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A novel *prfA* mutation that promotes *Listeria monocytogenes* cytosol entry but reduces bacterial spread and cytotoxicity

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Abstract

Listeria monocytogenes is an environmental bacterium that becomes a pathogen following ingestion by a mammalian host. The transition from environmental organism to pathogen requires significant changes in gene expression, including the increased expression of gene products that contribute to bacterial growth within host cells. PrfA is a *L. monocytogenes* transcriptional regulator that becomes activated upon bacterial entry into mammalian cells and induces the expression of gene products required for virulence. How PrfA activation occurs is not known, however several mutations have been identified that increase PrfA activity in strains grown *in vitro* (*prfA** mutations). Here we describe a novel *prfA* mutation that enhances extracellular PrfA-dependent gene expression but in contrast to *prfA** mutants inhibits the cytosol-mediated induction of virulence genes. *prfA* Y154C strains entered cells and escaped from phagosomes with an efficiency similar to wild type bacteria, however the mutation prevented efficient *L. monocytogenes* actin polymerization and reduced spread of bacteria to adjacent cells. The *prfA* Y154C mutation severely attenuated bacterial virulence in mice but the mutant strains did generate target antigen specific $CD8^+$ effector cells. Interestingly, the *prfA* Y154C mutant was significantly less cytotoxic for host cells than wild type *L. monocytogenes*. The *prfA* Y154C mutant strain may therefore represent a novel attenuated strain of *L. monocytogenes* for antigen delivery with reduced host cell toxicity.

Keywords

PrfA; ActA; virulence regulation; gene expression

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1. Introduction

Listeria monocytogenes is a gram-positive bacterium that survives and flourishes in a myriad of environments that range from soil and decaying vegetation to the cytosol of infected mammalian cells. The bacterium mediates the transition from environmental organism to mammalian pathogen via the regulated expression of gene products that promote survival in specific environmental niches. In the environment of a human host *L. monocytogenes* is responsible for serious food-borne infections resulting in bacteremia, meningitis, and neonatal death [1–4]. Fundamental to the transition of *L. monocytogenes* from environmental organism to pathogen is the transcriptional up-regulation of a number of gene products that facilitate host cell invasion, bacterial entry into the cytosol, intracellular replication, and spread to adjacent cells [2,5–8]. The central virulence regulatory protein PrfA appears to function as a key switch to help mediate the transition from bacterial survival in the outside environment to replication within the human host [9]. It is required for the expression of most known *L. monocytogenes* virulence determinants [9–13].

PrfA is a 27 kD protein that is a member of the Crp/Fnr family of transcriptional activators [14]. Members of this family require the binding of small molecule co-factors (such as cAMP or carbon monoxide) or some form of post-translational modification for full activity [15,16]. In some cases, mutations have been identified in these regulatory proteins that enable activation in the absence of co-factor binding or modification. For example, several mutations have been identified within *crp* (known as *crp** mutations) that provide for activation of Crp-dependent gene expression in the absence of co-factor cAMP binding [17,18]. No co-factor or post-translation modification has as of yet been identified for PrfA, but mutationally activated forms of PrfA (PrfA* mutants) have been isolated that provide for increased levels of PrfA-dependent gene expression outside of mammalian cells [19–22]. Strains containing *prfA** mutations are fully virulent in mouse models of infection [21,23]. In contrast, *L. monocytogenes* strains lacking functional PrfA remain trapped within host cell vacuoles and are severely attenuated for virulence in mice [24]. This study describes the isolation of a unique *prfA* mutant that facilitates *L. monocytogenes* entry into the cytosol of infected host cells but inhibits bacterial cell-to-cell spread and attenuates bacterial virulence.

2. Results

2.1 Isolation and identification of a L. monocytogenes isolate containing the prfA Y154C mutation

As part of an approach to study the effects of PrfA activation on *L. monocytogenes* pathogenesis, a genetic screen was undertaken to identify *L. monocytogenes prfA* mutant strains with altered expression of the PrfA-dependent virulence gene *actA*. *actA* is normally expressed at low to undetectable levels during bacterial growth in broth culture but its expression is highly induced (> 200-fold) in the mammalian cell cytosol [30,37,42,43]. A plasmid (pNF1019) [19] containing the *prfA* promoters and the *prfA* coding region was maintained for several generations within a hypermutator strain of *Escherichia coli* and then introduced into a *L. monocytogenes* Δ*prfA* strain containing an *actA* transcriptional fusion to the reporter gene *gus*, encoding β-glucuronidase (GUS) [44]. Bacterial colonies with altered *actA* expression were identified via their blue color (indicating GUS activity) on indicator media plates. Under these conditions bacteria containing wild type *prfA* form white colonies (M. Miner, unpublished results). Approximately 40,000 bacterial colonies were examined, and one mutant (NF-L1215) was selected for further characterization based on its intermediate blue color. Mutant NF-L1215 exhibited an approximately four-fold increase in levels of *actA* expression in broth culture as measured by GUS activity in comparison to isogenic strains containing wild type *prfA* (Fig. 1A and B), and sequencing of the NF-L1215 *prfA* gene revealed a coding change resulting in the substitution of a cysteine for tyrosine at amino acid position 154 (Y154C). To

confirm that the *prfA* Y154C mutation was responsible for the mutant phenotype observed, the mutation was introduced into the chromosome of the *L. monocytogenes* in place of the wild type copy of *prfA* by allelic exchange (resulting in strain NF-L1213) and the patterns of *actA* expression in NF-L1213 were found to be identical to those of the originally isolated NF-L1215 plasmid-insertion mutant, thus confirming the role of *prfA* Y154C in stimulating a moderate enhancement of *actA* expression *in vitro* (Fig. 1A and B).

2.2 The prfA Y154C mutation results in enhanced expression of additional PrfA-dependent gene products in vitro

To determine if the *prfA* Y154C mutation influenced the expression of genes in addition to *actA*, two additional PrfA-dependent gene products were examined. Levels of phosphatidylcholine phospholipase C (PC-PLC), encoded by *plcB*, were enhanced (Fig. 1C) as anticipated given that *plcB* is co-transcribed with *actA*. The expression of listeriolysin O (LLO, encoded by *hly*) was also enhanced approximately three-fold in comparison to wild type strains [NF-L1124 (wild type *prfA*), 17 units +/− units; NF-L1213 (*prfA* Y154C), 50 units +/− 5 units] (Fig. 1D).

The levels of PrfA-dependent gene expression observed in BHI broth culture for the *prfA* Y154C strain were found to be modestly higher than wild type levels of expression but lower than those observed for the *prfA** mutant *prfA* G155S [21] (Fig. 1A). When the mutant strains were grown in BHI broth treated with charcoal (BHIC), a condition known to enhance PrfA-dependent gene expression *in vitro* [23,45], strains containing *prfA* Y154C exhibited only a modest increase in *actA* expression (an approximate 7 to 9-fold increase compared to BHI levels as measured by GUS activity) in comparison to the large increase (up to 93-fold) observed for the *prfA** strain *prfA* E77K and a 33-fold increase observed for wild type *L. monocytogenes* (Table 3). The maximum *actA* expression levels as measured by GUS activity for both *prfA* G155S and *prfA* E77K strains grown in BHIC were significantly higher than either wild type or *prfA* Y154C strains (7312 units ± 137units and 7031 units ± 84 units versus 848 units ± 52 units and 1175 units ± 61 units, respectively) (Table 3). The *prfA* Y154C mutation thus differs from the *prfA** mutants *prfA* G155S and *prfA* E77K in that it failed to stimulate high level expression of *actA* in BHIC. The *prfA* Y154C mutant also exhibited a lower fold induction of *actA* than wild type in BHIC but was similar to wild type with regards to the maximum levels of *actA* expression achieved in this media.

2.3 PrfA Y154C fails to induce L. monocytogenes virulence gene expression within the cytosol of mammalian cells

While bacterial growth in BHIC for wild type strains results in a significant induction of *actA* expression in comparison to strains grown in BHI, expression levels are still low in comparison to the levels of *actA* expression achieved by bacteria located in the cytosol of infected host cells [38]. To determine if strains containing the *prfA* Y154C mutation were capable of fully inducing *actA* expression in the cytosol, mutant bacteria were first examined for their ability to gain entry and replicate within the cytosol of infected host cells (Fig. 2). Following bacterial uptake by J774 murine macrophage-like tissue culture cells, the *prfA* Y154C mutant was indistinguishable from wild type *L. monocytogenes* for intracellular growth (Fig. 2A). Based on rate and amount of intracellular bacterial replication, the *prfA* Y154C strain appeared to enter host cells and escape from cell vacuoles with an efficiency similar to that of wild type *L. monocytogenes* (Fig. 2A) and this same pattern was observed for the infection of epithelial cells (PtK2 cells) (Fig. 2B). In contrast, *L. monocytogenes* strains lacking functional PrfA remain trapped within host cell phagosomes and are unable to replicate [24]. However, when the NF-L1213 *prfA* Y154C strain was used to infect monolayers of murine fibroblast L2 cells, defects in bacterial cell-to-cell spread were readily observed (Fig. 2C). It has previously been demonstrated that defects in bacterial cell-to-cell spread can be visualized by the failure

of a mutant strain to form plaques in infected monolayers or by an overall reduction in plaque size [27]. Surprisingly, despite the enhanced expression of *hly* and *actA* in BHI broth culture, the *prfA* Y154C mutant failed to form any visible plaques in cell monolayers after three days incubation (Fig. 2C). Only after prolonged incubation (seven days) were tiny plaques detectable (M. Miner, unpublished data). In contrast, wild-type *L. monocytogenes* and the *prfA** mutants *prfA* G155S and *prfA* E77K {[19,21] and M. Miner, unpublished results} formed multiple plaques following a three day incubation in cell monolayers.

Consistent with its inability to form visible plaques after three days in cell monolayers, the mutant exhibited a profound delay in bacterial actin accumulation and polymerization in infected cells (Fig. 3A–G). At three hours post infection, approximately 70% of wild type bacteria were observed surrounded by actin clouds while 13% had progressed to having associated actin tails (Fig. 3A). In contrast, the *prfA* Y154C mutant at the same time point failed to accumulate actin of any form (Fig. 3B), and a trend of delayed actin association was seen for each time point examined (Fig. 3G). At 7 hours post-infection, small numbers of bacteria were observed associated with short actin tails (Fig. 3F), indicating that the *prfA* Y154C strain did eventually express sufficient intracellular *actA* to promote low amounts of actin polymerization.

The ability of *prfA* Y154C mutant strains to gain access to and replicate within the host cytosol enabled direct comparison of the intracellular levels of *actA* expression between wild type and mutant strains. The ability of *L. monocytogenes* to direct intracellular actin-based bacterial motility is dependent upon the abundant expression of the *actA* gene product in the host cytosol [35] and *actA* expression has been shown to increase at least 200-fold following bacterial escape from the phagosome [30,37,42,43]. Intracellular *actA* expression was therefore measured in wild type and *prfA* Y154C strains following the infection of PtK2 epithelial cells. Although the *L. monocytogenes prfA* Y154C mutant entered the cytosol and replicated with kinetics that were comparable to the wild type strain (Fig. 3A – F and M. Miner, unpublished results), the mutant exhibited only a modest increase in *actA* expression (an approximately 10-fold increase in *actA* expression in comparison to BHI) (Fig. 3H). In contrast, levels of cytosolic *actA* expression increased in wild type strains over 1000-fold in comparison to BHI grown cultures (Fig. 3H)

2.4 The prfAY154C mutant is less cytotoxic to infected host cells

Interestingly, the *prfA* Y154C mutant was also found to be significantly less cytotoxic to infected host cells than wild type bacteria based on the observed release of cellular lactate dehydrogenase (LDH) ($P < 0.001$) and on microscopic examination of infected cells (Fig. 4A–C). This reduction in cytotoxicity was not associated with the failure of the mutant to efficiently spread to adjacent cells as an *actA* deletion mutant completely defective for cell-to-cell spread exhibited levels of cytotoxicity essentially equivalent to those observed for wild type strains ($P > 0.05$) (Fig. 4A). Two PrfA-dependent gene products, LLO and PC-PLC, have been demonstrated to be major contributors to *L. monocytogenes*-mediated host cell cytotoxicity in multiple cell types, including PtK2 cells [30, 40, 46]. *plcB* (encoding PC- PLC) is co-transcribed with *actA* and intracellular transcripts directed by the *actA* promoter are reduced in the PrfA Y154C mutant strain (Fig. 3H). It was hypothesized that a block in PrfA Y154C activation would result not only in a decrease in PC-PLC activity but also in intracellular LLO production. Immunoprecipitation of radioactively labeled LLO from infected cells indicated that intracellular LLO production was indeed reduced by approximately 64% in PrfA Y154C mutants in comparison to wild type bacteria (Fig. 4D). These data are consistent with the failure of *prfA* Y154C to up- regulate PrfA-dependent target gene expression within the cytosol of infected host cells.

2.5 The L. monocytogenes prfA Y154C mutant is severely attenuated for virulence in mice but generates antigen specific CD8+ T cells

Given that the *prfA* Y154C mutation reduced the ability of *L. monocytogenes* to spread within infected cell monolayers, the capacity of the mutant strain to cause disease in infected animals was examined by determining the LD_{50} value following intravenous infection of BALB/c mice. Whereas the LD_{50} for wild type *L. monocytogenes* was approximately 2×10^4 CFU, the LD_{50} for the *prfA* Y154C mutant was approximately 3×10^6 CFU, which equates to a 150-fold reduction in virulence. This level of attenuation differs from that observed for strains completely lacking *prfA* as the absence of *prfA* or of *actA* expression results in severe attenuation [LD_{50} values of 3×10^9 and 2×10^7 for Δ*prfA* and Δ*actA*, respectively [26,47]]. Consistent with low level PrfA activity (versus no PrfA activity) the *prfA* Y154C mutant was capable of bacterial replication in the spleens of infected mice at days one and two post-infection, with a decline in bacterial numbers occurring at day three (Fig. 5, left panel). In contrast, no bacterial replication was observed for the Δ*actA* mutant even when animals were inoculated with larger numbers of bacteria (Fig. 5, left panel). In order to assess whether diminished *in vivo* replication altered the subsequent stimulation of antigen specific CD8+ cells following injection, mice were immunized with either wildtype or the *prfA* Y154C strain, and 7 days later, the frequency of $LLO_{91–99}$ or $p60_{217–225}$ specific IFN-γ secreting cells determined by intracellular cytokine staining. Target antigen specific IFN-γ secreting CD8+ cells were found to be stimulated following immunization, and the levels of the responses were not statistically different from the levels detected following immunization with wild type (Fig. 5, right panel). However, the response stimulated by *prfA* Y154C strains is dependent upon the immunization dose, as indicated by negligible levels of peptide specific cells measured at the 520 CFU dose. Collectively these results show that the *prfA* Y154C mutation allows for *in vivo* bacterial replication and stimulation of target antigen specific CD8+ effector cells while reducing host cell cytotoxicity and bacterial virulence.

3. Discussion

This study describes the isolation of a novel *prfA* mutation that promotes *L. monocytogenes* entry into the cytosol but inhibits actin-based bacterial motility, thereby attenuating bacterial virulence. Although the PrfA Y154C mutation stimulates modestly enhanced levels of PrfA-dependent gene expression during bacterial growth in BHI, the mutation is distinct from all other *prfA* mutations in that it facilitates phagosome lysis but inhibits PrfA-dependent gene expression within the host cell cytosol. The reduced expression of bacterial virulence genes within the cytosol decreased bacterial toxicity to infected host cells. The *prfA* Y154C mutant therefore represents a novel attenuated form of *L. monocytogenes* that is capable of antigen delivery into the cytosol in the absence of LLO and PC-PLC-mediated cytotoxicity.

The *prfA* Y154C mutation is notably distinct from a *prfA* loss of function mutation as demonstrated by the ability of *prfA* Y154C strains to invade host cells and enter the cytosol at rates equivalent to wild type bacteria, a phenotype not shared by *prfA* loss of function strains [10,47]. This indicates that the *prfA* Y154C mutation provides sufficient expression of PrfA-dependent gene products for host cell invasion and phagosomal escape. How might the *prfA* Y154C mutation modify PrfA function so as to impede cytosolic induction of PrfA-dependent genes? The PrfA Y154C mutation is located in a region of the protein associated with amino acid substitutions resulting in the mutational activation of PrfA (PrfA* mutants), including the PrfA G145S, PrfA G155S, and PrfA L140F mutations [19,21,22,48,49]. Studies by Eiting *et al* [48] have compared the structures of wild type PrfA with the PrfA G145S mutant protein and their data suggests that PrfA activation results in a conformational change that repositions the helix-turn-helix DNA binding region of PrfA such that it becomes more accessible for substrate. Eiting *et al.* [48] and additional investigators [22] have shown that PrfA G145S has

a higher binding affinity for PrfA target promoters. It is thus possible, based on its location that the PrfA Y154C mutation interferes with or prevents the complete repositioning of the helix-turn-helix domain that occurs following co-factor binding. The potential failure of PrfA Y154C to undergo a conformational shift following bacterial entry into the cytosol could account for the low levels of *actA* expression observed for the mutant in infected cells. Alternatively, it is possible that the Y154C mutation may somehow decrease PrfA protein stability in cytosolic bacteria.

L. monocytogenes strains lacking functional ActA are also capable of reaching the host cell cytosol and are defective for cell-to-cell spread [50]. However, Δ*actA* strains differ from *prfA* Y154C in that these strains maintain wild type *L. monocytogenes* secretion levels of LLO and PC-PLC and show levels of cytotoxicity that are not significantly different from wild type strains ($P > 0.05$) (Fig. 4). In addition, Δ*actA* strains do not exhibit an increase in bacterial CFU in liver and spleen following infection of mice (Fig. 5A). The *prfA* Y154C strain may thus present a novel means of delivering antigen to the cytosol in that, in contrast to Δ*actA* (and presumably auxotrophic mutants as well), the mutant replicates but does not efficiently induce high levels of LLO or PlcB secretion thereby potentially extending the amount of time that antigen could be delivered in the absence of host cell lysis.

4. Materials and Methods

4.1 Bacterial strains

L. monocytogenes strains used in this study are listed in Table 1. All *L. monocytogenes* strains were derived from 10403S (serotype 1/2a) and unless specified were grown in brain-heart infusion (BHI) broth. Bacteria containing integrated copies of plasmid vector pPL2 [25]were grown in 7.5 ug/ml chloramphenicol for vector maintenance. α-select *E. coli* (Bioline) was used for cloning and maintenance of plasmid constructs and was grown in Luria Broth (LB) at 37° C. SM10 *E. coli* was used for pPL2 vector-derived plasmid conjugation into *L. monocytogenes*.

4.2 Tissue culture cells

The murine macrophage-like cell line J774 and L2 mouse fibroblasts were grown in DMEM plus 10% FBS plus mM glutamine at 37° C with 5% CO_2 [26,27]. The Potoroo rat kidney cell line PtK2 was grown in MEM plus 10% FBS plus 2 mM glutamine at 37° C with 5% CO_2 [28].

4.3 Genetic screen for prfA mutations that lead to enhanced actA expression

Plasmid pNF1019 containing *prfA* under the control of the *prfA*P1, *prfA*P2, and *plcA* promoters in the integrative plasmid vector pPL2 (pNF1019) [19] was transformed into chemically competent XL1 Red *E. coli* hypermutator bacterial cells (Stratagene). Selected transformants were inoculated into LB at 1:1000 dilution and grown with shaking to stationary phase at 37° C with shaking. Cultures were repeatedly diluted and grown to stationary phase for a total of four cycles. The pNF1019 plasmid was then purified from XL1 Red and introduced via electroporation into conjugation competent SM10 cells. Transfer of pNF1019 from SM10 into *L. monocytogenes* Δ*prfA* was carried out as described previously [19]. Transconjugant *prfA* mutants exhibiting enhanced *actA* expression were identified as blue colonies on BHI plates containing 7.5 ug/ml chloramphenicol, 200 ug/ml streptomycin, and 50 ug/ml 5-bromo-4-chloro-3-indolyl- beta-D-glucuronic acid (X-gluc).

4.4 Construction of plasmids and L. monocytogenes recombinant strains

pNF1118, containing transcriptional fusions of *gus* and *neo* to *L. monocytogenes actA* in the temperature sensitive shuttle vector pKSV7 (pKSV7-*actA-gus-neo-plcB*), was generated by cloning the gene for neomycin resistance derived from pBEST501 [29] downstream of *gus* in plasmid pNF470 [30] using primers NEO-RBS and NEO-END (Table 2). Strains NF-L1123 (Δ*prfA*, *actA-gus-neo-plcB*) and NF-L1124 (*actA-gus-neo-plcB*) were constructed by introducing the *actA-gus-neo-plcB* fusion into the *L. monocytogenes* chromosome by allelic exchange using pNF1118 in NF-L890 (Δ*prfA*) and 10304S respectively. For the construction of an isogenic *prfA* Y154C strain, pNF1146 (pKSV7-*prfA*) was generated by PCR amplification of *prfA* coding sequences (from nucleotide +3 to +841 relative to the ATG start codon) from genomic DNA isolated from 10403S using primers prfA-pstI-Amplify-+3F and prfA-pstI-Amplify-+841R (Table 2). The PCR product was digested with *Pst*I and ligated into *Pst*I-digested pKSV7. The *prfA* Y154C mutations was introduced into pNF1146 using the Quik Change site directed mutagenesis (Stratagene) with plasmid pNF1146 (pKSV7-*prfA*) serving as a template for primers Y154C quik 5' and Y154C quik 3' (Table 2). The resulting plasmid, pNF1232, contained the *prfA* Y154C mutation and was used to replace the wild type chromosomal copy of *prfA* in NF-L1124 via allelic exchange as previously described [21].

4.5 Phospholipase activity

Phospholipase activity was measured as previously described [31]. Egg yolk overlay plates were made by mixing egg yolks 1:1 with PBS and added to BHI agar at 5% (vol/vol). Bacteria were streaked on egg yolk agar plates and incubated at 37° C overnight.

4.6 Hemolytic activity

Hemolytic activity was measured as previously described [32,33]. Briefly, bacteria were grown without shaking overnight at 30° C, the bacterial cells were removed using centrifugation, and two-fold serial dilutions of bacterial supernatants were incubated with PBS-washed sheep red blood cells for 30 minutes at 37° C. After incubation, cells were recovered by centrifugation to measure 50% lysis and supernatants were read in a spectrophotometer plate reader at OD_{450}. Hemolytic units are reported in the text as the reciprocal of the supernatant dilution resulting in 50% lysis of red blood cells [34].

4.7 Measurement of in vitro GUS activity

GUS activity was measured as previously described [35]. In short, bacteria were grown overnight in BHI at 30° C without shaking, diluted 1:25 in fresh media and grown with shaking at 37° C. For media treated with activated charcoal, a heat-sterilized 5% (w/v) stock suspension of HCl washed activated charcoal powder (Sigma) in ultrapure water was added to BHI broth to 0.2% final volume before autoclaving, stirred for 1 h, then autoclaved and filtered through a 0.22 μm membrane. At the indicated time points, the OD_{600} was determined for each culture using a spectrophotometer (UV-1201 by Shimadzu). Bacterial pellets were harvested from 500ul of culture and resuspended in ABT buffer (0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl, .1% Triton-X 100). GUS activity was measured as described by Youngman [36] with the substitution of 4-methylumbelliferyl-β-D-glucuronide (Sigma) in place of 4-methylumbelliferyl- β-D-galactoside [37].

4.8 Intracellular GUS assay

Measurement of intracellular GUS activity was carried out as described, modified for PtK2 cells [37]. Monolayers of PtK2 cells were grown to confluency on treated 60-mm petri dishes (Nunclon by Nunc). PtK2 cells were also grown on coverslips in duplicate 60-mm dishes. Monolayers were lysed at 8 hours post-infection in 200ul ABT buffer. 4-methylumbelliferyl-β -D-galactoside was replaced with 4-methylumbelliferyl-β-D-glucuronide (Sigma). At 2, 4, 6,

and 8 hours post-infection, three coverslips were removed, cells were lysed, and bacteria plated for CFUs. GUS units were determined based on the number of bacterial CFU recovered as previously described [38].

4.9 Plaque formation

Plaque assays were performed with murine L2 fibroblasts as described, infecting 6-well plates of confluent cells with 20ul of 1:250 diluted overnight culture grown at 30° C in BHI without shaking [27].

4.10 Intracellular bacterial growth and fluorescence

Intracellular growth in J774 macrophage-like cells were performed as previously described [21,39] with an MOI of ~1:5. Intracellular growth and fluorescence in PtK2 cells were performed as previously described [21] with an MOI of ~5:1. Actin association was quantified by fluorescence microscopy with a 100X objective by counting bacterial cells either enveloped in actin clouds or associated with actin tails. Ten separate fields were counted for a minimum of fifty bacteria within infected cells for each strain and the numbers were averaged for two independent experiments.

4.11 Cytotoxicity measurement

Cytotoxicity was measured by lactate dehydrogenase (LDH) release from infected PtK2 cells as described [40] using CytoTox 96 Non- Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions. PtK2 cells were infected with an MOI of 100:1 and LDH release was measured 24 hours post-infection. Percent cytotoxicity = 100 × [(experimental LDH release) - (spontaneous LDH release)]/[(maximum LDH release) − (spontaneous LDH release)].

4.12 Intracellular metabolic labeling of LLO

[^{35}S]-methionine labeling of bacterial proteins was performed as previously described [37]. PtK2 cells were infected with an MOI of 100:1 in 35-mm dishes. At 1 hour post-infection, cells were washed 3 times with PBS and media replaced with gentamicin (10 ug/ml). At hours p.i. the media was replaced with methionine-free media (methionine, cysteine-free DMEM, 10% dialyzed FBS, gentamicin) that contained protein synthesis inhibitors cycloheximide (22.5 ug/ml) and anisomycin (30 ug/ml). Thirty minutes later, fresh methionine-free media was added containing 200 uCi of [^{35}S]-methionine (EasyTag Express^{35}S Protein Labeling Mix, NEN, PerkinElmer) for 1 hour. Monolayers were then washed 3 times with PBS and lysed with 500 microliters RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors (Mini Complete, Roche, and 10 mM EDTA) and incubated rotating at 4° for 30 minutes. Lysates were then passed through 25 5/8 gauge needle and then centrifuged for 30 minutes at 4° to pellet bacteria. LLO was then immunoprecipitated using monoclonal antibody B3–19 [41] as described previously [26]. Immunoprecipitated samples were subjected to SDS-PAGE, transferred to nitrocellulose and processed for autoradiography using Storm phosphorimager and ImageQuant software. In a parallel experiment, PtK2 monolayers on coverslips were infected, the monolayers were lysed at 5 hours post-infection, and bacterial CFUs were enumerated on solid media to enable normalization of bacterial numbers.

4.13 Mouse infections

Fifty percent lethal doses (LD_{50}) were determined in BALB/c mice by intravenous injection as described previously according to IACUC approved procedures [39]. To evaluate *in vivo* growth, naïve BALB/c mice were intravenously infected with *L. monocytogenes* wild type 10403S, *prfA* Y154C, or Δ*actA* strains. At various time-points post-infection, spleens from

three mice were homogenized and serial dilutions of the homogenate were plated onto BHI agar to enumerate the bacterial load.

4.14 Intracellular cytokine staining and flow cytometry

BALB/c mice were immunized with wildtype of NF-L1213, and 7 days later, spleens were removed and single cell suspensions prepared by passing the tissue over a cell strainer. Intracellular cytokine staining was performed by culturing splenocytes *ex vivo* for 5 h with 10^{-6} M peptide in the presence of Brefeldin A (BioLegend). Surface staining for CD3 and CD8 and intracellular staining for IFNγ was performed with the Cytofix/Cytoperm kit according to the manufacturer's directions (BD Biosciences). Cells were stained with FITC, PE, Pacific Blue and/or APC-conjugated antibodies specific for CD8α (clone 53–6.7, eBioscience), CD3 (clone 145-2C11, eBioscience), and IFNγ(clone XMG1.2, eBioscience). Data acquisition was performed on a BD LSR II flow cytometer, and was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

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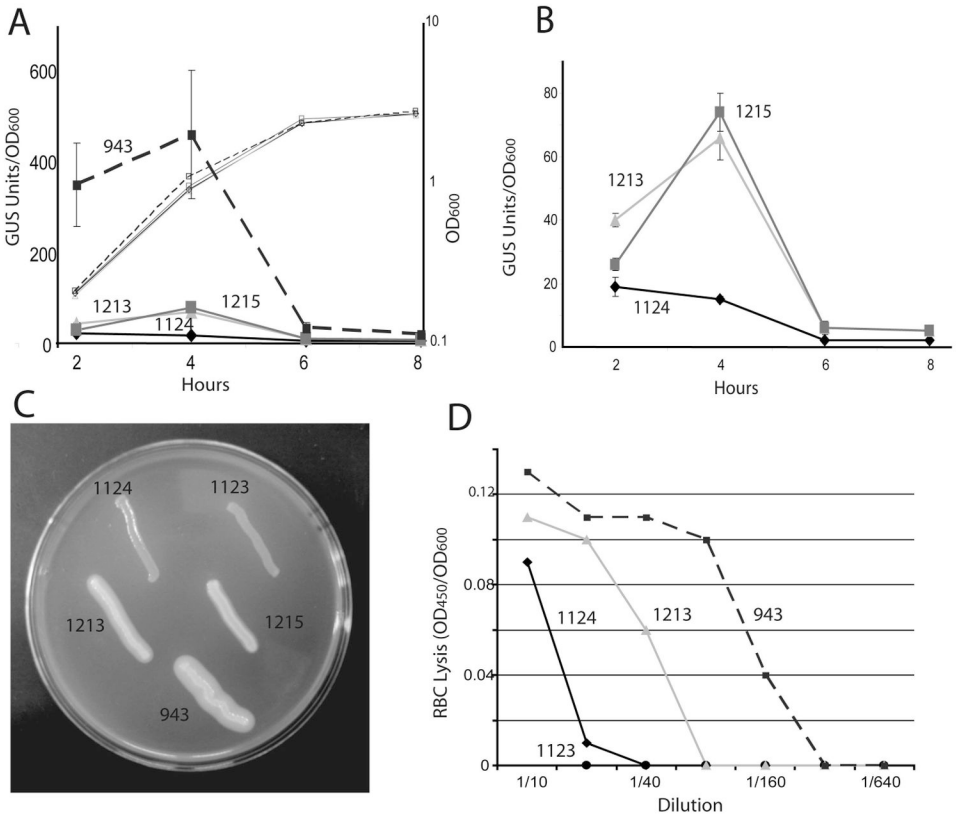

Fig. 1. *prfA* Y154C strains exhibit enhanced virulence gene expression *in vitro*

(A) Bacterial *actA* expression levels as measured by monitoring GUS activity. Levels of *actA* expression are indicated by symbols denoting GUS activity as measured at the indicated time points. GUS activity was measured for bacterial cultures grown in BHI broth and the units of GUS were normalized for optical density as described by Shetron-Rama *et al*., [38]. Each time point was measured in triplicate, and the data shown is representative of three experiments ± SEM. ◆, parent strain NF-L1124; ■, *prfA** mutant strain NF-L943 (*prfA* G155S); ■, original plasmid integration *prfA* Y154C mutant (NF-L1215); ▲, isogenic chromosomal *prfA* Y154C (NF-L1213). Large symbols indicate GUS activity, the smaller symbols reflect bacterial growth as measured by optical density. (B) Detailed view of NF-L1124, NF-L1213, and NF-L1215 from panel A. (C) Measurement of phospholipase activity. Bacteria were grown on media containing egg-yolk; the white precipitate surrounding bacterial growth is indicative of PC-PLC activity. Both NF- L1213 and NF-L1215 *prfA* Y154C strains exhibit enhanced PC-PLC activity in comparison to wild type (NF-L1124) or Δ*prfA* (NF-L1123) strains, but have lower levels than the *prfA** mutant strain NF-L943 (*prfA* G155S). Data shown is representative of two experiments. (D) Secreted hemolytic activity as measured by erythrocyte (RBC) lysis. Serial dilutions of bacterial supernatants were incubated with RBCs and cell lysis was determined by measuring absorbance at 450 nm (OD_{450}). Data shown is representative of three experiments. Symbols: ◆, parent strain NF-L1124; ▲, isogenic chromosomal *prfA* Y154C (NF-L1213); ● Δ*prfA* (NF-L1123); ■, *prfA* G155S (NF- L943).

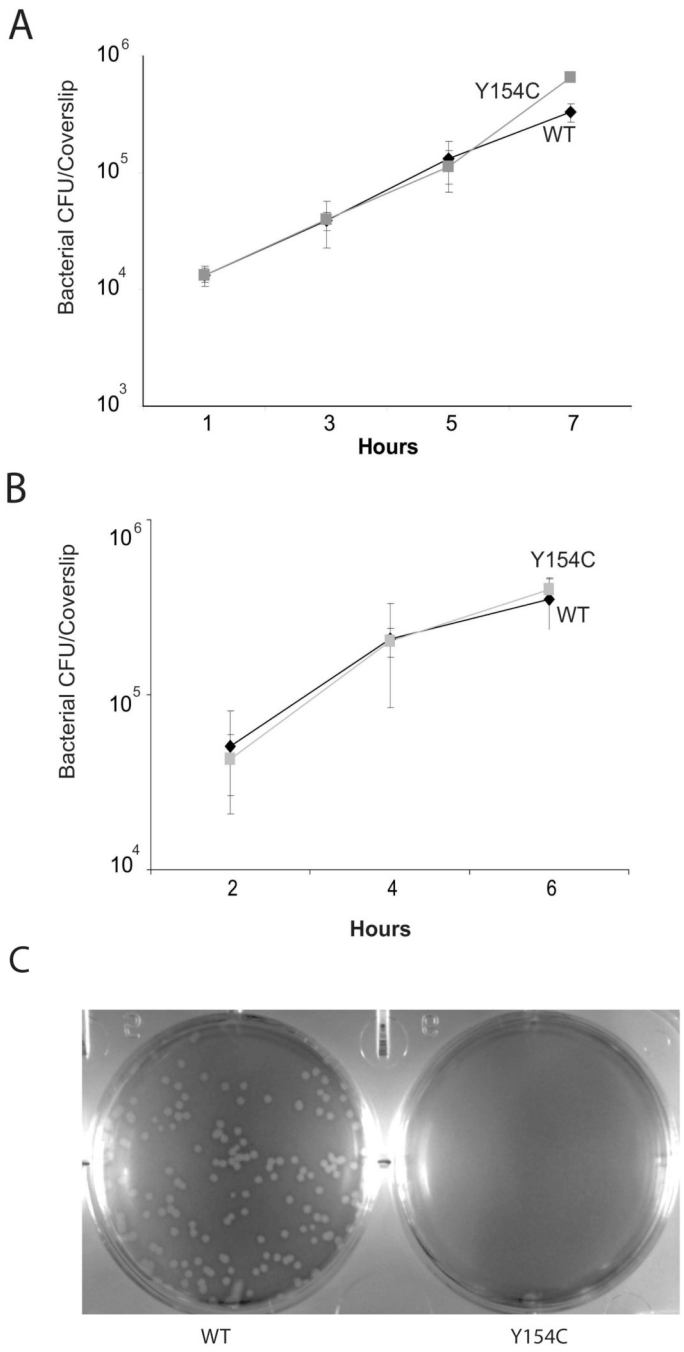

Fig. 2. *prfA* Y154C strains gain entry and replicate within the cytosol of host cells

L. monocytogenes strains were grown to stationary phase and used to infect tissue culture cells grown on glass coverslips. At 30 minutes (J774 cells) or 60 minutes (PtK2 cells) post-infection, monolayers were washed, gentamicin was added, and at the indicated time points cell monolayers were lysed and the number of bacteria per coverslip was determined. The results are expressed as the mean number of bacterial CFU + the standard deviation for three coverslips per time point. The data for one of three experiments with similar results is shown. (A) Bacterial intracellular growth in J774 macrophage-like tissue culture cells. (B) Bacterial intracellular growth in PtK2 epithelial cells. ◆, parent strain NF-L1124; ■, *prfA* Y154C (NF-L1213). (C) Intracellular growth and cell-to-cell spread of *L. monocytogenes* in murine L2 fibroblasts, as

indicated by plaque formation. The *prfA* Y154C strain failed to form visible plaques after three days of incubation, whereas multiple plaques were visible in monolayers infected with wild type bacteria. Data shown is representative of three experiments.

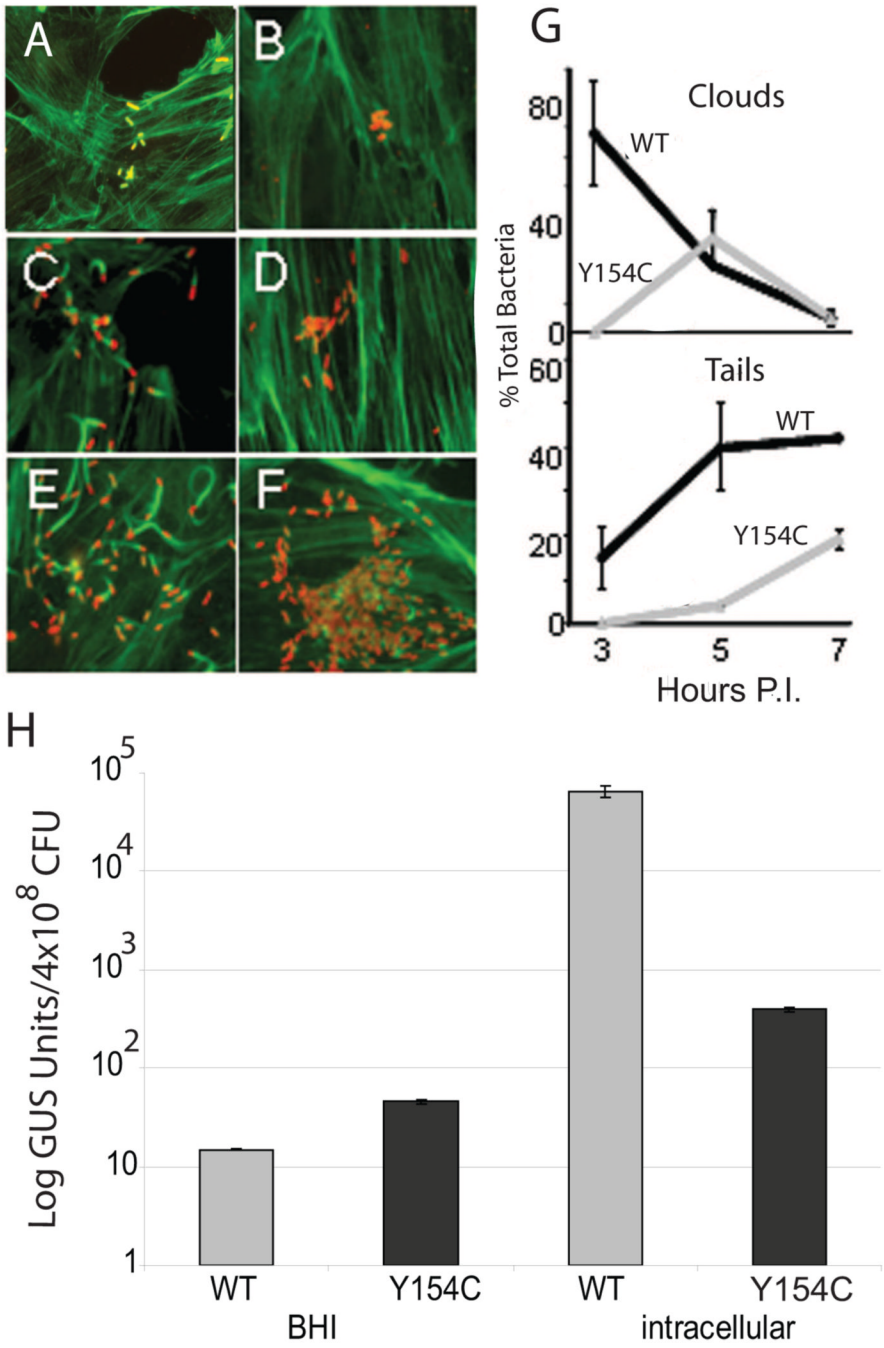

Fig. 3. The *prfA* Y154C mutation reduces intracellular *actA* expression and bacterial actin association

(A–F) Visualization by fluorescence microscopy of polymerized actin in PtK2 epithelial cells infected with *L. monocytogenes*. PtK2 cells were infected with either NF-L1124 (wild type *prfA*) (A, C, E) or NF-L1213 (*prfA* Y154C) (B, D, F) bacteria at an MOI of ~5:1. At one hour post-infection the monolayers were washed and gentamicin was added to kill any extracellular bacteria. At 3 hr (A, B), 5 hr (C, D), and 7 hr (E, F) post-infection the monolayers were fixed, permeabilized, and stained with NBD-phallicidin for filamentous actin (green) and an anti-*Listeria* antibody and tetramethylrhodamine goat anti-rabbit conjugated secondary antibody to detect bacteria (red) as described in Materials and Methods. Results shown are representative

of two independent experiments. (G) Quantitation of actin association for wild type NF-L1124 and *prfA* Y154C strain NF-L1213. Actin association in the form of clouds (polymerized actin surrounding bacteria) or tails was scored for approximately 50 bacteria in infected cells from at least 10 independent microscopic fields for each time-point listed. Numbers listed represent the mean ± standard error derived from two independent experiments. (H) Bacterial intracellular *actA* expression as measured by GUS activity. Tissue culture dishes containing monolayers of PtK2 cells were infected with the indicated *L. monocytogenes* strains for 8 h as described in Materials and Methods. GUS activity was determined following lysis of the monolayers. The number of CFU per dish was determined by lysing infected PtK2 cells grown on coverslips in duplicate dishes and plating a portion of the lysates. Units of GUS are as described by Shetron-Rama *et al*. [38]. Each assay was done in triplicate and the data represent the mean ± standard deviation for at least three individual experiments.

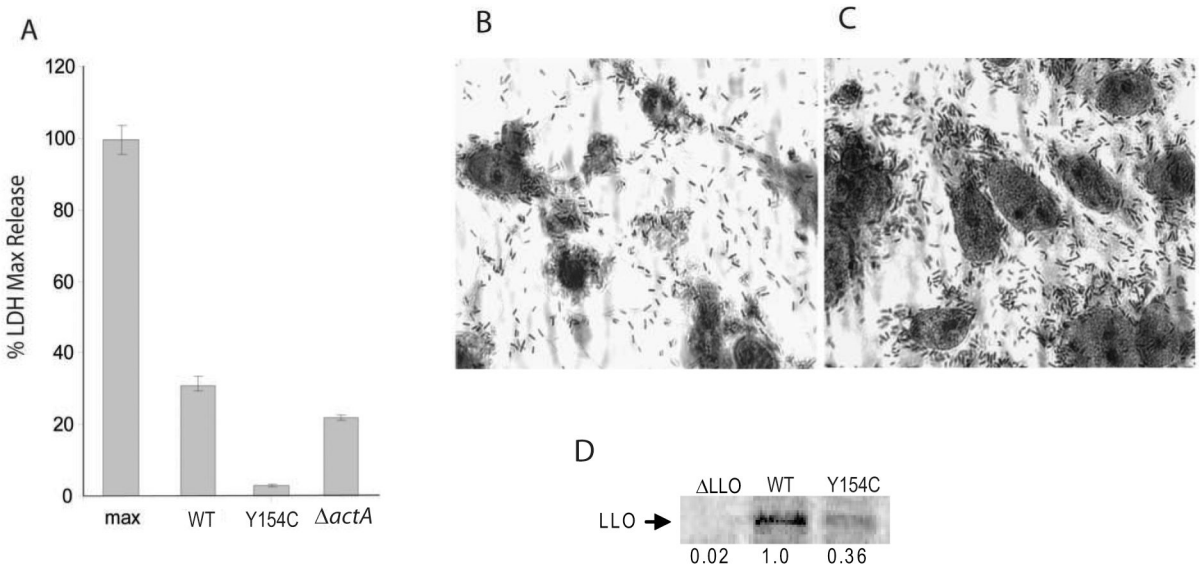

Fig. 4. The *prfA* Y154C mutation reduces bacterial cytotoxicity for host cells and intracellular LLO expression

(A) Cytotoxicity was measured by lactate dehydrogenase (LDH) release from bacteria-infected PtK2 epithelial cells at 24 hours post-infection with the indicated strains. Maximum release (max) was determined from complete host cell lysis and set to 100%; all values are expressed as a percentage of max ± standard deviation. The data shown is representative of three independent experiments. (B–C) Cytotoxicity visualized by microscopy of PtK2 cells infected with the parent strain (NF- L1124) (B) or *prfA* Y154C (NF-L1213) (C) at 24 hours post-infection. PtK2 cells were infected with bacteria at an MOI of ~5:1. At one hour post-infection the monolayers were washed and gentamicin was added to kill any extracellular bacteria. At 24 hr post-infection the monolayers were fixed and stained with Diff Quik stain. Data shown is representative of three independent experiments. (D) Intracellular expression of LLO by *L. monocytogenes* in PtK2 cells. PtK2 cells were infected with *L. monocytogenes*: at 5 hr post-infection the infected cells were starved for methionine and cysteine and host protein synthesis was blocked as described in Materials and Methods. Infected cells were incubated with [^{35}S]-methionine for 1 hr prior to host cell lysis and immunoprecipitation with monoclonal anti-LLO antibody B3–19 [41]. The amount of sample loaded per lane was normalized for the number of bacteria. An autoradiograph representative of two independent experiments is shown, which was exposed for 100 hours using the Storm Phosphorimager and analyzed with ImageQuant software. The arrow indicates the position of the LLO band, which is approximately 58 kD.

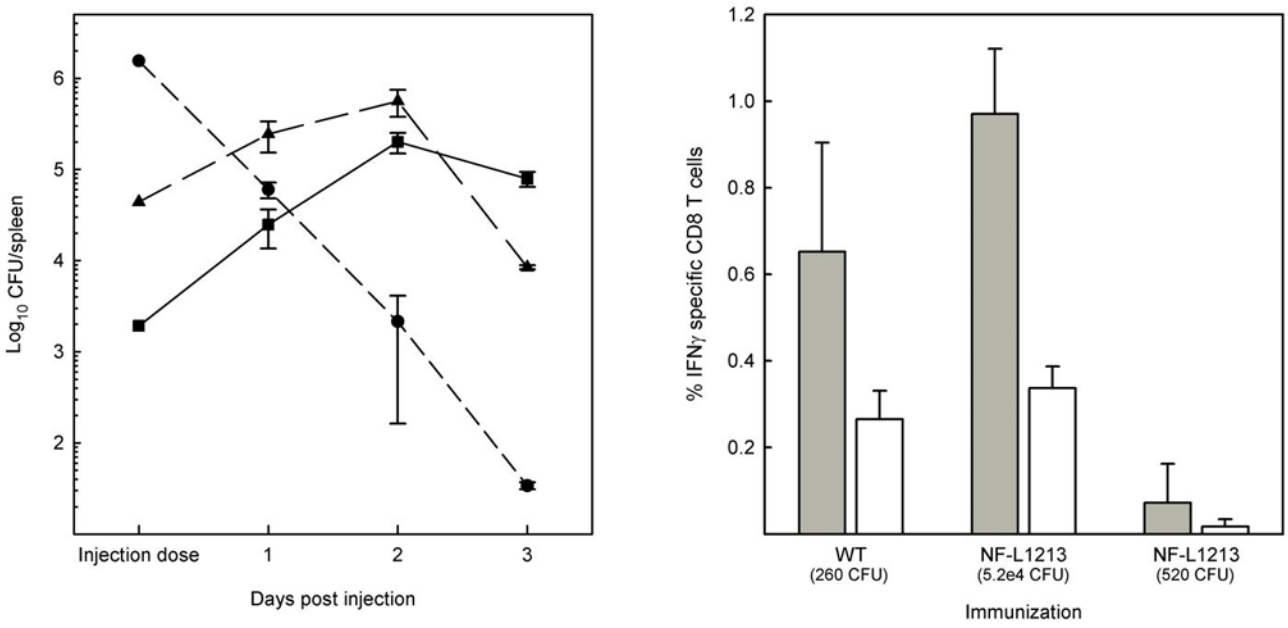

Fig. 5. The *prfA* Y154C mutant is capable of limited growth within the spleens of infected animals and stimulates target antigen specific CD8$^+$ effector cells

Left panel: BALB/c mice were injected intravenously with wild type (■, 1900 CFU), Δ*actA* (●, 1.55×10^6 CFU), or *prfA* Y154C (▲, 4.4×10^4 CFU). On the days indicated the spleens from 2–3 mice were homogenized individually and numbers of CFU/spleen determined by dilution plating. Right panel. BALB/c mice were immunized with either wildtype *L. monocytogenes* or *prfA* Y154C (NF-L1213) at the indicated doses. Seven days later, spleens were removed, a single cell suspensions prepared and the frequency of $LLO_{91–99}$ (grey bars) or $p60_{217–225}$ (white bars) specific IFN-γ producing CD8+ cells by determined by intracellular cytokine staining.

Table 1

L. monocytogenes and *E. coli* strains used in this study

Strain	Genotype	Reference
NF-L100	10403S WT	[39]
DP-L1492	10403S Δ*actA*	[26]
DP-L1261	10403S Δ*hly*	[51]
NF-E470	*E. coli* pKSV7-*actA-gus-plcB*	[30]
NF-L476	NF-L100 *actA-gus-plcB*	[30]
NF-L890	NF-L100 Δ*prfA*	[19]
NF-E908	*E. coli* pBEST501	[29]
NF-L924	NF-L476 *prfA* E77K	[21]
NF-L943	NF-L476 *prfA* G155S	[21]
NF-L1003	NF-L890 *actA-gus-plcB*	[19]
NF-E1019	*E. coli* pPL2-*pplcA-pprfA-prfA*	[19]
NF-L1123	NF-L890 *actA-gus-neo-plcB*	This study
NF-L1124	NF-L100 *actA-gus-neo-plcB*	This study
NF-L1215	NF-L1003 *pPL2-pplcA-pprfA-prfA* Y154C	This study
NF-L1213	NF-L1124 *prfA* Y154C	This study
NF-E1232	*E. coli* pKSV7-*prfA* Y154C	This study
NF-E1118	*E. coli* pKSV7-*actA-gus-neo-plcB*	This study
NF-E1146	*E. coli* pKSV7-*prfA*	This study

Table 2

Oligonucleotide primers used in this study

Name	Sequence 5'-3'	Characteristics
NEO-RBS	GCGCTGCAGAGGAGGAAAAATATGAATGGACCAATAATAATGACT	Neo 5' RBS and PstI site
NEO-END	GCGCTGCAGCGTTCAAAATGGTATGCGTTTTGA	Neo 3' PstI site
Y154C quik 5'	TCCTGACCTATGTGTGTGGTAAAGAAACTCCTG	Y154C 5' quik change
Y154C quik 3'	CAGGAGTTTCTTTACCACACACATAGGTCAGGA	Y154C 3' quik change
prfA-PstI-amplify-+3F	GCGCTGCAGGAACGCTCAAGCAGAAGAATTC	prfA 5' PstI site
prfA-PstI-amplify-+841R	GCGCTGCAGGGAACAACTATCTGTTGCAGCTC	prfA 3' PstI site

Table 3

Fold change of *actA/gus* expression in BHIC compared to BHI and maximum GUS units produced during exponential growth

Time (hours)[a]	OD600[b]	NF-L1123 (Δ*prfA*)	NF-L1124 (*prfA*)	NF-L924 (*prfA* E77K)	NF-L943 (*prfA* G155S)	NF-L1213 (*prfA* Y154C)
1	~0.03	0.5[c] (26 ± 9.2)[d]	1.1 (107 ± 13)	3.8 (2980 ± 98)	2.5 (5205 ± 409)	0.9 (659 ± 0)
3	~0.2	0.5 (6.7 ± 0)	11 (404 ± 8.7)	36 (7031 ± 84)	20 (7312 ± 137)	5.1 (735 ± 3.1)
5	~1.2	0.5 (3.8 ± 0.4)	33 (848 ± 52)	77 (3764 ± 25)	10 (4377 ± 184)	6.9 (1175 ± 61)
7	~2.0	1 (2.3 ± 0)	17 (87 ± 0.4)	93 (2654 ± 232)	31 (874 ± 19)	8.8 (190 ± 0.7)

[a] Hours growth in broth culture

[b] Approximate bacterial culture optical density at 600 nm

[c] Fold increase in GUS activity observed for growth in BHIC compared to growth in BHI at the same time point.

[d] (Maximum GUS units in BHIC + SD)