). Lipoate-starved *L. monocytogenes* InlAm* required supplementation with at least 0.25 nM lipoic acid to reach late exponential phase (Fig. 5B). As expected, none of the lipoate-starved bacteria grew in minimal medium lacking lipoate, and all three strains reached similar optical densities after the addition of 25 nM lipoic acid (Fig. 5C). To verify that the lipoate-starved Δ*lplA1* mutant did not replicate inside mammalian cells, we conducted intracellular growth assays with J774 macrophages.

![FIG 4](https://iai.asm.org/iai.83.8.3152-3167/Fig4.png)
Lipoate-starved \textit{L. monocytogenes} \(\Delta\text{lplA1}\) strains were able to survive but not grow in these cells (Fig. 5D). In contrast, the complemented mutant grew exponentially in \(J774\) cells after an extended lag phase.

Since lipoate-starved \textit{L. monocytogenes} had an extended lag phase both in liquid medium and in \(J774\) cells, we were concerned that this might reduce the ability of the bacteria to establish infection in mice. To test this, BALB mice were fed an equal ratio of antibiotic-tagged parental strain \textit{L. monocytogenes} InlA\(^{\text{TM}}\) that was grown either in rich medium (BHI) or under lipoate starvation conditions (IMM without lipoic acid). These bacteria were fully capable of scavenging lipoate from host cells but were transmitted to the gastrointestinal tract in a lipoate-starved state that might occur. The ileum and colon from each mouse were harvested 20 h later and flushed extensively, and the total number of each bacterial strain present in the flushed tissue was determined by plating on BHI supplemented with either erythromycin or kanamycin. As shown in Fig. 5E, the lipoate-starved \textit{L. monocytogenes} InlA\(^{\text{TM}}\) did not have a defect in establishing intestinal invasion and, in fact, had a slight advantage compared to \textit{L. monocytogenes} InlA\(^{\text{TM}}\) grown in BHI. Therefore, although lipoate starvation did cause \textit{L. monocytogenes} to have an extended lag phase during growth in lipoate-limiting conditions, it did not reduce bacterial fitness to colonize the intestinal tract in mice.

Next, to establish the importance of intracellular growth during the gastrointestinal phase of foodborne listeriosis, BALB mice were fed lipoate-starved \textit{L. monocytogenes} \(\Delta\text{lplA1}\) mutant and the complemented strain mixed in a 1:1 ratio. The total number of each strain present in either the ileum or the colon was determined and is presented as a competitive index (CI) ratio in Fig. 5F. The \(\Delta\text{lplA1}\) strain had very little defect in establishing infection in the colon, but on average, 14-fold fewer \(\text{lplA1}\) mutant bacteria were recovered from the ileum 1 day postinfection. By 3 days after infection, however, the complemented strain outcompeted the mutant by an average of \(~500\)-fold in the ileum and \(~30\)-fold in the colon (Fig. 5F). Together, these results suggested that intracellular replication was not necessary for \textit{L. monocytogenes} to establish intestinal infection in mice but that the ability to grow inside a host cell strongly promoted persistence, particularly in the small intestine.

\textbf{Intracellular replication of \textit{L. monocytogenes} was vital for spread beyond the intestine.} To find out if intracellular replication was essential for \textit{L. monocytogenes} to disseminate beyond the intestine, CFU counts in the MLN, spleen, and liver were determined 3 days after coinfection with a 1:1 mixture of lipoate-starved \textit{L. monocytogenes} \(\text{lplA1}\) and the complemented strain. As shown in Fig. 6A, \textit{L. monocytogenes} \(\text{lplA1}\) had a dramatic defect (2,500-fold) in reaching the MLN compared to the complemented (+\text{lplA1}) strain. The MLN is thought to be a bottleneck for further dissemination to the spleen and liver via the bloodstream, and accordingly, no \textit{L. monocytogenes} \(\text{lplA1}\) was recovered from the liver 3 days postinfection, while an average of \(10^6\) CFU of the +\text{lplA1} complemented strain was detected in the spleen (Fig. 6B).

FIG 5 Lipoate-starved \textit{L. monocytogenes} grew slowly in minimal medium but was able to establish intestinal infection in mice. Freshly streaked colonies of \textit{L. monocytogenes} were incubated in IMM(-) overnight to deplete lipoate reserves and then diluted into fresh medium with or without lipoic acid (A to C) or frozen at \(-80^\circ\text{C}\) prior to infection of cells (D) or mice (E). (A) The rates of growth in a rich medium (BHI) with shaking at 37°C were similar for \textit{L. monocytogenes} SD2301 (\(\Delta\text{lplA1}\) mutant), the complemented mutant \textit{L. monocytogenes} SD2302 (+\text{lplA1}), and the parental strain (\textit{L. monocytogenes} SD2000). (B) Lipoate-starved \textit{L. monocytogenes} SD2000 had a long lag phase in IMM but achieved exponential growth in at least 0.25 nM lipoic acid. (C) The \textit{IplA1} deletion strain (\(\Delta\text{lplA1}\)) and the complemented mutant (+\text{lplA1}) did not grow in the absence of exogenous lipoate but reached growth densities similar to those of the parental strain in IMM supplemented with 25 nM lipoic acid. (D) \(J774\) cells were infected in triplicate, and the mean number (=SD) of gentamicin-resistant (10 \(\mu\text{g}/\text{ml}\) CFU per well was determined over time. Statistical significance was determined by Mann-Whitney analysis. For panels A to D, data from one of at least two separate experiments are shown.

(E) Mice were infected with a 1:1 ratio of \textit{L. monocytogenes} SD2001 (Kan’ InlA\(^{\text{TM}}\) prepared in IMM) and \textit{L. monocytogenes} SD2002 (Ery’ InlA\(^{\text{TM}}\) prepared in BHI) for a total inoculum of \(2 \times 10^8\) CFU. The population of tissue-associated \textit{L. monocytogenes} in the ileum or colon was determined 24 h later and is shown both as a competitive index (CI) and as the absolute number of cell-associated CFU recovered from each mouse. (F) BALB mice were infected with a 1:1 mixture of \textit{L. monocytogenes} SD2301 (\(\Delta\text{lplA1}\)) and the complemented mutant \textit{L. monocytogenes} SD2302 (+\text{lplA1}) for an average total inoculum of \(8 \times 10^8\) CFU. The tissue-associated \textit{L. monocytogenes} population was determined and is shown as a CI; the fold difference from the hypothetical value of 1.0 is shown in the parentheses above. Pooled data from at least two separate experiments are shown.

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As shown in Fig. 6C, very few of the both in the gut and in peripheral tissues 4 days after coinfection. Therefore, it was possible dissemination to the MLN was greatly reduced for 

Likewise, only a few mice had any 

FIG 6 


discussion

Invasion of mammalian cells is considered to be the main virulence strategy of 

ports using other small animal models of listeriosis (4, 5). We conclude from these studies that intracellular is actually a minimal component of the bacterial load during the early stages of infection. Silva and Pestana recently suggested that the extracellular phase of many facultative intracellular pathogens could be important for virulence and that the presence of extracellular bacteria was greatly underappreciated in most infection models (24). The data presented here suggest that virulence strategies used by extracellular may be particularly important for initial colonization and survival in the gastrointestinal tract. In that regard, Travier et al. recently reported that ActA, the sole 

In this study, intracellular was not crucial for bacterial survival in the gastrointestinal tract until relatively late in the infection (3 days after foodborne challenge). During this time frame, 

The primary strategy used here for determining the proportion of intracellular was treatment with gentamicin, an aminoglycoside that does not penetrate mammalian cells at low concentrations. Although this is a widely used technique in the field of bacterial pathogenesis, one must be cautious in interpreting data from gentamicin protection assays, since excessively high concentrations of gentamicin can kill or stress intracellular bacteria, possibly due to the pinocytosis of extracellular fluid containing 

During this time frame, 

The fate of once the bacteria cross the mucosal barrier is not well understood and may depend on the route used for invasion. For example, uptake via M cells in the small intestine would result in deposition within a lymphoid follicle or Peyer’s patch, where rapid phagocytosis is likely to occur by a unique subset of dendritic cells localized in the subepithelial dome (28, 29). In contrast, 

The peritoneal cavity is a functionally distinct environment with concentrations of 

The MLN during foodborne infection.

Invasion of mammalian cells is considered to be the main virulence strategy of 

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Invasion of mammalian cells is considered to be the main virulence strategy of
tissue was 198 ng/mg protein (41,42). Therefore, it is likely that combined results from both the defects in fragile, heavily infected cells that lysed in vitro. However, the combined results from both the in vitro and in vivo gentamicin treatments strongly suggest that a large proportion of the L. monocytogenes burden in the gut is extracellular during the first few days following foodborne challenge.

To address the role of intracellular L. monocytogenes during the early stages of infection in the gut, we used lipoate-starved L. monocytogenes ΔplA1 mutants. These bacteria were able to invade cells, escape from the vacuole, and mediate actin-based motility to avoid autophagy (34–36) but could not replicate efficiently due to an inability to scavenge lipoate from the host cell cytosol. A more common approach to study L. monocytogenes that cannot survive intracellularly has been to use listeriolysin O (LLO) mutants that are killed following invasion of murine cells because they cannot mediate escape from the vacuole (37, 38). But our primary objective was to determine if intracellular localization of L. monocytogenes was needed for dissemination to the MLN, and use of an LLO mutant could abort infection prior to colonization of the lamina propria (39, 40), making it difficult to distinguish invasion defects from dissemination defects. When grown in minimal defined medium, lipoate-starved L. monocytogenes ΔplA1 required a minimum of 0.25 nM lipoate to replicate (Fig. 5B). Although the concentration of lipoic acid present in the tissues of mice is unknown, it was previously reported that concentrations of lipoic acid in plasma in healthy humans ranged from 3.1 to 50 ng/ml (~15 to 242 nM) and the concentration in normal human liver tissue was 198 ng/mg protein (41, 42). Therefore, it is likely that during in vivo growth in mice, extracellular L. monocytogenes ΔplA1 can readily obtain free lipoate from the host and that the persistence and dissemination defects that we observed were due to a lack of intracellular replication.

It is possible that invasion of a particular cell type in the intestinal lamina propria is critical for L. monocytogenes to be transported to the MLN. For example, InLA may enhance invasion into a subset of dendritic cells that express E-cadherin. E-cadherin-positive dendritic cells were shown to be recruited to the intestine and MLN using a T cell-mediated model of colitis in mice (46), but their role in inflammation induced by infection has not yet been explored. However, the ΔplA1 mutant data reported here suggest that it is replication, and not just intracellular localization of L. monocytogenes, that is important for persistence and spread beyond the gut. Exponential replication in the cytosol of a more permissive cell type in the gut, such as a tissue-resident macrophage, may serve as an amplification step to increase L. monocytogenes burdens above a particular threshold that is needed for efficient dissemination. These results further highlight the importance of studying dissemination in the context of natural foodborne transmission, using relatively low doses, because innate immune defenses that limit bacterial spread can be overwhelmed by excessively large intraepithelial inocula. In support of this idea, Gonzalez et al. showed that the dermis represented a significant bottleneck for the spread of Y. pestis to the skin-draining lymph nodes, but this bottleneck was partially ablated when higher doses of bacteria were used (10).

Extracellular L. monocytogenes predominated in the gut whether we infected mice with wild-type L. monocytogenes EGDe or with a mouse-adapted derivative of this strain. Tsai et al. recently raised the concern that InLA-expressing strains of L. monocytogenes may cause more inflammation than wild-type L. monocytogenes during intestinal infection and that this could lead to prolonged colonization in the gut (16). In this regard, we showed that foodborne transmission of wild-type L. monocytogenes resulted in a more transient infection of the intestines than was previously observed with L. monocytogenes InLA-expressing strains. Likewise, we did find that approximately twice as much L. monocytogenes InLA as wild-type L. monocytogenes EGDe was extracellular, and it is possible that this was due to increased cellular damage in the inflamed gut. However, during infection with the wild-type strain, we still found that the vast majority of L. monocytogenes in the MLN (70 to 80%) was extracellular, and the kinetics of systemic spread and clearance in peripheral tissues was similar to that previously published for the mouse-adapted strain (3). We propose that extracellular localization of L. monocytogenes during the early stages of intestinal infection is a feature that is likely to be shared by all L. monocytogenes strains and that the large proportion of extracellular bacteria in the gut may be involved in promoting dissemination.

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