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Oxygen-Dependent Regulation of SPI1 Type Three Secretion System by Small RNAs in *Salmonella enterica* serovar Typhimurium

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Summary

Salmonella Typhimurium induces inflammatory diarrhea and uptake into intestinal epithelial cells using the *Salmonella* pathogenicity island 1 (SPI1) type III secretion system (T3SS). Three AraC-like regulators, HilD, HilC, and RtsA, form a feed-forward regulatory loop that activates transcription of *hilA*, encoding the activator of the T3SS structural genes. Many environmental signals and regulatory systems are integrated into this circuit to precisely regulate SPI1 expression. A subset of these regulatory factors affect translation of *hilD*, but the mechanisms are poorly understood. Here, we identified two sRNAs, FnrS and ArcZ, which repress *hilD* translation, leading to decreased production of HilA. FnrS and ArcZ are oppositely regulated in response to oxygen, one of the key environmental signals affecting expression of SPI1. Mutational analysis demonstrates that FnrS and ArcZ bind to the *hilD* mRNA 5' UTR, resulting in translational repression. Deletion of *fnrS* led to increased HilD production under low aeration conditions, whereas deletion of *arcZ* abolished the regulatory effect on *hilD* translation aerobically. The *fnrS arcZ* double mutant has phenotypes in a mouse oral infection model consistent with increased expression of SPI1. Together, these results suggest that coordinated regulation by these two sRNAs maximizes HilD production at an intermediate level of oxygen.

Graphical Abstract

Salmonella is a leading cause of gastrointestinal disease worldwide. Proper temporal and spatial expression of the *Salmonella* SPI1 type-three secretion system is critical for invasion of the host intestinal epithelium. Here, we show that two oxygen-dependent sRNAs, FnrS and ArcZ, regulate

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Author contributions

KK designed and performed experiments, interpreted and analyzed the data, and wrote the manuscript.

YAG and KK designed and performed all animal experiments, interpreted the data and wrote the manuscript.

CKV reviewed experimental design and data interpretation, and edited the manuscript.

JMS supervised and reviewed experimental design, data analyses, and wrote and edited the manuscript.

Ethics statement

All animal work was reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC). Procedures were performed in our AAALAC accredited facility in accordance with University and PHS guidelines under protocol 15214. All efforts were made to minimize animal suffering.

Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

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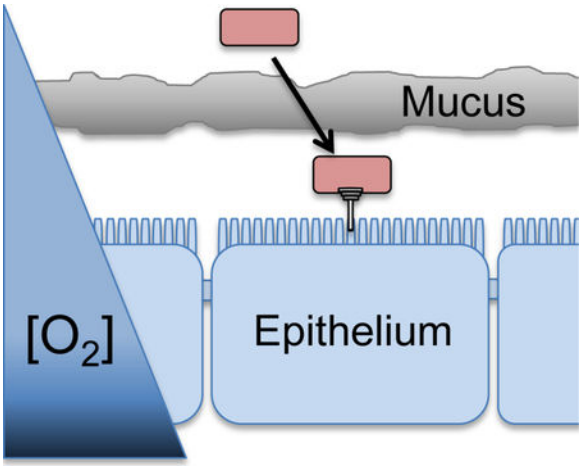
production of the invasion machinery, tuning SPI1 expression to a particular oxygen level consistent with that at the epithelial surface.



Keywords

Salmonella infection; SPI1; HilD; FnrS; ArcZ

Introduction

Bacterial pathogens encounter various host environments and adapt their physiology and virulence traits accordingly. *Salmonella enterica* serovar Typhimurium preferentially infects epithelial cells in the distal ileum of the small intestine, causing inflammatory diarrhea and initiating systemic infection (Savidge *et al.*, 1991; Clark *et al.*, 1994; Penheiter *et al.*, 1997; Jepson and Clark, 2001). Both invasion and induction of inflammation is dependent on the type three secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI1), which directly injects bacterial effectors into the host cell cytosol to initiate cytoskeletal rearrangement and alter signal transduction (Penheiter *et al.*, 1997; Galan and Collmer, 1999; Galan, 2001; Ellermeier and Slauch, 2007).

Production of the SPI1 T3SS is tightly regulated in response to numerous environmental cues (Jones, 2005; Ellermeier and Slauch, 2007; Golubeva *et al.*, 2012). HilA (hyperinvasion locus A), encoded within the SPI1 locus, directly activates the T3SS structural genes (Bajaj *et al.*, 1996; Darwin and Miller, 1999; Eichelberg and Galan, 1999). Transcription *of hilA* is activated by three AraC-like proteins, HilD, HilC, and RtsA, which each bind the promoter of *hilA* to directly enhance transcription. In addition, HilD, HilC, and RtsA activate their own transcription and the transcription of each other, forming a complex feed-forward regulatory loop (Olekhnovich and Kadner, 2002; Ellermeier *et al.*, 2005; Olekhnovich and Kadner, 2006; Olekhnovich and Kadner, 2007) (Fig 1).

A number of environmental signals and regulatory systems are integrated into the SPI1 regulatory circuit primarily at the level of *hilD* translation or HilD protein activity (Golubeva *et al.*, 2012), while HilC and RtsA act to amplify these inducing signals (Ellermeier and

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Slauch, 2007; Saini *et al.*, 2010; Golubeva *et al.*, 2012) (Fig. 1). For example, FliZ and HilE control HilD protein activity (Baxter *et al.*, 2003; Chubiz *et al.*, 2010; Golubeva *et al.*, 2012; Grenz *et al.*, 2018). Several systems have been shown to control *hilD* mRNA translation or stability, but the underlying mechanism is understood in only a couple of cases (Martinez *et al.*, 2011). Given the role of HilD as signal integrator and dominant activator of *hilA* transcription, characterizing the direct regulation of *hilD* translation is crucial to understanding SPI1 regulation at a systems level.

Small regulatory RNAs (sRNAs) are increasingly recognized as key regulators of bacterial physiology and virulence (Frohlich and Vogel, 2009; De Lay *et al.*, 2013; Desnoyers *et al.*, 2013; Jagodnik *et al.*, 2017). Ranging from 50 to 500 nucleotides in length, sRNAs control gene expression by base pairing with target mRNAs. The RNA chaperones Hfq or ProQ facilitate base pairing-dependent regulation by sRNAs on mRNA targets (Moller *et al.*, 2002; Geissmann and Touati, 2004; Mandin and Gottesman, 2009; Vogel and Luisi, 2011; Henderson *et al.*, 2013; Smirnov *et al.*, 2016). The outcomes of sRNA-mRNA base pairing vary, but usually the translation and/or stability of the mRNA is affected either positively or negatively (Majdalani *et al.*, 1998; Majdalani *et al.*, 2001; Vanderpool and Gottesman, 2004; Masse *et al.*, 2005; McCullen *et al.*, 2010). In *Salmonella,* both experimental and bioinformatic approaches have been used to identify more than 325 sRNAs, but few of them are characterized (Kroger *et al.*, 2012; Kroger *et al.*, 2013; Srikumar *et al.*, 2015; Colgan *et al.*, 2016; Smirnov *et al.*, 2016). Dramatic changes in expression of all SPI1 genes in *hfq* mutants compared to wild-type *Salmonella* implicate sRNAs in direct or indirect control of SPI1 gene expression (Sittka *et al.*, 2007; Storz *et al.*, 2011).

Low oxygen tension and high osmolarity are conditions traditionally used to induce the SPI1 T3SS (Ni Bhriain *et al.*, 1989; Lee and Falkow, 1990; Tartera and Metcalf, 1993; Ellermeier and Slauch, 2007; Jennewein *et al.*, 2015). *Salmonella* primarily utilizes two major regulators, Fnr and the ArcAB two-component system, to adapt to oxygen-related environmental changes (Fink *et al.*, 2007; Lim *et al.*, 2013; Troxell and Hassan, 2016). Fnr is directly inactivated by oxygen via oxidation of its 4Fe-4S cluster (Khoroshilova *et al.*, 1997). Fnr is known to affect expression of the SPI1 T3SS (Fink *et al.*, 2007; Golubeva *et al.*, 2012). Our previous data suggest that Fnr negatively regulates SPI1 by repressing *hilA* expression, but the mechanism is unclear (Golubeva *et al.*, 2012). In the mouse model, *fnr* mutants show a mild loss of virulence (Craig *et al.*, 2013).

The activity of the ArcAB two-component system is influenced by oxygen availability (Georgellis *et al.*, 2001; Lu *et al.*, 2002). ArcB is a histidine sensor kinase, and controls the phosphorylation of ArcA, the response regulator. ArcB activity responds to the oxidation state of the quinone pool of the respiratory chain, thus acting as an indirect oxygen sensor (Bauer *et al.*, 1999). ArcB phosphorylates ArcA under anaerobic conditions and dephosphorylates ArcA-P under aerobic conditions. ArcA-P controls expression of various genes including some involved in aerobic metabolism. ArcA also affects *hilA* expression under aerobic growth conditions, but the mechanism is unclear (Lim *et al.*, 2013). Despite several links between oxygen sensing and regulation of SPI1 gene expression, the overall mechanisms of oxygen-mediated control of SPI1 remain enigmatic.

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Two sRNAs, FnrS and ArcZ, are produced in response to changes in oxygen tension. Data from *E. coli* and *Salmonella* show that FnrS is produced under anaerobic conditions due to Fnr-mediated activation of *fnrS* transcription, whereas ArcZ is produced primarily under aerobic conditions; its transcription is repressed anaerobically by ArcA-P (Papenfort *et al.*, 2009; Durand and Storz, 2010; Mandin and Gottesman, 2010). FnrS represses genes related to aerobic metabolism and genes encoding products that protect cells from reactive oxygen species (Boysen *et al.*, 2010; Durand and Storz, 2010). ArcZ regulates *rpoS*, *csgD* and *eptB* mRNAs along with several others encoding products related to aerobic metabolism (Papenfort *et al.*, 2009; Mandin and Gottesman, 2010; Moon *et al.*, 2013; Mika and Hengge, 2014).

Here, we show that these two sRNAs, FnrS and ArcZ, provide missing links between environmental oxygen changes and changes in SPI1 gene expression. FnrS and ArcZ both inhibit *hilD* translation, thus leading to decreased expression of *hilA* and the SPI1 T3SS (Fig 1). We suggest that this dual repression of *hilD* by two sRNAs produced at different oxygen concentrations optimizes production of the SPI1 T3SS to intermediate oxygen tensions. This may be one mechanism by which *Salmonella* cells precisely control the production of the SPI1 T3SS at the right time and place within the host GI tract.

Results

Screening an E. coli *sRNA library for regulators of* hilD *translation initiation*

We previously showed that multiple regulatory signals feed into the SPI1 T3SS system by affecting *hilD* at the post-transcriptional level (Golubeva *et al.*, 2012). We hypothesized that some of this regulation is mediated through sRNAs. To identify direct sRNA regulators of *hilD* translation, we took advantage of an *E. coli* sRNA library that includes key sRNA regulators of cellular physiology (Mandin and Gottesman, 2010), and virulence gene control (Vogel, 2009). The sRNAs included in this library are highly conserved between *E. coli* and *Salmonella.* We created a P_{BAD}-*hilD'-'lacZ* translational fusion in *E. coli* under the control of an arabinose-inducible promoter (Mandin and Gottesman, 2009). The *hilD* translational fusion contains the 35-nt 5' UTR and the first 11 codons of *hilD* fused in-frame to *lacZ* (Fig S1). This is analogous to the in-locus *lacZ* fusion that we have used in *Salmonella*, and our previous studies showed that this region of *hilD* is sufficient for the observed post-transcriptional regulation (Golubeva *et al.*, 2012). Performing our initial screening in *E. coli* reduced the complications imposed by the complex feed-forward loop controlling SPI1 gene expression in *Salmonella* (Ellermeier *et al.*, 2005; Golubeva *et al.*, 2012).

Plasmids encoding one of 23 sRNAs from *E. coli* under control of a *lac* promoter (Mandin and Gottesman, 2009) were each introduced into an *E. coli* P_{BAD}-*hilD'-'lacZ* fusion strain. Strains were cultured in low salt LB (LSLB) with 100 μM IPTG to induce expression of the sRNA and 0.001% arabinose to induce the reporter fusion and β-galactosidase activity was measured. Five of the sRNAs in the library, RydC, GcvB, MicC, ArcZ, and FnrS, caused an ~2-fold or greater decrease in expression of the *hilD'-'lacZ* fusion (Fig S2). Overexpression of either FnrS or ArcZ had the greatest effect and we have focused on these sRNAs.

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Both FnrS (122 nt) and the processed ArcZ (57 nt) are highly conserved between *E.coli* and *Salmonella*, each differing by only 2 nucleotides in the stem-loop of the terminators (Papenfort *et al.*, 2009; Durand and Storz, 2010; Chao *et al.*, 2017). Despite this conservation, we cloned *fnrS* and *arcZ* from *Salmonella* Typhimurium strain 14028 into the pBRpLac. Overexpression of either the *E.coli* or *Salmonella* FnrS and ArcZ sRNAs led to decreased expression of the *hilD'-'lacZ* fusion in *E. coli* (Fig 2A). We then introduced the plasmids producing the *Salmonella* and *E. coli* FnrS and ArcZ sRNAs into a *Salmonella* strain containing the in locus *hilD'-'lacZ* fusion (similar fusion joint but under the *hilD* promoter; Fig S1). Overexpression of each of the sRNAs caused a >2-fold decrease in activity of the reporter fusion (Fig 2B). These data show that FnrS and ArcZ negatively regulate *hilD* translation.

We then tested whether overexpression of FnrS or ArcZ also affected the expression of the SPI1 regulators HilC and RtsA, which act with HilD in the feed-forward loop controlling *hilA* expression. Translational *lacZ* fusions to *hilC* and *rtsA* were created in *E. coli*. Overexpression of neither sRNA affected *hilC* or *rtsA* translation in *E. coli* (Fig S3A and B), suggesting that these sRNAs only regulate *hilD* translation.

To further examine effects of the sRNAs on SPI1 expression, we overexpressed FnrS or ArcZ in a *Salmonella* strain carrying a *hilA'-lacZ*$^{+}$ transcriptional fusion (Fig S1). Both FnrS and ArcZ dramatically repressed *hilA* expression (Fig 2C). This enhanced repression is consistent with a direct regulatory effect of each sRNA on *hilD* translation, which, due to the feed forward regulatory loop (Fig. 1), is expected to decrease expression of *rtsA*, *hilC* and dampen *hilD* autoregulation, all of which would lead to decreased transcription of *hilA*. Importantly, neither FnrS nor ArcZ affect expression of a *hilA'-'lacZ* translational fusion in *E coli* (Fig S3C), showing that these sRNAs do not directly control *hilA* expression. These data suggest that FnrS and ArcZ are direct sRNA regulators of *hilD* translation in *Salmonella.*

FnrS and ArcZ basepair directly with the hilD *mRNA*

We hypothesized that FnrS and ArcZ regulate SPI1 via base pairing interactions with the *hilD* mRNA. Computational prediction suggested that both FnrS and ArcZ bind to the *hilD* mRNA near the ribosome binding site (RBS) (Fig 3A). To test these predictions, we performed mutational analyses using the P_{BAD}-*hilD'-'lacZ* fusion in *E. coli*. FnrS nts 37 to 49 are predicted to basepair with *hilD* mRNA from nts −8 to +5 relative to the initiation AUG (Fig 3A). Consistent with this prediction, a mutant FnrS G47C was not able to regulate wild type *hilD'-'lacZ* (Fig 3B). A compensatory mutation at position −6 in the *hilD* fusion restored the regulation by mutant FnrS (Fig 3B). These genetic data support the model that FnrS requires this base pairing interaction to regulate *hilD* translation.

ArcZ nts 65–76 are predicted to interact with *hilD* nts −5 to −21 relative to the AUG (Fig 3A). Although several single mutations in this region failed to disrupt the interaction, mutating the boxed ArcZ nts disrupted the interaction with *hilD* mRNA and the resulting mutant ArcZ failed to regulate wild-type *hilD* translation (Fig 3C). Introduction of compensatory mutations in the *hilD* fusion restored the regulation by the mutant ArcZ, suggesting that ArcZ requires this base pairing interaction to regulate *hilD* translation.

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Oxygen-dependent regulation of sRNAs

Oxygen is regarded as one of the key regulatory signals for control of the SPI1 system (Ni Bhriain *et al.*, 1989; Lee and Falkow, 1990; Tartera and Metcalf, 1993; Ellermeier and Slauch, 2007; Jennewein *et al.*, 2015). Since FnrS and ArcZ are known to be differentially produced in response to oxygen availability (Papenfort *et al.*, 2009; Boysen *et al.*, 2010; Durand and Storz, 2010; Mandin and Gottesman, 2010), we hypothesized that these sRNAs provide one link between changes in environmental oxygen concentrations and control of SPI1 through regulation of *hilD* translation. To test this, we created *fnrS* or *arcZ* deletion mutations in both the *hilD'-'lacZ* translational and the *hilA'-lacZ*$^{+}$ transcriptional fusion backgrounds, and measured β-galactosidase activity under high-aeration and low-aeration conditions. We observed higher activity from both the *hilD'-'lacZ* and *hilA'-lacZ*$^{+}$ fusions under low aeration conditions (Fig 4), in agreement with previous data (Ellermeier *et al.*, 2005). Deletion of *fnrS* led to increased *hilD* translation when strains were grown in low aeration (Fig 4A); the effect on *hilA* transcription was slightly greater than that on *hilD* (Fig 4B). However, in high aeration, wildtype and *fnrS* mutants showed similar levels of activity for both the *hilD'-'lacZ* and *hilA'-lacZ*$^{+}$ fusions (Fig 4). In contrast, deletion of *arcZ* caused increased levels of *hilD* translation only when the strains were incubated under high aeration conditions, with a concomitant increase in *hilA* expression (Fig 4). There was no phenotype conferred by the *arcZ* deletion in low aeration (Fig 4). These data show that both FnrS and ArcZ affect *hilD* translation under conditions known to induce their expression, high aeration for ArcZ and low aeration for FnrS. The phenotypes are consistent with a model in which these sRNAs act to optimize *hilD* translation, and thus SPI1 expression, in response to some intermediate oxygen level.

Mechanism of FnrS or ArcZ mediated regulation of hilD *translation in* Salmonella

sRNA-mRNA base pairing interactions can induce mRNA degradation by RNase E (Masse *et al.*, 2003; Prevost *et al.*, 2011; Lalaouna *et al.*, 2013). To determine whether FnrS or ArcZ promote *hilD* mRNA turnover via RNase E, we created an *rne131* mutation in *Salmonella* (Vanzo *et al.*, 1998; Lopez *et al.*, 1999; Viegas *et al.*, 2007). The *rne131* mutation truncates RNase E, maintaining enzymatic activity, but preventing assembly of the degradosome, which in many cases also eliminates sRNA-dependent mRNA turnover (Lopez *et al.*, 1999; Masse *et al.*, 2003; Rice and Vanderpool, 2011). RNaseE enzymatic activity is required for processing of the ArcZ sRNA (Chao *et al.*, 2017). Significant levels of processed ArcZ were present in the mutant background (Fig S4). The *rne131* mutation caused a moderate increase in the basal level of *hilD'-'lacZ* fusion activity in *Salmonella*. However, both FnrS and ArcZ still repressed *hilD* translation in the *rne131* mutant background (Fig 5). These data support the idea that both FnrS and ArcZ directly block translation of the *hilD* mRNA and that repression does not require mRNA degradation.

To further examine the mechanisms of regulation, we determined the *hilD* mRNA half-life in wild type, *arcZ*, *fnrS*, or *rne131* mutant backgrounds under high aeration and low aeration conditions. We monitored expression of the *hilD'-'lacZ* fusion indicating that the mutations conferred the expected phenotype under the exact conditions in which we isolated RNA (High aeration: WT, 50±4; *arcZ*, 69±4; Low aeration: WT, 57±3; *fnrS*, 94±8 β-gal units). The *hilD* mRNA had a half-life of approximately 1.8 min under low aeration conditions (Fig

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6). Interestingly, the half-life decreased to 0.85 min under high aeration conditions, suggesting that control of mRNA degradation is one mechanism of regulation in response to oxygen. However, the half-life was essentially unaffected by loss of either ArcZ or FnrS (Fig 6), consistent with interpretation above that these sRNAs act by directly blocking translation rather than inducing degradation of the message. The half-life of the *hilD* message was significantly increased in the *rne131* background, showing the involvement of the degradosome in overall mRNA stability (Fig 6).

FnrS acts independently of Fur to regulate SPI1 expression

Anaerobic activation of *fnrS* is mainly dependent on Fnr, but is also affected by ArcA and CRP (Durand and Storz, 2010). It has been reported that Fur, the ferric uptake regulator, also affects *fnrS* expression (Colgan *et al.*, 2016). Fur is known to positively regulate HilD expression. This regulation requires both the *hilD* promoter and HilD protein and is likely the result of Fur affecting H-NS dependent repression of *hilD* transcription (Lavrrar *et al.*, 2002; Olekhnovich and Kadner, 2006; Olekhnovich and Kadner, 2007; Ellermeier and Slauch, 2008; Teixido *et al.*, 2011; Troxell *et al.*, 2011). To better understand the relationship between these global regulators and the potential link to SPI1 gene expression via FnrS, we tested whether the expression of *fnrS* depends on Fnr or Fur in response to oxygen concentration and iron availability in *Salmonella*. We created a transcriptional fusion to *fnrS*, and deleted either *fnr* or *fur*. All strains were tested under high and low aeration and in the presence or absence of dipyridyl, an iron chelator that induces iron starvation and causes Fur derepression of target genes (Ikeda *et al.*, 2005; Ellermeier and Slauch, 2008). Under high aeration conditions, transcription of *fnrS* was very low (Fig 7A, note scale). Expression of *fnrS* was strongly induced under low aeration conditions (Fig 7B), and this activation was predominantly dependent on Fnr. Neither addition of dipyridyl nor deletion of *fur* had a substantial impact on *fnrS* transcription (Fig 7B). These data suggest that the production of FnrS is oxygen-dependent and controlled primarily by Fnr in *Salmonella* under our experimental conditions.

We then examined the regulation of *hilA* by FnrS and Fur. We constructed *fnrS* and *fur* mutations in *Salmonella* strains with a *hilA'-lacZ*$^{+}$ transcriptional fusion. For a control, we examined expression of a *sodB'-'lacZ* translational fusion. Fur regulates *sodB* indirectly via repressing transcription of the two paralogous sRNAs, RyhB-1 and RyhB-2 in *Salmonella* (Troxell *et al.*, 2011). FnrS also regulates *sodB* in *E. coli* (Durand and Storz, 2010). When we measured β-galactosidase activity of these fusions in SPI1-inducing conditions, the *sodB* fusion behaved as expected; *sodB* is independently regulated by Fur and FnrS (Fig 7D). Likewise, we observed increased levels of *hilA'-lacZ*$^{+}$ in the *fnrS* mutant compared to wild-type. Deletion of *fur* in these strains reduced the expression of the *hilA'-lacZ*$^{+}$ fusion. However, the effect of *fnrS* was still observed in the *fur* background (Fig 7C). These data suggest that the regulation of *hilA* by FnrS is independent of the regulation by Fur.

Fnr regulates SPI1 expression independent of FnrS

We have previously shown that Fnr represses *hilA* expression independent of HilD (Golubeva *et al.*, 2012), in seeming contrast to repression of *hilD* translation by FnrS. This suggests that Fnr controls expression of SPI1 via more than one mechanism. To distinguish

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between these mechanisms, we determined the activity of the *hilA'-lacZ*$^{+}$ transcriptional fusion in strains containing *fnr* and *fnrS* deletions. As shown in figure 8A, deletion of *fnrS* increased *hilA* transcription only in the *fnr*$^{+}$ background. This is as expected, given that FnrS expression is dependent on Fnr. However, deletion of *fnr* increased *hilA-lacZ* transcription in both *fnrS*$^{+}$ and *fnrS*$^{-}$ backgrounds. This suggests that Fnr has an additional FnrS-independent regulatory effect on *hilA* transcription.

Expression of *hilA* in all of these backgrounds was dependent on HilD, making it impossible to distinguish how Fnr and FnrS feed into the regulatory circuit. To separate the effects of FnrS on *hilD* expression, we placed *rtsA* under control of a tetracycline inducible promoter. In this background, we can activate expression of *hilA* in the absence of HilD. As shown in figure 8B, adding 50 ng anhydrotetracycline in this background restored *hilA* expression to approximately wild type levels in the absence of HilD. Deletion of *fnr* in this background increased *hilA* expression, showing that this effect is independent of HilD. Deletion of *fnrS* in these strains had no significant effect, proving that the sRNA acts solely by controlling *hilD* translation. These data are consistent with a model in which Fnr controls *hilA* via both FnrS (translation of *hilD*) and some mechanism that affects *hilA* transcription independent of HilD.

The ArcAB two-component system feeds into the system via multiple pathways

The expression of ArcZ is repressed by ArcA under anaerobic conditions (Mandin and Gottesman, 2010). Inhibition of ArcAB by the oxidized quinone pool in the respiratory chain leads to derepression of *arcZ* under aerobic growth conditions (Georgellis *et al.*, 2001; Mandin and Gottesman, 2010). In a recent study, ArcA was reported to have a regulatory effect on *hilA* expression (Lim *et al.*, 2013). We tested how ArcAB and ArcZ each feed into the SPI1 regulatory circuit. We deleted *arcZ* and/or *arcA* in either *hilD'-'lacZ* or *hilA'-lacZ*$^{+}$ backgrounds and performed β-galactosidase assays under high aeration conditions. The data (Fig 9A) indicate that, as above, deletion of *arcZ* increased *hilD'-'lacZ* expression. In contrast, deletion of *arcA* decreased expression of the *hilD'-'lacZ* fusion. Further deletion of *arcZ* in the *arcA* background led to the expected increase in *hilD'-'lacZ* expression. We also observed a similar decrease of a *hilD'-lacZ*$^{+}$ transcriptional fusion in absence of ArcA (Fig S5), showing that this effect is at the transcriptional level. (Note that these fusion strains are *hilD* null, negating any effects of autoregulation.) These data suggest that ArcAB positively controls *hilD* via two separate pathways: repressing *arcZ* expression and activating *hilD* transcription by some unknown mechanism. These effects on *hilD* are reflected in *hilA* expression, which mirrors expression of HilD. Both effects are HilD dependent as reflected by the absence of any significant phenotype in a *hilD* null strain where *hilA* is activated by RtsA (Fig 9C).

The ArcAB system was reported to control *hilD* transcription through activation of LoiA, a LysR type regulator encoded in SPI14 that presumably binds directly to the *hilD* promoter (Jiang *et al.*, 2017). To test this possibility, we deleted *loiA* or the entire SPI14 island in both *hilD'-lacZ*$^{+}$ and *hilA'-lacZ*$^{+}$ fusion strains. We confirmed these deletions by PCR analysis and performed β-galactosidase assays under both low and high aeration conditions. We

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observed no effect on either *hilD'-lacZ*$^{+}$ or *hilA'-lacZ*$^{+}$ expression (Fig S6). Therefore, we conclude that phenotypes that we observe are independent of LoiA.

Impact of sRNA regulation of SPI1 in mouse models of infection

Both FnrS and ArcZ repress translation of *hilD*, resulting in decreased *hilA* expression under low or high aeration conditions. We suppose that this regulation defines an oxygen "window" for optimal activation of SPI1. Mouse competition assays were used to ask if this regulation is relevant in the animal model of infection. We created a mutant deleted for both *fnrS* and *arcZ* in a *hilA'-lacZ*$^{+}$ background. (The fusion has no significant effect on virulence, Table S3, and was present in both strain backgrounds.) Before testing the strain in mice, we measured the phenotype conferred by the double deletion in low aeration and high aeration conditions. Compared to wild type and individual deletion mutants, the double deletion of both sRNAs increased *hilA* expression independent of aeration levels (Fig 10).

To address the contribution of the FnrS- and ArcZ-mediated regulation to virulence in the host, we used oral mouse competition assays (dependent on the SPI1 T3SS), and intraperitoneal (IP) infection (bypassing the need for SPI1). Streptomycin treatment of mice leads to availability of anaerobic terminal electron acceptors and as well as oxygen (Clark and Barrett, 1987; Stecher and Hardt, 2008; Winter *et al.*, 2010; Thiennimitr *et al.*, 2011; Lopez *et al.*, 2012; Winter *et al.*, 2013; Faber *et al.*, 2017). Given the role of these sRNAs in regulating aspects of respiration (Papenfort *et al.*, 2009; Durand and Storz, 2010; Mandin and Gottesman, 2010), we tested the effects of removing FnrS and ArcZ in in normal (Strep −) or streptomycin-treated (Strep+) mice. To determine whether any observed effects were due to changes in SPI1 expression, we also performed competition assays in a *spi1* null background (Δ*spi1* Δ*rtsA*).

There was marked variability in the competitive indices between individual mice, making it difficult to make significant conclusions. However, there was a trend that was particularly evident in the normal (Strep−) mice. Given the fact that SPI1 is not required systemically after invasion, the ratio of strains in the spleen after oral infection reflects the ratio of invasion (Ellermeier *et al.*, 2005). The *fnrS arcZ* double mutant competed equally with the wild-type strain in both oral and IP infections (Fig 11). In contrast, in the Δ*spi1* mutant background, *fnrS arcZ* mutants were at a competitive disadvantage compared to the *fnrS*$^{+}$*arcZ*$^{+}$ strain in orally infected mice. We interpret these data to suggest that, whereas loss of FnrS and ArcZ confers a SPI1-independent competitive disadvantage, the increased expression of SPI1 in the sRNA mutant leads to an overall increase in invasion. These phenotypes are largely negated in Strep+ mice, consistent with significant changes in the intestinal environment. Interestingly, deletion of each of the sRNAs alone did not confer a phenotype suggesting, perhaps, that it is the requirement to adapt to subtly different oxygen concentrations that is critical. This could also explain the large variability in individual mice, possibly reflecting variations in oxygen concentrations in different niches in the intestine.

Discussion

Multiple environmental signals influence expression of the SPI1 T3SS and the integration of these signals presumably allows the cell to determine the appropriate time and place to

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produce the invasion machinery. Our long-term goal is to understand the relative impact of these various environmental factors and how these signals are mechanistically integrated into the SPI1 regulatory circuit (Ellermeier and Slauch, 2007; Saini *et al.*, 2010; Golubeva *et al.*, 2012). Much of the regulatory input is integrated at HilD, affecting either the translation or activity of the HilD protein (Saini *et al.*, 2010; Golubeva *et al.*, 2012). Consistent with this dominant role, we identified two sRNAs, FnrS and ArcZ, that directly affect SPI1 T3SS activation via regulating *hilD* translation. Both FnrS and ArcZ repress *hilD* translation resulting in significant inhibition of *hilA* transcription (Fig 1). These sRNAs have no direct effects on *hilC*, *rtsA,* or *hilA* translation. Bioinformatic prediction and genetic analyses proved that both FnrS and ArcZ sRNAs basepair near the ribosome binding site of the *hilD* message (Fig 4) leading to direct translational inhibition; the RNase E degradosome was not required for *hilD* translational regulation by these sRNAs. The *hilD* mRNA also has an unusually long ~300 nt 3' UTR, which apparently affects the stability of the message. Several mechanisms of regulation at the 3' UTR have been characterized (Lopez-Garrido *et al.*, 2014; Gaviria-Cantin *et al.*, 2017; El Mouali *et al.*, 2018).

Oxygen and osmolarity have been regarded as key environmental signals that control SPI1 T3SS activation, but it is clear that oxygen concentrations impact the SPI1 regulatory circuit through several mechanisms. For example, we have previously shown that the *rtsA, hilC,* and *hilD* promoters each respond to oxygen levels in the absence of all known regulators (Ellermeier *et al.*, 2005). Our results here show that the half-life of the *hilD* mRNA is decreased under high aeration by an unknown mechanism (Fig 6). It is also clear from our results that Fnr and ArcAB affect SPI1 expression in complex ways that are independent of the sRNAs (see below). Available iron, the levels of which are significantly affected by oxygen (Ikeda *et al.*, 2005) affect SPI1 expression via Fur (Ellermeier and Slauch, 2008). Further investigations are required to completely understand how oxygen influences SPI1 gene expression.

Both FnrS and ArcZ repress *hilD* translation and the their production is differentially controlled by oxygen levels. We propose that these sRNAs tune expression of SPI1 to be maximally produced at some optimal intermediate oxygen concentration. Studies in *E. coli* suggest that Fnr is active at O_2 concentrations below ~20 μM, whereas the ArcA shifts toward the dephosphorylated state at about this same concentration or above (Tseng *et al.*, 1996). The intestine has both longitudinal and radial gradients of oxygen. Recent studies show that the oxygen concentration in the lumen of conventional mice drops from >50 μM in the duodenum to ~9 μM in the terminal ileum (Friedman *et al.*, 2018). But there is also a considerable radial gradient. The midpoint of the lumen is the most anoxic, with any available oxygen being quickly reduced by resident bacteria or other chemical reactions (Friedman *et al.*, 2018). The concentration rises steeply below the mucus and at the surface of the intestinal epithelial cells. Interestingly, data suggest that the oxygen concentration in this region is on the order of 20 μM (Espey, 2013; Albenberg *et al.*, 2014), the concentration at which neither FnrS nor ArcZ should be produced.

There are also chemical gradients along the intestine that act as signals to optimize SPI1 expression. For example, we and others have provided data showing that short and long chain fatty acids control SPI1 gene expression. Diet-derived long chain fatty acids negatively

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regulate expression by directly binding to HilD to prevent DNA binding (Golubeva *et al.*, 2016). Presumably, these fatty acids are absorbed along the small intestine, being at their lowest concentration in the distal small intestine. Both acetate and proprionate activate SPI1 gene expression (Lawhon *et al.*, 2002; Hung *et al.*, 2013). These short chain fatty acids are at their highest concentration in the distal small intestine (Argenzio *et al.*, 1974; Cummings *et al.*, 1987; Macfarlane *et al.*, 1992). In contrast, butyrate, produced by the strict anaerobes in the colon, negatively regulates SPI1 expression (Gantois *et al.*, 2006; Bronner *et al.*, 2018; Gillis *et al.*, 2018). We believe that these concentration gradients are such that SPI1 is optimally expressed in the distal small intestine.

Pretreatment of mice with oral streptomycin and subsequent infection with *Salmonella* leads to SPI1-mediated induction of inflammatory diarrhea (Que and Hentges, 1985; Barthel *et al.*, 2003). *Salmonella* benefits from the inflammatory response by taking advantage of newly available carbon sources and terminal electron acceptors, including tetrathionate and nitrate, thereby out-competing fermenting bacteria (Clark and Barrett, 1987; Stecher and Hardt, 2008; Winter *et al.*, 2010; Thiennimitr *et al.*, 2011; Lopez *et al.*, 2012; Winter *et al.*, 2013; Faber *et al.*, 2017). FnrS and ArcZ both affect expression of numerous genes, including those encoding central metabolic and respiratory enzymes (Papenfort *et al.*, 2009; Durand and Storz, 2010; Mandin and Gottesman, 2010). Therefore, it is not surprising that there were significant differences observed between normal and streptomycin-treated mice infected with the *fnrS arcZ* double mutant. To separate the pleiotropic effects of the sRNAs from their specific role in SPI1 regulation, we tested loss of the sRNAs in a *spi1* mutant background. Although normally such a test is straightforward, in this case interpretation is complicated by the fact that the inflammatory response in the streptomycin-treated mice is largely SPI1 dependent (Que and Hentges, 1985; Barthel *et al.*, 2003).

FnrS is anaerobically induced by the major anaerobic transcriptional regulator, Fnr (Boysen *et al.*, 2010; Durand and Storz, 2010). However, it was reported that Fur also activates the expression of FnrS in exponential growth (Colgan *et al.*, 2016). Because oxygen affects iron availability, Fur-mediated regulation is oxygen dependent (Kehres *et al.*, 2002). However, under the conditions that we tested, FnrS expression is dominantly regulated by environmental oxygen and Fnr. Iron depletion or deletion of *fur* did not affect the FnrS expression (Fig 6-C).

In addition to activating *fnrS* expression leading to translational inhibition of *hilD*, Fnr decreases *hilA* transcription independently of FnrS and HilD (Fig 7). Of the two effects, FnrS plays a slightly greater role. We could not identify any obvious Fnr binding sites in the *hilA* promoter, suggesting that this regulation may be indirect. Such indirect effects would not be surprising given the response of the SPI1 system to numerous physiological factors and the pleiotropic role of Fnr in central metabolism (Fink *et al.*, 2007; Golubeva *et al.*, 2012). But direct or indirect, Fnr induction in low oxygen leads to a decrease in *hilA* transcription and hence SPI1 expression through both HilD-dependent and -independent mechanisms.

Like Fnr, the ArcAB two-component system regulates SPI1 through multiple mechanisms. Above 20 μM, ArcB begins to shift ArcA to the de-phosphorylated state (Tseng *et al.*, 1996).

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But this is not a sharp cutoff and ArcA-P levels are significant under a wide range of oxygen concentrations (Rolfe *et al.*, 2011). Different genes will require varying levels of ArcA-P to be regulated. Deletion of *arcA* leads to decreased *hilD* transcription independent of ArcZ, suggesting that there is still significant ArcA-P under our aerobic conditions, even though *arcZ* is not being significantly repressed. As with Fnr, this is almost certainly an indirect effect on *hilD* transcription. It was recently proposed that the transcriptional regulator LoiA is transcriptionally repressed by ArcA in low oxygen and that LoiA directly activates *hilD* transcription (Jiang *et al.*, 2017). However, in our hands, loss of LoiA had no effect on *hilD* or *hilA* expression under either low- or high-aeration conditions (Fig S3). Therefore, ArcA is controlling *hilD* transcription by an unknown mechanism. It is also interesting that the two effects are seemingly in opposition.

FnrS and ArcZ play an important role in the regulation of SPI1 in response to oxygen, adding to our overall understanding of the mechanisms by which environmental signals relevant in the intestine feed into the regulatory network. Although oxygen levels have long been considered an important parameter controlling SPI1, the system responds to oxygen via multiple pathways and more studies will be required to fully understand how these various mechanisms are integrated.

Experimental procedures

Strain construction

Bacterial strains and plasmids are described in Table S1. All *Salmonella enterica* serovar Typhimurium strains created for this study are isogenic derivatives of strain 14028 [American Type Culture Collection (ATCC)] and were constructed using P22 HT105/1 *int*-201 (P22)-mediated transduction (Maloy *et al.*, 1996). Deletion of various genes and concomitant insertion of an antibiotic resistance cassette was carried out using lambda Red-mediated recombination (Datsenko and Wanner, 2000; Yu *et al.*, 2000; Ellermeier *et al.*, 2002). The end-points of each deletion are indicated in Table S1. In all cases, the appropriate insertion of the antibiotic resistance marker was confirmed by polymerase chain reaction analysis. In each case, the constructs resulting from this procedure were moved into an unmutagenized background by P22 transduction. When appropriate, antibiotic resistance cassettes were removed using the temperature sensitive plasmid pCP20 carrying the FLP recombinase (Cherepanov and Wackernagel, 1995). To create transcriptional *lacZ* fusions of FnrS or ArcZ, the insertion mutations in FnrS and ArcZ were converted to transcriptional *lac* + fusions using an FLP/FRT-mediated site-specific recombination method as previously described (Ellermeier *et al.*, 2002).

Construction of translational lacZ *reporter fusions in* E. coli

The translational *lacZ* reporter fusions were constructed using lambda Red recombination in the *E. coli* strain PM1205 as described previously (Mandin and Gottesman, 2009). All fusions are under P_{BAD} control. The 5' UTR and early coding regions of *hilD, hilC, rtsA,* or *hilA* were fused in-frame to *lacZ* to create translational fusions (Fig S1). The corresponding DNA fragments were amplified from purified genomic DNA of *Salmonella* using the primers in Table S2 with homology to the P_{BAD} promoter or to *lacZ*. The PCR fragments

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were purified using a PCR purification Kit (Qiagen) and competent cells were prepared as described (Mandin and Gottesman, 2009). Recombinants were selected on sucrose minimal plates (M63 salts, 0.2% glycerol, 5% sucrose) containing 40 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). To create fusions with mutations in the *hilD* 5' UTR, the nucleotide changes were encoded in the amplifying primers (Table S2).

Media, reagents, enzymatic assays, and growth conditions

Lysogeny Broth medium containing 10 g tryptone, 5 g yeast extract, and 0 g NaCl per liter (designated no salt LB, NSLB), 5 g NaCl per liter (low salt LB, LSLB) or 10 g NaCl per liter (high salt LB, HSLB) were used as indicated. Superoptimal broth with catabolite repression (SOC) was used for the recovery of transformants (Maloy *et al.*, 1996). Bacterial strains were normally grown at 37°C except for the strains containing the temperature sensitive plasmids, pCP20 or pKD46, which were grown at 30°C. When required, antibiotics were used at the following concentrations: 100 μg ml^{-1} ampicillin (Ap), 20 μg ml^{-1} chloramphenicol (Cm), 50 μg ml^{-1} kanamycin (Kn), 25 μg ml^{-1} tetracycline (Tet), and 50 μg ml^{-1} apramycin (Apr). Primers were purchased from IDT. Enzymes were purchased from New England Biolabs or Invitrogen.

β-Galactosidase assays were performed using a microtiter plate assay as previously described on strains grown under the indicated conditions (Slauch and Silhavy, 1991). β-Galactosidase activity units are defined as (mmol of ortho-nitrophenyl-β-galactoside formed min-1) x106/(OD600 X ml of cell suspension) and are reported as mean ± standard deviation where $n = 4$. Cultures used to measure β-galactosidase activity in *Salmonella* were initially inoculated into NSLB and grown overnight, then subcultured 1/100 and grown under one of the following conditions: (i) statically overnight in 3 ml of HSLB in a 13 × 100 mm tube, referred to as either Low Aeration or SPI1 inducing; (ii) on a platform shaker at 225 r.p.m. in 4 ml of HSLB in a 125 ml baffled flask to an OD600 of 0.8, referred to as High Aeration. Cultures used to measure β-galactosidase activity in *E. coli* were initially inoculated into LSLB, grown overnight, and subcultured 1/100 into 2 ml of LSLB with 100 μM IPTG and 0.001% Arabinose in a 13 × 100 mm tube and incubated on a roller drum to an OD_{600} of 0.5.

Plasmid construction and site-directed mutagenesis of sRNA constructs

The pFnrS and pArcZ plasmids were constructed by PCR amplifying *fnrS* or *arcZ* from strain 14028 using primers F-AatII-FnrS and R-EcoRI-FnrS or F-AatII-ArcZ and R-EcoRI-ArcZ, respectively (Table S2). The PCR products were subsequently cloned into the pBR-plac vector after digestion with AatII and EcoRI (Mandin and Gottesman, 2009).

Various bioinformatics tools (Zuker, 2003; Kruger and Rehmsmeier, 2006; Busch *et al.*, 2008) were used to predict the region of FnrS or ArcZ that base pairs with the *hilD* mRNA. The Quick Change Lightening Site Directed Mutagenesis Kit (Stratagene) was used to create the corresponding mutant constructs with the primers listed in Table S2.

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Northern analysis

High aeration cultures were grown as described above. For low aeration conditions, overnight NSLB cultures were subcultured 1/100 into 16 ml of HSLB in a 20 × 150 mm tube and incubated for 3 hours on a platform shaker at 225 rpm. For the measurement of the half-life of *hilD* mRNA, 500 μg of rifampicin was added into the bacterial cultures (Holmqvist *et al.*, 2018). Immediately upon addition of rifampicin (time 0) and at 1, 2, 4, 8 and 16 minutes after the treatment, 800 μl aliquots of bacterial cells were collected. Bacterial cells were immediately suspended in 915 μl of 65°C phenol solution (15–594-047, Invitrogen) with 120 μL of Lysis buffer (0.3M NaOAc (pH 5.2), 8% SDS, and 0.02 M EDTA (pH 8.0)) and then incubated at 65°C for 10 minutes while shaking (Ares, 2012). After centrifugation at 13,000 rpm for 10 min, the aqueous fraction was collected and added to 500 μl of a phenol: chloroform: isoamylalchol (P:C:I, 25:24:1) solution (pH 6.6, AM9732, Invitrogen) for further purification. After centrifugation, the subsequent aqueous portion were transferred into 1.3 ml of ice-chilled ethanol and incubated at −80°C for at least 2 hours. After centrifugation at 13,000 rpm for 10 min, the supernatant was carefully removed and the pellets were washed with 1 ml 70% ethanol. The RNA pellets were allowed to air-dry and suspended in 25 μl DEPC-treated H_2O.

For each sample, 20 μg total RNA was denatured in 3X volume of Formaldehyde Loading Dye (AM8552, Ambion) at 95°C for 3 min, and separated on a 1.2% agarose gel with 1X MOPS buffer and 7% formaldehyde for 1 h at 85 V. RNA was transferred to a BrightStar™-Plus Positively Charged Nylon Membrane (AM10104, Ambion) by capillary transfer with Northern MAX transfer buffer (AM8672, Ambion)(2005). To probe *hilD* mRNA, a radiolabeled random-primed probe was generated from 25 ng of PCR fragment corresponding to the *hilD* orf following the manufacturer's instructions (18187–013, Life Technologies).

To examine ArcZ processing, we used aliquots from the 0 time point wild type and *rne131* samples grown under high aeration. Total RNA (20 μg) was denatured in 1× RNA loading buffer II (AM8546G, Ambion) at 95°C for 3 min, and separated by 6% polyacrylamide gel with 7 M urea for 2 h at 300 V (Chao *et al.*, 2017). RNA was transferred to BrightStar™-Plus Positively Charged Nylon Membrane (AM10104, Ambion) by electro- blotting (1 h, 50 V, 4°C) in 1× TBE buffer. To produce a radiolabeled oligo probe antisense to ArcZ , 10 pmol of ArcZ oligonucleotide was incubated with 25 μCi of [$\gamma-^{32}$P]-ATP and 1 U T4 polynucleotide kinase (M0236S, NEB) at 37°C for 1 hour.

After crosslinking by 0.12 J/cm2 UV light, the membranes were hybridized with radiolabeled DNA probes at 42°C overnight in ULTRAhyb hybridization buffer (AM8670, Invitrogen). In all cases, as a loading control, 5S RNA was detected by radio-labeled oligo probe, synthesized as for the ArcZ probe. Signal was visualized on a phosphorimager (Fuji FLA-3000) and quantified using the Image Quant image analyzer (ImageGauge V4.22). Decay curves corresponding to rifampicin chase experiments were generated by using GraphPad Prism version 8.0 (Sinha *et al.*, 2018).

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Mouse and in vitro competition assays

Bacteria were initially inoculated into 2 ml LSLB, grown overnight, then subcultured 1/35 in 4 ml HSLB (1% NaCl) in 125 mL flasks and grown for 4 h with aeration at 200 rpm. BALB/c mice (Harlan) (10 to 13 weeks old) were inoculated either orally or intraperitoneally (i.p.) with 0.2 ml of a bacterial suspension. For oral infections of normal mice, the bacteria were washed and suspended at 5×10^8 (wt background) or 10^9 (*spi1* background) cells per 0.2 ml in sterile 0.1 M sodium phosphate buffer, pH 8.0. Before infection, food and water were withheld for 4 hours and mice were orally inoculated with the indicated number of bacteria, after which the food and water were provided immediately. For oral infections of streptomycin-treated mice, the bacteria were washed and suspended at 5×10^7 (wt background) or 5×10^8 (*spi1* background) cells per 0.2 ml in sterile 0.1 M sodium phosphate buffer, pH 8.0. For streptomycin treatment, food and water were withheld for 4 hours; then mice were treated with 20 mg of streptomycin delivered intragastrically, after which the food and water were provided immediately. At 20 hours after the streptomycin treatment, food and water were withheld for 4 hours and mice were orally inoculated with the indicated number of bacteria, after which the water was provided immediately and food was provided at 2 hours post infection. For intraperitoneal infections, the cells were diluted to 10^3 cells per 0.2 ml in sterile PBS. For oral infections, mice were sacrificed by CO_2 asphyxiation at 3.5 days after inoculation and the spleens, small intestines, and large intestines were harvested. For i.p. infections, the mice were sacrificed by CO_2 asphyxiation between 4 and 5 days after inoculation and spleens were harvested. These organs were homogenized, and serial dilutions of the homogenates were plated on the appropriate medium to determine the number of CFU per organ. The relative percentage of each strain recovered was determined by replica plating to the appropriate antibiotic-containing medium. In all competition assays, the inoculum consisted of a 1:1 mix of two bacterial strains. The actual CFU and relative percentage represented by each strain was determined by direct plating of the inoculum. The competitive index (CI) was calculated as (percentage of strain A recovered/percentage of strain B recovered)/(percentage of strain A inoculated/percentage of strain B inoculated). All strains were independently reconstructed and the competition assays were repeated to ensure that any phenotypes were the result of the designated mutations. To measure competitive growth in vitro, strains were initially inoculated into 2 ml LSLB, grown overnight, then mixed 1:1, diluted and inoculated into indicated medium/condition at 10^3 bacteria per tube/flask, then grown for 16h in indicated conditions. In all cases, the Student *t* test was used to determine whether the output ratio was significantly different from the input ratio or to compare groups of mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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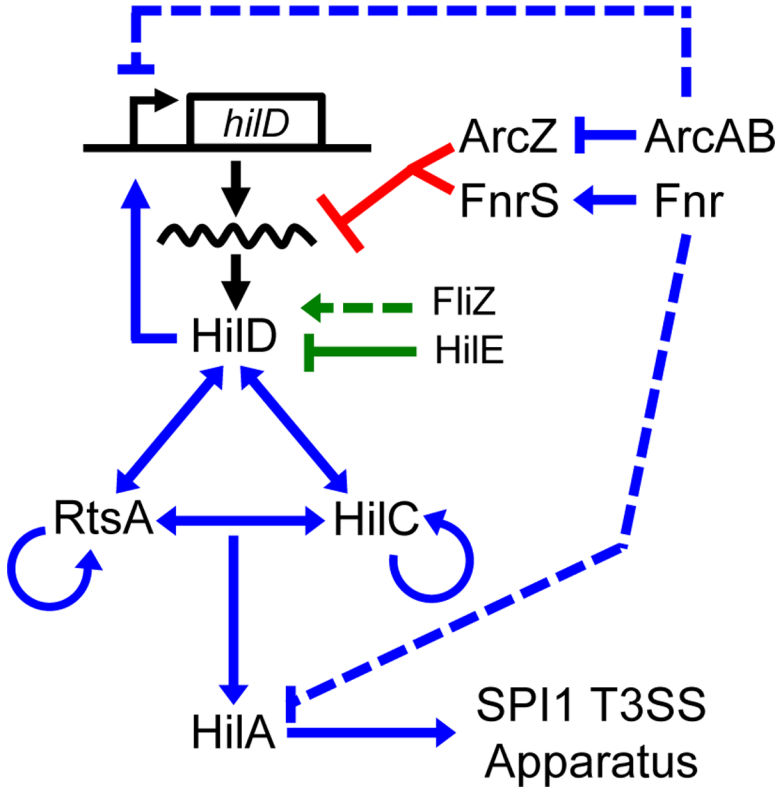

Figure 1. Simplified model of the SPI1 T3SS regulatory circuit.

Blue lines indicate transcriptional regulation, green lines indicate regulation of HilD at the protein level, and red lines indicate regulation of *hilD* translation. Dotted lines indicate that the exact mechanism of regulation is not known and is likely indirect.

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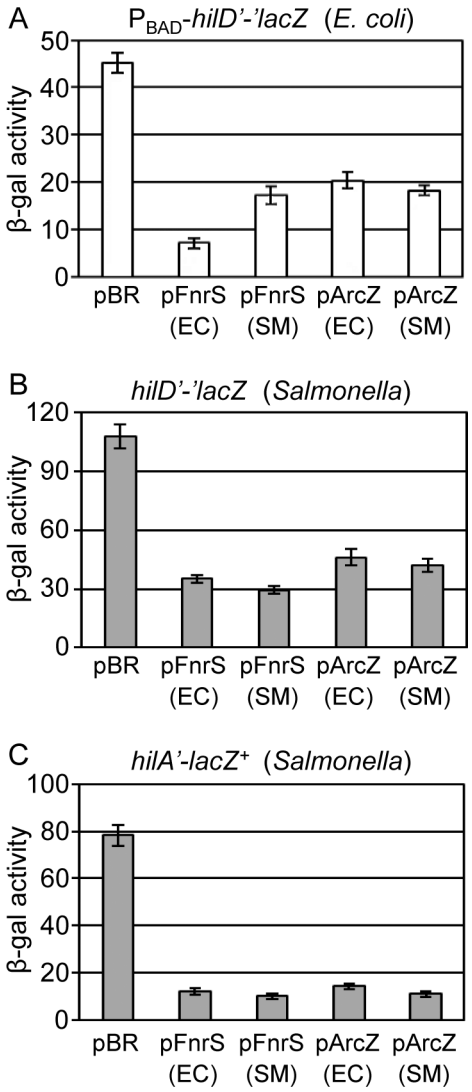

Figure 2. FnrS and ArcZ downregulate SPI1 expression by repressing *hilD* translation.

(A) β-galactosidase activity in *E. coli* strains containing the *hilD'-'lacZ* translational fusion and plasmids overexpressing FnrS or ArcZ from either *E. coli* (EC) and *Salmonella* (SM) grown in the presence of 100 μM IPTG and 0.001% arabinose to induce the sRNA expression and the fusion *lacZ* protein expression, respectively. β-galactosidase activity in *Salmonella* strains containing (B) a *hilD'-'lacZ* translational fusion, or (C) a *hilA'-lacZ*$^{+}$ transcriptional fusion and plasmids overexpressing FnrS or ArcZ from either *E. coli* (EC) or *Salmonella* (SM) grown in SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^{6}/(OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JMS6500, JS892, or JS749, each with plasmid pBRplac, pFnrS-EC, pFnrS-SM, pArcZ-EC, or pArcZ-SM.

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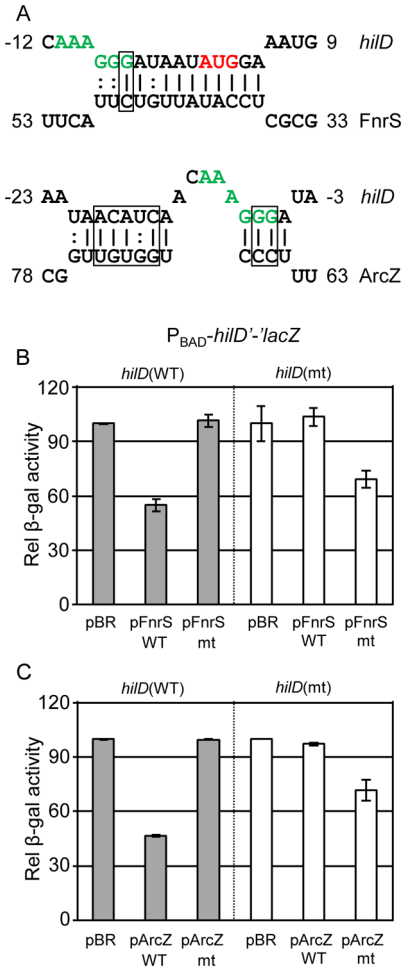

Figure 3. FnrS and ArcZ regulate *hilD* translation by direct base-pairing interactions.

(A) Predicted base-pairing interactions between each sRNA and *hilD* mRNA. For *hilD*, nucleotides are numbered from the translational start site. The ribosome binding site of *hilD* mRNA is highlighted in green, and the translational start site of *hilD* is highlighted in red. Boxes mark nucleotides for which complementary mutations were created in *hilD* and each sRNA. (B) Relative β-galactosidase activity in *E. coli* strains containing the wild type or mutated *hilD'-'lacZ* translational fusion and plasmids overexpressing either the wild type (pFnrS) or mutated (pFnrS mt) sRNA grown as indicated in Figure 2A. (C) Relative β-galactosidase activity in *E. coli* strains containing the wild type or mutated *hilD'-'lacZ* translational fusion and plasmids overexpressing either the wild type (pArcZ) or mutated (pArcZ mt) sRNA grown as indicated in Figure 2A. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^6/(OD_{600} × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JMS6500, JMS6501, and JMS6502 with plasmid pBRplac, pFnrS-SM, pFnrS-mt, pArcZ-SM, or pArcZ-mt.

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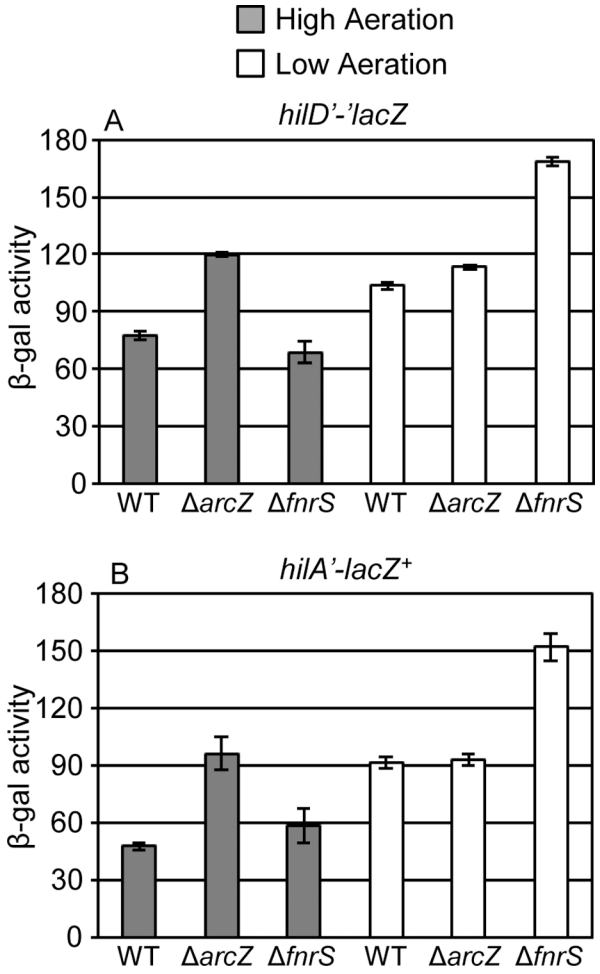

Figure 4. Loss of FnrS or ArcZ results in the increased level of *hilA* expression due to the abolished repression of *hilD* translation.

β-galactosidase activity in *Salmonella* strains containing a (A) *hilD'-'lacZ* translational or (B) *hilA'-lacZ*$^{+}$ transcriptional fusion in the wildtype, Δ*arcZ*, or Δ*fnrS* background grown under either high aeration or low aeration conditions as described in Experimental procedures. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^{6}/(OD_{600} × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS892, JS2123, JS2124, JS749, JS2125 and JS2126.

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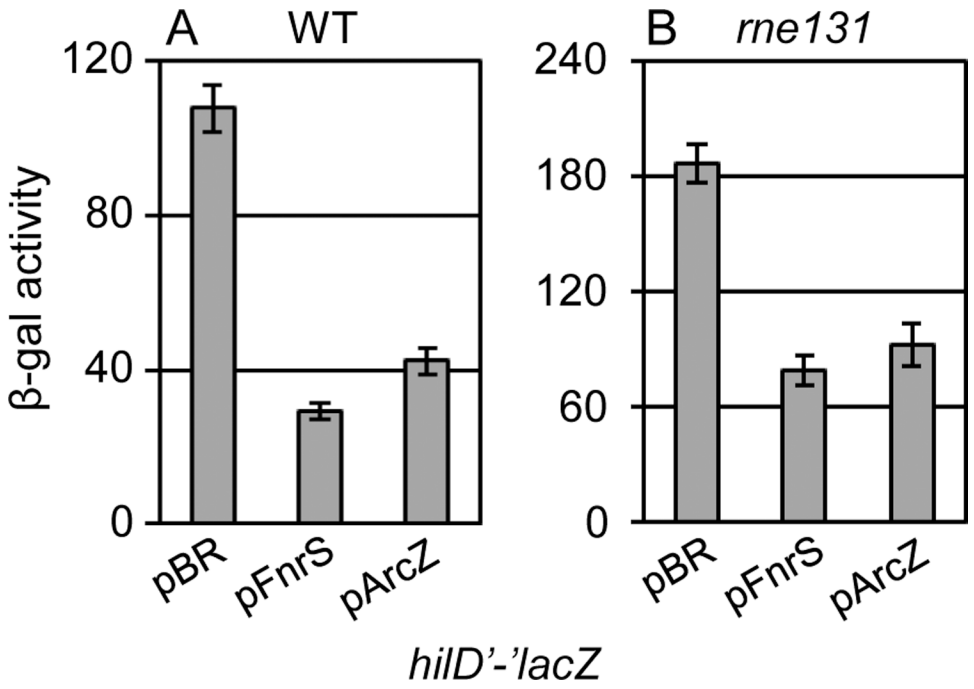

Figure 5. Mechanism of FnrS and ArcZ regulation of *hilD* mRNA translation in *Salmonella*.
β-galactosidase activity in *Salmonella* strains containing the *hilD'-'lacZ* translational fusion and either the empty vector or plasmids overexpressing FnrS or ArcZ from *Salmonella* in a (A) wildtype or (B) *rne131* background grown in SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^6/(OD_{600} × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS892, JS2118 or JS2119, each with plasmid pBRplac, pFnrS-SM, or pArcZ-SM.

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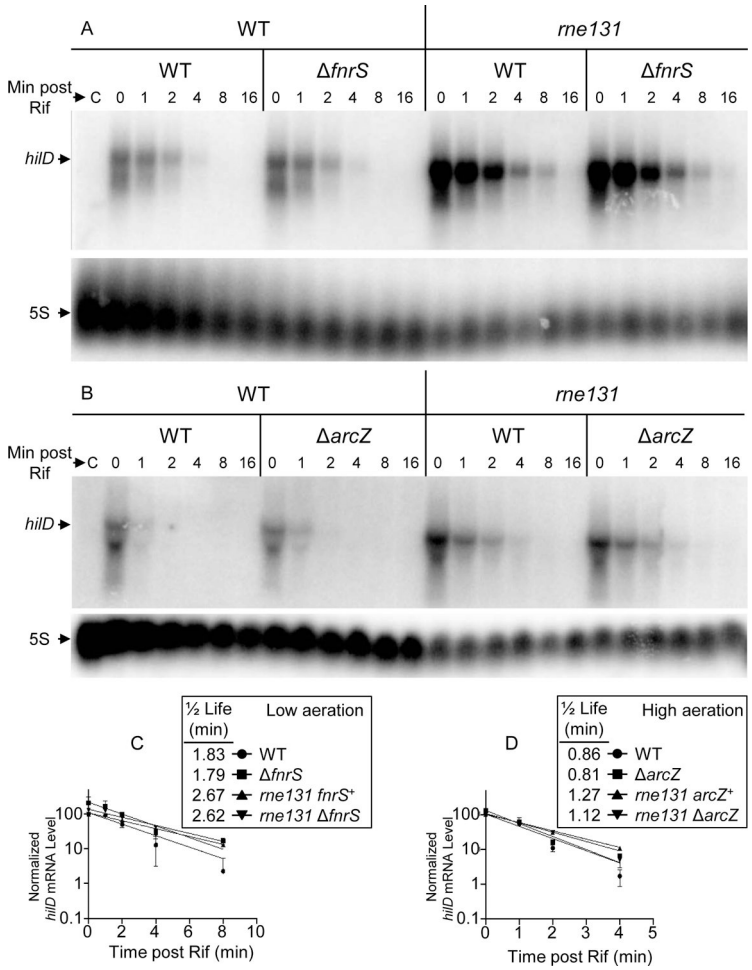

Figure 6. The half-life of *hilD* mRNA.

Cells were grown under (A) low aeration or (B) high aeration conditions and RNA was isolated at various time points after addition of rifampicin, and processed as described in Experimental procedures. ("C" indicates a sample from a Δ*hilD* strain.) The northern blots are representative of two independent experiments. The intensities of the *hilD* mRNA bands were quantified and normalized to the 5S bands. The WT bands at 0 min was considered 100%. mRNA decay curves represent the means and the standard errors (SEM) for the two experiments Strains used: 14028, JS481, JS2117, JS2121, JS2165, JS2166, and JS2167.

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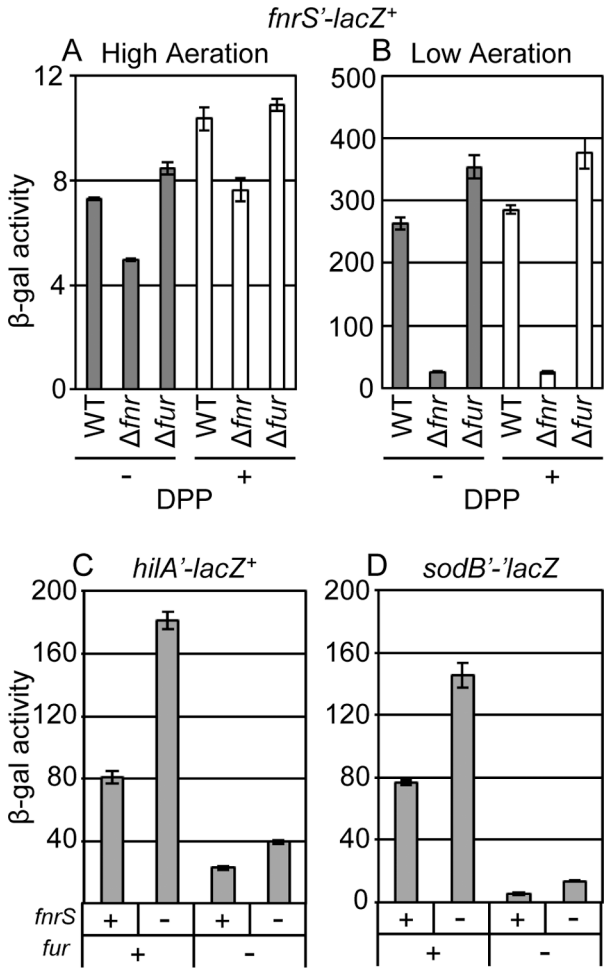

Figure 7. FnrS regulation of SPI1 expression is independent of Fur.

β-galactosidase activity in *Salmonella* strains containing an *fnrS'-lacZ*$^{+}$ transcriptional fusion in the wild type, Δ*fnr*, or Δ*fur* background grown in (A) high aeration or (B) low aeration conditions in the presence or absence of 200 μM dipyridyl (DPP). β-galactosidase activity in *Salmonella* strains containing (C) *hilA'-lacZ*$^{+}$ transcriptional fusion or (D) *sodB'-'lacZ* translational fusion in the wild type or the indicated mutant background grown in SPI1 inducing conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^{6}/(OD_{600} × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS2129, JS2130, JS2131, JS749, JS2125, JS583, JS2132, JS619, JS2133, JS620, and JS2134.

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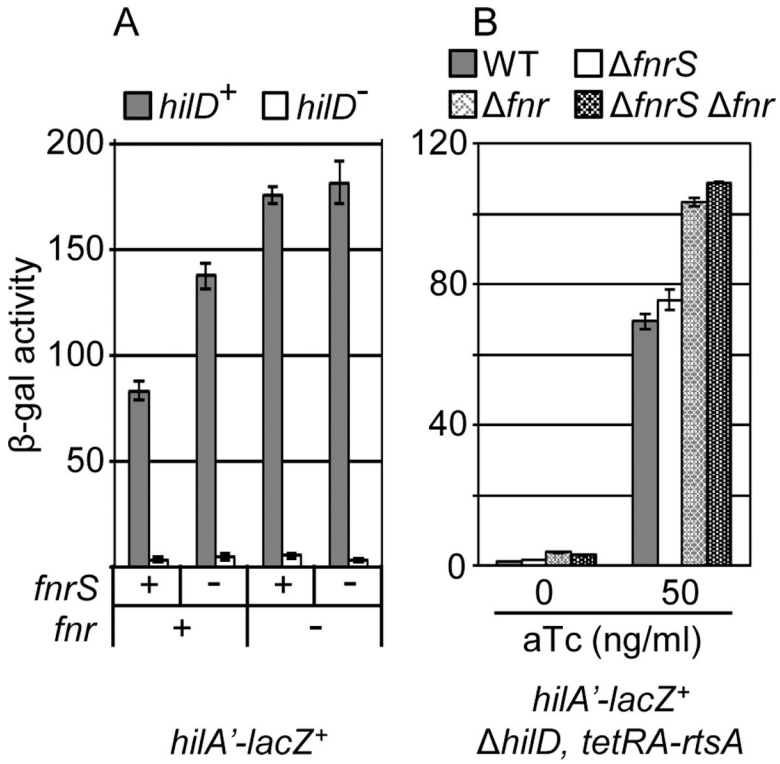

Figure 8. Fnr regulates SPI1 expression independent of FnrS.

(A) β-galactosidase activity in *Salmonella* strains containing the *hilA'-lacZ*$^{+}$ transcriptional fusion in the wild type, Δ*fnrS*, or Δ*fnr* background in the presence or absence of HilD grown in low aeration conditions. (B) β-galactosidase activity in *Salmonella* strains containing *hilA'-lacZ*$^{+}$ transcriptional fusion and the indicated mutations in the Δ*hilD* background with RtsA protein produced under a tetracycline regulated promoter grown under low aeration conditions with the indicated a-tetracycline concentrations. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^{6}/(OD$_{600}$ × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS749, JS2125, JS2135, JS2136, JS2137, JS2138, JS2139, and JS2140.

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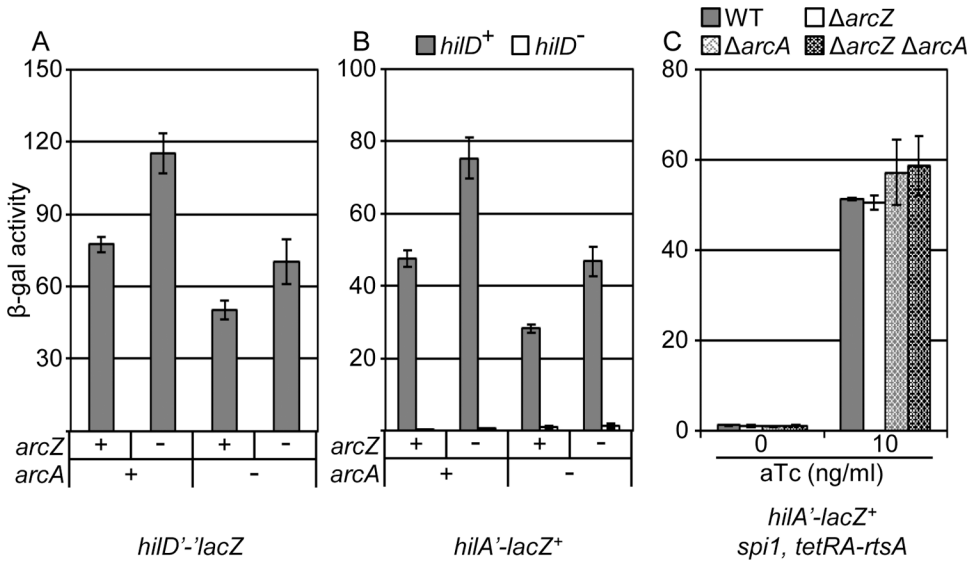

Figure 9. ArcA regulates SPI1 expression independent of ArcZ.

β-galactosidase activity in *Salmonella* strains containing the (A) *hilD'-'lacZ* translational fusion or the (B) *hilA'-lacZ* transcriptional fusion in a wild type, Δ*arcZ*, and/or Δ*arcA* background grown in high aeration conditions. (C) β-galactosidase activity in *Salmonella* strains containing *hilA'-lacZ*$^{+}$ transcriptional fusion and the indicated mutations in the *spi1* (Δ*spi1* Δ*rtsA*) background with RtsA protein produced under a tetracycline regulated promoter grown under high aeration conditions with the indicated a-tetracycline concentrations. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^{6}/(OD_{600} × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS892, JS2124, JS2143, JS2144, JS749, JS2126, JS2145, JS2146, JS2147, JS2148, JS2149, JS2150, JS2077, JS2151, JS2152, and JS2153.

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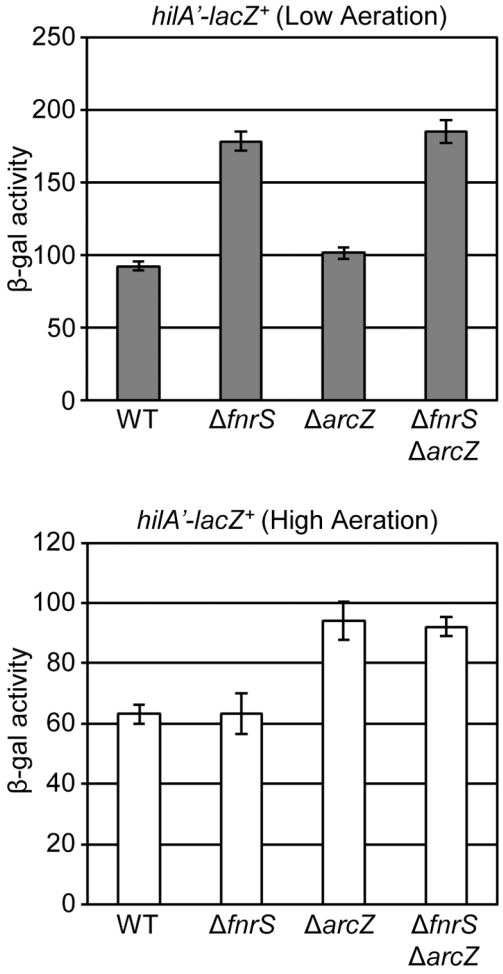

Figure 10. Loss of both FnrS and ArcZ abolishes oxygen-mediated regulation of *hilA* expression.

β-galactosidase activity in *Salmonella* strains containing the *hilA'-lacZ*$^{+}$ transcriptional fusion in the wild type, Δ*fnrS*, Δ*arcZ, or* Δ*fnrS* Δ*arcZ* background grown in either high aeration or low aeration conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) × 10^{6}/(OD$_{600}$ × ml of cell suspension) and are reported as mean ± standard deviation where n=3. Strains used: JS749, JS2125, JS2126. and JS2160.

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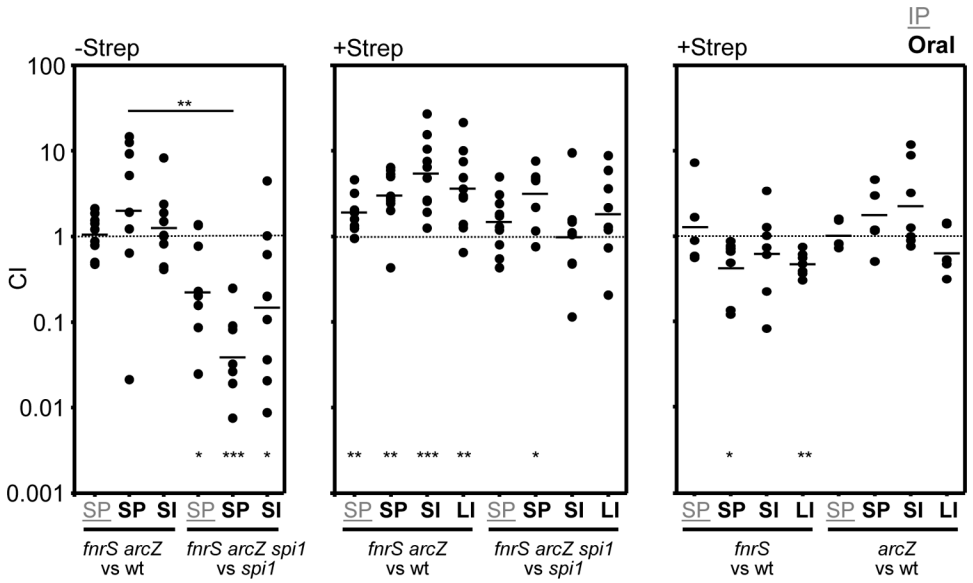

Figure 11. Mouse competition assays.

Mice were infected (intraperitoneally) IP or orally (as noted) with a 50:50 mix of the indicated strains. Competition assays were performed in streptomycin-treated (+Strep) or untreated (−Strep) mice (see Experimental procedures). Bacteria were recovered from the spleen (designated SP) in the case of IP competition assays or from the spleen (SP), small intestine (SI), and large intestine (LI) in oral competitions. The competitive index (CI) was calculated as described in experimental procedures and is shown for each mouse. The line indicates the geometric mean for each set. The Student t test was used to compare the CIs to the inocula or between groups. p=<0.05 (*), p=<0.005 (**), p=<0.0005 (***). The strains used were JS749, JS2125, JS2164, JS2160, JS2162, JS2075.