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Listeria monocytogenes adapts to long term stationary phase survival without compromising bacterial virulence

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Abstract

Bacteria withstand starvation during long-term stationary phase through the acquisition of mutations that increase bacterial fitness. The evolution of the Growth Advantage in Stationary Phase (GASP) phenotype results in the ability of bacteria from an aged culture to outcompete bacteria from a younger culture when the two are mixed together. The GASP phenotype was first described for *Escherichia coli* but has not been examined for an environmental bacterial pathogen which must balance long-term survival strategies that promote fitness in the outside environment with those that promote fitness within the host. *Listeria monocytogenes* is an environmental bacterium that lives as a saprophyte in soil but is capable of replicating within the cytosol of mammalian cells. Here we demonstrate the ability of *L. monocytogenes* to express GASP via the acquisition of mutations during long-term stationary growth. *L. monocytogenes* GASP occurred through mechanisms that were both dependent and independent of the stress responsive alternative sigma factor SigB. Constitutive activation of the central virulence transcriptional regulator PrfA interfered with the development of GASP, however *L. monocytogenes* GASP cultures retained full virulence in mice. These results indicate that *L. monocytogenes* can accrue mutations that optimize fitness during long-term stationary growth without negatively impacting virulence.

Keywords

PrfA; bacterial virulence; stationary phase survival; SigB; GASP

Introduction

Bacteria exhibit a remarkable ability to adapt to disparate conditions that would otherwise limit growth. A simple yet compelling example of bacterial adaptation can be observed during the distinct phases of growth in liquid culture. The lag, logarithmic, and stationary phases of bacterial growth have been well described (Perry & Staley, 1997), however the phases of growth following stationary phase have only recently been investigated in detail. Following entry into stationary phase, a death phase occurs during which a greater than 90% loss of bacterial viability is observed (Perry & Staley, 1997). The amount of viable bacteria then levels off and remains relatively constant. This second stable stationary phase is known as the long-term stationary phase (Finkel *et al.*, 2000; Steinhaus & Birkeland, 1939). The timing of bacterial growth phases varies depending on the growth medium and on the bacterial species being studied. For *Escherichia coli* in LB, the death phase starts 2–3 days after the initial inoculation and lasts for about a day while the long-term stationary phase of

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growth lasts for several weeks or longer (Finkel, 2006). Studies of long term stationary phase growth and survival of *E. coli* led to the discovery of the growth advantage in stationary phase or GASP phenotype, which reflects the ability of bacteria from an aged culture to outcompete the same strain of bacteria from a younger culture when the two are grown together (Zambrano *et al*., 1993). For *E. coli* grown in LB, the aged culture must be at least 8-days old and in the long-term stationary phase of growth to effectively outcompete a younger 1-day old culture (Finkel, 2006; Zambrano *et al*., 1993; Zambrano & Kolter, 1993). The GASP phenotype of *E. coli* results from a dynamic and continuous acquisition of mutations that increase bacterial fitness during periods of long-term stationary growth (Farrell & Finkel, 2003; Zambrano *et al*., 1993; Zambrano & Kolter, 1993; Zinser & Kolter, 1999; Zinser & Kolter, 2000; Zinser *et al.*, 2003; Zinser & Kolter, 2004).

Listeria monocytogenes is a Gram-positive environmental bacterial pathogen that has evolved to survive in disparate environments both inside and outside of mammalian hosts (Czuprynski, 2005; Gray *et al.*, 2006; Vazquez-Boland *et al.*, 2001). As an intracellular pathogen, the bacterium invades mammalian cells, escapes from host cell phagosomes, replicates within the cytosol, and spreads into neighboring cells (Freitag *et al.*, 2009; Hamon *et al*., 2006). A number of bacterial factors are required for *L. monocytogenes* intracellular replication and cell-to-cell spread (Goebel *et al.*, 2000; Vazquez-Boland *et al*., 2001), and the expression of a majority of these gene products is regulated by the transcriptional regulator known as PrfA (Kreft & Vazquez-Boland, 2001; Scortti *et al.*, 2007). The fitness of *L. monocytogenes* inside of the host is severely compromised in the absence of PrfA (Freitag, 2006).

Outside of mammalian hosts, *L. monocytogenes* is widely distributed and is believed to live as a saprophyte off of decaying plant material (Czuprynski, 2005; Freitag *et al.*, 2009; Gray & Killinger, 1966; Vazquez-Boland *et al.*, 2001). *L. monocytogenes* has been isolated from soil, silage, ground water, sewage, and vegetation (Thevenot *et al.*, 2006) and, although it does not form spores, the bacterium can become firmly established in food processing environments and persist for long periods of time, even years (Orsi *et al.*, 2011; Lunden *et al.*, 2002). Based upon an anticipated requirement for *L. monocytogenes* to be able to balance survival under nutrient poor conditions in the outside environment with life within the infected host, we assessed the bacterium for its ability to adapt to periods of long-term stationary phase growth through the development of GASP. Our results indicate that *L. monocytogenes* is capable of stably adapting itself for long-term survival without compromising its ability to cause disease.

Materials and Methods

Bacterial strains

The bacterial strains and plasmids are listed in Table 1. Antibiotics were used as follows: erythromycin (1 μg/ml), chloramphenicol (10 μg/ml), and streptomycin (200 μg/ml).

Monoculture growth experiments

Overnight cultures grown in BHI were added at a 1:250 (vol:vol) dilution to fresh BHI and incubated at 37°C with aeration. CFU/mL were determined by plating dilutions of culture aliquots on BHI agar.

Stationary phase mixing experiments

Competitive indices of mixed bacterial cultures during stationary phase were performed as previously described (Bruno & Freitag, 2010; Finkel *et al.*, 2000; Zambrano *et al.*, 1993) (Fig 1A). Aliquots of 12-day old cultures were stored at −80°C. For each experiment, an

aliquot of frozen cells was thawed and 50 μL was added to 12.5 mL of BHI and grown overnight at 37°C. 125 μL of the overnight 12-day old culture was added to 12.5 mL of a 1-day old culture at a ratio of 1:100 and incubated at 37°C for 10 days. 12-day old and 1-day old were distinguished based on chloramphenicol resistance of the 1-day old cultures containing the site-specific integration vector pPL2 which conferred chloramphenicol resistance without influencing bacterial growth (Lauer *et al.*, 2002). Every 24 hours, an aliquot of the mixed culture was removed, diluted, and plated onto BHI agar to enumerate bacterial CFUs. 150 of the resulting colonies were then patched onto BHI agar containing chloramphenicol, selecting for the original 1-day old chloramphenicol resistant bacteria; this was found to be the most reliable method for clearly distinguishing drug resistant colonies. The competitive index (CI) value was determined as follows: CI = (test strain CFU)/(reference strain CFU).

Intravenous mouse infections

Mid-log *L. monocytogenes* were washed and diluted in PBS to a final concentration of 1×10^5 CFU/ml. 7–8 week old ND4 Swiss Webster mice (Harlan Laboratories, Inc., Madison, WI) were infected via tail vein with 2×10^4 CFU. Forty-eight hours post infection homogenized tissue dilutions were plated on BHI agar to determine CFU/organ.

For competitive index experiments, mice were infected via tail vein with a 1:1 mixture of a reference and test strains. The reference strain was DP-L390, a wild type strain with a Tn*917*-LTV3 insertion that confers erythromycin resistance and has been confirmed to have no affect *L. monocytogenes* virulence [(Auerbuch *et al.*, 2001) and Fig 5B]. Strains were grown to mid-log phase and mixed together in PBS. 200 μL of 2×10^4 CFU mixed bacterial suspension was used for infection. After 48 hours, livers and spleens were harvested and homogenized. The CI value for each organ was determined as previously described (Auerbuch *et al.*, 2001).

Statistics

Statistical analysis was performed using Prism Software (GraphPad v.2.0). Where appropriate, a Student's T-test was used to identify statistically significant differences. In all cases, a p-value <0.05 was considered significant.

Results and Discussion

L. monocytogenes expresses GASP

The GASP phenotype reflects bacterial adaptation to long term nutrient starvation (Finkel, 2006) and, as an environmental organism, *L. monocytogenes* would be anticipated to encounter periods of sustained nutrient deprivation. The development of the GASP phenotype is marked by the ability of bacteria from an aged culture to outcompete bacteria from a younger culture during long-term stationary phase growth (Finkel, 2006). GASP thus requires that a bacterial strain be capable of surviving for an extended period of time following its inoculation into growth medium. To measure the survival of *L. monocytogenes* during nutrient starvation, bacteria grown in nutrient-rich broth (BHI) were assessed for viability following incubation for 12 days at 37°C. Cultures exhibited a characteristic lag, logarithmic, and stationary growth phase during the first 24 hours of growth (Fig 2A). After remaining in stationary phase for 1–2 days, *L. monocytogenes* entered a death phase during which an approximate 90% loss of cell viability was observed over 24 hours. The subsequent bacterial population then maintained a stable cell density representative of a long-term stationary growth phase that persisted for the remaining days (Fig 2A).

The ability of *L. monocytogenes* to express the GASP phenotype was next assessed. As *E. coli* cultures need to be at least 8 days old (when cultured in LB under aerobic conditions) to express the GASP phenotype (Finkel, 2006; Zambrano *et al.*, 1993), we aged *L. monocytogenes* cultures for 12 days prior to the assessment for GASP as an arbitrary staring point. Bacteria from a *L. monocytogenes* 12-day old culture were added to a 1-day old culture at a ratio of 1:100 (Fig 1). Bacteria from the 12-day old culture outcompeted bacteria of the 1-day old culture over 10 days, such that the ratio at day 10 was 10:1 of 12-day old cells to 1-day old cells (Fig 2B). In contrast, when bacteria from a 1-day old culture of *L. monocytogenes* were added to another 1-day old culture at a ratio of 1:100, no change in this ratio was observed over 10 days (Fig 2B). The competitive advantage exhibited by the bacteria from a 12-day old culture was thus reflective of culture age, and indicated that *L. monocytogenes* is capable of expressing GASP.

To determine if the *L. monocytogenes* GASP phenotype was the result of a stable genetic change, bacteria from a 12-day old culture were grown in BHI to a high cell density, diluted 1:100 into fresh media, and once again grown to high cell density. This process was repeated every 24 hours for a total of 12 cycles of dilution and outgrowth or passages (Fig 1B). Bacteria from the passaged 12-day old culture were then added to a 1-day old culture of wild type *L. monocytogenes* at a ratio of 1:100. Just as with bacteria from a non-passaged 12-day old culture, bacteria from the passaged 12-day old culture outcompeted bacteria of the 1-day old culture over 10 days (Fig 2B), thus indicating that *L. monocytogenes* GASP resulted from a stable genetic change.

Constitutive activation of PrfA interferes with GASP expression

The transcriptional activator PrfA regulates the expression of most of the gene products that have been associated with *L. monocytogenes* pathogenesis (Scortti *et al.*, 2007). PrfA exists in both low activity and high activity forms, and constitutive activation of PrfA via *prfA** mutations enhances *L. monocytogenes* virulence while compromising the fitness of bacteria in broth culture (Bruno & Freitag, 2010). To evaluate the impact of PrfA activation on *L. monocytogenes* long-term survival, the mutationally activated *prfA** G145S mutant was grown for 12 days in BHI at 37°C. Cultures of the *prfA* G145S mutant exhibited death and long-term stationary growth phases (Fig 3A), indicating that the *L. monocytogenes prfA** mutant was capable of long-term survival. However, cultures of the *prfA* G145S mutant exhibited final bacterial cell densities that were two to three-fold lower than those of wild type cultures in the same growth phase (Fig 3A). The constitutive activation of PrfA thus reduced the overall numbers of *L. monocytogenes* that were capable of surviving long-term in exhausted media.

To determine if constitutive activation of PrfA affected the development of GASP, *prfA* G145S mutant bacteria from a 12-day old culture were added to a 1-day old culture of *prfA* G145S at a ratio of 1:100 (Fig 3B). Over the course of 10 days, bacteria from the *prfA** 12-day old culture outcompeted the *prfA** 1-day old culture such that the ratio at day 10 was a little less than 1:10 (Fig 3B). While the competitive advantage of the aged culture indicates that the *L. monocytogenes prfA** mutant was indeed capable of exhibiting a GASP phenotype, the phenotype was weaker than that exhibited by wild type bacteria (Fig 3B).

The failure of the *prfA* G145S mutant to express a robust GASP phenotype could reflect an impaired ability of bacteria to develop GASP, or may indicate that PrfA activation contributed to the development of a partial GASP phenotype in the 1-day old cultures. To help distinguish whether the presence of the *prfA** mutation impaired or enhanced the expression of GASP, the competitive index between wild type 12-day old cultures and 1-day old wild type or *prfA* G145S cultures was assessed. Because *prfA** mutants exhibit a competitive defect with wild type strains during short periods of growth in BHI [(Bruno &

Freitag, 2010) and Fig. 3C], this fitness defect would be anticipated to contribute to the magnitude of any GASP-related fitness effect observed between 12-day old wild type and 1-day old *prfA** cultures. If the *prfA* G145S mutant expresses a partial GASP phenotype as the result of PrfA activation, then the competitive advantage of a wild type 12-day old culture should be less in comparison to 1-day *prfA** than in comparison to 1-day old wild type. Alternatively, if *prfA** interferes with GASP, the overall defect observed between wild type 12-day old cultures and 1-day old *prfA** mutants should reflect both the *prfA**-associated fitness defect in BHI broth culture as well as an impaired GASP phenotype. When bacteria from a 12-day old wild type culture were added to a 1-day old culture of the *prfA* G145S mutant at a ratio of 1:100, after 10 days the ratio of wild type to mutant shifted from 1:100 to 100:1, representing a 10,000-fold increase in 12-day aged wild type bacteria (Fig. 3C). This shift was larger than the 1,000-fold increase in 12-day aged bacteria observed when bacteria from a 12-day old wild type culture were added to a 1-day wild type old culture (Fig. 3C). This enhanced fitness advantage was nearly equal to the sum of the fitness advantage observed for wild type versus *prfA** strains for one day old cultures (Fig. 3C, 1dWT v 1dG145S) plus the magnitude of wild type GASP expression (Fig. 3C, 1dWT v 12dWT), suggesting that PrfA activation impedes the development of GASP.

Activation of PrfA via a *prfA** mutation has been shown to influence the metabolic capacity of *L. monocytogenes*, enhancing bacterial growth in the presence of some carbon sources while decreasing growth in the presence of others (Bruno & Freitag, 2010; Chico-Calero *et al.*, 2002; Deutscher *et al.*, 2005; Deutscher *et al.*, 2006; Goetz *et al.*, 2001; Joseph *et al.*, 2006; Joseph & Goebel, 2007; Joseph *et al.*, 2008). It is possible that the metabolic shift that occurs in *L. monocytogenes* as a result of PrfA activation interferes with efficient nutrient acquisition during the conditions of long-term stationary phase. However, activation of PrfA has also been shown to increase the sensitivity of *L. monocytogenes* to osmotic and acid stresses (Bruno & Freitag, 2010), thus there may be multiple mechanisms functioning simultaneously to reduce bacterial fitness during long term stationary phase. Finally, as the *prfA** strains exhibited a 2 to 3-fold lower cell density at stationary phase, it is possible that the reduced GASP phenotype reflects a reduction in overall cell numbers available for the accumulation of potential GASP mutations.

The stress related alternative sigma factor SigB contributes to long-term stationary phase survival and influences the development of GASP

The most common mutations resulting in the *E. coli* GASP phenotype are mutations within *rpoS* (Farrell & Finkel, 2003; Finkel & Kolter, 1999; Hengge-Aronis, 2000; Zinser & Kolter, 2004), which encodes a member of the σ^{70} family of sigma factors that contribute to bacterial stress responses in *E. coli* and other bacteria (Hengge-Aronis, 2000; Loewen *et al.*, 1998; Zinser & Kolter, 2004). *rpoS* is not essential for the expression of the *E. coli* GASP phenotype, as aged Δ*rpoS* mutants out-compete younger Δ*rpoS* mutants (Finkel, 2006) and mutations associated with GASP have been mapped to other genes unrelated to *rpoS* (Zinser & Kolter, 1999; Zinser & Kolter, 2000; Zinser *et al.*, 2003). However, the most common mutations associated with *E. coli* GASP are mutations within *rpoS* that result in the attenuation of RpoS activity; these mutations are sufficient to confer the GASP phenotype (Farrell & Finkel, 2003; Finkel & Kolter, 1999; Hengge-Aronis, 2000; Zinser & Kolter, 2004). *L. monocytogenes* harbors a stress-responsive σ^{70} sigma factor, known as SigB (Abram *et al.*, 2008; Chaturongakul & Boor, 2006; Garner *et al.*, 2006; Kazmierczak *et al.*, 2003; O'Byrne & Karatzas, 2008), thus it seemed logical to assess if SigB function contributed to the development of *L. monocytogenes* GASP. When examined for long-term survival in culture, a Δ*sigB* mutant exhibited the expected death and long-term stationary growth phases during the course of a 12-day incubation in BHI at 37°C (Fig 4A). Similar to the *prfA** mutant, Δ*sigB* long-term stationary phase cultures exhibited final stable bacterial

CFU numbers that were approximately two-fold lower than those maintained by wild type *L. monocytogenes* (Fig 4A). SigB is thus required for the optimal fitness of *L. monocytogenes* during the long-term stationary growth phase.

Δ*sigB* mutant bacteria from 12-day old cultures were added to 1-day old mutant cultures at a final ratio of 1:100. Over 10 days, bacteria from the 12-day old culture outcompeted bacteria of the 1-day old culture such that the ratio at day 10 was 1:1 (Fig 4B), indicating that the Δ*sigB* mutant retained its ability to express the GASP phenotype. However, similar to the phenotype expressed by the *prfA** mutant, the GASP phenotype exhibited by the Δ*sigB* strain was not as robust as that exhibited by wild type *L. monocytogenes* (Fig. 4B). Whereas bacteria derived from 12-day old wild type cultures increased 1,000-fold in comparison to 1-day old wild type bacteria (Fig. 4B), the bacterial numbers of a 12-day old Δ*sigB* culture increased approximately 100-fold in comparison to those of the 1-day old Δ*sigB* culture (Fig 4B).

Similar to the situation described above for *prfA** strains, the failure of the Δ*sigB* mutant to express a robust GASP phenotype could reflect an impaired ability to develop GASP, or may indicate that the loss of SigB contributed to a partial GASP phenotype for 1-day old cultures. To distinguish between these two possibilities, the competitive index between wild type 12-day old cultures and 1-day old wild type or Δ*sigB* cultures was assessed. If the Δ*sigB* mutant expresses a partial GASP phenotype as the result of the loss of SigB, then the competitive advantage of a wild type 12-day old culture should be less in comparison to 1-day old Δ*sigB* than in comparison to 1-day old wild type. Interestingly, the difference in the competitive advantage of wild type 12-day old cultures observed versus 1-day old wild type or 1-day old Δ*sigB* was minimal (Fig. 4C). SigB contributes to *L. monocytogenes* fitness in broth culture, based on the competitive advantage of 1-day old wild type strains versus 1-day old Δ*sigB* mutants (Fig. 4A). Thus, in spite of Δ*sigB* mutants exhibiting a broth culture fitness defect, the overall magnitude of the competitive defect observed between 12-day old wild type *L. monocytogenes* and 1-day old wild type strains and Δ*sigB* mutants was similar rather than exacerbated for Δ*sigB*, suggesting that the loss of SigB may indeed contribute to the development of the GASP phenotype. Taken together, these data indicate a role for SigB in *L. monocytogenes* long-term stationary phase survival and in the expression of GASP.

GASP cultures of *L. monocytogenes* remain fully virulent

The GASP mutation(s) that enable *L. monocytogenes* to adapt to long-term stationary growth and to nutrient starvation could potentially impact other aspects of *L. monocytogenes* physiology, including those relating to bacterial virulence. As an environmental pathogen, *L. monocytogenes* would presumably encounter conditions in which long-term stationary growth survival would be required prior to human or animal infection. To determine if adaptation to nutrient starvation affected the virulence of *L. monocytogenes*, bacteria from 12-day old cultures were used to intravenously infect mice. At forty-eight hours post infection, the bacterial loads of the livers and spleens from mice infected with bacteria from 12-day old wild type *L. monocytogenes* cultures were not statistically different from those of mice infected with bacteria from 1-day old *L. monocytogenes* cultures (Fig 5A). To further examine the age-adapted bacteria for subtle fitness defects *in vivo* that might be detectable in comparison to 1-day old bacterial cells, competition experiments were performed (Fig. 5B). Mice were intravenously infected with a 1:1 mixed bacterial suspension of bacteria from 12-day old and 1-day old cultures, and 48 hours post infection the competitive index (CI) values for bacteria isolated from the murine livers and spleens were determined. CI values remained very close to 1 (Fig 5B), indicating that genetic alterations that promote *L. monocytogenes* long-term stationary phase survival under nutrient limited conditions do not appear to impact bacterial virulence in systemic models of animal infection.

Based on observations made with *E. coli* (Finkel & Kolter, 1999; Finkel, 2006), the bacteria from 12-day old *L. monocytogenes* cultures likely reflect dynamic and evolving populations of cells. If a GASP mutation within a sub-population of cells attenuates bacterial virulence, the presence of the other bacteria with different mutational adaptations could potentially mask sub-population defects. It has recently been reported that the phenomena of GASP is complex, with mutant and wild type strains cooperating within the population to maximize bacterial fitness (Keymer et al., 2008). Cooperation between GASP mutant and wild type bacteria may thus ensure that *L. monocytogenes* effectively adapts for long-term stationary phase survival while maintaining bacterial virulence under nutrient poor conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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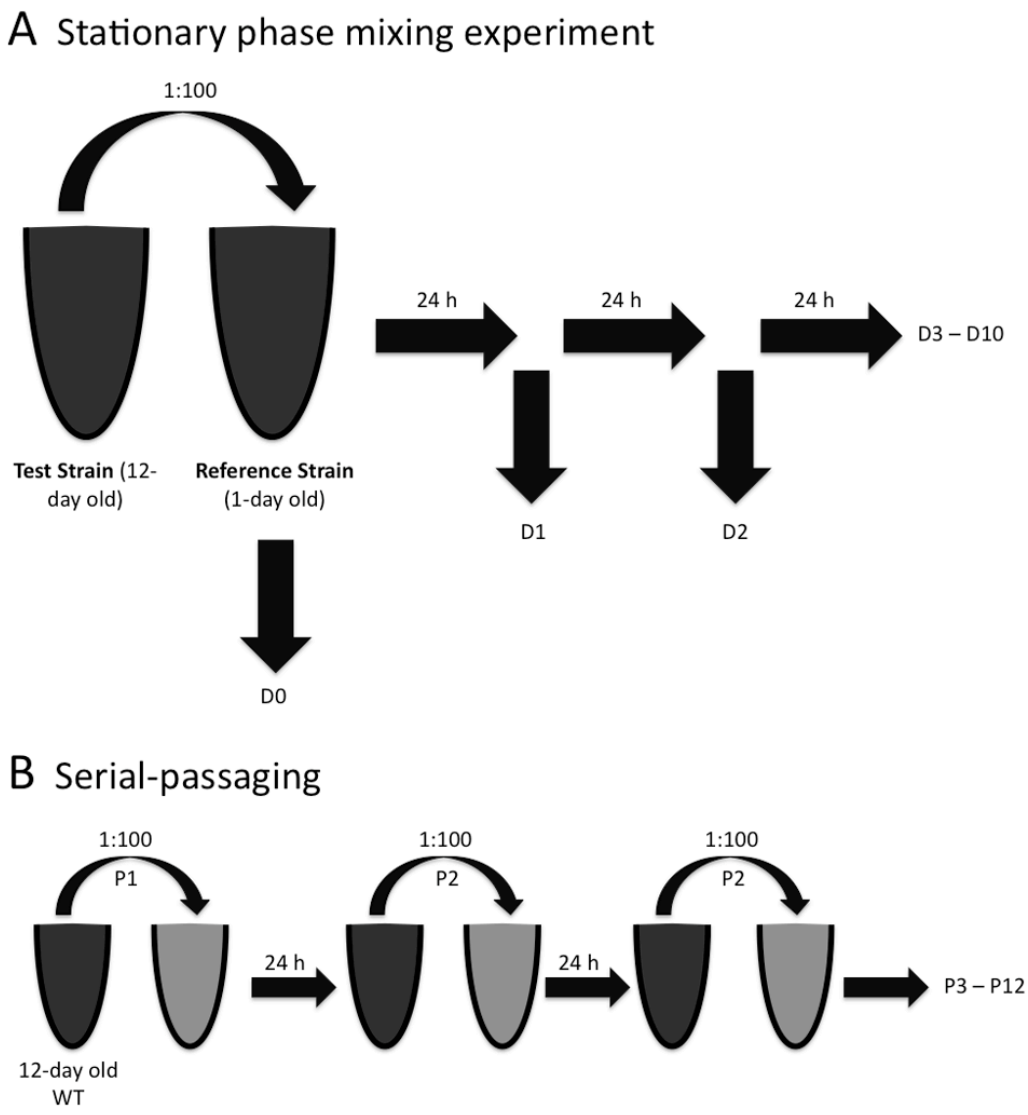

Figure 1. Overview of stationary phase culture mixing experiments and of repeated cycles of culture dilution and outgrowth

(A) Stationary phase mixing experiments. 125 μL of a test strain culture was added to a 12.5 mL of a reference strain culture. Every 24 hours, the CI value of the mixed culture was determined as described in Materials and Methods, with Day 0 (D0) representing the initial mixture of the two cultures. For experiments that assessed the capacity of a strain to express the GASP phenotype, the test strain culture was an aged (12-day old) culture and the reference strain culture was a younger (1-day old) culture of the same bacterial strain. (B) Culture dilution and outgrowth. 125 μL of a culture was added to 12.5 mL of fresh media and grown for 24 hours. This process constituted one cycle of growth (or passage). Then, 125 μL of the culture was added to another 12.5 mL of fresh media and grown for 24 hours. This repeated cycle of inoculating fresh media with a fraction of a culture and propagating the fraction is referred to as ‘serial-passaging’. Bacteria from a 12-day old *L. monocytogenes* culture that was serially-passaged for a total of 12 passages are referred to as passaged 12-day old *L. monocytogenes* (12d-P) in subsequent figures.

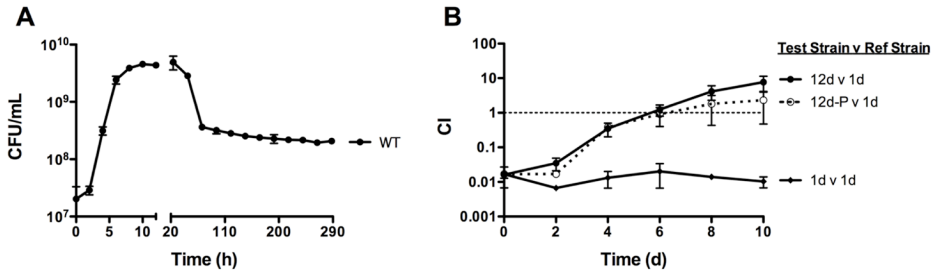

Figure 2. Long-term growth and the expression of the GASP phenotype by *L. monocytogenes*

(A) Long term culture growth curve of *L. monocytogenes* in BHI at 37°C. Bacterial growth and survival was determined by measuring CFU/mL at the indicated time points. Data shown represents the mean ± the standard error of three independent experiments. (B) *L. monocytogenes* expression of GASP. Bacteria from a 12-day old (12d), a passaged 12-day old (12d-P), or a 1-day old (1d) *L. monocytogenes* culture (test strains) were added to a 1-day old *L. monocytogenes* culture (reference strain). Mixed bacterial cultures were incubated in BHI at 37°C. Passaged 12-day old bacteria are bacteria from a culture that was aged 12 days and then diluted 1:100 in fresh BHI every 24 hours for a total of 12 passages (Fig 1B). The reference strains contained the stable integrated plasmid vector pPL2, which conferred chloramphenicol resistance. The test strains were chloramphenicol sensitive. The competitive index (CI) values were determined at the indicated time points as described in Materials and Methods, with Day 0 being the time point immediately after bacteria from a test strain culture were added to the reference strain culture. The data represent the means ± standard errors of three independent experiments.

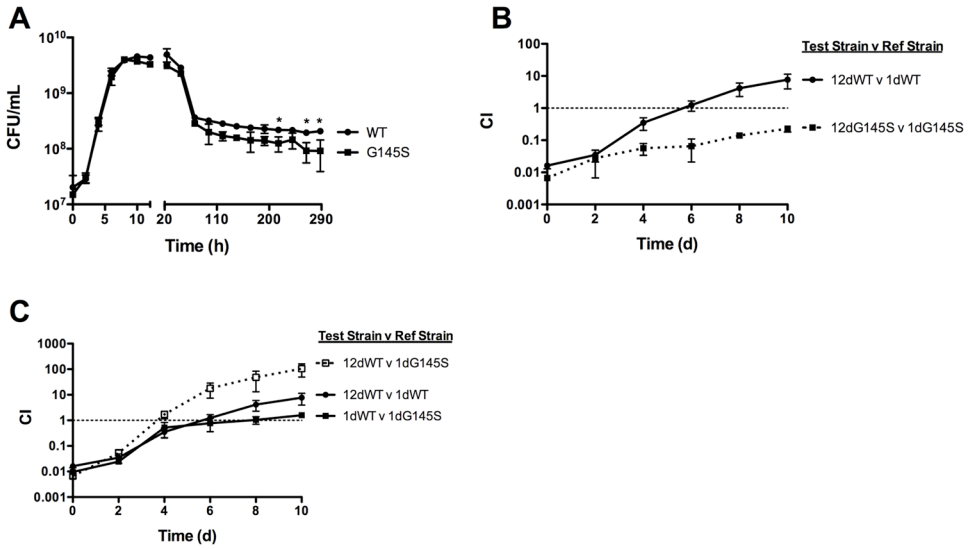

Figure 3. Constitutive PrfA activation reduces bacterial fitness during long-term stationary growth and impairs the expression of the GASP phenotype

(A) Long term stationary phase growth of wild type and *prfA** *L. monocytogenes*. Bacterial growth in BHI at 37°C was measured by enumerating CFU/mL at the indicated time points. Each growth curve represents the mean ± standard error of three independent experiments with the degree of statistical significance indicated for the last four time points (* = $p < 0.05$). (B) Mutationally activated *prfA* G145S mutants express a reduced GASP phenotype. Bacteria from 12-day old chloramphenicol sensitive cultures (test strains) were added to a 1-day old chloramphenicol resistant cultures (reference strains). Mixed cultures were maintained in BHI at 37°C and the competitive index (CI) values were determined at the indicated time points. The data represent the means ± standard errors of three independent experiments. (C) Mutational activation of *prfA* interferes with the development of GASP. Bacteria from 12-day old wild type chloramphenicol sensitive cultures (test strains) were added to a 1-day old wild type or *prfA* G145S chloramphenicol resistant cultures (reference strains). Mixed cultures were maintained in BHI at 37°C and the competitive index (CI) values were determined at the indicated time points. The data represent the means ± standard errors of three independent experiments.

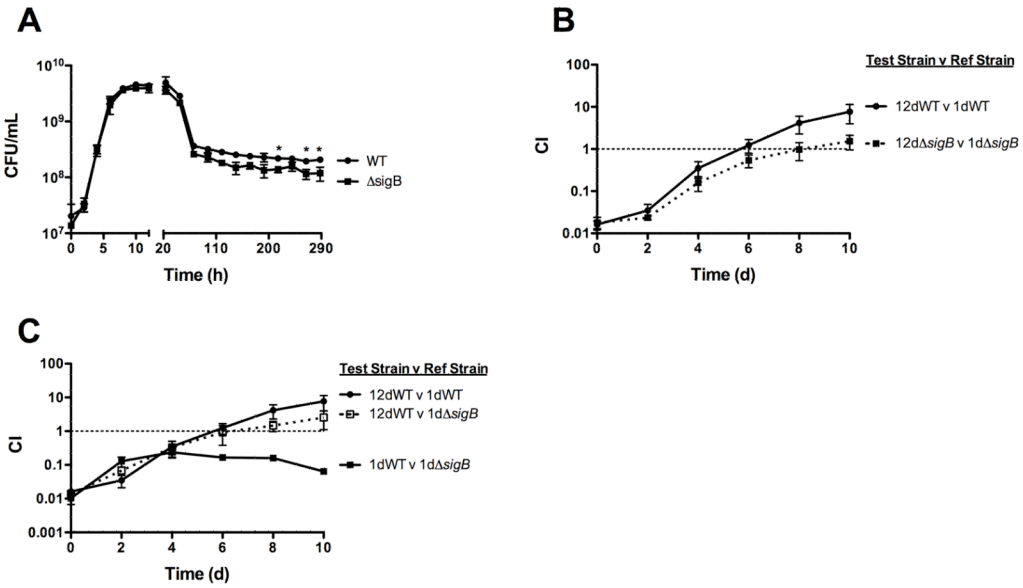

Figure 4. SigB contributes to bacterial fitness during long-term stationary growth but negatively impacts the development of GASP

(A) Comparison of long term stationary phase growth and survival of wild type and Δ*sigB* cultures. Bacterial growth in BHI at 37°C was measured by enumerating CFU/mL at the indicated time points. Each growth curve represents the mean ± standard error of three independent experiments with the degree of statistical significance indicated for the last four time points (* = $p < 0.05$). (B) Δ*sigB* mutants express a reduced GASP phenotype. Bacteria from 12-day old chloramphenicol sensitive cultures (test strains) were added to a 1-day old chloramphenicol resistant cultures (reference strains). Mixed cultures were maintained in BHI at 37°C and the competitive index (CI) values were determined at the indicated time points. The data represent the means ± standard errors of three independent experiments. (C) Loss of SigB function contributes to GASP. Bacteria from 12-day old wild type chloramphenicol sensitive cultures (test strains) were added to a 1-day old wild type or Δ*sigB* chloramphenicol resistant cultures (reference strains). Mixed cultures were maintained in BHI at 37°C and the competitive index (CI) values were determined at the indicated time points. The data represent the means ± standard errors of three independent experiments.

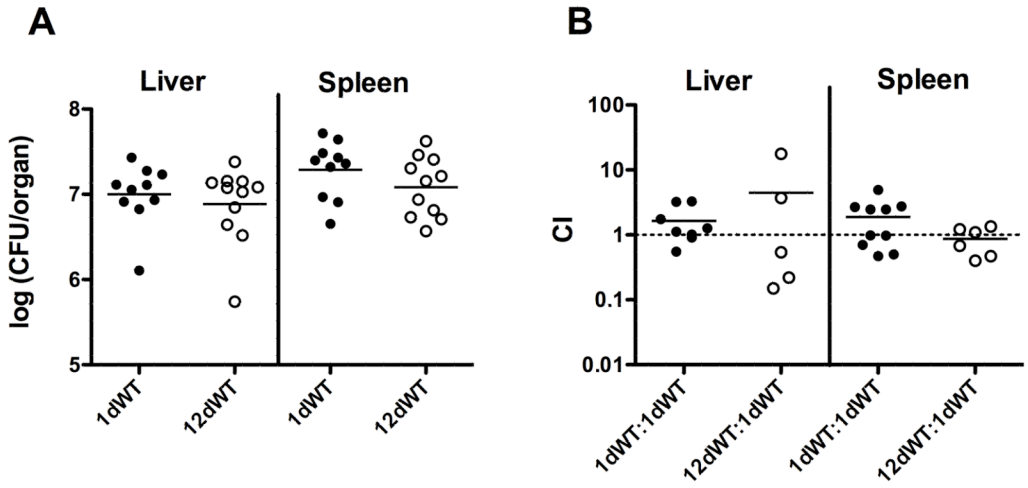

Figure 5. The expression of GASP does not significantly impact *L. monocytogenes* virulence

(A) Growth of bacteria from 12-day old *L. monocytogenes* cultures in the livers and spleens of intravenously infected mice. 7–8 week old ND4 Swiss Webster mice were infected with 2×10^4 CFU of bacteria from either 1-day old or 12-day old *L. monocytogenes* cultures via tail vein injections. 48 hours post infection, the bacterial loads of the livers and spleens were determined. A Student's T-test was performed to compare the bacterial loads of mice infected with 12-day old bacteria to those of mice infected with 1-day old bacteria. Two-tailed p-values were greater than 0.05 for both the liver and spleen, indicating an absence of statistically significant difference. (B) 12-day old *L. monocytogenes* GASP cultures exhibit no significant competitive defect in comparison to bacteria derived from 1-day old cultures. Erythromycin-sensitive bacteria from either 1-day old or 12-day old *L. monocytogenes* cultures and erythromycin-resistant bacteria from 1-day old *L. monocytogenes* cultures were mixed 1:1 for a total bacterial suspension of 2×10^4 CFU and then intravenously injected into mice. 48 hours post infection, the mice were sacrificed and the competitive index (CI) values of the livers and spleens were determined as described in Materials and Methods. Horizontal lines represent the mean CI values. A one-sided Student's T-test was performed to determine if any statistically significant difference was present between a mean CI value and 1. All two-tailed p-values were greater than 0.05, indicating an absence of statistically significant differences.

Table 1

Bacterial strains and plasmid used in this study.

Strain	Description/Genotype	Designation	Reference
SM10	*E. coli* strain for harboring plasmids		
NF-L100	10403S		(Bishop & Hinrichs, 1987)
NF-L476	NF-L100 *actA-gus-plcB*		(Shetron-Rama *et al.*, 2002)
NF-L1124	NF-L100 *actA-gus-neo-plcB*	WT	(Miner *et al.*, 2008)
NF-L1177	NF-L1124 $prfA^{G145S}$	*prfA* G145S	(Miner *et al.*, 2008)
NF-L1006	NF-L476 $tRNA^{Arg}$::pPL2	WT cam^R	
NF-E1000	SM10 with pPL2		
FSL A1-254	10403S Δ*sigB*	Δ*sigB*	(Wiedmann *et al.*, 1998)
NF-L1823	FSL A1-254 $tRNA^{Arg}$::pPL2	Δ*sigB* cam^R	This study
NF-L1824	NF-L1177 $tRNA^{Arg}$::pPL2	*prfA* G145S cam^R	This study
DP-L3903	10403S with Tn*917* insertion	WT erm^R	(Auerbuch *et al.*, 2001)
Plasmid	**Description/Genotype**	**Designation**	**Reference**
pPL2	Site-specific phage integration vector		(Lauer *et al.*, 2002)