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Using a Bacterial Pathogen to Probe for Cellular and Organismic-level Host Responses

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We are submitting our revised manuscript for publication in JoVE.

The authors declare no conflicts of interest.

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Sincerely,
Kurt Schesser
University of Miami Miller School of Medicine

TITLE:

Using a Bacterial Pathogen to Probe for Cellular and Organismic-level Host Responses

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Infection; mice; microbial pathogens; *Listeria monocytogenes*; flow cytometry; cell biology

SUMMARY:

We describe both in vitro and in vivo infection assays that can be used to analyze the activities of host-encoding factors.

ABSTRACT:

There are a variety of strategies bacterial pathogens employ to survive and proliferate once inside the eukaryotic cell. The so-called 'cytosolic' pathogens (*Listeria monocytogenes*, *Shigella flexneri*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Rickettsia* spp.) gain access to the infected cell cytosol by physically and enzymatically degrading the primary vacuolar membrane. Once in the cytosol, these pathogens both proliferate as well as generate sufficient mechanical forces to penetrate the plasma membrane of the host cell in order to infect new cells. Here, we show how this terminal step of the cellular infection cycle of *L. monocytogenes* (*Lm*) can be quantified by both colony-forming unit assays and flow cytometry and give examples of how both pathogen- and host-encoded factors impact this process. We also show a close correspondence of *Lm* infection dynamics of cultured cells infected in vitro and those of hepatic cells derived from mice infected in vivo. These function-based assays are relatively simple and can be readily scaled up for discovery-based high-throughput screens for modulators of eukaryotic cell function.

INTRODUCTION:

Infection-based experimental models are inherently challenging due to their dependence on the starting state conditions of the host and pathogen, the various pathogen infection strategies, and the difficulty of attributing pathogen- and host-driven processes based on outcomes. The

bacterium *Listeria monocytogenes* (*Lm*) has become an ideal pathogen to probe host defense responses because of its genetic and microbiological tractability, its rapid and processive cellular infection strategy, and the relatively clear relationship between its cellular- and organismic-level infection phenotypes. The cellular infection of *Lm* proceeds through four distinct phases¹: (i) cellular invasion that concludes with *Lm* being enclosed within a vacuole; (ii) *Lm*-directed dissolution of the vacuole membrane and release of *Lm* into the cytosol; (iii) intracytosolic replication; and (iv) physical penetration of the plasma membrane that results in either the infection of directly adjacent cells (such as in an epithelial sheet) or, in solitary cells, release of *Lm* into the extracellular milieu. Each of these phases are promoted by specific *Lm*-encoded factors (referred to as ‘virulence factors’) that, when deleted, cause infection defects in both cellular and animal models. This general infection strategy has been independently evolved by a number of the so-called ‘cytosolic’ pathogens².

Colony-forming unit (CFU) assays are widely employed to evaluate both in vitro (i.e., cellular) as well as in vivo (i.e., organismic) infection outcomes. In addition to their high sensitivity, particularly for in vivo infections, CFU assays provide an unambiguous readout for pathogen invasion and intracellular survival/proliferation. CFU assays have been extensively used to analyze both *Lm* and host cell determinants that impact infection. As informative as these prior studies have been to analyze cellular invasion and intracytosolic replication, CFU assays have not, to the best of our knowledge, been used to track the fourth phase of the *Lm* infection process: cellular escape. Here, we describe relatively simple means of how cellular escape (hereafter referred to as ‘emergence’) can be monitored by CFU assay (as well as by flow cytometry) and show examples of how both pathogen- and host-encoded factors regulate this phase of the *Lm* infection cycle. The analysis of the terminal phase of the cellular *Lm* infection cycle may make it possible to identify additional pathogen and host cell infection-specific factors and activities.

PROTOCOL:

Mice were treated humanely in accordance with all appropriate government guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and their use was approved for this entire study by the University of Miami Institutional Animal Care and Use Committee (protocol 16-053).

1. Preparing cells for infection

1.1 Propagate mouse macrophage-like cell line RAW 264.7 in DMEM tissue culture media supplemented with 10% fetal calf serum (henceforth referred to as DMEM/FCS) using a 125 mL flask and a tissue culture incubator maintained at 37 °C/5% CO₂.

1.2 The day before the infection, use a cell scraper to remove cells from the expansion flask and collect in a 15 mL screw cap conical tube. Centrifuge cells at 800 x *g* for 10 min, decant supernatant and suspend the cell pellet in 1 mL of DMEM/FCS (without antibiotics) and determine the titer using a hemocytometer or an automated cell counter.

1.3 Adjust titer to 2×10^6 cells/mL and add 0.10 mL (2×10^5 cells) to wells of a 48-well tissue culture dish. Ensure that the cell suspension covers the entire surface of the well.

1.3.1 Plate cells in additional wells for a later source of conditioned media. Also place 0.10 mL of DMEM/FCS in three additional wells to serve as a 'no cell control'.

1.4 Incubate overnight in a tissue culture incubator at 37 °C/5% CO₂. The next day, do not remove from incubator until the bacterium inoculum is prepared and ready to use.

2. Preparing *Listeria monocytogenes* (*Lm*) for infection

2.1 Inoculate 2 mL of brain-heart infusion (BHI) with a single colony from a freshly-streaked agar plate (<2 days) of *Lm* and incubate overnight at 37 °C with shaking at 130 rotations/min.

2.2 The next day, add 0.10 mL of overnight culture to 2 mL pre-warmed BHI and continue incubating at 37 °C with shaking until the optical density (OD₆₀₀) is between 0.4 and 0.6.

2.3 Determine approximate bacterial titer using an empirically-derived conversion formula. In our lab, an exponentially growing *Lm* culture in BHI is approximately 1.3×10^9 colony forming units (CFU)/OD₆₀₀. Minimize the amount of time bacterial cultures are exposed to room temperature.

2.4 Just prior to infection adjust the titer to 5×10^7 CFU/mL using pre-warmed DMEM/FCS as diluent.

3. Infection

3.1 Working quickly and precisely, add 20 µL (1×10^6 CFU; multiplicity of infection, MOI = 5) of the freshly-prepared *Lm* inoculum (step 2.4 above) to wells by slightly tilting the dish and carefully placing the pipet tip in the overlying media; **avoid touching the walls of the well with the pipet tip.**

3.2 Gently pipet the contents of each well to ensure complete mixing; do not shake or significantly tilt the plate as this will spread *Lm* over the walls of the well. Return cell culture dish to 37 °C/5% CO₂ incubator.

3.3 Prepare a 10 µg/mL gentamicin solution using conditioned media from uninfected wells as diluent.

3.4 At 30 minutes post infection (mpi), carefully add 30 µL of the gentamicin solution (final concentration 2.5 µg/mL) and gently pipet the contents of the well as before and return cell culture dish to 37 °C/5% CO₂ incubator.

3.5 At 60 mpi, harvest appropriate wells for either CFU assay (60 mpi time point) or FCM

analysis (as described below in sections 4 and 5, respectively).

3.6 For remaining wells, at various times thereafter either harvest cells as before or, for the *Lm* emergence assay, entirely remove media from well and replace with 150 μ L of gentamicin-free conditioned media.

4. Sampling processing and analysis of intracellular and emergent *Lm* by CFU assay

4.1 To harvest, slightly tilt the dish and carefully remove the entire media from the well. Add 0.50 mL of distilled sterile water (dsH₂O) to the well. After 30 s, transfer the resulting water lysate (10^0) to a 1.5 mL microcentrifuge tube and vortex vigorously for 10 s.

4.2 Prepare 10^{-1} and 10^{-2} dilutions by adding either 4.5 μ L or 50 μ L of the water lysate to 450 μ L of dsH₂O and briefly vortex to ensure thorough mixing.

4.3 Spread 50 μ L of the 10^0 , 10^{-1} and/or 10^{-2} samples onto lysogeny broth (LB) agar plates.

4.4 To assay for emergent *Lm* (step 3.6 above), remove 10 μ L of the overlaying media and divide it into two 5 μ L aliquots: to one aliquot add 5 μ L of DMEM/FCS and to the second aliquot add 5 μ L of DMEM/FCS containing 5 μ g/mL gentamicin. The latter control distinguishes between extracellular *Lm* (gentamicin sensitive) and intracellular *Lm* (gentamicin resistant) in the subsequent CFU assay.

4.5 After 5 min at room temperature, add 90 μ L of dsH₂O, vortex vigorously for 10 s, and spread 50 μ L on LB agar plates.

4.6 Enumerate *Lm* colonies on LB agar plates following 2 days of incubation at 37 °C.

5. Sampling processing and analysis of intracellular and emergent *Lm* by flow cytometry (FCM)

5.1 Prepare RAW 264.7 cells as described in steps 1.1-4. Adjust cells to 1×10^6 cells/mL and add 1 mL (1×10^6 cells) to wells of a 6-well tissue culture dish.

5.2 Prepare bacterium inoculum as described in step 2.1-2.3 and infect cells with volume of inoculum corresponding to a MOI of 50 (5×10^7 CFU).

5.3 At 1.5 hours post infection (hpi), collect media from first set of wells and place in a 1.5 mL microcentrifuge tube with 500 μ L of 4% paraformaldehyde (PFA) and place on ice until all wells have been processed.

5.4 To collect intracellular *Lm*, add 1 mL of dsH₂O to the well and after 60 s, transfer the resulting water lysate to a 1.5 mL microcentrifuge tube with 500 μ L of 4% PFA. Vortex vigorously for 10 s and place on ice until all wells have been processed. (Sample processing is described in

step 5.8 below.)

5.5 For remaining wells, remove media and replace with 1 mL of DMEM/FCS with 5 µg/mL gentamicin to kill extracellular *Lm* and promptly return cells to incubator.

5.6 After 30 min (2 hpi) remove media and replace with DMEM/FCS without gentamicin.

5.7 After 2 and 4 hours (4 and 6 hpi) take second and third time points by collecting media and lysates as steps 5.3 and 5.4.

5.8 Centrifuge tubes at 10,000 x *g* for 7 min. Remove supernatant without disturbing pellet.

5.9 Suspend each pellet in staining cocktail of 50 µL of 4% PFA and 15 µL of phalloidin. Incubate tubes in the dark at 4 °C for 20 min.

5.10 After incubation add 1 mL of FACS buffer to each tube and pipette up and down to mix.

5.11 Spin tubes at 10,000 x *g* for 7 min. Remove supernatant without disturbing pellet.

5.12 Suspend each pellet in 400 µL of FACS buffer for immediate use, or 200 µL of FACS buffer and 200 µL of 4% PFA if storing for later analysis.

5.13 Run samples on a flow cytometry and analyze the collected data.

6. Sampling processing and analysis of *Lm* colonization and host responses in the liver

6.1 Prepare *Lm* for infection as described in step 2.1-2.3.

6.2 Calculate the necessary amount of subculture for desired titer of 3 x 10⁶ CFU/mL.

6.3 Just prior to infection dilute the amount of calculated bacteria with pre-warmed Hank's Balanced Salt Solution (HBSS) in a final volume of 1 mL. This is the input inoculum that is injected into the mouse.

6.4 Using a 28 G½ 100U insulin syringe, draw 100 µL (3 x 10⁵ CFU) of input inoculum and remove any visible air bubbles.

6.5 Place the mouse (strain C57BL/6; males between 6 and 12 weeks old) into the holding restraint and use warm water (no more than 45 °C) to dilate the tail vein, then inject the input inoculum into the vein.

6.6 Plate 10⁻² and 10⁻³ dilutions of the input inoculum onto LB agar 10bcm plates and incubate overnight at 37 °C to determine the actual input dosage.

6.7 At 18 hpi, humanely euthanize mouse (CO₂ asphyxiation followed by cervical dislocation) and using sterile scissors cut open mouse peritoneum to collect frontal lobe of the liver using separate pairs of tweezers and scissors for the outside and inside of the mouse. Place the liver in a 6 cm Petri dish on ice.

6.8 Cut the liver into small cubes and place into a glass vial with a magnetic stir bar. Add 2 mL of 2 mg/mL collagenase D reconstituted in DMEM without FCS. Incubate vials at 37 °C on a magnetic stirrer for 30–45 min. Once the tissue is homogenized, add 3 mL of DMEM/FCS to inactivate the collagenase.

6.9 Filter the cells through a 70 µm cell strainer into a conical tube, adding more DMEM/FCS if necessary.

6.10 Centrifuge cells at 800 x *g* to pellet at 4 °C for 10 min, remove supernatant and suspend cell pellet in 500 µL of ACK buffer to lyse red blood cells.

6.11 Incubate cells at room temperature for 5 min in ACK buffer. Suspend cells in 1 mL of DMEM/FCS.

6.12 Centrifuge cells at 800 x *g* to pellet at 4 °C for 10 min, remove supernatant and suspend in 1 mL of DMEM/FCS. Then, count the cells using a hemocytometer or an automated cell counter.

6.13 Transfer 1 x 10⁶ cells to 1.5 mL microcentrifuge tube and spin to pellet. Then suspend in 1 mL of dsH₂O and vortex to lyse the cells. Plate 50 µL on LB plates at undiluted and 10⁻² dilutions and incubate at 37 °C to enumerate *Lm* for CFU analysis.

6.14 Transfer 1 x 10⁶ cells into FACS tubes for each tissue sample according to the cell counts and wash with 1 mL of FACS buffer.

6.15 Centrifuge cells at 800 x *g* to pellet at 4 °C for 10 min and remove supernatant.

6.16 Prepare an antibody cocktail containing the antibodies of interest based on the recommended concentration for each antibody and FACS buffer. See **Table of Materials** for recommended amounts used in the analysis.

6.17 Suspend pellet in 100 µL of antibody cocktail and incubate tubes in the dark at 4 °C for 30 min.

6.18 After incubation, add 2 mL of FACS buffer to each tube and suspend cells.

6.19 Centrifuge cells at 800 x *g* to pellet at 4 °C and discard the supernatant. Then suspend the pellet in 400 µL of FACS buffer for immediate use, or 200 µL of FACS buffer and 200 µL of 4% PFA if storing for later.

265
266 6.20 Analyze cells by flow cytometry (see step 5.13).
267

268 REPRESENTATIVE RESULTS:

269 Assessing the role of pathogen and host-encoded factors impacting cellular infection

270 Using the infection conditions described above, 0.15% of the input wild-type *Lm* is recovered
271 after 1.5 h of co-incubation with cultured macrophages (**Figure 1A**). In the subsequent 1.5 h of
272 co-incubation (3 h post infection, hpi), there was a 4-fold increase in recovery of viable *Lm* and
273 from 3 to 6 hpi there was an additional 7.5-fold increase in the recovery of viable *Lm*. Since the
274 cell-impermeable antibiotic gentamicin is added to the co-cultures at 1 hpi to eliminate
275 extracellular *Lm*, the 30-fold increase in viable *Lm* levels between 1.5 and 6 hpi exclusively
276 represents intracellular proliferation.
277

278 *Lm* rapidly gains access to the cytosol following its internalization within eukaryotic cells (see
279 *Introduction*). Cytosolic *Lm* associates with the actin polymerization machinery by which means
280 it propels itself capable of penetrating the plasma membrane. In the in vitro infection assay
281 described above, if gentamicin is removed from the wells at 6 hpi, extracellular *Lm* can be readily
282 detected 1 h later (7 hpi) in the overlying media (**Figure 1B**). No CFUs were recovered from control
283 wells that were not seeded with macrophages but that were otherwise treated identically as
284 seeded wells thereby showing that the appearance of extracellular *Lm* was entirely dependent
285 on the presence of macrophages and not a result of incomplete antibiotic killing (*not shown*).

286 The capability of *Lm* to survive and proliferate intracellularly is largely dependent on a suite of
287 genes whose expression are controlled by the transcription factor Positive Regulatory Factor A
288 (PrfA)¹. Initially, there is a comparable recovery of the wild-type *Lm* strain and a *Lm* mutant strain
289 deleted for this factor ($\Delta prfA$) (**Figure 1A**). However, there is a subsequent reduction in the
290 recovery of the $\Delta prfA$ strain after prolonged co-incubation such that the recovery at 6 hpi is 3-
291 fold less than the recovery at 1.5 hpi. Relatedly and expectedly, $\Delta prfA$ bacteria were not detected
292 in the overlying media at 7 hpi (**Figure 1B**).
293

294 The activity of PrfA is regulated at multiple levels. A number of constitutively active PrfA variants,
295 encoded by hyper-virulent *prfA** alleles, have been isolated³. A *Lm* strain possessing a *prfA** has
296 an initial infection profile that is comparable to the isogenic wild-type strain at 1.5 and 3 hpi
297 (**Figure 1A**). However, between 3 and 6 hpi, the fold-increase of the *prfA** strain significantly
298 differed from that of the wild-type strain. Again, relatedly and expectedly, the *prfA** strain was
299 readily detected in the overlying media at 7 hpi at levels that significantly exceeded that observed
300 for the wild-type *Lm* strain (**Figure 1B**).
301

302 Following its release into the cytosol, *Lm* associates with the cellular actin polymerization
303 machinery that results in the bacterium being enveloped by polymerized actin. To confirm that
304 extracellular *Lm* is derived from the actin-rich cytosol of the infected host cell, macrophages were
305 either left uninfected or infected with a GFP-expressing *prfA** strain and the overlying media was
306 collected at 1.5, 4, and 6 hpi, stained with phalloidin that binds polymerized actin, and analyzed
307 by flow cytometry. Media overlaying both uninfected and infected macrophages contained light-
308 scattering bacterium-sized particulate matter (**Figure 2A**), however, only media from infected

macrophages possessed GFP-positive signals within this size gating (**Figure 2B**; left panel). There was a time-dependent increase in the appearance of phalloidin-positive *Lm* in the media overlaying infected macrophages (**Figure 2B**; right three panels). Phalloidin-positive GFP-expressing *Lm* were also detected in lysates prepared from infected macrophages (**Figure 2C**; left panel). However, no phalloidin-positive *Lm* was detected in lysates prepared from macrophages infected with a GFP-expressing *Lm* mutant strain that lacked the virulence factor ActA that is required to recruit the actin-polymerization machinery to the outer surface of *Lm* following its release into the cytosol (**Figure 2C**; right panel). These data support a scenario that in an in vitro infection model 'emergent' extracellular *Lm* are derived from the infected cell cytosol.

In addition to *Lm*-encoding factors, a number of host factors have been identified that impact *Lm* cellular infection (see *Discussion*). Recently, the host factor Perforin-2 (P2) has been shown to be a critical component of cell-level defenses against a number of bacterial pathogens. Peritoneal exudate macrophages (PEMs) isolated from wild-type ($P2^{+/+}$) and $P2^{-/-}$ mice are initially comparably infected by *Lm* as measured by CFU assay at 1 hpi (**Figure 3A**). In $P2^{+/+}$ PEMs the number of CFUs recovered at 6 hpi is 10-fold greater compared to the levels recovered at 1 hpi. Similar to previously published findings, in $P2^{-/-}$ PEMs the number of CFUs recovered at 6 hpi is 80-fold greater compared to the levels recovered at 1 hpi⁴. Enhanced *Lm* intracellular proliferation in $P2^{-/-}$ PEMs was accompanied with greater levels of viable *Lm* detected in the overlying media compared to levels detected in $P2^{+/+}$ PEMs (**Figure 3B**). These data show that in an in vitro assay that the appearance of emergent extracellular *Lm* can also report on host factors that impact infection.

In vivo infection and analysis of the liver

In addition to the in vitro infection assays described above, the relative infectivity of the wild-type, attenuated, and hyper-virulent *Lm* strains can also be assessed by in vivo infection assays. Mice were intravenously co-infected with an equal mixture of the wild-type and $\Delta prfA$ *Lm* strains and 18 h later were humanely euthanized, livers excised, and single cell preparations were made. Isolated liver cells were lysed and the resulting lysates subjected to a CFU assay that could distinguish between the wild-type and $\Delta prfA$ *Lm* strains. Compared to the 1:1 ratio of these two strains in the original inoculum (the 'input'), the $\Delta prfA$ /wild type ratio at 18 hpi (the 'output') was considerably less than unity (for 6 mice range was 0.001 to 0.330; mean 0.100) (**Figure 4**). In contrast, mice co-infected with an equal mixture of the wild-type and $prfA^*$ *Lm* strains the $prfA^*$ /wild type ratios ranged from 2.4 to 10.2 (mean 7.1). This experiment shows that the relative infectivity of these three *Lm* strains (wild type, $\Delta prfA$, and $prfA^*$) in an intact organism resembles that observed in in vitro infection assays.

The host can also be monitored to determine whether the varying level of infectivity of the wild-type, $\Delta prfA$, and $prfA^*$ strains are associated with a corresponding gradation of the innate immune response. Previously it has been shown that resident macrophages (Küpferr cells) and infiltrating inflammatory monocytes and neutrophils play critical and interrelated roles in protecting the liver during *Lm* infection⁵⁻⁸. Cells prepared from uninfected mice and mice infected with the GFP-expressing wild-type *Lm* strain for 18 h were stained with cell type-specific markers and analyzed by flow cytometry. In terms of non-immune cells of the liver (e.g., hepatocytes and

endothelial cells), there were no detectable differences between uninfected and infected mice. In contrast, there were notable infection-related changes in the liver in terms of the proportion of immune cells that positively stained for the leukocyte common antigen CD45 (**Figure 5A**). Specifically, mice infected with the wild-type and *prfA*^{*} *Lm* strains had significantly higher levels of CD45⁺ cells in the liver compared to uninfected mice whereas the levels of CD45⁺ liver cells in mice infected with the attenuated Δ *prfA* *Lm* strain were not notably different than levels observed in uninfected mice. To further characterize both resident and infiltrating immune cell populations, CD45⁺ cells can be furthered subtyped. As observed by others⁹, a notable shift with infection is the almost complete disappearance of resident K  pffer Cells (CD11b^{mid} F4/80⁺) in the liver and the concomitant appearance of a distinct population of CD11b^{hi} cells that represent infiltrating neutrophils (**Figure 5B; lower middle panel**). Also appearing in the liver at 18 hpi, are CD11b^{mid} F4/80⁻ cells appear that, since they also are Ly6C^{hi}, represent infiltrating inflammatory monocytes (**Figure 5B; lower middle and right panels**). In infected mice these latter cells are the only cells in which appreciable GFP signal was detected following 18 hs of infection (**Figure 5C**). This latter finding is similar to that recently reported by Jones and D’Orazio who showed that in mice, *Lm* primarily is associated with Ly6C^{hi} monocytes that infiltrate the gut following food-borne infection¹⁰. The profile of liver CD45^{hi} cells of mice infected with the *Lm* Δ *prfA* strain resembled that of uninfected mice whereas mice infected with the hyper-virulent *Lm* *prfA*^{*} strain had modestly enhanced levels of infiltrating neutrophils and inflammatory monocytes compared to mice infected with the wild-type *Lm* strain (*data not shown*).

FIGURE AND TABLE LEGENDS:

Figure 1: Infection of cultured macrophages by *Listeria monocytogenes* (*Lm*). The mouse macrophage cell line RAW 264.7 were infected in vitro with either wild-type *Lm* (wt; filled circles), attenuated *Lm* (Δ *prfA*; open circles), or hyper-virulent (*prfA*^{*}; open squares) *Lm* strains (see text for details). **(A)** At 1.5, 3, and 6 hours post infection (hpi), macrophages were lysed and the cellular contents were analyzed by colony-forming unit (CFU) assay. **(B)** Infected macrophages were briefly treated with the antibiotic gentamicin to eliminate non-internalized *Lm* and at 6 hpi the overlaying media was collected and analyzed by CFU assay. Dotted line represents the detection limit of the assay. Each data point represents an independent well/infection and P values were determined by Student’s T test. Shown are the results of a single representative experiment performed three times with similar outcomes.

Figure 2: Actin-bound *Lm* analyzed by flow cytometry. The mouse macrophage cell line RAW 264.7 were either left uninfected or infected in vitro with GFP-expressing *Lm*. The overlying media was removed, centrifuged, and the collected material stained with actin-binding phalloidin. The stained material was analyzed by flow cytometry and shown is the light-scattering plots **(A)** indicating the gating used for the GFP- and phalloidin-associated levels of extracellular (i.e., media-derived) *Lm* shown in **(B)**. **(B)** The indicated area represents the GFP/actin double positive *Lm* and their abundance as a percentage of the total events within the light-scattering gates. **(C)** Mouse macrophages were infected with either GFP-expressing *Lm* or a GFP-expressing *Lm* strain lacking the gene encoding ActA (Δ *actA*) that recruits the actin-polymerization machinery of the host cell to the *Lm* cell surface. At 6 hpi, infected cells were lysed and the released cellular contents, including the intracellular *Lm*, were stained for actin and analyzed by

flow cytometry. Shown are the results of a single representative experiment performed three times with similar outcomes.

Figure 3: Infection of wild-type and *Perforin-2* knockout macrophages by *Lm*. Peritoneal macrophages isolated from either a wild-type ($P2^{+/+}$; filled circles) or knockout ($P2^{-/-}$; dotted circles) were infected in vitro with *Lm*. (A) At 1 and 6 hpi, macrophages were lysed and the cellular contents analyzed by CFU assay. (B) Infected peritoneal macrophages were briefly treated with the antibiotic gentamicin and at 2.5 and 6 hpi the overlaying media was collected and analyzed by CFU assay. Each data point represents an independent well/infection and P values were determined by Student's T test. Shown are the results of a single representative experiment performed three times with similar outcomes.

Figure 4: *Lm* infection of the mouse. Groups of 6 mice were infected with an equal mixture of either the wild-type *Lm* (wt) and attenuated *Lm* ($\Delta prfA$) strains or an equal mixture of the wild-type *Lm* and hyper-virulent ($prfA^*$) strains. At 18 hpi mice the *Lm* burden in the liver was determined by CFU assay. In this experiment the wild-type *Lm* strain possesses an antibiotic-resistance gene that allows it to be distinguished from the antibiotic-sensitive *Lm* mutant strains. Liver homogenates were plated on both antibiotic-containing and antibiotic-free media and the ratio of mutant and wild-type *Lm* were plotted. The dotted line represents the mutant/wild-type ratio of the infecting dose. The absolute levels of the wild-type *Lm* in the two cohorts of mice were 12 and 15 CFU/ 10^6 liver cells, respectively. Shown are the results of a single representative experiment performed two times with similar outcomes.

Figure 5: Immunological response to *Lm* infection in the mouse liver. Mice were either uninfected or systemically infected with 2×10^5 CFU of GFP-expressing *Lm* strains for 18 h. Single-cell preparations of livers were analyzed by flow cytometry for expression of immune- and myeloid-specific markers. (A) The percentage of cells staining positive for the immune-specific cell surface marker CD45⁺ per 10^6 isolated total live liver cells shown for uninfected mice and mice infected with either the wild-type (wt *Lm*), attenuated ($\Delta prfA$), or hyper-virulent ($prfA^*$) *Lm* strains. Plotted is the mean of three mice per group and P values were determined by Student's T test. (B) Shown in the left panels are the staining profile of CD45-staining liver cells from an individual uninfected mouse (top) and an individual wt *Lm*- infected (bottom); in the middle panels is shown the expression levels of myeloid-specific markers CD11b and F4/80 of the CD45⁺ cells; and on the right panels is shown the Ly6C expression levels of the indicated gated cells. In the far right panel is shown an overlay of the Ly6C⁺ cells from the uninfected and infected mice including the percentage and mean fluorescence intensities (MFI) of the CD11b⁺ F4/80⁺ cells positive for the indicated Ly6C gate. Shown is a representative mouse from 3 mice per group. (C) Ly6C⁺ cells derived from livers of uninfected and infected mice (described (B); middle and right panels) were analyzed for GFP expression by flow cytometry.

DISCUSSION:

Due to its rapid and processive cellular infection program, *Lm* is an ideal pathogen to probe cellular activities that impact infection. A number of host factors have been identified that either positively or negatively affect *Lm* cellular infection¹¹⁻¹⁴. Two such host factors characterized in

our laboratory, Perforin-2 and the Heme Regulated Inhibitor (P2 and hI), regulate distinct features of the *Lm* infection process. As shown in **Figure 3**, P2 deficient cells are highly susceptible to *Lm* and this enhanced susceptibility also is true of the P2 knockout mice⁴. Detailed comparative studies of P2-expressing and P2-deficient macrophages showed that the latter cells formed highly acidified *Lm*-containing phagosomes that resulted in enhanced virulence gene expression. The hypothesis that differential virulence gene expression contributes to the enhanced susceptibility of P2-deficient macrophages was supported by the finding that the hyper-virulent *prfA** strain, that, as mentioned earlier, expresses virulence genes at a constitutively high level, infected P2-expressing and P2-deficient macrophages equally well⁴. Thus, the availability of a hyper-virulent *Lm* strain made it possible to test the hypothesis that the P2 host factor negatively regulates pathogen virulence factor deployment. For hI, we showed that this host factor regulates the intracellular trafficking of *Lm* such that its emergence from the infected cell is restricted; this cellular activity is likely the bases for the enhanced susceptibility of hI-deficient mice for *Lm* infection^{15,16}.

In studies that documented cellular activities occurring within minutes of infection, it is crucial that prior to their co-incubation, that both the bacteria and the host cells are not excessively exposed to fluctuating temperatures or other environmental conditions that may activate stress responses that would overlap (and confound) infection-induced activities. For the studies described above, this includes such seemingly simple points as minimizing the handling time of *Lm* just prior to initiating infection. For example, if a bacterial culture is removed from a shaking 37 °C incubator and allowed to sit on a bench top, shifts in both temperature and oxygen availability (among other things) will activate adaptations that may affect the initial phase of the subsequent interaction with the host cell. Similarly, host cells are also sensitive to temperature and media shifts that may affect initial infection responses. Although in practice it is impossible to completely eliminate all fluctuations in bacterial and host cell culturing conditions, by reducing them as much as possible and establishing a standard operating plan, variation between experiments will be minimized.

An additional variable, that in our opinion the importance of which is also underappreciated, is that of the use of antibiotics in cell culture infection models. Gentamicin is often the antibiotic of choice because it is relatively impermeable to the eukaryotic plasma membrane. In both older and current literature, it is generally used at 50 µg/mL to kill extracellular (i.e., non-internalized) bacteria. Recently it has been directly demonstrated that when present at 25 µg/mL, there is sufficient diffusion of gentamicin across the eukaryotic membrane to kill intracellular *Lm*¹⁷. In our experience, this level of gentamicin kills *Lm* immediately upon contact, whereas much lower levels, in the 2 to 5 µg/mL range kill >99.9% *Lm* in less than 5 minutes⁴. Lawrenz and colleagues have shown that the widely-used gentamicin concentrations of 25 – 50 µg/mL are also sufficient to kill intracellular *Yersinia pestis*¹⁸. Interestingly, this group also showed considerable differences between cell types in terms of gentamicin permeabilization highlighting that optimal conditions should be determined for each pathogen-host cell pairing.

DISCLOSURES:

The authors have nothing to disclose.

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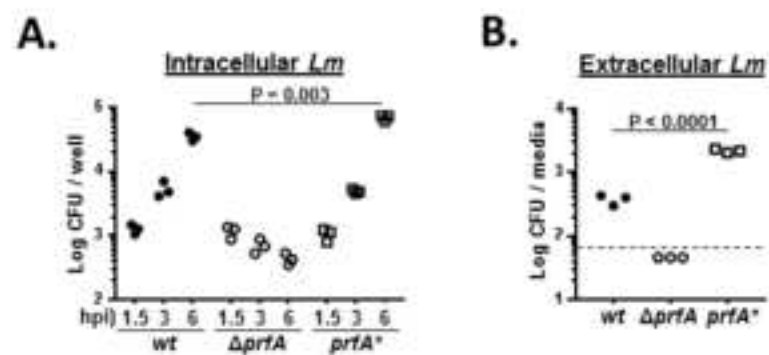
Fig. 1

Fig. 2

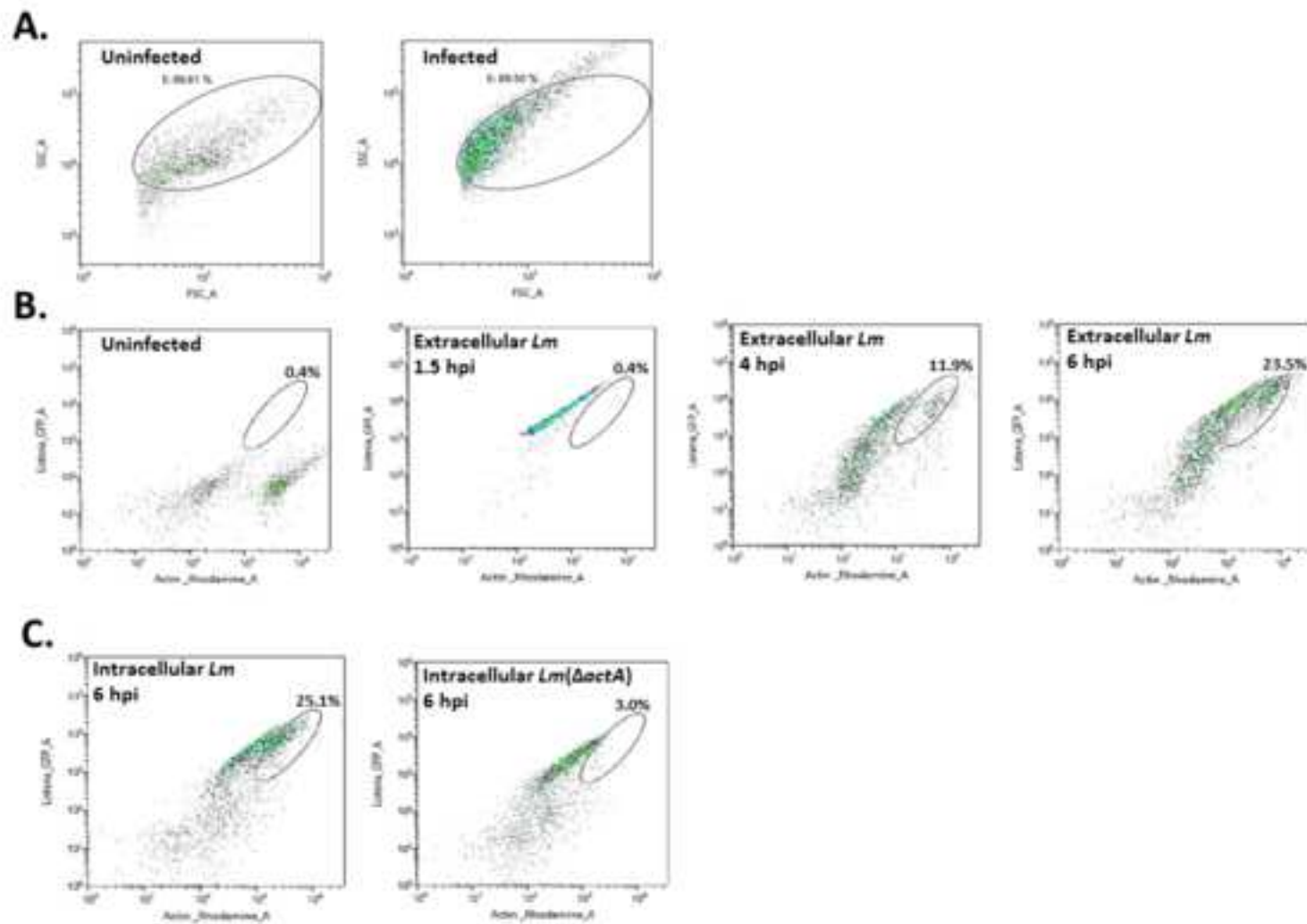


Fig. 3

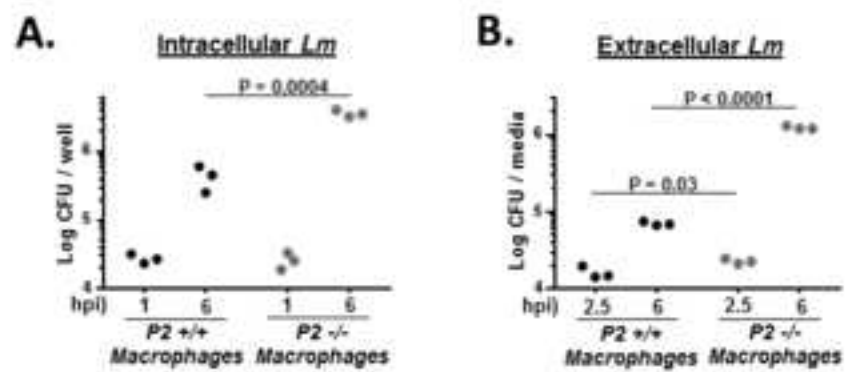


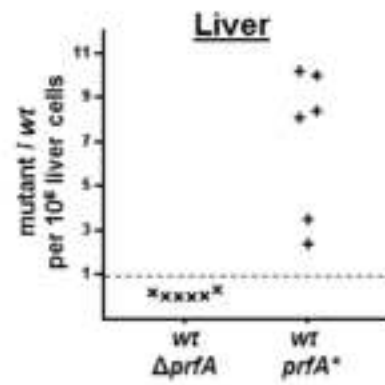
Fig. 4

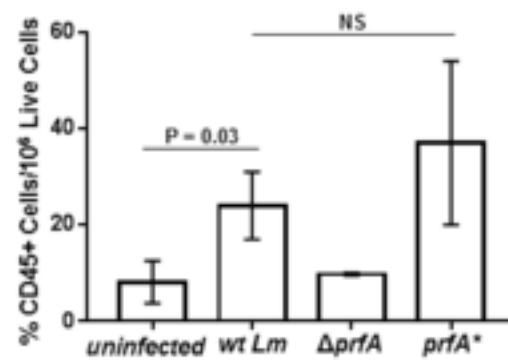
Fig. 5**A.**

Fig. 5

B.

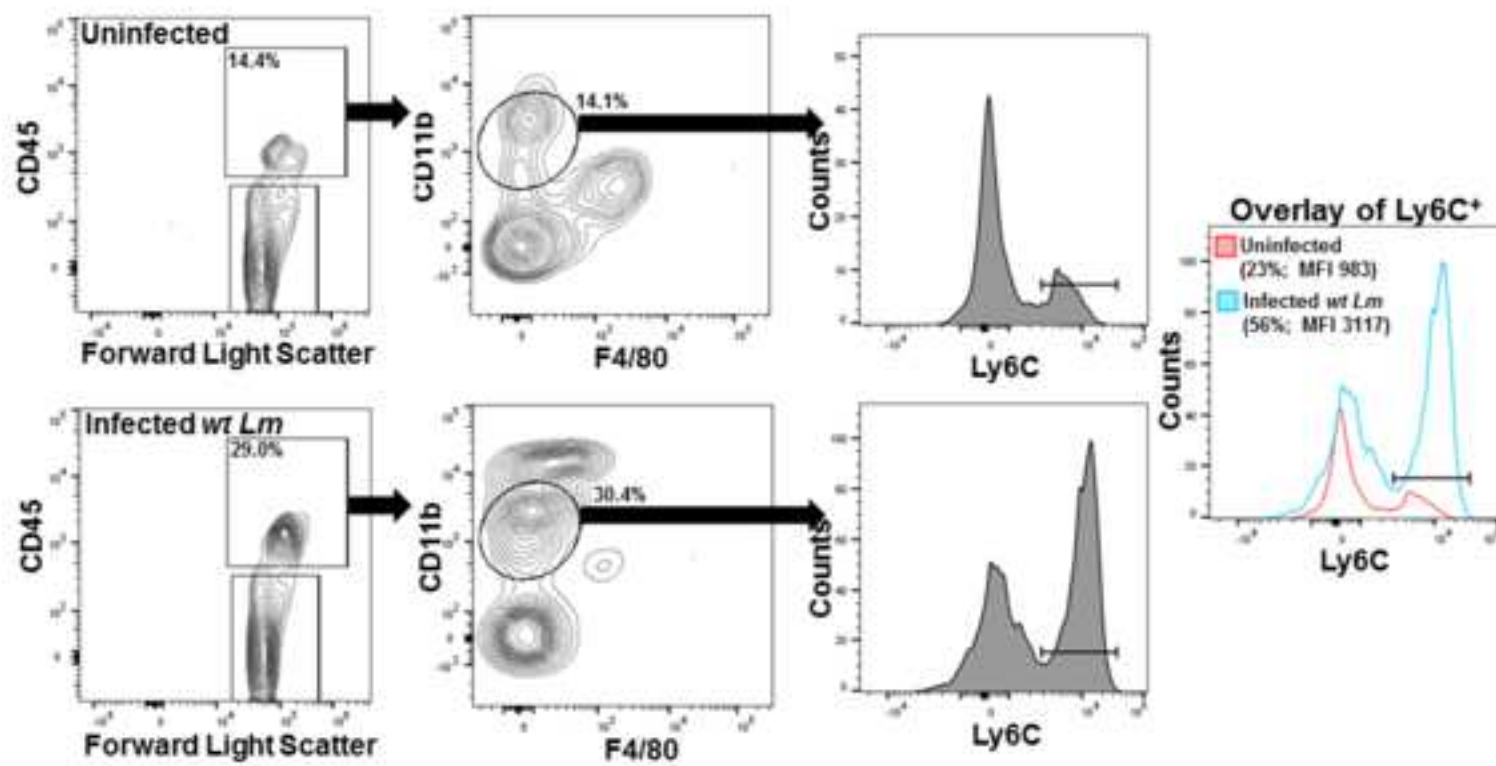
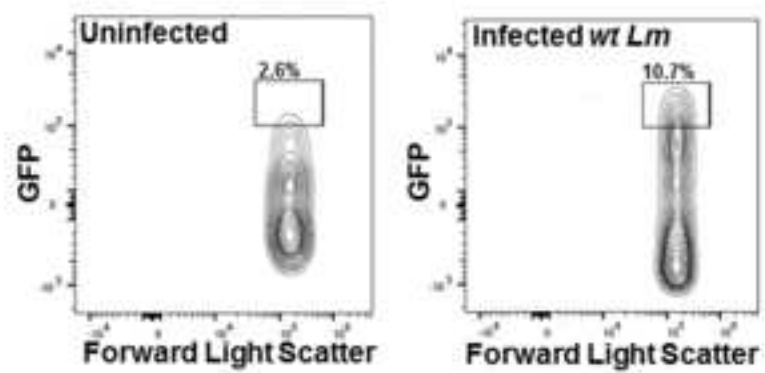


Fig. 5**C.**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description	
ACK Lysing Buffer	Gibco	A1049201		
ArC Amine Reactive Compensation Beads	Life Technologies	A10346		
BHI (Brain Heart Infusion) broth	EMD Milipore	110493		
Cell Strainer, 70 µm	VWR	10199-656		
Collagenase D	Roche	11088858001		
DMEM media	Gibco	11965-092		
FACS tubes	BD Falcon	352054		
FBS - Heat Inactivated	Sigma-Aldrich	F4135-500ML		
Hanks' Balanced Salt solution	Sigma-Aldrich	H6648-6X500ML		
LB agar	Grow Cells MS	MBPE-4040		
LIVE/DEAD Fixable Yellow Dead Cell Stain kit	Life Technologies	L349S9		
Rhodamine Phalloidin	Thermo Fischer	R415		
SP6800 Spectral Analyzer	Sony			
Syringe 28G 1/2" 1cc	BD	329461		
TPP Tissue Culture 48 Well Plates	MIDSCI	TP92048		
TPP Tissue Culture 6 Well Plates	MIDSCI	TP92406		
UltraComp eBeads	eBioscience	01-2222-42		
Antigen	Fluorochrome	Dilution	Manufacturer	Clone
CD11b	PE Cy5	1/100	Biolegend	M1/70
CD11c	AF 647	1/100	Biolegend	N418
CD45	APC Cy7	1/100	Biolegend	30-F11
F4/80	PE	1/100	Biolegend	BM8
Live/Dead	AmCyN	1/100	Invitrogen	
Ly6C	PacBlue	1/200	Biolegend	HK1.4
MHC II	AF 700	1/200	Biolegend	M5/114.15.2
NK 1.1	BV 605	1/100	Biolegend	PK136



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 Line 162: Please specify the age, gender and strain of mouse used. **Done**
 Line 166: Please specify the euthanasia method. **Done**
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15. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available. **Done**

Reviewers' comments:

Reviewer #1:

This manuscript provides a protocol and representative data to evaluate how *Listeria monocytogenes* escapes from cells after replicating in the cytoplasm. This is an important step in *Listeria* pathogenesis and the authors provide a reasonable description of the protocol and provide data to support most of their conclusions. There are, however, a few major and multiple minor comments that should be addressed.

Major Comments to the authors:

1) In figure 2A, the authors state that there is no phalloidin signal in the uninfected samples, but clearly there is a fair amount of Actin-Rhodamine staining. Additionally, since the dot plot is showing S/N from uninfected samples, there should not be any events at all. Showing the FSC vs. SSC would be helpful, because in the uninfected S/N the events must be coming from debris, exosomes, air bubbles, etc. and not from *Listeria*. To strengthen these findings, microscopy should be shown.

We have added the FSC vs SSC plot in Fig. 2A

2) In this reviewer's opinion, figures 5 and 6 do not add very much value to the manuscript. However, if they are shown, a better description should be provided and the gating for figure 6 should be shown. Additionally, are other cells, including DC's macrophages, neutrophils, and hepatocytes infected with *Listeria*? Previous publications have implicated all of these cell types in either phagocytosing *Listeria* or becoming infected. Without comparisons to other cells, showing monocytes in isolation does not allow for a proper interpretation.

In lines 265-266 we have done a better job of explaining why the in vivo infections fit with the in vitro infection model. We only detect *Listeria*-derived GFP signal in the Ly6C monocytes.

Minor Comments to the authors:

1) List the other supplements in the DMEM media. **There were no other supplements other than serum.**

2) When describing centrifugation steps, provide the RCF instead of stating "low-speed centrifugation". **Done**

3) Clearly state when media contains antibiotic or not. **Done**

4) On line 106, describe how to "harvest some wells". Does this mean S/N, cells or everything? **Clarified**

5) On line 113, will cells be harvested? **Clarified**

6) Define PFA. **Done**

- 7) Describe and/or list the flow cytometer and analysis software used. **Done**
- 8) List the vendors and clone names/numbers for all antibodies used. **Done**
- 9) Line 245 should say "left panel". **Done**
- 10) Describe how to isolate the peritoneal exudate macrophages. **The appropriate literature is cited**
- 11) The protocol used for figure 4 should be described in the protocol section, not the figure legend. **The experiment is somewhat complicated and, in our opinion, would not fit well in the protocol section.**
- 12) All of the strains of *Listeria* should be listed in a table with a description, antibiotic resistance and a reference to the original article. **These strains are extensively described in the Results section and are cited appropriately.**
- 13) The original paper showing that specific depletion of neutrophils reduces host resistance to *Listeria* (EJI: 2011: vol 41, pp. 2666-2676) should be referenced on line 279. **Done**
- 14) How reproducible are the data? How many times was each experiment repeated? Triplicate wells do not equal "n=3" and should not be used for statistical analysis purposes. **This information is add to the figure legend.**
- 15) In the figure legends, the use of the term mouse macrophages is ambiguous. Are these primary cells or cell lines? **The cell line is now identified (RAW 264.7).**

Reviewer #2:

Manuscript Summary:

The manuscript describes methods to monitor *Lm* emergence in in vitro and in vivo samples. The manuscript is well written and provides detailed procedures that can be easily followed by others and adapted to other intracellular, cytoplasmic pathogens.

Major Concerns:

The authors should consider adding some description of flow cytometry approaches to the introduction to compliment the introduction to CFU assays.

Minor Concerns:

1. Line 83: Add speed of shaker. **Done**
2. Line 128: Clarify what you mean by step 1. **Done**
3. Line 142: Clarify what you mean by described above (list step numbers). **Done**
4. Line 164: Add an "and" between "plates" and "incubate". **Done**
5. Line 230: It would be helpful for the reader to include a description of prf* strain here (e.g., that this is a hypervirulent strain). This will help understand the results without reading the discussion. **Done**
6. Figure 4: The legend describes this a competitive index that would result in a ratio of WT to mutant strains. However the data in the graph is represented as the total number of bacteria (CFU) per 10^6 cells (not sure which bacteria of the mixed population are shown). Please reconcile. Recommend showing as a competitive index, as this would be the most appropriate format to display the data.. **Mistake on our part, has been corrected.**

Several places in the results, Ref. numbers need to be processed through your ref. management

software. As these are different format from rest of the manuscript, I am not sure they refer to the correct reference as listed in Bibliography. Please verify.