Constitutive Activation of PrfA Tilts the Balance of Listeria monocytogenes Fitness Towards Life within the Host versus Environmental Survival

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Abstract

PrfA is a key regulator of Listeria monocytogenes pathogenesis and induces the expression of multiple virulence factors within the infected host. PrfA is post-translationally regulated such that the protein becomes activated upon bacterial entry into the cell cytosol. The signal that triggers PrfA activation remains unknown, however mutations have been identified (prfA* mutations) that lock the protein into a high activity state. In this report we examine the consequences of constitutive PrfA activation on L. monocytogenes fitness both in vitro and in vivo. Whereas prfA* mutants were hyper-virulent during animal infection, the mutants were compromised for fitness in broth culture and under conditions of stress. Broth culture prfA*-associated fitness defects were alleviated when glycerol was provided as the principal carbon source; under these conditions prfA* mutants exhibited a competitive advantage over wild type strains. Glycerol and other three carbon sugars have been reported to serve as primary carbon sources for L. monocytogenes during cytosolic growth, thus prfA* mutants are metabolically-primed for replication within eukaryotic cells. These results indicate the critical need for environment-appropriate regulation of PrfA activity to enable L. monocytogenes to optimize bacterial fitness inside and outside of host cells.

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Introduction

The environmental bacterial pathogen Listeria monocytogenes is an intriguing example of a microorganism that has become well adapted to life in the soil as well as to life within the cytosol of mammalian host cells. This bacterium is widespread in the environment where it is believed to live as a saprophyte on decaying plant material [1]. Upon ingestion by a susceptible mammalian host, L. monocytogenes transitions into a physiological state that facilitates bacterial survival and replication within host cells [2,3]. While disease caused by L. monocytogenes in healthy individuals is usually restricted to a self-limiting gastroenteritis, in immunocompromised individuals and pregnant women L. monocytogenes is capable of causing systemic infections that lead to meningitis, encephalitis, and in the case of pregnant women, infection of the developing fetus leading to abortion, stillbirth, or neonatal infections [4,5]. L. monocytogenes contamination of food products has resulted in some of the most expensive food recalls in U.S. history [2,6–12] and this is thought to reflect the bacterium’s widespread environmental distribution and its ability to withstand a variety of stress conditions [13–16].

A significant amount of research has focused on the mechanisms used by L. monocytogenes to establish its replication niche within mammalian host cells. L. monocytogenes invades a wide variety of cell types and is capable of escaping from the phagosome following cell entry, of replicating within the cytosol, and of utilizing host cell actin polymerization machinery to propel itself through the cytosol and into neighboring cells [3,5,17]. To survive and flourish within eukaryotic cells the bacterium requires the regulated expression of a number of secreted virulence factors, and the expression of most of these gene products is regulated by a transcriptional regulator known as PrfA [18]. PrfA is an essential regulator of L. monocytogenes pathogenesis, and bacterial mutants that lack functional PrfA are severely attenuated in animal infection models [19,20].

PrfA is a member of the Csr/Fnr family of transcriptional activators, and members of this family appear to require post-translational modification or the binding of a small molecule co-factor for full activity [21–23]. PrfA activation occurs upon bacterial entry into the host cell cytosol and is required for the increased expression of gene products that promote bacterial cell-to-cell spread [19,24–29]. L. monocytogenes strains that encode a mutant form of prfA (prfA Y154C) whose product fails to become activated following cytosol entry are severely attenuated for virulence [30]. The signal that induces PrfA activation remains unknown, however L. monocytogenes strains have been isolated that contain mutations within prfA resulting in constitutive PrfA activation (prfA* alleles) [31–36]. prfA* strains exhibit enhanced invasion of host cells, rapid escape from the phagosome, and an apparent increase virulence following intravenous injection of mice [32,37]. In broth culture prfA* mutants exhibit the same high
levels of PrfA-dependent gene expression normally observed for bacteria during intracellular growth [32,30–40]. While a number of mutations have been identified in prfA that confer activation, the absolute level of activation observed for different amino acid substitution mutants can vary, with mutants exhibiting the highest level of activation most closely resembling the levels of activation observed for cytotoxic bacteria [32,34,41,42].

Given that L. monocytogenes has evolved specific mechanisms to regulate PrfA activity in response to environmental conditions found inside and outside of host cells, we sought to determine the impact of constitutive PrfA activation on the fitness of L. monocytogenes by comparing the growth of strains in broth culture and in tissue culture and mouse infection models. Our results indicate that PrfA activity must be carefully modulated in response to environmental signals so as to enable L. monocytogenes to optimize bacterial fitness both inside and outside of the infected host.

Results

Constitutive activation of PrfA reduces the fitness of L. monocytogenes in nutrient-rich broth

Until recently, it has proven difficult to construct isogenic L. monocytogenes prfA* mutant strains containing the alleles that confer the highest PrfA activity by standard methods. As a result, these high activity prfA* mutations have been introduced into ΔprfA strains on plasmids [30,31,34,40,43,44]. While these approaches have been informative, there are associated caveats that include multicopy plasmid effects or altered gene expression profiles resulting from the use of integrated plasmids in ectopic locations. We recently reported the successful construction of high activity prfA* isogenic mutants in strains containing promoterless copies of the genes encoding β-glucuronidase (gus) and neomycin resistance (neo) located in the chromosome downstream of the PrfA-dependent gene actA [41]. Isogenic prfA* mutants constructed via allelic exchange were isolated based on the PrfA*-dependent increase in actA-gus-neo expression that enabled selection for prfA* colonies on selective media containing neomycin and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (x-gluc), a substrate for GUS activity. This approach now enables the direct comparison of independently isolated L. monocytogenes prfA* mutants with strains containing the wild type allele.

To assess if the constitutive activation of PrfA influences the fitness of L. monocytogenes outside of host cells, strains containing mid-level (prfA G155S) or high-level (prfA G145S and prfA L140F) prfA* activity mutations [41] were compared with a wild type strain for growth in BHI broth. Consistent with previous reports, prfA* mutations conferred high levels of PrfA activity in broth culture as indicated by actA expression levels (Fig. 1A). Expression from the actA promoter for the prfA G155S and prfA G145S mutants was 230-fold and 1870-fold respectively than the levels observed for wild type prfA strains after 24 hours of growth in BHI (Fig. 1A). Overall growth of the prfA* mutants was very similar to that of the wild type strain, although the doubling times of the prfA* mutants during logarithmic growth were slightly longer (Table S1) and the final bacterial cell densities at stationary phase were slightly lower in the prfA* monocultures than in the wild type monocultures (Fig. 1B).

In contrast to monoculture growth, pronounced fitness effects were observed for high activity prfA* strains when the mutants were mixed and grown with wild type bacteria in BHI. Each prfA* mutant exhibited a competitive defect when cultures were inoculated in equal numbers with wild type bacteria and grown to stationary phase with subsequent cycles of dilution and outgrowth (Fig. 1C). After nine sequential cycles of overnight growth and dilution, wild type bacteria were observed in two-fold greater numbers in comparison to the mid-level prfA G155S mutant and 200-fold greater numbers in comparison to the high-level prfA G145S and prfA L140F mutant strains (Fig. 1C). A direct correlation thus appeared to exist between the level of PrfA activation conferred by a prfA* mutation and the magnitude of the competitive defect observed in broth culture. In addition, the identical phenotypes observed for independently derived prfA G145S and prfA L140F strains confirmed that the observed defect resulted from the mutational activation of prfA and did not reflect a second site mutation; an additional independently derived prfA* mutant (prfA Y63C) likewise exhibited an identical competitive defect [J. Bruno, unpublished]. Bacterial supernatants derived from wild type cultures did not inhibit the growth of prfA* mutants [J. Bruno, unpublished], indicating that there is no apparent inhibitory substance produced by wild type bacteria that compromised mutant growth.

To determine if the competitive defects exhibited by the prfA* mutants occurred during logarithmic growth or whether the defects were associated with entry into or survival during stationary phase, mixed cultures were diluted into fresh BHI upon reaching late-logarithmic phase (OD600 of 0.8–1.0), prior to bacterial entry into stationary phase. When mixed cultures of wild type and prfA G145S bacteria were grown under these conditions, the resulting competitive defect was essentially identical to the competitive defect observed for mixed cultures grown to stationary phase (Fig. 1D). This indicates that constitutive activation of PrfA impairs the competitive fitness of L. monocytogenes in broth culture during logarithmic growth.

The presence of glucose exacerbates the competitive defect exhibited by L. monocytogenes prfA* strains. It has been previously reported that multicopy plasmid-based over-expression of constitutively activated PrfA (prfA G145S) interferes with bacterial utilization of glucose as a carbon source [43,44]. To examine if isogenic prfA* mutants exhibited a fitness defect in the presence of glucose, the prfA G145S mutant was grown in LB buffered to pH 7.4 and supplemented with 55 mM of glucose. LB was selected for monitoring growth as L. monocytogenes requires an added carbon source for optimal growth in this medium. Similar to the observations made for prfA* monocultures in BHI, cultures grown in LB and glucose-supplemented LB resembled the wild type strain with only subtle growth differences, indicating that the isogenic prfA* strains were able to efficiently use glucose as a carbon source (Fig. 2A and Table S1). However, when prfA G145S cultures were mixed with the wild type strain and grown in LB or in LB supplemented with glucose, the competitive defect exhibited by the prfA G145S mutant in LB with glucose was of greater magnitude than that exhibited in LB alone (Fig. 2B). After seven cycles of dilution and outgrowth, wild type bacteria outnumbered the prfA G145S mutants by 30-fold and 170-fold in LB and in glucose-supplemented LB, respectively (Fig. 2B). The presence of glucose thus exacerbated the competitive defect associated with PrfA activation in broth culture.

Constitutive activation of PrfA increases the fitness of L. monocytogenes in the presence of glycerol

Stoll et al. have reported that plasmid-based over-expression of prfA decreased the fitness of L. monocytogenes in the presence of glycerol based on reduced growth in media where glycerol was the main or sole carbon source [44]. To determine if isogenic prfA* mutants were compromised for growth in the presence of glycerol, the prfA G145S mutant was grown in LB buffered to pH 7.4 and supplemented with 55 mM glycerol. Surprisingly, monocultures of the prfA G145S mutant in glycerol-supplemented LB grew to five-fold higher cell densities in comparison to wild type strains (Fig. 2A).
Consistent with this growth advantage, the pfrA G145S mutant exhibited a competitive advantage when it was mixed and grown with wild type L. monocytogenes in glycerol-supplemented LB. After seven cycles of dilution and outgrowth, pfrA G145S outnumbered wild type bacteria by more than 20-fold (Fig. 2B). These findings indicate that constitutive activation of PrfA increases the fitness of L. monocytogenes in the presence of glycerol. The findings further indicate that competitive defects associated with the pfrA* strains in other media cannot simply be attributed to the metabolic burden of increased PrfA-dependent gene product expression, as high expression levels are maintained by pfrA* strains in the presence of glycerol ([44,45] and J. Bruno, unpublished).

Constitutive activation of PrfA increases the sensitivity of L. monocytogenes to osmotic stress and acid stress

The ability of L. monocytogenes to withstand a variety of stresses is vital for its survival and replication in disparate environments [5,13,46,47], including food processing facilities [9,48] and the gastrointestinal tract [3,17,49,50]. To determine if constitutive activation of PrfA influences the ability of L. monocytogenes to respond to stress, monoculture and mixed culture growth of the pfrA* mutants and wild type L. monocytogenes was examined under two different stress conditions, osmotic stress and acid stress. Although no dramatic differences were observed for mutant and wild type strains with respect to growth in monoculture (Fig. 3AC), the pfrA G145S mutant and the pfrA G145S mutant exhibited more severe competitive defects when mixed with the wild type strain and grown in BHI supplemented with 5% NaCl in comparison to BHI lacking additional NaCl (Fig. 3B). After nine cycles of dilution and outgrowth, wild type bacteria outnumbered mutants by more than 150-fold (pfrA G155S) and 200,000-fold (pfrA G145S) in the presence of additional NaCl, in comparison to differences of 2-fold (pfrA G155S) and 200-fold (pfrA G145S) in growth media lacking added NaCl (Fig. 3B).
Figure 2. Effects of different carbon sources on monoculture growth and competitive index of a prfA* mutant. (A) Growth curves of the wild type and prfA G145S L. monocytogenes strains in buffered LB (pH 7.4) with and without 55 mM of either glucose (glu) or glycerol (gly) at 37 °C with shaking were determined by measuring CFU/mL at the specified time points. Each growth curve is representative of two independent experiments. (B) The wild type camR strain was mixed with the chloramphenicol-sensitive prfA G145S mutant in buffered LB with and without 55 mM of either glucose (glu) or glycerol (gly) at 37 °C with shaking. Mixed cultures were subjected to repeated cycles of growth and dilution (1:100) into fresh media every 24 hours. CI values were determined immediately prior to each dilution. The data represent the means ± standard errors of three independent experiments.

Figure 3. Stress conditions exacerbate the competitive defects exhibited by prfA* mutants. (A) Monoculture growth curves of L. monocytogenes strains in BHI supplemented with 5% NaCl at 37 °C were determined by measuring CFU/mL at the specified time points. The growth curves of wild type and prfA G145S L. monocytogenes in BHI without additional NaCl are included. (B) Competitive index of wild type camR strain mixed with a chloramphenicol-sensitive prfA* mutant in BHI supplemented with 5% NaCl at 37 °C. Mixed cultures were subjected to repeated cycles of growth and dilution (1:100) into fresh media every 24 hours. CI values were determined immediately prior to each passage. The data represent the means ± standard errors of three independent experiments. (C) Monoculture growth curves of L. monocytogenes strains in BHI buffered to pH 7.4 or pH 5.5 at 37 °C were determined by measuring CFU/mL at the specified time points. (D) Competitive index of the wild type camR strain mixed with a chloramphenicol-sensitive prfA* mutant in BHI buffered to pH 7.4 or 5.5 at 37 °C. Mixed cultures were subjected to repeated cycles of growth and dilution (1:100) into fresh media every 24 hours. CI values were determined immediately prior to each dilution. The data represent the means ± standard errors of three independent experiments.
The prfA G145S mutant exhibited a similarly exacerbated competitive defect under acid stress. When L. monocytogenes was grown in unbuffered BHI broth at 37°C, the pH was observed to decrease from approximately 7.2 to 6.0 after 24 hours of growth (J. Bruno, unpublished). When grown with wild type L. monocytogenes strains, the prfA G145S mutant initially exhibited a more severe competitive defect in BHI buffered to pH 5.5 than in unbuffered BHI. After three cycles of dilution and outgrowth, wild type bacteria were present in 400-fold greater numbers than the prfA G145S mutant in BHI pH 5.5 in comparison to 15-fold greater numbers in unbuffered BHI (Fig. 3D). Interestingly, the largest competitive defect exhibited by the prfA G145S mutant during the first three cycles of dilution and outgrowth in BHI pH 5.5 shifted to a competitive advantage with subsequent cycles, reducing the wild type advantage from 400-fold to 20-fold after nine cycles (Fig. 3D). This ratio was similar to the ratio observed after nine cycles of dilution and outgrowth in BHI buffered to pH 7.4 (Fig. 3D). These findings suggest that the prfA G145S mutant goes through an adaptation or acid tolerance response [51] that increases its tolerance to acid stress to wild-type levels or even beyond. Overall, these findings indicate that constitutive activation of PrfA impaired the ability of L. monocytogenes to respond to osmotic stress as well as its initial response to acid stress conditions.

The impaired stress response of prfA* mutants does not result from impaired function of the stress-associated sigma factor, SigB

The exacerbated decrease in the bacterial fitness of prfA* mutants when subjected to two different stress conditions suggested that a general response related to stress tolerance may be compromised by constitutive activation of PrfA. A central regulatory component that contributes to the ability of L. monocytogenes to survive various stress conditions is the alternative RNA polymerase sigma factor SigB [16,52]. SigB contributes to prfA expression [25], and several previous studies have suggested the existence of functional overlap between SigB and PrfA in regulating the expression of L. monocytogenes genes that contribute to virulence and/or stress tolerance [40,53–60]. While ΔsigB growth in monoculture resembled that of the wild type strain (Supplemental Fig. S2), ΔsigB mutants exhibited a competitive defect when mixed with the wild type strain in BHI, indicating that loss of SigB function decreases the competitive fitness of L. monocytogenes (Fig. 4). Interestingly, the magnitude of the competitive defect exhibited by the ΔsigB mutant closely resembled that observed for prfA G145S mutants in BHI (Fig. 4C).

To determine if the competitive defect associated with prfA* strains was related to an impairment of SigB function, prfA G155S ΔsigB and prfA G145S ΔsigB double mutants were tested in broth competition assays. If PrfA* impairs SigB function, one would anticipate that the magnitude of the competitive defect exhibited by a prfA* ΔsigB double mutant would be equivalent to that exhibited by either single mutant (Fig. 4A). If however the magnitude of the competitive defect exhibited by a prfA* ΔsigB double mutant was equivalent to the sum of the defects exhibited by the prfA* and ΔsigB single mutants (Fig. 4A), this would suggest that PrfA and SigB alter stress resistance through separate pathways. The competitive defect of a prfA* ΔsigB double mutant was found to be equivalent to the sum of the defects of the prfA* and the ΔsigB single mutants, and the additive effect of prfA* and ΔsigB in the double mutant strain was evident throughout the course of mixed growth (Fig. 4BC). Therefore, the stress related competitive defect associated with the constitutive activation of PrfA appears distinct from the defect associated with the loss of SigB function.

Constitutive activation of PrfA enhances L. monocytogenes virulence following intravenous and intragastric infection of mice

Previous studies have reported that the prfA* mutants with mid-level PrfA activity (prfA G155S mutants) were fully virulent when intravenously inoculated into mice based on the bacterial CFU required for a 50% lethal dose (LD50) [32]. Consistent with this observation, mice intravenously infected with 2 × 10^5 CFU had significantly higher numbers of prfA* bacteria (prfA G155S, prfA G145S, and prfA L140F) recovered from the livers and spleens at 24 hours post-infection compared to those infected with wild type bacteria (Fig. 5A) (the liver and spleen are the primary organ targets for L. monocytogenes replication [5]). Although the difference was not statistically significant at 48 hours post-infection, the bacterial burdens of the livers and spleens from mice infected with the prfA* mutant tended to be higher than in organs associated with wild type infection (Fig. 5B and J. Bruno, unpublished). The hyper-virulent phenotype of the prfA* mutants was more apparent when the infectious dose was reduced by ten-fold to 2 × 10^3 CFU: the bacterial burdens of the livers and spleens from mice infected with the prfA G145S mutant at 48 hours post-infection were approximately 300-fold higher and 4.5-fold higher in liver and spleen than those of mice infected with wild type bacteria (P<0.01 for both organs) (Fig. 5B). In mixed infection, the prfA* mutants consistently exhibited a competitive advantage over wild type strains (Fig. 5C). The competitive index values determined for each liver and spleen for intravenously infected mice at 48 hours post-infection showed that, on average, 2- to 7-fold more prfA* bacteria were recovered from each organ in comparison to wild type (Fig. 5C).

Although constitutive activation of PrfA enhanced bacterial infection following intravenous injection of mice, the increased sensitivity to both osmotic stress and acid stress observed for the mutant strains (Fig. 3) suggested that the virulence of the prfA* mutants might be attenuated if administered orally, the more natural route of infection. We therefore examined the consequences of constitutive PrfA activation on the fitness of L. monocytogenes within an animal host following intragastric inoculation. Intragastric infection with either the prfA G145S mutant or wild type L. monocytogenes strain was carried out following the introduction of the inlA” mutation into each strain background to enhance bacterial interaction with mouse E-cadherin and translocation of bacteria across the intestinal epithelium [61]. Surprisingly, 2- to 7-fold more bacteria were recovered from the livers, spleens, stomachs, and intestines of mice infected with the prfA G145S inlA” mutant than from the organs of mice infected with the wild type prfA inlA” strain at infectious doses of either 5 × 10^4 CFU or 5 × 10^5 CFU (Fig. 6). These findings indicate that constitutive activation of PrfA enhances the fitness of L. monocytogenes inside of the host following either intravenous or intragastric inoculation.

Discussion

Central to the ability of L. monocytogenes to flourish in a wide variety of environments is the appropriate expression of gene products that facilitate bacterial survival and replication within a given niche. L. monocytogenes occupies disparate environments that range from soil and food-processing plants to the gastrointestinal tract and cell cytosol of infected mammals [3,5,9,13,17,46–50]. It has been previously demonstrated that dramatic increases in PrfA activity and PrfA-dependent gene expression occur following entry of L. monocytogenes into the cytosol [18,42,62,63]. We therefore sought to investigate the importance of appropriate regulation of
PrfA activity under different environmental conditions in the context of *L. monocytogenes* fitness inside and outside of infected host cells. Our results indicate that while constitutive activation of PrfA serves to enhance bacterial virulence within the infected host, in most cases PrfA activation decreases bacterial fitness outside of host cells. *L. monocytogenes* therefore regulates PrfA activity so as to optimally balance life in the outside environment with life inside of the host.

Environmental regulation of PrfA activity suggests that the high levels of PrfA activity required for intracellular life are detrimental to the fitness of *L. monocytogenes* outside of a host cell, and limited analyses of *L. monocytogenes* field strains appear to support this
hypothesis. Although far from being exhaustively examined, no field strain reported in the literature has been found to contain a prfA* mutation nor to exhibit a PrfA* phenotype; all of the prfA* mutations reported have arisen spontaneously in laboratory media [30–33,35,37]. Moreover, field strains have been isolated containing missense mutations or small deletions within the coding region of the prfA gene that decrease or eliminate PrfA activity, indicating that PrfA activity is not required for optimal bacterial fitness outside of a host cell [64,65] although a recent report indicates that some activity is required for efficient biofilm formation [66].

The link between carbon source utilization and PrfA regulation of L. monocytogenes virulence gene products has long been recognized but has remained poorly defined. Previous studies have demonstrated that when L. monocytogenes was grown in the presence of glucose or other carbon sources taken up by the phosphoenolpyruvate (PEP) transport system (PTS), the expression levels of PrfA-dependent genes were decreased [29,67,68]. Other studies have reported that over expression of prfA* on a multicopy plasmid in L. monocytogenes significantly impaired bacterial growth and glucose uptake in media where glucose was the main or sole carbon source [43,44]. Although the isogenic prfA* strains used in

Figure 5. Growth of the prfA* mutants in the livers and spleens of intravenously infected mice. 7–8 week old ND4 Swiss Webster mice were infected with L. monocytogenes via tail-vein injections, and at the specified times post-infection (pi), the bacterial loads of the livers and spleens were determined as described in Experimental Procedures. Data are presented as scatter dot plots, with horizontal bars representing means. (A) Infection of mice with 2 × 10^6 CFU wild type, prfA G155S, prfA L140F, or prfA G145S mutants. Organs were harvested 24 hours pi. Asterisks denote statistically significant differences between the amounts of prfA* mutant and wild type CFU recovered using a one-way analysis of variance with Dunnett’s post-test (*, P < 0.05; ***, P < 0.001). (B) Comparison of infection with 2 × 10^5 or 2 × 10^6 CFU of wild type and prfA G145S mutant. Organs were harvested 48 hours pi. Asterisks denote statistically significant differences between the amounts of prfA G145S mutant and wild type CFU recovered using an unpaired t test with a two-tailed P value (**, P < 0.01). (C) Competitive index of wild type and prfA* strains. Prior to intravenous injection, the wild type Ermr reference strain and the indicated test strain were mixed 1:1 for a total bacterial suspension of 2 × 10^6 CFU. For each organ, the competitive index (CI) value (CI = test strain CFU/reference strain CFU) was determined as described in Experimental Procedures. Asterisks denote statistically significant CI values compared to 1 using a one-sample t test with a two-tailed P value (*, P < 0.05; **, P < 0.01).

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In addition to the glucose-related growth defects reported for L. monocytogenes strains containing multicopy plasmid-encoded prfA*, it has also been reported that similar strains exhibited a subtle monoculture growth defect when grown in media where glycerol (a non-PTS carbon source) was the main carbon source [44]. In contrast to this finding, our data indicate that isogenic prfA* mutants were enhanced for glycerol utilization and exhibited a competitive advantage over wild type in the presence of glycerol (Fig. 2). While differences related to prfA* copy number may influence the ability of L. monocytogenes prfA* mutants to utilize glucose, the results in either case indicate that the presence of glucose decreases the fitness of L. monocytogenes when PrfA is activated.

In addition to the glucose-related growth defects reported for L. monocytogenes, the addition of glucose to LB was found to exacerbate the competitive defect exhibited by the prfA G145S mutant in LB (Fig. 2B). While differences related to prfA* copy number may influence the ability of L. monocytogenes prfA* mutants to utilize glucose, the results in either case indicate that the presence of glucose decreases the fitness of L. monocytogenes when PrfA is activated.

In summary, the findings presented in this study emphasize the critical need for L. monocytogenes to regulate PrfA activity dependent
on its environmental location. While experiments in broth culture indicate a competitive fitness defect for prfA* mutants, it remains possible that PrfA activation contributes to L. monocytogenes outside of mammalian infection, for example by promoting bacterial survival in the presence of lower eukaryotes or other soil dwellers. PrfA activation clearly enhances bacterial virulence in mammalian hosts, however the need for down modulation of PrfA activity in other settings might well be a reflection of the yin-yang nature of the L. monocytogenes saprophyte-pathogen balance.

Materials and Methods

Bacteria and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. All L. monocytogenes strains used were derived from the 1/2a serotype 10403S L. monocytogenes strain, which is a streptomycin-resistant derivative of strain 10403 [80,81]. The phenotypes reported for strains containing prfA* mutations were verified in independent isolates constructed by allelic exchange and/or by phage transduction, and by comparison of different prfA* alleles (prfA G145S, prfA L140F, prfA Y369S). L. monocytogenes strains were grown in brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) or Lysogeny Broth (LB) (Invitrogen Corp., Grand Island, NY). Escherichia coli strains were grown in LB. When appropriate, LB was supplemented with 55 mM of either glucose or glycerol. To test strains were grown in LB. When appropriate, LB was supplemented with 55 mM of either glucose or glycerol. To increase medium acidity, BHI or LB was buffered to pH 7.4 (Sigma Chemical Co., St. Louis, MO) or to pH 5.5 with 100 mM of 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5 (Sigma), respectively. To increase medium osmolarity, BHI was supplemented with 5% sodium chloride (NaCl). The antibiotics (and concentrations) used in this study were: neomycin (5 μg/mL), chloramphenicol (10 μg/mL), erythromycin (1 μg/mL), and streptomycin (200 μg/mL).

Construction of L. monocytogenes mutant strains via bacteriophage transduction

L. monocytogenes strain NF-L1775 (prfA G145S ΔsigB) was constructed by bacteriophage transduction as previously described [33,82,83]. Briefly, 10^7–10^8 PFU of Listeria phage U153 lysates [82] prepared from NF-L1777 (prfA G145S actA-gus-neo-plcB) [41] were mixed with 10^8 CFU of mid-log FSL A1-254 (ΔsigB, a kind gift of Dr. Kathryn Boor, Cornell University, Ithaca, NY) [84]. The prfA G145S ΔsigB double mutant was confirmed to contain both the prfA G145S mutation and the downstream actA-gus-neo-plcB transcriptional fusion from the prfA G145S mutant [41] by isolating transductants that exhibited neomycin resistance and a blue colony appearance on BHI agar containing 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (x-gluc).

Construction of L. monocytogenes mutant strains via allelic exchange

L. monocytogenes strains NF-L1774 (prfA G145S ΔsigB), NF-L1772 (inlA^D), and NF-L1773 (prfA G145S inlA^D) were constructed using derivatives of the temperature-sensitive integration vector pKSV7

Table 1. Bacterial strains and plasmids used in this study.

| Strain | Description/Genotype | Designation | Reference |
|--------|-----------------------|-------------|-----------|
| TOP10, SM10 | E. coli strains for constructing recombinant plasmids | | |
| NF-L100 | 10403S wild type | [80,81] |
| NF-L890 | NF-L100 ΔprfA | [33] |
| NF-L476 | NF-L100 actA-gus-plcB | [89] |
| NF-L1124 | NF-L100 actA-gus-neo-plcB | WT 10403S | [30] |
| NF-L1123 | NF-L890 actA-gus-neo-plcB | ΔprfA | [30] |
| NF-L1493 | NF-L476 prfA G155S | prfA G155S | [32] |
| NF-L1177 | NF-L1124 prfA G145S | prfA G145S | [41] |
| NF-L1166 | NF-L1124 prfA L140F | prfA L140F | [41] |
| NF-L1006 | NF-L476 tRNAArg::pPL2 WT | WT cam^R |
| NF-E1613 | TOP10 with pTJA-57 | | |
| FSL A1-254 | 10403S ΔsigB | ΔsigB | [84] |
| NF-L1774 | NF-L943 ΔsigB | prfA G155S ΔsigB | This study |
| NF-L1775 | NF-L1177 ΔsigB | prfA G145S ΔsigB | This study |
| DP-L3903 | 10403S with Tn917 insertion | WT erm^R | [90] |
| NF-E1458 | E. coli with HEL-913 | | [38] |
| NF-L1772 | NF-L1124 inl^D::Km^R | WT 10403S inl^D | This study |
| NF-L1773 | NF-L1177 inl^D::Km^R | prfA G145S inl^D | This study |

Plasmid Description/Genotype Reference

| Plasmid | Description/Genotype | Reference |
|---------|----------------------|-----------|
| pPL2 | Site-specific phage integration vector | [87] |
| pKSV7 | Temperature-sensitive integration vector for allelic exchanges | [85] |
| HEL-913 | pKSV7::inl^D::Km^R | [38] |
| pTJA-57 | pKSV7::ΔsigB | [84] |

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[85]. The inlA<sup>+</sup> mutation enhances the intestinal translocation of <i>L. monocytogenes</i> but has no impact on the outcome of intravenous infection [61]. Plasmid vector pTJA-57 (pKSV7::ΔsigB; kind gift of Dr. Kathryn Boor) [84], a pKSV7 derivative designed for the construction of ΔsigB mutations, was introduced into NF-L1543 (pJTA G1545S) by electroporation as previously described [86]. Chromosomal integration of pTJA-57 and subsequent allelic exchange and plasmid curing were carried out as previously described [85]. The introduction of the ΔsigB mutation into the pJTA G1545S mutant background was confirmed by PCR amplification of the sigB open reading frame using primers LmsigB-15 and LmsigB-16 [84] (Table 2).

To facilitate the investigation of intragastric infections of mice, the inlA<sup>+</sup> (inlA<sup>Y360S</sup>) mutation described by Wollert et al. [61] was introduced into a wild type 10403S strain (NF-L1124) and the pJTA G1454S mutant by electroporation, allelic exchange, and plasmid curing of the plasmid vector pHEL-913 (pKSV7-inlA<sup>+</sup>), a kind gift of Dr. Helene Marquis, Cornell University, Ithaca, NY [83]. Strains NF-L1772 (inlA<sup>+</sup>) and NF-L1773 (pJTA G145S; inlA<sup>+</sup>) were generated. The introduction of the inlA<sup>+</sup> mutation was confirmed by PCR amplification and DNA sequencing of the inlA open reading frame using primers MARQ403 and MARQ408 [38] (Table 2).

### Monoculture growth experiments

50 μL or 100 μL of an overnight culture grown in BHI were added to 12.5 mL or 25 mL, respectively, of fresh broth culture medium (a 1:250 dilution) and incubated at 37°C with vigorous shaking and aeration. At specified time points, the optical density at 600 nm (OD<sub>600</sub>) of the culture was measured using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and CFU/mL were determined by plating equal amounts of bacteria from overnight cultures of wild type camR into BHI agar containing the appropriate antibiotic. For example, the prfA G1454S mutant is neomycin-resistant because of the actC-gus-neo<sup>r</sup> transpositional fusion it contains, but the WT camR strain is neomycin-sensitive, so aliquots from mixed cultures of the prfA G1454S and WT cam<sup>R</sup> strains were also plated on BHI agar containing neomycin. For graphic representation, the CI value of a mixed culture was plotted as a function of the mixed culture’s dilution cycle number or passage number (passage #, P#), with ‘passage 0’ representing the initial mixture of two monocultures, ‘passage 1’ representing the mixed culture after the initial 24 hours of growth immediately prior to the first passage, ‘passage 2’ representing the mixed culture after the 24 hours of growth following the first passage and immediately prior to the second passage, etc. (Figure S1). pPL2 integration did not affect the competitive index of wild type 10403S in any growth condition as the WT cam<sup>R</sup> strain never exhibited a competitive advantage nor disadvantage when mixed with the 10403S strain lacking the pPL2 inserted plasmid (CI values of ~1 throughout the course of the mixing experiment) [41].

### Measurement of β-glucuronidase (GUS) activity

GUS activity was measured by an enzymatic assay as previously described [88]. Briefly, overnight cultures grown in BHI were diluted 1:50 into fresh media and grown with shaking at 37°C. CFU/mL were measured at specified time points and two 500 μL culture aliquots were collected (for the pJTA G1454S and pJTA L140F mutants, two 50 μL culture aliquots were collected because of their increased prfA expression) [41]. The aliquots were centrifuged (16,100 <i>x</i>g) for 5 minutes, supernatants were removed, and one pellet from each two aliquots was suspended in 100 μL of ABT buffer (0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl, 0.1% Triton) while the other was suspended in 1 mL of ABT buffer. Two 50 μL aliquots of each ABT bacterial suspension were pipetted into separate wells of a 96-well plate. 10 μL of 0.4 mg/mL of the GUS substrate 4-methylumbelliferyl-β-D-glucuronide (Sigma) were added to each 50 μL aliquot, and these mixtures were incubated at 37°C for 60 minutes. Substrate conversion was measured with a Barnstead/Turner QuanTech FM109515 Fluorometer (Dubuque, IA). Units of GUS activity were calculated as previously described [88].

### Intravenous infections of mice

Animal procedures were IACUC approved by the UIUC Animal Care Committee [Approval #09-153] and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Mid-log <i>L. monocytogenes</i> growing in BHI were washed, suspended, and diluted in PBS to reach a final concentration of 1 × 10<sup>9</sup> CFU/mL or 1 × 10<sup>6</sup> CFU/mL. 7–8 week old ND4 Swiss Webster mice (Harlan Laboratories, Inc., Madison, WI) were infected via tail vein injections with 200 μL of the bacterial suspensions, achieving an infectious dose (ID) of 2 × 10<sup>5</sup> CFU or 2 × 10<sup>6</sup> CFU, respectively. 24 or 48 hours post infection, the mice were sacrificed, and their livers and spleens were harvested. Each organ was placed in 5 mL of sterile Milli-Q water and homogenized with a Tissue Master-125 Watt Lab Homogenizer.

Immediately prior to each dilution an aliquot of the mixed culture was removed, diluted, and plated onto BHI agar to obtain bacterial CFU counts. 150 of the resulting colonies were then patched onto BHI agar containing chloramphenicol to select for wild type cam<sup>R</sup> bacteria. The competitive index (CI) value of the mixed culture was determined using the following equation: CI = (test strain CFU)/WT cam<sup>R</sup> reference strain CFU. When the test strain was resistant to an antibiotic to which the WT cam<sup>R</sup> reference strain was sensitive, aliquots were also plated on BHI agar containing the appropriate antibiotic. For example, the prfA G1454S mutant is neomycin-resistant because of the actC-gus-neo<sup>r</sup> transpositional fusion it contains, but the WT cam<sup>R</sup> strain is neomycin-sensitive, so aliquots from mixed cultures of the prfA G1454S and WT cam<sup>R</sup> strains were also plated on BHI agar containing neomycin. For graphic representation, the CI value of a mixed culture was plotted as a function of the mixed culture’s dilution cycle number or passage number (passage #, P#), with ‘passage 0’ representing the initial mixture of two monocultures, ‘passage 1’ representing the mixed culture after the initial 24 hours of growth immediately prior to the first passage, ‘passage 2’ representing the mixed culture after the 24 hours of growth following the first passage and immediately prior to the second passage, etc. (Figure S1). pPL2 integration did not affect the competitive index of wild type 10403S in any growth condition as the WT cam<sup>R</sup> strain never exhibited a competitive advantage nor disadvantage when mixed with the 10403S strain lacking the pPL2 inserted plasmid (CI values of ~1 throughout the course of the mixing experiment) [41].

### Table 2: Oligonucleotides used in this study.

| Primer | Sequence (5’→3’) | Reference |
|--------|----------------|-----------|
| LmsigB-15 | AATATATATAGAAACGAGTGGAG | [84] |
| LmsigB-16 | ATGAACTTATTGCAATCGTCCCT | [84] |
| MARQ403 | CAGACTAGACCAAGTACAA | [38] |
| MARQ408 | CAGACTAGAATGTGACAGTGGTGC | [38] |

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Oral infections of mice

Mol-log \( L.\) monocytogenes growing in BHI were washed, suspended, and diluted in PBS to reach a final concentration of \(2.5 \times 10^{8} \) CFU/mL or \(2.5 \times 10^{10} \) CFU/mL. 0–10 week old C57BL/6 mice (Harlan) were infected orally with 200 \( \mu \)L of the bacterial suspensions, achieving an ID of \(5 \times 10^{8} \) CFU or \(5 \times 10^{10} \) CFU, respectively. 72 hours post infection, mice were sacrificed, and their livers, spleens, stomachs, and intestines were harvested. The organs were homogenized and their bacterial loads were determined as described above.

Supporting Information

Table S1 Logarithmic doubling times of \( L.\) monocytogenes strains under various conditions at 37°C. (DOC)

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