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RESEARCH ARTICLE

Role of RpoS in Regulating Stationary Phase *Salmonella* Typhimurium Pathogenesis-Related Stress Responses under Physiological Low Fluid Shear Force Conditions

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ABSTRACT The discovery that biomechanical forces regulate microbial virulence was established with the finding that physiological low fluid shear (LFS) forces altered gene expression, stress responses, and virulence of the enteric pathogen *Salmonella enterica* serovar Typhimurium during the log phase. These log phase LFS-induced phenotypes were independent of the master stress response regulator, RpoS (σ^S). Given the central importance of RpoS in regulating stationary-phase stress responses of *S.* Typhimurium cultured under conventional shake flask and static conditions, we examined its role in stationary-phase cultures grown under physiological LFS. We constructed an isogenic *rpoS* mutant derivative of wild-type *S.* Typhimurium and compared the ability of these strains to survive *in vitro* pathogenesis-related stresses that mimic those encountered in the infected host and environment. We also compared the ability of these strains to colonize (adhere, invade, and survive within) human intestinal epithelial cell cultures. Unexpectedly, LFS-induced resistance of stationary-phase *S.* Typhimurium cultures to acid and bile salts stresses did not rely on RpoS. Likewise, RpoS was dispensable for stationary-phase LFS cultures to adhere to and survive within intestinal epithelial cells. In contrast, the resistance of these cultures to challenges of oxidative and thermal stresses, and their invasion into intestinal epithelial cells was influenced by RpoS. These findings expand our mechanistic understanding of how physiological fluid shear forces modulate stationary-phase *S.* Typhimurium physiology in unexpected ways and provide clues into microbial mechanobiology and nuances of *Salmonella* responses to microenvironmental niches in the infected host.

IMPORTANCE Bacterial pathogens respond dynamically to a variety of stresses in the infected host, including physical forces of fluid flow (fluid shear) across their surfaces. While pathogens experience wide fluctuations in fluid shear during infection, little is known about how these forces regulate microbial pathogenesis. This is especially important for stationary-phase bacterial growth, which is a critical period to understand microbial resistance, survival, and infection potential, and is regulated in many bacteria by the general stationary-phase stress response protein RpoS. Here, we showed that, unlike conventional culture conditions, several stationary-phase *Salmonella* pathogenic stress responses were not impacted by RpoS when bacteria were cultured under fluid shear conditions relevant to those encountered in the intestine of the infected host. These findings offer new insight into how physiological fluid shear forces encountered by *Salmonella* during infection might impact pathogenic responses in unexpected ways that are relevant to their disease-causing ability.

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Salmonella Typhimurium is a leading cause of bacterial foodborne illness with transmission occurring through consumption of contaminated food or water (1–4). Infection is often associated with self-limiting gastroenteritis but can progress to serious systemic disease in those who are immunocompromised (5–11). As a facultative intracellular pathogen, *S.* Typhimurium faces multiple environmental stresses in the infected host, including acidic pH, bile salts, and oxidative and thermal stresses (12). While many studies have long focused on these stresses as regulators of microbial pathogenesis and virulence, the contribution of biomechanical/physical forces in regulating the infectious disease process was not broadly recognized until 2000, when it was discovered that fluid shear altered the virulence and pathogenesis-related stress responses of *S.* Typhimurium (13, 14).

In vitro recapitulation of physiological fluid shear forces can be accomplished using the rotating wall vessel (RWV) bioreactor (Fig. 1A). In the low fluid shear (LFS) orientation, the RWV maintains cultures in suspension in a gentle fluid orbit that creates a sustained low fluid shear, low turbulence environment with an average fluid shear force of $<$0.01 dynes/cm^2 (15). This fluid shear level is relevant to that encountered by *S.* Typhimurium near the brush border microvilli of epithelial cells before infection (14, 16). In the reoriented RWV control, the LFS optimized suspension culture environment is disrupted and bacterial cells sediment to the bottom of the reactor (14).

Our team has used the RWV extensively to profile the molecular genetics and phenotypic responses of *S.* Typhimurium to physiological fluid shear forces (13–15, 17–24). The majority of our RWV studies have characterized the LFS response of *S.* Typhimurium during the logarithmic (log) phase (13–15, 17, 18, 21, 22, 25). Collectively, these findings revealed that LFS culture caused extensive reprogramming of transcriptomic, proteomic, and phenotypic responses in log phase *S.* Typhimurium (13, 17, 18). Pertinent to this study, log phase LFS cultures of wild-type (wt) *S.* Typhimurium χ3339 exhibited increased virulence in orally infected mice (decreased LD_{50}, decreased time to death, increased colonization of liver and spleen) and increased resistance to acid stress, osmotic stress, thermal stress, and increased survival within macrophages compared to reoriented control conditions (13, 15, 17). Surprisingly, while LFS increased virulence and pathogenesis-related stress resistance in log phase cultures, the expression of known virulence genes was either downregulated or unchanged compared to control conditions (18). We subsequently showed that the alternative sigma factor, RpoS (σ^S/σ^{38}/KatF), did not regulate the LFS response in log phase *S.* Typhimurium (17).

RpoS is the master regulator of the general stationary-phase stress response in *Salmonella* and protects the bacteria against a variety of harmful conditions encountered both in the infected host and the environment (26–33). Although RpoS can also function in other growth phases under stressful conditions (29–32, 34–36), much of the work to profile its role in *Salmonella* stress resistance and pathogenesis has focused on the stationary-phase, which likely represents the stage of *Salmonella* before ingestion (37).

The regulatory actions of RpoS confer cross-resistance to multiple stresses in stationary-phase *Salmonella* and other Gram-negative bacteria, including nutrient deprivation (26, 38, 39), temperature variation (38–41), hyperosmolarity (39, 40), oxidative stress (26, 38, 39), and changes in pH (26, 28, 41). RpoS also regulates the expression of *Salmonella* virulence factors necessary for establishing intracellular replication and systemic infections in mice (26, 28, 33, 35, 42–49).

As RpoS directly or indirectly regulates diverse pleiotropic *Salmonella* phenotypes, it is not surprising that RpoS-mediated gene expression is controlled at multiple levels, including mRNA and protein via modification of transcriptional and translational activity,

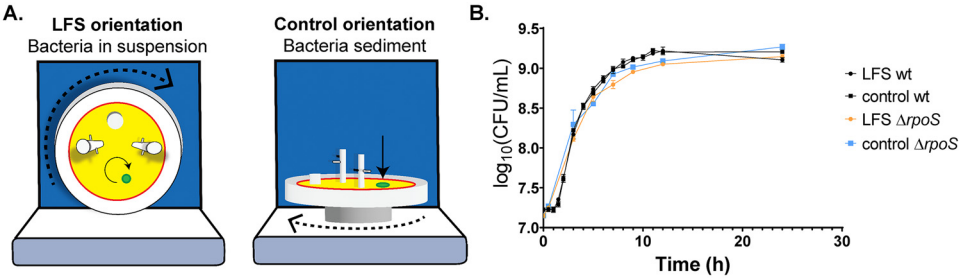


FIG 1 Rotating wall vessel (RWV) culture conditions and growth curve analysis. (A) The low fluid shear (LFS) orientation of the RWV maintains bacteria in a constant state of suspension in a gentle fluid orbit. In the control orientation, cultures sediment thus disrupting the LFS condition. (B) Growth curves for both the wild-type and isogenic *rpoS* mutant strains in the RWV (LFS and control) at 37°C, 25 rpm, for 24 h of incubation period; mean ± SEM. For each strain, at least two growth curves were done, each plated with a minimum of three technical replicates per time point. No significant differences were observed between RWV cultures (Student's *t* test $P < 0.05$).

as well as abundance and stability of the respective biological products (46, 47, 50–58). In addition, the expression of RpoS itself is associated with complex regulation, including translational regulation by the RNA binding protein Hfq (30, 59–67). Hfq interacts with several small regulatory RNAs, which in turn, promotes or inhibits translational activation of the *rpoS* transcript (68–76). *S.* Typhimurium strains carrying a deletion in the *hfq* allele are impaired in their ability to form biofilms, infect mice, survive within macrophage cell cultures, invade and survive within HeLa cervical epithelial cells, and are defective in motility compared to their wild-type parent strains (19, 77–79). Pertinent to the current study, we previously reported that Hfq coordinates the phenotypic and molecular genetic responses of stationary-phase *S.* Typhimurium when cultured under an LFS environment (19, 24). Given the importance of RpoS in regulating the stationary-phase stress response in conventionally cultured *S.* Typhimurium (26–28, 33, 43, 44), the likelihood that *S.* Typhimurium is in stationary-phase before ingestion (37), the knowledge that Hfq regulates key aspects of the stationary-phase LFS response in this pathogen (19), and the recent discovery that LFS differentially regulates genes in the RpoS regulon in stationary-phase *S.* Typhimurium (24), we investigated the role of RpoS in stationary-phase stress resistance and *in vitro* colonization profiles of *S.* Typhimurium grown under physiological LFS.

Although *S.* Typhimurium is one of the best-characterized pathogens at the molecular genetics and phenotypic levels and significant advances have been made in understanding its pathogenic mechanisms, we still have an incomplete understanding of how this organism mediates gastrointestinal disease in humans, including the role that physical forces play in the infection process. This is due to multiple reasons, ranging from the lack of *in vitro* and *in vivo* infection models that fully recapitulate human physiology (80–84) to the conventional culture of pathogens under physical force conditions that do not mimic those encountered in the infected host. The findings from our current study address the latter point and add to our mechanistic understanding of how biomechanical forces relevant to those encountered by *Salmonella* during the natural course of infection potentiate microbial pathogen responses in unexpected ways.

RESULTS

Growth characteristics of the wild-type and *rpoS* mutant strains in the RWV. To determine whether RpoS regulates the LFS response in *S.* Typhimurium at stationary-phase, we constructed an isogenic *rpoS* deletion mutant (Fig. 2A and B). PCR was used to confirm that the *rpoS* coding sequence was successfully deleted from the χ3339 genome (Fig. 2C). Additionally, a catalase-negative phenotype was confirmed for the *rpoS* mutant in the presence of 30% H_2O_2 (Fig. 2D). To ensure that strains were in the same phase of growth, we compared the RWV growth profiles for the wild-type and *rpoS*

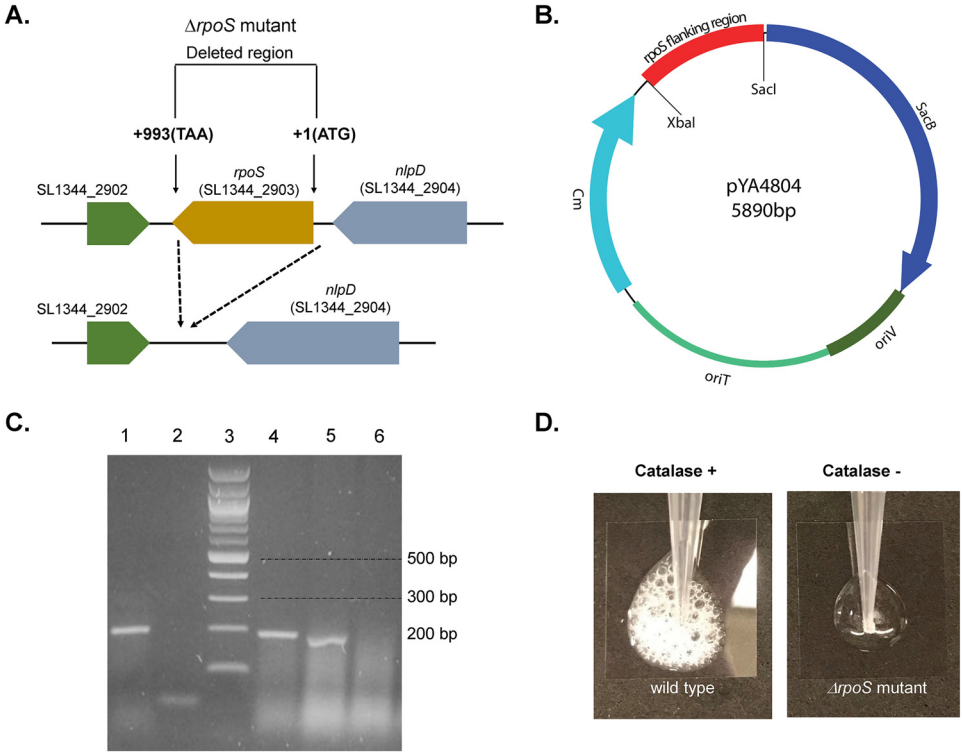
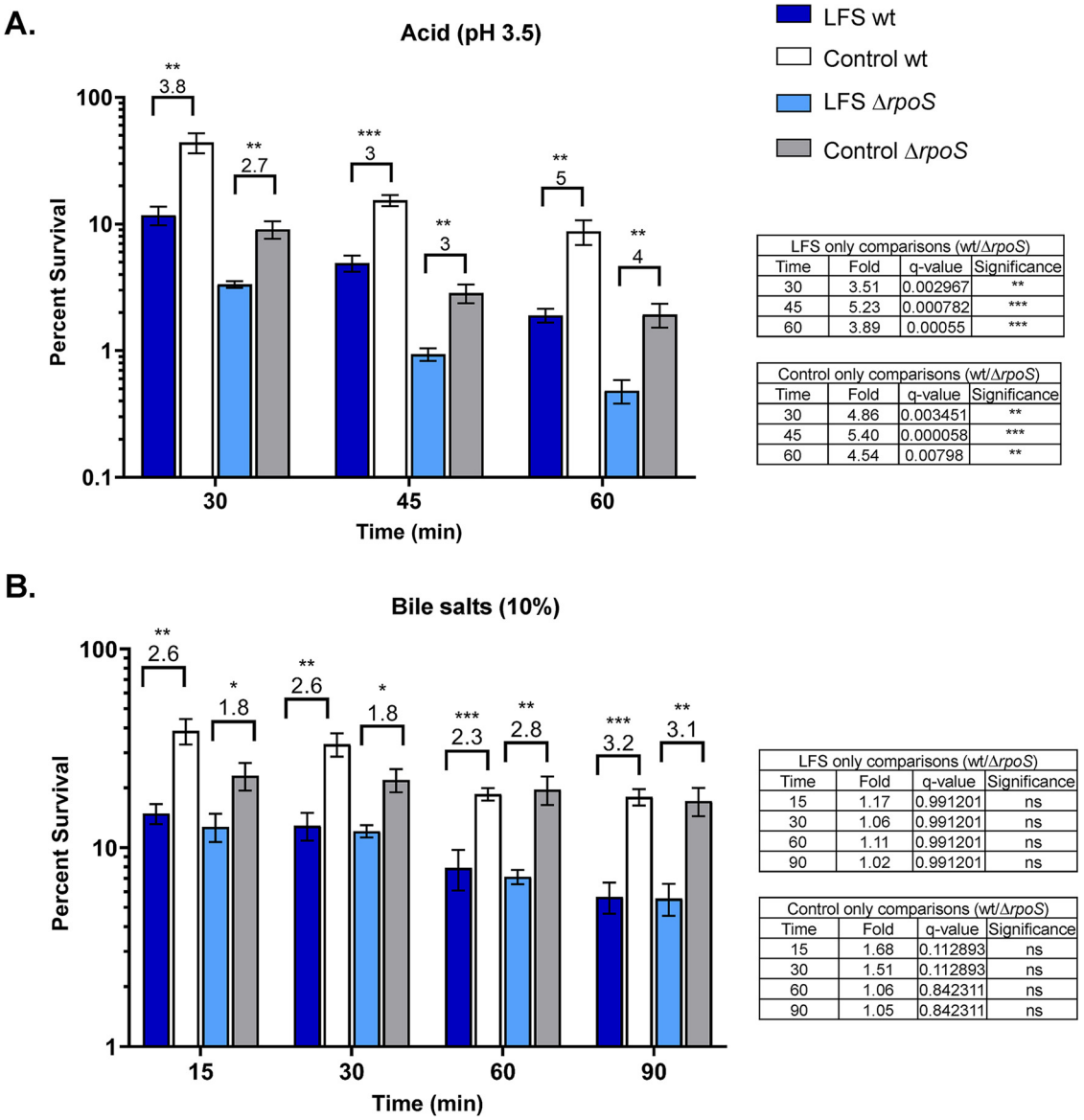


FIG 2 Construction of *rpoS* mutant and molecular and phenotypic strain validation. (A) Deletion of the *rpoS* gene from the *S.* Typhimurium χ3339 genome; deletion start site +1, end site +993 bp. In the absence of an available sequenced genome for χ3339, the sequenced genome of strain SL1344 (the parental strain of χ3339) (85) was used as a proxy. (B) Suicide vector pYA4804 was engineered by cloning a 717 bp fragment encompassing up-and downstream regions of the *rpoS* gene from *S.* Typhimurium χ3761 (wild-type UK-1) into the XbaI-SacI cloning sites of pRE112. (C) PCR analysis was used to confirm the deletion of *rpoS*. Lane 1, wild-type strain χ3339; lane 2, PCR negative control; lane 3, NEB 100 bp DNA ladder; lanes 4 and 5 show strains that did not have a successful double-crossover event to delete the *rpoS* gene and, thus, were not selected for use in this study. Lane 6 shows no PCR amplification of *rpoS*, thus demonstrating the successful deletion of the *rpoS* gene by the 2nd crossover. This strain was selected as the *rpoS* mutant for use in this study. (D) The χ3339 *rpoS* mutant exhibited reduced catalase activity compared to the wild-type strain.

mutant strains under LFS and control conditions. As shown in Fig. 1B, no differences were observed in growth profiles or final cell densities between the wild-type and *rpoS* mutant. Likewise, no colony morphological differences were observed on LB agar plates between wild-type and mutant strains (data not shown).

LFS stationary-phase wild-type and *rpoS* mutant strains show the same survival trend when challenged with acid and bile salts. We evaluated the ability of LFS stationary-phase cultures of the wild-type and *rpoS* mutant strains to survive challenges with acidic pH and bile salts, as these stressors are encountered by *S.* Typhimurium during the natural course of infection in the stomach (acidic pH) and intestine (bile salts). In addition, RpoS is known to regulate the stationary-phase response to these stresses under conventional culture conditions (26, 28, 86). Wild-type and *rpoS* mutant strains were challenged with these stressors immediately after removing them from the RWV bioreactor. As shown in Fig. 3, LFS cultures of either the wild-type or *rpoS* mutant strains were more susceptible to acid (pH 3.5) and the detergent-like activity of bile salts (10%), compared to control cultures. The LFS stationary-phase response for wild-type *S.* Typhimurium χ3339 to acid stress corroborates previous work from our team, and others, reported for this strain, wherein LFS cultures displayed decreased resistance to this stressor compared to control cultures (19–21, 25). In addition, comparisons specifically made between the wild-type and *rpoS* mutant strains grown under LFS or control conditions (Fig. 3 insets) showed an expected decrease in acid stress resistance for the *rpoS* mutant. Interestingly, however, this same trend was not observed in response to challenge with bile salts, where no difference was observed between



LFS only comparisons (wt/Δ*rpoS*)			
Time	Fold	q-value	Significance
30	3.51	0.002967	**
45	5.23	0.000782	***
60	3.89	0.00055	***

Control only comparisons (wt/Δ*rpoS*)			
Time	Fold	q-value	Significance
30	4.86	0.003451	**
45	5.40	0.000058	***
60	4.54	0.00798	**

LFS only comparisons (wt/Δ*rpoS*)			
Time	Fold	q-value	Significance
15	1.17	0.991201	ns
30	1.06	0.991201	ns
60	1.11	0.991201	ns
90	1.02	0.991201	ns

Control only comparisons (wt/Δ*rpoS*)			
Time	Fold	q-value	Significance
15	1.68	0.112893	ns
30	1.51	0.112893	ns
60	1.06	0.842311	ns
90	1.05	0.842311	ns

FIG 3 RpoS does not regulate the stationary-phase LFS-induced response of *S.* Typhimurium to either acid or bile stresses. χ3339 and Δ*rpoS* strains were independently grown to stationary-phase in the RWV in the LFS and control orientations. Immediately after harvesting from the RWV, bacteria were challenged with (A) acid (pH 3.5) or (B) bile salts (10%). Viable CFU per mL was obtained by removing samples at each of the indicated time points, serially diluting, and plating on LB agar plates. Percent survival was calculated by normalizing all samples to T0 (number of bacteria before exposure to the stress). Results represent the average of a minimum of three independent biological replicates, each with a minimum of three or more technical replicates per time point; mean percent survival ± SEM are plotted for each of the stresses. A Shapiro-Wilk test was used to test for the normality of the data and an F-test was used to compare variances between groups. Statistical significance at each time point was calculated using an unpaired *t* test with Welch correction, followed by multiple comparisons using the two-stage step-up method of Benjamini, Krieger, and Yekutieli (*, $q < 0.05$; **, $q < 0.01$; ***, $q < 0.001$; ns = no significance); values beneath asterisks represent fold changes. For each of the stresses, additional direct strain comparisons are summarized as shown in the table format; fold values were calculated by dividing percent survivals for the wild-type by those of the Δ*rpoS* strain.

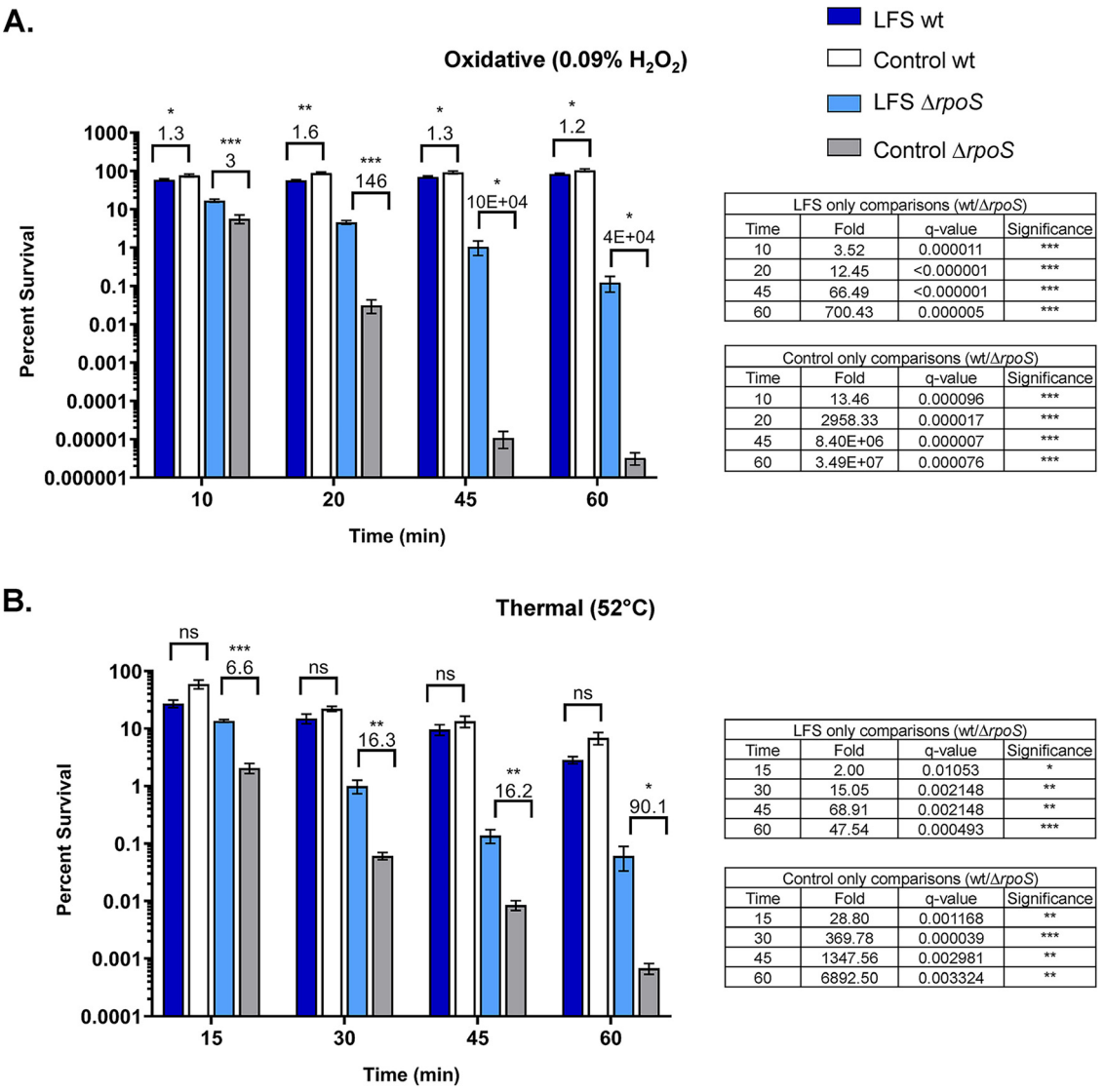
the wild-type and *rpoS* mutant. Furthermore, an evaluation of the wild-type to mutant ratio (wt/Δ*rpoS*) indicates the fold changes between the two strains for either LFS or control comparisons under either stress response were comparable in magnitude (Fig. 3, insets). This observation suggests that *rpoS* affects *S.* Typhimurium survival equally across LFS and control culture conditions. Together, these data suggested that the mechanism driving the physiological fluid shear-induced phenotypes observed for both acid and bile salt stressors was not dependent upon a functional RpoS protein in stationary-phase *S.* Typhimurium.

LFS stationary-phase wild-type and *rpoS* mutant strains exhibited different survival trends in response to oxidative or thermal stress. We next evaluated the role of RpoS in regulating the stationary-phase LFS response of *S.* Typhimurium to survive challenges with reactive oxygen species (ROS) and thermal stress. These conditions were relevant to those to which *Salmonella* was exposed during its natural life cycle both in the infected host and the environment. Moreover, when cultured conventionally, RpoS is known to regulate the stationary-phase response of *S.* Typhimurium to these stresses (26, 38, 39). Because the *rpoS* mutant was extremely sensitive to hydrogen peroxide concentrations that we normally use for the wild-type strain (0.24%), we lowered the concentration to 0.09% to allow recovery of the mutant. Our data showed that LFS cultures of the *rpoS* mutant exhibited enhanced survival in response to challenges with oxidative stress compared to control conditions, which is the opposite trend observed with the wild-type strain (Fig. 4A). Interestingly, a similar pattern of survival was observed for both the wild-type and *rpoS* mutant strains in response to thermal stress (52°C) (Fig. 4B). Comparisons made between the wild-type and *rpoS* mutant strains cultured under LFS or control orientations (Fig. 4, insets), revealed an expected decrease in the stress resistance of the *rpoS* mutant to oxidative and thermal stressors. In addition, the absence of *rpoS* had a much greater impact on the survival of control cultures than LFS cultures. Because the wild-type and *rpoS* mutant displayed different fluid shear survival trends, we concluded that the LFS response of stationary-phase *S.* Typhimurium to both oxidative and thermal stressors was dependent on RpoS.

RpoS regulated invasion, but not adherence or intracellular survival, of LFS stationary-phase *S.* Typhimurium in human intestinal epithelial cells. To determine if RpoS regulated the ability of LFS cultured *S.* Typhimurium to colonize (adhere to, invade into, and survive within) intestinal epithelial cells, HT-29 colonic epithelial monolayers were infected with RWV-cultured wild-type or *rpoS* mutant strains grown to stationary-phase. The noninvasive strain, *E. coli* HB101, was used as a control in these studies. LFS significantly enhanced the ability of both *S.* Typhimurium strains to adhere, invade, and survive in HT-29 cells, compared to control cultures (Fig. 5). A comparison of LFS stationary-phase colonization profiles between the two strains showed that the *rpoS* mutant exhibited wild-type ability to adhere to (Fig. 5A) and to survive within intestinal epithelial cells (Fig. 5C). In contrast, the *rpoS* mutant demonstrated enhanced ability to invade host cells compared to the wild-type strain grown under LFS conditions (Fig. 5B). Interestingly, direct comparisons between the wild-type and *rpoS* mutant under control conditions indicated no significant differences in the colonization of host cells at all time points analyzed (Fig. 5). Thus, even though the wild-type and *rpoS* mutant strains exhibited conserved fluid shear phenotypes, direct strain comparisons for the invasion time point indicated that LFS cultures have a RpoS-dependent regulatory component not observed in control cultures. Collectively, our infection assay results further highlight the complex association between RpoS and LFS responses in *S.* Typhimurium.

DISCUSSION

Studies with *S.* Typhimurium were the first to document that physical forces serve as environmental regulators of microbial virulence. Specifically, these initial studies and subsequent follow-up research showed that low fluid shear forces relevant to those encountered by *Salmonella* in the intestinal tract of the infected host regulated virulence, stress resistance, and gene expression (transcriptomic and proteomic) during the logarithmic phase (13, 14, 17, 18, 22, 25). While the LFS log phase stress response in *S.* Typhimurium was shown to be independent of RpoS (17), the role of RpoS regulation of LFS pathogenesis-related phenotypes and gene expression in stationary-phase remains largely unexplored. Given the central importance of RpoS in regulating the general stationary-phase stress response during conventional culture of *S.* Typhimurium (26–28, 33, 43, 44), combined with the knowledge that *S.* Typhimurium was likely in stationary-phase before ingestion (37), that Hfq, a master regulator of RpoS, regulated gene expression and stress responses of *S.* Typhimurium LFS cultures (19, 20), recent work from our team showing that LFS regulated multiple genes in the RpoS regulon in stationary-phase *S.* Typhimurium



LFS only comparisons (wt/Δ*rpoS*)

Time	Fold	q-value	Significance
10	3.52	0.000011	***
20	12.45	<0.000001	***
45	66.49	<0.000001	***
60	700.43	0.000005	***

Control only comparisons (wt/Δ*rpoS*)

Time	Fold	q-value	Significance
10	13.46	0.000096	***
20	2958.33	0.000017	***
45	8.40E+06	0.000007	***
60	3.49E+07	0.000076	***

LFS only comparisons (wt/Δ*rpoS*)

Time	Fold	q-value	Significance
15	2.00	0.01053	*
30	15.05	0.002148	**
45	68.91	0.002148	**
60	47.54	0.000493	***

Control only comparisons (wt/Δ*rpoS*)

Time	Fold	q-value	Significance
15	28.80	0.001168	**
30	369.78	0.000039	***
45	1347.56	0.002981	**
60	6892.50	0.003324	**

FIG 4 The LFS stationary-phase response of *S.* Typhimurium to oxidative and thermal stresses is dependent on RpoS. *S.* Typhimurium χ3339 and the Δ*rpoS* strain were grown in the RWV for 24 h at 37°C in the LFS or control orientations. Immediately after harvesting from the RWV, bacterial strains were challenged with (A) oxidative stress (0.09% H_2O_2) or (B) thermal stress (52°C). CFU/mL was calculated by removing samples at each of the indicated time points, serially diluting, and plating on LB agar plates. Percent survival was calculated by normalizing all samples to T0 (number of bacteria before the addition of the stress). An average of two (A) and three (B) biological replicates are represented, each with a minimum of 3 or more technical replicates per time point. Mean percent survival ± SEM is plotted for each of the stresses. A Shapiro-Wilk test was used to test for the normality of the data and an F-test was used to compare variances between groups. Statistical significance at each time point was calculated using an unpaired *t* test with Welch correction, followed by multiple comparisons using the two-stage step-up method of Benjamini, Krieger, and Yekutieli (*, $q < 0.05$; **, $q < 0.01$; ***, $q < 0.001$; ns = no significance); values beneath asterisks represent fold changes (E = exponent). For each of the stresses, additional direct strain comparisons are summarized in table format; fold values are calculated by dividing percent survivals for the wild-type by those of the Δ*rpoS* mutant strain.

(24), and RpoS was essential for the LFS stationary-phase stress response in *Escherichia coli* (87–89), we reasoned that RpoS may broadly regulate LFS stationary-phase *S.* Typhimurium pathogenesis-related phenotypes.

In this study, we investigated the role of RpoS in stationary-phase stress resistance and *in vitro* colonization profiles of human intestinal epithelial cells for *S.* Typhimurium grown under physiological LFS. Specifically, we compared the ability of wild-type and isogenic *rpoS* mutant strains to survive challenges with *in vitro* stresses that mimic either those encountered in the infected host or before ingestion. We focused on acid, oxidative, and bile salt stresses relevant to conditions encountered by *Salmonella* during the infection process, as well as thermal stress associated with processed/cooked

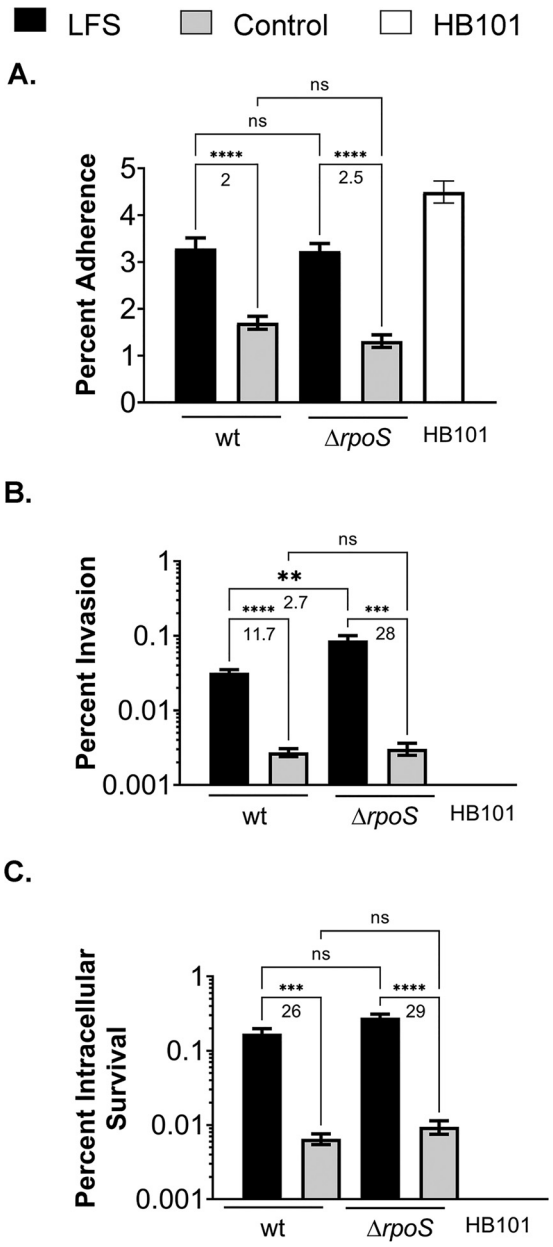


FIG 5 Assessing the effect of LFS and RpoS on stationary-phase *S.* Typhimurium colonization profiles using human intestinal epithelial cells. (A) Adherence of *S.* Typhimurium strains χ3339 and Δ*rpoS* to HT-29 cells was evaluated 30 min after infection. (B) Invasion (3 h) and (C) intracellular survival (24 h) were assayed using a standard gentamicin protection assay. At each time point, bacteria were quantified by plating for CFU/mL. The results depicted represent the average of two biological replicates, each in technical triplicate. Data were normalized at each time point to the number of initial inocula. *E. coli* strain HB101 was included as a noninvasive control. Each bar graph represents the mean percent ± SEM. Data were assessed for normality using the D'Agostino-Pearson test and homogeneity of variance using Bartlett's test. Statistical significance was calculated using Welch ANOVA followed by Dunnett T3 for multiple comparisons (**, $q < 0.01$; ***, $q < 0.001$; ****, $q < 0.0001$; ns = no significance); values beneath asterisks represent fold changes.

foods. In addition, human colonic intestinal epithelial cells were used in bacterial colonization studies to profile potential differences in adherence, invasion, and intracellular survival of this enteric pathogen.

All the phenotypes tested in this study have previously been shown to be regulated by RpoS in stationary-phase *Salmonella enterica* pathovars cultured conventionally (26, 28, 33, 40–42, 44–47, 86, 90). However, there is surprisingly little information regarding

the impact of physiological fluid shear conditions on RpoS regulation of stationary-phase *Salmonella* pathogenesis-related responses.

Unexpectedly, the *rpoS* mutant behaved similarly to the wild-type parent strain following a challenge with either acid stress or bile salts stress. Specifically, we observed no difference in the magnitude or direction of the survival trends for either comparison (either between the same strains or between the two culture conditions). These data indicate that RpoS does not play a role in the *S.* Typhimurium LFS stationary-phase response to these stresses. Likewise, our data showed that adherence to and survival within intestinal epithelial cells did not require RpoS. Our finding that a functional *rpoS* allele was dispensable for the ability of LFS cultured *S.* Typhimurium to survive inside host cells was surprising, given that RpoS regulates the *spv* genes which are required for intracellular survival of *S.* Typhimurium when cultured conventionally (26, 33, 35, 55, 91, 92). Collectively, our findings indicate that stationary-phase *S.* Typhimurium cultured under fluid shear forces relevant to those encountered in the infected host exhibited phenotypes that are relevant to the pathogenesis of this organism, but which are not regulated by RpoS, thus differing from observations in conventional culture conditions.

Our evaluation of stationary-phase *S.* Typhimurium LFS phenotypic responses to oxidative and thermal stresses, as well as invasion of human intestinal epithelial cells, confirmed that RpoS played a regulatory role in these phenotypes.

A study by Pacello et al. (93), which used different *S.* Typhimurium strains than those in our current study, reported that the ability of (likely stationary-phase) LFS cultures to survive oxidative stress was not dependent on RpoS. The authors further identified the RpoS-regulated gene, *katN*, together with *katG*, as mediators of the enhanced resistance of the pathogen to oxidative stress during LFS culture. It is important to note there were key experimental design differences between this study and ours that could explain these divergent trends, including the use of different bacterial strains, culture conditions, and pH. These different responses of closely related strains of *S.* Typhimurium to the LFS environment suggest that the involvement of RpoS may be strain specific.

In comparison, the role of RpoS in the LFS response of stationary-phase cultures of *E. coli* has been well documented (87–89). For example, *rpoS* was shown to be essential for the LFS response of stationary-phase (but not log phase) cultures of *E. coli* to survive challenges with acid and osmotic stresses (87). The difference between the log phase and stationary-phase dependent responses in *E. coli* was attributed to the effects of LFS on mRNA translational efficiency and RpoS protein stability (87). Furthermore, RpoS was also found to regulate *E. coli* LFS biofilm-mediated resistance to sodium chloride stress and ethanol stress (88). A separate study also revealed the importance of RpoS in regulating adherence and invasion profiles of LFS stationary-phase cultures of *E. coli* to Caco-2 human colonic intestinal epithelial cells (89).

An essential step in the early stages of human enteric salmonellosis is the colonization of intestinal epithelial cells (attachment, invasion, and intracellular survival). Most studies that have greatly advanced our understanding of this process have relied on culturing *Salmonella* under fluid shear conditions in shake flasks and static culture, which do not mimic those physical forces found in the infected host. In this study, we showed that physiological LFS enhanced *S.* Typhimurium *in vitro* colonization profiles for intestinal epithelial cells for both the wild-type and *rpoS* mutant. This increased colonization of host cells by wild-type cultures was in alignment with our recent finding of enhanced colonization of a three-dimensional (3-D) biomimetic intestinal tissue coculture model using LFS-grown stationary-phase cultures of the same wild-type *S.* Typhimurium χ3339 (24).

Recent transcriptomic characterization of *S.* Typhimurium χ3339, grown under the same conditions as this study, revealed that LFS differentially regulated the expression of several RpoS-regulated genes associated with host cell colonization (24). These included gene transcripts associated with *Salmonella* Pathogenicity islands 1 and 2, fimbriae, motility, and chemotaxis. These changes in gene expression suggested an active role of RpoS in regulating stationary-phase *S.* Typhimurium LFS cell infection phenotypes (adherence, invasion, and intracellular survival). In our current study,

S. Typhimurium displayed a RpoS-dependent response during an invasion, but not during adherence or survival. While our phenotypes did not fully align with the observed changes in gene expression, the increase in invasion observed for the LFS stationary-phase *rpoS* mutant relative to wild-type, agreed with previous findings using both an *S.* Typhimurium *rpoS* mutant and wild-type strains cultured statically (44).

This study leverages previous infectious disease mechanobiology research with *S.* Typhimurium (13, 15, 17–22, 24, 25, 93, 94) and further validates the importance of considering the influence of physiological fluid shear forces on microbial pathogen responses in the context of host-pathogen interactions and infectious disease outcome. Our work continues to add to our mechanistic understanding of the mechanobiology of infectious disease and the significance of simulating *in vivo* like physical forces to characterize their impact on shaping physiological adaptations of human pathogens to their infected hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S.* Typhimurium wild-type strain χ3339 (an animal-passaged isolate of SL1344) (85) and an isogenic *rpoS* mutant derivative of χ3339 (construction described below and in Fig. 2) were used in this study. All strains were initially grown overnight in Lennox broth (LB) in flask cultures with aeration (250 rpm) at 37°C for 14 to 16 h. Overnight cultures were diluted 1:200 into fresh LB and loaded into RWV bioreactors positioned in the LFS and control orientations (Fig. 1A) such that the bioreactors were completely filled with the bacterial cultures. Care was taken to remove all bubbles from the reactors before starting their rotation at 25 rpm in a 37°C incubator for 24 h. Cultures in both orientations are aerated through a gas permeable membrane at the back of the reactor. Growth curves were performed for both the wild-type and *rpoS* mutant strains in the LFS and control orientations of the RWV by determining viable CFU per mL (CFU/mL) (Fig. 1B).

Construction of rpoS mutant. The *rpoS* gene (993 bp, from +1 to +993) in χ3339 was deleted to generate the isogenic *rpoS* mutant strain used in this study (Fig. 2A). To construct this mutant, we used recombinant suicide vector pYA4804 (5.89 kb) carrying DNA fragments from *S.* Typhimurium strain χ3761 (wild-type UK-1) up-and downstream regions of the *rpoS* gene inserted into the XbaI-SacI cloning sites of vector pRE112 (Addgene) (Fig. 2B). An *E. coli* strain (F+, λ*pir*) carrying pYA4804 was then conjugated with *S.* Typhimurium χ3339. The resulting conjugants were resuspended in sterile PBS and plated onto LB agar containing chloramphenicol (30 μg/mL) to select recombinant colonies from the first crossover event. Isolated colonies were subsequently inoculated into fresh LB and grown until reaching an optical density at 600 nm (OD_{600}) between 0.4 and 0.6. The culture was serially diluted and plated on LB agar containing 5% sucrose without NaCl or chloramphenicol to select for double-crossover mutants. The mutant candidates were further screened for *rpoS* deletion by PCR (forward 5′-ttaccaccagacgcaggttact-3′ and reverse 5′-taacgacctggctgaagaagag-3′ primer set designed using *S.* Typhimurium strain SL1344 as the reference genome) (Fig. 2C) and confirmed to be chloramphenicol sensitive (data not shown). The Δ*rpoS* mutant was phenotypically verified by the catalase test (Fig. 2D). For the catalase test, both strains were exposed to 100 μL of 30% H_2O_2. The lack of bubble formation by the Δ*rpoS* mutant grown to stationary-phase confirmed the absence of *rpoS*-dependent catalase gene expression.

Stress responses. Strains were grown in the LFS and control orientations of the RWV under aerated conditions as described above. Cultures were harvested at 24 h and divided into 10 mL aliquots (unless otherwise noted) immediately before the introduction of the stress. For acid stress assays, the culture pH was lowered to 3.5 by the addition of a predetermined volume of concentrated citrate buffer (stock concentration 1 M sodium citrate, 0.513 M sodium phosphate dibasic heptahydrate; Sigma-Aldrich). The final pH was confirmed at the end of each assay with a pH electrode. For bile salts stress assays, bile salts (sodium cholate and sodium deoxycholate mix, Sigma-Aldrich) were added at a final concentration of 10%. For thermal stress assays, a 1 mL aliquot of the culture was transferred into a tube held in a heating block maintained at 52 ± 1°C. For oxidative stress assays, fresh hydrogen peroxide (stored at 4°C for less than a month) was added to the culture from a 30% stock solution to a final concentration of 0.09%. For each stress condition, bacterial viability was assessed over a range of time points by serially diluting the cultures in Dulbecco's Phosphate Buffered Saline (DPBS) and plating for CFU/mL on LB agar plates. Percent survival was calculated by dividing the number of CFU at each time point by the number of bacteria at time zero (before the addition of the stress).

Infection of intestinal epithelial cell cultures. Human colonic intestinal epithelial cells (HT-29; ATCC HTB-38) were seeded at 2×10^5 cells/mL into 24-well tissue culture plates. Monolayers used for infection studies were ~90% confluent, with ~1×10^6 cells/well. Infection studies were performed as described previously using a modified approach (44). Specifically, cultures were maintained at 37°C under 10% CO_2 in GTSF-2 medium (HyClone) for all studies. Cells were infected with 25 μL of either the wild-type or *rpoS* mutant grown in the RWV as described above, at a multiplicity of infection (MOI) of ~2:1 (bacteria:host cell). Infected cultures were incubated for 30 min, 3 h, and 24 h to monitor for bacterial adherence, invasion, and intracellular survival, respectively. Gentamicin was added after the 30 min (50 μg/mL) and 3 h (10 μg/mL) time points to eliminate extracellular bacteria. At each time point, the cells were washed in triplicate with Hanks' Balanced Salt Solution (HBSS) and lysed with 1 mL of 0.1% sodium deoxycholate. Samples were concentrated 10× by 10 min centrifugation (7,500 rpm) followed by resuspension of the cell pellet in a final volume of 100 μL of 0.1% sodium deoxycholate. Samples were vortexed, serially diluted in DPBS, and plated for viable CFU/mL. Percent adherence, invasion, and intracellular survival were

calculated at each time point by normalizing to the initial bacterial inoculum. *E. coli* strain HB101 was included as a noninvasive control. For HB101, 14 to 16 h overnight cultures (shaking, 250 rpm, 37°C) were diluted 1:200 into 5 mL sterile LB and then grown for 24 h at 37°C before use for infection studies.

Statistical analysis. For each experiment, the number of biological replicates is specified in the corresponding figure legends. For growth curves, stress response profiles, and invasion assays, graphs represent the mean percent survival ± standard error of the mean (SEM). For stress responses and infection assays, both the equality of variance and normality of the data were assessed before applying a parametric statistical test. An unpaired *t* test with Welch correction or analysis of variance (ANOVA) with Welch correction was performed to determine significant differences. Additional details of each statistical procedure are described in the figure legends. All statistical analyses were performed using GraphPad Prism version 9.3.1.

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